Ihh Signaling and Muscle Forces are Required for Enthesis Development

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Ihh Signaling and Muscle Forces are Required for Enthesis Development

by

Andrea Gitomer Schwartz

A dissertation presented to the Graduate School of Arts and Sciences of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Professor Stavros Thomopoulos, Chairperson

Tendon-to-bone repair is clinically challenging and plagued by high failure rates. The attachment of relatively stiff bone (~20GPa) to more compliant tendon (~200MPa) represents a fundamental engineering challenge. In the native tendon-bone attachment, termed the “enthesis,” transitional tissue contains gradients of structure and composition that effectively reduce stress concentrations at the boundary between hard and soft tissue. This transitional tissue is replaced by scar after injury and repair, resulting in a mechanically inferior attachment. The goal of this thesis is to study biological and mechanical cues that are critical to the development of the structure and function of the native tendon, which could inspire novel repair strategies to improve tendon-to-bone healing.

To accomplish this, we characterized mineralization patterns in the murine supraspinatus enthesis throughout postnatal development on the micro-scale using Raman spectroscopy and at the nano-scale using transmission electron microscopy – electron energy loss spectroscopy. Mineralization of this tissue occurred postnatally via endochondral ossification. We observed a
constant and approximately linear increase in the mineral-to-collagen ratio at the mineralizing front within the enthesis at all developmental stages. Using a multi-scale linear elastic model of the tendon enthesis, we demonstrated that the mineral gradient amplifies stresses near mineralizing cells early in development while reducing stress concentrations at the mature tendon-bone interface.

Next, we investigated tendon enthesis development in the absence of muscle forces. This localized paralysis model resulted in joint level deformities and mineralization defects. We observed a dramatic decrease in the enthesis biomechanical properties accompanied by structural and compositional changes. Collagen fiber alignment was reduced and mineralization defects were observed using Raman spectroscopy and X-ray diffraction.

In order to probe the biological mechanisms that might influence development of this tissue, we hypothesized that factors critical to endochondral bone formation will also influence enthesis mineralization. Using a murine reporter of active Indian hedgehog (Ihh) signaling, we identified a population of cells present early in development that populate the mature enthesis. Lineage tracing analyses indicated that this cell population remained at the mature enthesis while down-regulating Ihh signaling in mineralized regions. In the case of reduced muscle forces, Ihh signaling was slightly elevated in this model compared to controls. Eliminating Ihh signaling throughout development using a conditional Smoothened (Smo) knockout mouse model specific to tendon lineage cells resulted in dramatic mineralization defects in the enthesis and reduced biomechanical behavior of the attachment.
Taken together, this thesis demonstrates that Ihh signaling and muscle loading are necessary for mineralization and maturation of a mechanically robust tendon-to-bone attachment.


Chapter 1 – Introduction


Summary

The attachment of dissimilar materials is a major engineering challenge. Stress concentrations would arise at the interface of two disparate materials unless the interface is tuned to the mechanical mismatch. An effective biologic solution to this problem can be seen at the attachment of a relatively compliant tendon to a relatively stiff and brittle bone. A functionally graded tissue develops between tendon and bone to provide a robust attachment (the “enthesis”) that alleviates potential stress concentrations. This thesis will demonstrate that development and maintenance of this tissue is driven by mechanobiology, the cellular response to the physical loading environment. We show that muscle loading is necessary for the formation of a transitional tissue between tendon and bone and explore some of the biological factors that mediate this process. This unique transitional tissue is not recreated during healing, so surgical reattachment of these two tissues often fails. This chapter describes the structure-function relationships at the tendon enthesis and reviews previous work investigating the role of mechanobiology in the development and homeostasis of the enthesis. Previous studies have demonstrated that the tendon enthesis is a functionally graded material with regard to its
extracellular matrix composition, its structural organization, its mineral content, and its mechanical properties. The fetal and post-natal developmental process leading to these functional gradients requires muscle loading. Less well understood are the biological signals that transduce mechanical signals into changes in tissue properties during these scenarios. A better understanding of mechanobiology at the insertion may help guide rehabilitation strategies (e.g., protective immobilization followed by active motion) and tissue engineering protocols (e.g., tissue specific bioreactor designs) for enhancing tendon-to-bone healing.

1.1 Structure-function relationships at the attachment of tendon to bone

The attachment of tendon to bone represents a fundamental engineering challenge. Bone is a stiff, brittle material with an elastic modulus near 20GPa in both tension and compression (Bostrom MPG 2000). Conversely, tendon is relatively tough and extensible with an elastic modulus of 200MPa in tension, while buckling in compression (Woo SL 2000). The attachment of two dissimilar materials lends itself to stress singularities at the interface and an increased risk of fracture. In order to overcome these challenges, the tendon enthesis is uniquely adapted to provide a smooth transfer of load between two dissimilar tissues. To accomplish this, the enthesis has transitional tissue characterized by structural and compositional gradients over a range of length scales that give rise to graded tissue mechanical properties.

The risk of elevated stresses leading to failure has important implications for orthopaedic interfaces, which have high rates of rupture and tearing. Two examples interfaces prone to injury are the rotator cuff in the shoulder and the anterior cruciate ligament in the knee. Surgical repair of these tissues is particularly difficult because the surgeon must overcome the challenge
of attaching two materials with vastly different mechanical properties. This contributes to documented rates of re-rupture as high as 20% for minor rotator cuff tears and up to 94% for massive rotator cuff tears (Harryman, Mack et al. 1991; Galatz, Ball et al. 2004). Of note is that the functionally graded transitional tissue of uninjured insertion is typically not recreated after surgical reattachment and healing (Thomopoulos, Hattersley et al. 2002; Thomopoulos, Williams et al. 2003; Galatz, Sandell et al. 2006).

1.1.1. Functionally graded morphology of the mature tendon-to-bone insertion

From an anatomic perspective, two major types of attachments have been described for tendon and bone (Benjamin, Kumai et al. 2002). The first type of attachment is termed “fibrous”; these attachments insert into bone across a wide footprint, presumably to distribute loads over a large area and reduce stresses. Characteristic to these insertions are perforating mineral fibers (“Sharpey’s fibers”) that interdigitate into the underlying bone (Quain 1856). This type of attachment is found at the insertion of the deltoid tendon and at the tibial insertion of the medial collateral ligament. The second type of attachment is termed “fibrocartilaginous”; these attachments are found at the bony insertions of the rotator cuff and Achilles tendons (Figure 1.1) (Benjamin, Kumai et al. 2002; Benjamin, Toumi et al. 2006). Fibrocartilaginous attachments, which contain a unique transitional tissue adapted to withstand a complex stress environment, will be the primary focus of this chapter.

Fibrocartilaginous insertions are typically characterized by the presence of four distinct zones easily identified under the light microscope (Figure 1.1) (Benjamin, Kumai et al. 2002). The first zone is tendon proper, the second zone is un-mineralized fibrocartilage, the third zone is
mineralized fibrocartilage, and the fourth zone is bone (Benjamin, Kumai et al. 2002; Benjamin, Toumi et al. 2006). Each zone is characterized by a unique profile of cell morphology and extracellular matrix composition. Zone I consists of tendon fibroblast cells with a matrix rich in type I collagen and the proteoglycans decorin and biglycan (Waggett, Ralphs et al. 1998; Woo SL 2000). The second zone transitions to a more fibrocartilaginous morphology with rounder cell morphologies. The most abundant collagens are types II and III, but collagen types I, IX, and X are also present along with the proteoglycans decorin and aggrecan (Kumagai, Sarkar et al. 1994; Visconti, Kavalkovich et al. 1996; Fukuta, Oyama et al. 1998; Waggett, Ralphs et al. 1998; Thomopoulos, Williams et al. 2003). The third zone is characterized by the onset of mineralization but it is distinct from the underlying bone. The matrix is still characteristic of cartilage; rich in types II and X collagen and aggrecan (Kumagai, Sarkar et al. 1994; Visconti, Kavalkovich et al. 1996; Fukuta, Oyama et al. 1998; Ralphs, Benjamin et al. 1998; Thomopoulos, Williams et al. 2003). The final zone, the underlying bone, consists of highly mineralized type I collagen.
Figure 1.1: Tendon attaches to bone across a functionally graded fibrocartilaginous transition site (toluidine blue stained section from a rat supraspinatus tendon-to-bone insertion is shown; scale bar = 200μm).

While useful for making qualitative comparisons, this description of the enthesis as discrete “zones” is likely an oversimplification. Rather than discrete zones with abrupt transitions, the insertion consists of a graded morphology that is critical to transferring muscle forces from tendon to bone without large elevations in stress at the interface. Instead of an abrupt transition at the interface between the un-mineralized fibrocartilage and mineralized fibrocartilage regions, there is a gradual increase in mineral content. This mineral gradient was first described in a rat rotator cuff model as a nearly linear increase over a narrow region within the enthesis (Wopenka, Kent et al. 2008). A graded tissue has also been identified at the cartilage-bone interface (Gupta, Schratter et al. 2005), suggesting that this strategy of using
mineral gradients to dissipate stress at bone-soft tissue interfaces is also a feature common to other types of orthopaedic interfaces.

Microstructural variation in collagen fiber alignment is also a feature of the mature insertion. Using a rat rotator cuff model, collagen fiber alignment was measured with quantitative polarized light microscopy (Thomopoulos, Williams et al. 2003). This experiment demonstrated that collagen fiber alignment varies across the insertion from well-aligned in tendon through an increasingly disordered region of fibrocartilage before becoming again more aligned in the underlying bone.

The structure of the mature enthesis shares some similar features to the growth plates of developing bone (Gao, Messner et al. 1996). The growth plate is commonly divided into regions based on mineralization state and cell morphology (Provot and Schipani 2005; Villemure and Stokes 2009). The reserve zone consists of small chondrocytes with little organization that are induced to proliferate in response to growth inducing stimuli. Cells of the proliferative zone are organized into columns parallel to the long axis of the bone. Near the top of the columns, cells become flattened in morphology while cells closer to the metaphysis become hypertrophied and more spherical in morphology. In this hypertrophic zone, cells become filled with calcium that begins to mineralize the matrix surrounding the cells. The late hypertrophic zone is characterized by chondrocytes undergoing cell death, interspersed with newly formed vascular channels that allow osteoclasts and osteoblasts to remodel the mineralized matrix to form bone. The fibrocartilage at the tendon enthesis also contains cells with a more rounded chondrocyte-like morphology that are arranged into stacks perpendicular to and spanning the mineralized interface (Benjamin and Ralphs 2004). Similar to the enthesis, the growth plate hypertrophic zone is
characterized by graded variations of cell morphology, structure and extracellular matrix composition, including mineralization.

**1.1.2. The mechanical consequences of morphological gradients**

The structural and compositional variations described above have mechanical consequences. Strategies for reducing stress concentrations at the enthesis include a shallow tendon attachment angle at the bone interface, shaping of transitional tissue morphology (i.e. splaying), interdigitation of transitional tissue into bone, a compliant region, and functional grading of the transitional tissue (Thomopoulos, Williams et al. 2003; Thomopoulos, Marquez et al. 2006; Liu, Birman et al. 2011; Liu, Thomopoulos et al. 2012). These factors alone or in concert are all capable of reducing stress singularities at the tendon-bone interface. The microstructural arrangement of collagen fibers across the enthesis reduces stress and strain concentrations at the attachment (Thomopoulos, Marquez et al. 2006). Using a finite element model, it was demonstrated that the pattern of fiber alignment at the rotator cuff enthesis reduces stresses at the interface via a disorganized compliant region. Whereas engineering practice would be to interpolate between the mechanical properties of dissimilar materials such as tendon and bone, experimental evidence indicates that natural insertions contain a region that is more compliant than either the soft tissue or bone. The existence of this compliant region has been verified in several orthopaedic tissues. Biomechanical tensile tests on rat supraspinatus tendons indicated that the tissue near the insertion had a lower stiffness than the tendon midsubstance (Thomopoulos, Williams et al. 2003). Stouffer *et al.* measured increased tensile strain in human patellar tendon-to-bone insertions compared to the tendon midsubstance (Stouffer, Butler et al. 2008).
Micro-compression experiments in the anterior cruciate ligament insertion and meniscal attachments demonstrated that regions of uncalcified cartilage had lower compressive moduli than calcified regions (Villegas, Maes et al. 2007; Moffat, Sun et al. 2008; Hauch, Oyen et al. 2009). A recent optimization study has provided a rationale for such a seemingly illogical interfacial system (Liu, Thomopoulos et al. 2012). Through numerical optimization of a mathematical model of an insertion site, it was shown that stress concentrations can be reduced by a biomimetic grading of material properties (i.e., a compliant zone between tendon and bone) (Figure 1.2).

**Figure 1.2:** The distribution of material properties for minimization of radial stress concentration factor contains a biomimetic compliant band between tendon and bone (Liu, Thomopoulos et al. 2012).

The growth plate hypertrophic zone also contains similar structural, compositional and morphological gradients to the tendon enthesis. These gradients also contribute to variations in mechanical behavior that likely influence the tissue response to mechanical loading. In rat growth plates, higher compressive strains were detected in the hypertrophic region compared to
the resting/proliferative region (Villemure, Cloutier et al. 2007; Amini, Veilleux et al. 2010). The resting zone is also stiffer than the combined proliferative and hypertrophic zones (Cohen, Lai et al. 1998; Sergerie, Lacoursiere et al. 2009). This result is consistent with the more compliant region detected at the tendon- and ligament-to-bone interfaces.

The presence of a gradient in mineral content at the enthesis also serves to modulate the mechanical behavior of the tissue. Partially mineralized collagen fibers result in a stiffness increase only for mineral concentrations above a “percolation threshold” that corresponds to the formation of a continuous mineral network. Modeling results demonstrate that an effective mechanical attachment is created by combining the effects of a gradual increase in mineral content with variations in collagen organization (Genin, Kent et al. 2009).

1.2 Mechanobiology in musculoskeletal tissue homeostasis and development

A complex synergy between biophysical cues and biological processes gives rise to the gradations in structure and composition observed at the tendon enthesis. An improved understanding of the mechanotransduction mechanisms by which the loading environment is linked to extracellular matrix production and ultimately to tissue functional behavior will help guide the development of novel repair strategies. Based primarily on mechanical cues, musculoskeletal tissues are continually remodeled throughout the lifespan. This enables tissues to heal after injury and to adapt to environmental stimuli. The ability of musculoskeletal tissues to remodel in response to the loading environment is critical, not only to maintain mature tissue homeostasis, but also to direct the complex patterning necessary for fetal and postnatal development.
Abnormal loading conditions that result from muscle paralysis, prolonged bed rest, or overuse lead to pathological changes in the musculoskeletal system. Examples of these conditions include repetitive loading-induced tendinopathies, unloading-induced osteoporosis, joint instability-induced osteoarthritis, and paralysis-induced developmental defects. For example, neonatal brachial plexus palsy often occurs during difficult childbirth, resulting in paralysis and muscle imbalance in the shoulder (Birch 2002; Mehta, Blackwell et al. 2006). This paralysis leads to defects in glenohumeral joint development leading to severe functional impairments (Moukoko, Ezaki et al. 2004; Smith, Rowan et al. 2004; Kirkos, Kyrkos et al. 2005).

1.2.1. Mechanobiology in adult musculoskeletal tissue homeostasis

It is well established that musculoskeletal tissues are sensitive to changes in the mechanical loading environment. This is apparent for all tissues (bone, fibrocartilage, and tendon) and cell types (osteoblasts, osteoclasts, chondrocytes, and fibroblasts) at the tendon-to-bone insertion. Bone is in a constant state of remodeling. Old bone is resorbed by osteoclasts and new bone is deposited by osteoblasts. Control of the relative rates of bone formation and resorption determine whether bone mass is maintained, gained, or lost. The idea that bones respond to biomechanical stimuli is attributed to what is now referred to as Wolff’s Law of bone remodeling (Wolff 1892). Bone structure adapts in response to the loading environment; regions of lower stress are resorbed, leading to a net bone loss, and regions of higher stresses are reinforced, leading to a net increase in bone. This results in bone trabeculae that are aligned with the direction of principal stresses. Studies in bone demonstrated that cortical thickness and
trabecular architecture are both modulated by the loading environment (Goldstein 1987; Mullender and Huiskes 1995). For example, astronauts lose 2% of hip bone density per month in space and tennis players have increased bone density in their dominant arm (Priest, Jones et al. 1977; Lang, LeBlanc et al. 2004). While it is clear that mechanical forces are critical to bone maintenance, the exact biological mechanisms that transduce changes in the loading environment to changes in the rate of tissue formation, which ultimately affects tissue mechanical behavior, are less well understood. Moreover, extremes of unloading and overloading of musculoskeletal tissues lead to pathological conditions.

Articular cartilage serves to cushion the ends of bones and to reduce friction during movement. Mature articular cartilage has a zonal structure through its depth and is essentially an arrested bone growth front at the ends of long bones. The morphology is classified into surface, middle, and deep zones, with variations in structure, composition, and mechanical properties. The tensile modulus of the superficial zone is higher than the deep zone, while the deep zone has a greater compressive modulus (Guilak, Ratcliffe et al. 1995; Krishnan, Park et al. 2003). The two primary loading modes experienced by chondrocytes in articular cartilage due to joint loading are hydrostatic stress and shear stress. Chondrocytes subjected to high levels of compressive hydrostatic stress produce type II collagen and aggrecan while suppressing vascularization; all characteristics of a stable chondrocyte phenotype (Mizuno, Tateishi et al. 2002). Shear stresses in cartilage promote fibrillar collagen production. Tensile stress promotes vascular invasion and ossification that result in advancement of the growth front (O'Connor 1997). Cyclic compressive forces induced by joint loading are required to maintain the properties of articular cartilage. Joint immobilization eliminates cyclic compressive stress, activating the subchondral growth front and inducing degradation of the cartilage layer, a
characteristic of osteoarthritis (Carter, Beaupre et al. 2004). This also occurs in regions of joints that experience low levels of loading.

Tendons and ligaments are responsible for transmitting forces from muscle to bone in the case of tendons and from bone to bone in the case of ligaments. Both are essential for joint stabilization and movement. Like bone, tendon and ligaments are remodeled in response to mechanical stimuli. Changes in tendon due to exercise include an increase in the cross-sectional area and an increase in collagen turnover (Magnusson, Hansen et al. 2003). Immobilization leads to a decrease in collagen synthesis and a decrease in stiffness and tensile strength (Amiel, Woo et al. 1982; Woo, Gomez et al. 1982). Unloading a ligament results in changes to the composition and mechanical behavior of the tissue (Amiel, Woo et al. 1982; Woo, Gomez et al. 1982; Woo, Gomez et al. 1987; Walsh, Frank et al. 1992). Moreover, localized changes in the tendon loading mode (e.g., tension vs. compression) due to wrapping around a bone pulley in a joint induces a more chondrogenic phenotype, with increases the local proteoglycan content and down regulation of angiogenic factors (Vogel and Koob 1989; Vogel, Ordog et al. 1993; Vogel, Sandy et al. 1994; Petersen, Pufe et al. 2002; Pufe, Petersen et al. 2003; Thomopoulos, Das et al. 2011). Based on these observations, it is clear that mechanical stimuli are critical to the maintenance of skeletal tissues.

1.2.2 Mechanobiology in fetal and postnatal development

Biophysical cues also drive developmental patterning and growth in the fetal and postnatal musculoskeletal system (Carter, Beaupré et al. 2007). Bones, tendons, muscles, and joints are patterned in utero but development continues through postnatal stages. During the
embryonic stage of development, loss of muscle loading leads to severe musculoskeletal defects (Nowlan, Sharpe et al. 2010). The Hueter-Volkmann law states that in developing bone, compressive stress reduces the bone growth rate, while tensile loads accelerate it.

The impact of muscle loading on embryonic development has been examined in several animal models. Chicken embryos have the advantage of an externally laid egg and are thus particularly easy to manipulate with chemical paralysis agents (Osborne, Lamb et al. 2002). Mice are the most common model of mammalian development and are well established for genetic manipulations. Mice originally developed to examine the role of specific genes in development provide useful models of muscle-less mice. Examples of this include mutations in Pax3 or a combination of Myf5 and MyoD1, which both result in limbs without skeletal muscle (Brent, Braun et al. 2005; Nowlan, Bourdon et al. 2010). Muscle contractions in utero begin early in embryonic development. The magnitude of in utero forces increases dramatically when increases in muscle volume are coupled with the forces that result from bone elongation (Figure 1.3) (Sharir, Stern et al. 2011). In the absence of muscle forces, there are defects in bone size, shape, and mineralization (Mikic, Johnson et al. 2000; Osborne, Lamb et al. 2002; Gomez, David et al. 2007; Sharir, Stern et al. 2011). Joint cavitation does not occur, leading to bone fusion (Mikic, Johnson et al. 2000). This is the result of de-differentiation of joint progenitor cells, which return to a cartilage phenotype (Kahn, Shwartz et al. 2009). Bone shaping is altered in the absence of muscle loading, with defects observed in the knee joint, long bone cross-section, and the deltoïd tuberosity (Kahn, Shwartz et al. 2009).
Figure 1.3: (Top left) The ratio of the maximum and minimum second moment of inertia was lower for paralyzed (darker shade) compared to control (lighter shade) femora (schematics of the maximum and minimum cross-sectional moment of inertias are shown to the right of the plot for control [top] and paralyzed [bottom] mice). (Top right) Micro-CT cross-sectional images are shown for bones from paralyzed (right) and control (left) mice. (Bottom) The appositional radius, as a function of angular coordinate, is shown for paralyzed and control mice (Sharir, Stern et al. 2011).

Eliminating embryonic muscle forces also leads to defects in tendon and cartilage. Tendon development is initiated, but tendon precursors are not maintained, in the absence of muscle forces (Kardon 1998; Brent, Braun et al. 2005). Chondrocyte proliferation is decreased, altering bone growth rates and contributing to the observed bone defects (Germiller and Goldstein 1997). Furthermore, the lack of joint cavitation results in the complete loss of articular cartilage surfaces (Pacifici, Koyama et al. 2005). Compositional changes occur in developing
cartilage as a result of immobilization that lead to altered mechanical properties (Mikic, Isenstein et al. 2004).

The importance of mechanical loading to development increases after birth. At this point, the effects of joint contact forces due to body weight are added to the effects of increasing muscle forces. Bone growth in postnatal chicks is significantly arrested by adding only 10% of body weight using an external harness (Reich, Jaffe et al. 2005; Reich, Sharir et al. 2008). The reduction in bone length is reversed by removing the weight, but the resulting bones have impaired structural and mechanical behavior.

The rate of long bone growth by endochondral ossification is sensitive to the mechanical environment. Compression maintains the cartilage phenotype and slows bone growth, while tensile loads along the long axis of the bone increase elongation (Stokes, Aronsson et al. 2006; Stokes, Clark et al. 2007). Stokes and coworkers used three different animal models to demonstrate a linear relationship between the applied axial stress and the bone growth rate using externally applied loading plates pinned to the bones (Stokes, Aronsson et al. 2006; Stokes, Clark et al. 2007). This relationship held for both tensile and compressive loading of proximal tibial growth plates and vertebral bodies. The bone growth rate depends on the combined rates of cell division in the proliferative zone, cell volume increases in the hypertrophic zone, and the rate of chondrocyte maturation leading to mineralization (Villemure and Stokes 2009). The biological mechanisms that enable cells to transduce forces into cell fates, and the matrix production processes that control rates of bone growth are less well understood. It is likely that the mechanisms described above that influence endochondral ossification also drive the development of the unique transitional tissue at the enthesis.
1.2.3. Biological molecules critical to skeletal mechanotransduction

Members of many distinct families of molecules have been implicated in mechanotransduction pathways, all of which likely play roles in enthesis development. These include, but are not limited to, extracellular matrix proteins (collagens, proteoglycans, and glycosaminoglycans), growth factors (TGFβ’s, BMP’s, FGF’s), cytokines (IL1, IL6), hedgehog family members (Ihh), matrix metalloproteinases (MMP-1, MMP-13), and angiogenic factors (VEGF) (Henderson and Carter 2002). Several mechanisms of cellular mechanotransduction have been identified. Strains in the ECM are coupled to cytoskeletal rearrangements through integrins in the cell membrane, which provide a direct structural connection between the ECM and the cytoskeleton (Ingber 2008). Other cellular mediators of mechanotransduction include cell surface G-protein coupled receptors, receptor tyrosine kinases, and stretch activated ion channels (Wang 2006).
Figure 1.4: Schematic of chondrocyte maturation (blue) showing the Ihh/PTHrP negative feedback loop and selected biomarkers. Arrows indicate positive regulation and bars indicate negative regulation.

Endochondral bone formation is regulated by the autocrine/paracrine signaling of Indian hedgehog (Ihh) and parathyroid hormone-related protein (PTHrP) (Figure 1.4) (Vortkamp, Lee et al. 1996; St-Jacques, Hammerschmidt et al. 1999; Broadus, Macica et al. 2007). Ihh is a member of the conserved Hedgehog (Hh) family of cell-secreted proteins. Ihh signals through the transmembrane receptors Patched (Ptch) and Smoothened (Smo), ultimately controlling the localization and processing of the glioma-associated oncogene homolog (Gli) family of transcription factors that regulate expression of downstream target genes including Gli1 and Ptch (Long and Ornitz 2013). In the growth plate, Ihh is expressed by pre-hypertrophic and early hypertrophic chondrocytes entering the early stages of terminal differentiation. This molecule
signals to cells in the surrounding perichondral layer and proliferating chondrocytes and stimulates synthesis of PTHrP (Vortkamp, Lee et al. 1996; St-Jacques, Hammerschmidt et al. 1999). Ihh directly stimulates chondrocyte proliferation but acts directly through stimulation of PTHrP to induce chondrocyte hypertrophy (Long, Zhang et al. 2001; Hilton, Tu et al. 2007). PTHrP suppresses chondrocyte maturation through the PTHrP receptor expressed by the pre-hypertrophic and hypertrophic chondrocytes, blocking further expression of Ihh, establishing a negative feedback loop to provide fine control over the rate of chondrocyte proliferation and maturation.

Ihh and PTHrP also directly or indirectly regulate many other molecules critical to endochondral bone formation. The transcription factor SRY (sex determining region Y)-box 9 (Sox9) is a target of PTHrP in the growth plate that likely influences maintenance of a stable proliferating chondrocyte phenotype (Huang, Chung et al. 2001). Sox9 has also been implicated in later stages of chondrocyte hypertrophy (Akiyama, Chaboissier et al. 2002). Ihh is also required to induce Runx2 expression, a molecule critical for osteoblast differentiation (Long, Chung et al. 2004). Precise spatial and temporal control over these and other extracellular signaling molecules and transcription factors is critical to endochondral bone development.

Ihh is mechanosensitive: its expression in chondrocyte cultures is upregulated in response to tensile stretching and is required for increased proliferation (Wu, Zhang et al. 2001). This mechanoresponse is mediated downstream by BMP 2 and 4 and does not involve PTHrP. PTHrP has also been implicated in mechanotransduction pathways independent of interactions with Ihh (Chen, Macica et al. 2007). In this study, when tendon insertion sites were unloaded by tail suspension or tendon transection, there was a significant decrease in PTHrP expression. This decrease was more dramatic in the transection group compared to the suspension group. Based
on this, it is suggested that PTHrP might play a role in the migration of tendon insertion sites in order to accommodate increases in bone length during development. In support of this, there is increased osteoclastic activity at the leading edge of the insertion site, while more osteoblasts are observed at the trailing edge to allow bone growth.

Type X collagen is a well described marker of chondrocyte maturation and of the early stages of mineralization in endochondral bone formation (Shen 2005). Expression of collagen X is limited to hypertrophic chondrocytes in the growth plate. There is some evidence that this molecule is mechanoresponsive. In one study, collagen X mRNA was upregulated after tensile stretch was applied to chondrocytes (Wu and Chen 2000). Other reports indicated that collagen X expression and protein levels were reduced by compressive loading in growth plate chondrocytes (Villemure, Chung et al. 2005; Cancel, Grimard et al. 2009). These results are consistent with the idea that tensile loading of developing bone increases mineralization, which leads to longitudinal bone growth, while compressive loads have an arresting effect on growth.

Scleraxis (Scx) is a transcription factor noted for its role in tendon development (Cserjesi, Brown et al. 1995; Schweitzer, Chyung et al. 2001; Brent, Schweitzer et al. 2003; Murchison, Price et al. 2007). Scx null mice have several defects in force transmitting tendons of the limbs and have severely compromised movement (Murchison, Price et al. 2007). Furthermore, this mutation results in structural changes: increased disorder and decreased amounts of tendon extracellular matrix likely contribute to the noted functional losses. Recently, several studies have indicated a role for Scx in mechanotransduction. In vitro loading modulates Scx expression in tenocyte cultures (Scott, Danielson et al. 2011). More specifically, in tendons, mechanical force causes release of TGFβs from the extracellular matrix (Maeda, Sakabe et al. 2011). This activates the Smad 2 pathway through the TGFβ receptor leading to upregulation of Scx in
response to mechanical loading. Loss of loading due to tendon transection or botulinum toxin induced paralysis results in decreased Scx expression, as demonstrated using a ScxGFP reporter. In addition, Mendias et al. found that treadmill exercise upregulates Scx expression (Mendias, Gumucio et al. 2011).

1.3 Mechanobiology in the development of the tendon enthesis

Muscle loading is necessary for the development of a functionally graded tendon enthesis. This complex tissue has features of bone, ossifying cartilage, and tendon, and is typically subjected to a combination of compressive and tensile stresses. As described in the previous section, mechanical signals are capable of influencing the synthesis rate and composition of the extracellular matrix as well as cell fate in developing musculoskeletal tissues. It is likely that biological factors involved in mechanotransduction pathways in other orthopaedic tissues also impact enthesis development. In order to achieve the complex graded microstructure and morphological characteristics of the enthesis, it follows that there must also be spatial and temporal interplay between gene expression and synthesis of extracellular matrix molecules. These expression patterns are controlled by both genetic and biophysical cues from the environment (i.e., muscle loading) and produce the complex biomechanical gradients found at the mature insertion.

While neo-tendon and bone are established early in fetal development, the enthesis transitional tissue is often not evident until postnatal stages (Bland and Ashhurst 1997; Fujioka, Wang et al. 1997; Bland and Ashhurst 2001; Galatz, Rothermich et al. 2007). In an animal model, the supraspinatus neo-tendon was evident adjacent to developing humeral head bone at
embryonic (E) day 15.5 (E15.5) (Thomopoulos, Hattersley et al. 2002). In contrast, the mature insertion, defined by the appearance of fibrocartilaginous transitional tissue, was not identified until after birth. In mouse shoulders, an insertion region between the supraspinatus tendon and bone begins to appear by postnatal (P) day 7 (P7) and mature fibrocartilage is not in evidence until P21 (Galatz, Rothermich et al. 2007). During this period, the shoulder experiences large increases in limb loading as body weight and muscle mass increase along with activity levels. It follows that large muscle forces are likely necessary to drive the development of transitional tissues in the enthesis.

The enthesis develops adjacent to the mineralizing epiphyseal cartilage of the humeral head. Mineralization of the humeral head occurs through endochondral ossification, a process that proceeds by mineralization of a cartilage template followed by vascular invasion and remodeling of the mineralized cartilage template by osteoclasts and osteoblasts (Provot and Schipani 2005; Blitz, Viukov et al. 2009). Chondrocytes from the cartilage anlage are induced by biological and chemical factors to proliferate resulting in an increase in size or length of the cartilage template. Cells then enter a terminal differentiation process beginning with hypertrophy, followed by mineralization of the cartilage matrix, and finally cell death and matrix resorption and remodeling by recruitment of angiogenic factors. This bone formation process is characteristic of the growth plate region of long bones during fetal development that continues into postnatal development.

In one example, Blitz et al. described the development of the deltoid tendon-humeral tuberosity attachment (Figure 1.5) (Blitz, Viukov et al. 2009). It was observed that the deltoid tuberosity formed via endochondral ossification in a two-phase process: initiation was regulated by a signal from the tendons, whereas the subsequent growth phase was muscle (i.e., load)
dependent. Specifically, scleraxis regulated BMP-4 production in tendon cells at their bony insertion site. When BMP-4 expression was blocked in scleraxis expressing cells, the enthesis (and associated bone ridges) did not form. This implicates BMP-4 as a key mediator of tendon-specific signaling for enthesis formation. The key regulators of endochondral ossification, collagen II, Ihh, PTHrP, and collagen X were expressed at the developing enthesis.

**Figure 1.5:** Blitz *et al.* suggested the following model for the contribution of both tendons and muscles to enthesis formation. Through a biphasic process, tendons regulate enthesis initiation and muscles control its subsequent growth. Further research is necessary to determine the mechanism whereby muscle contraction regulates enthesis development (Blitz, Viukov et al. 2009).

### 1.3.1 Development of extracellular matrix gradients in the enthesis

This section will describe the developmental patterns that lead to the complex structure and composition of the tendon-to-bone insertion. The temporal expression of extracellular matrix molecules was described in detail using *in situ* hybridization experiments throughout
murine fetal and postnatal development (Galatz, Rothermich et al. 2007). At birth, the bone side of the insertion was largely made up of un-mineralized type 2 collagen, characteristic of cartilage. Neonatal collagen II gene expression was confirmed by in situ hybridization. The tendon side of the insertion expressed type I collagen, which is characteristic of tendon and bone extracellular matrix. As mineralization of the secondary ossification center proceeded over the first few weeks of postnatal development, expression of collagen II became restricted to a narrow zone in the transitional tissue of the insertion. Type I collagen was expressed by cells adjacent to the band of collagen II expressing cells on the tendon side of the insertion.

Type X collagen was evident in a band of cells on the bone side of the insertion, adjacent to the mineralizing front. Collagen X is typically localized to hypertrophic chondrocytes in the growth plate prior to mineralization. At the enthesis, expression of this marker persists after the large hypertrophic cells associated with mineralization are no longer seen, suggesting that this molecule may play a role in maintaining the mineralized interface. This result is consistent with the development of the rat Achilles tendon (Fujioka, Wang et al. 1997).

### 1.3.2. Biological factors necessary for insertion development

Development of the complex transitional tissue found at the tendon enthesis requires precise spatial and temporal control of a range of biological factors (Figure 1.6). The development of transitional tissue occurs during postnatal development when tendon and bone are already established, but still actively growing and remodeling. In order for transitional tissue to develop at the interface, there must be concurrent regulation of and interaction between the biological signals of tendon, bone, and cartilage. Individually, all three tissue types are
responsive to the mechanical environment. Specific biological molecules native to each tissue type have been implicated in the cellular response to the mechanical loading environment.

The mineralized side of the insertion is not yet mature bone during the early stages of enthesis development. Instead, it is an immature epiphysial cartilage template undergoing endochondral ossification. At this stage of development, the insertion shares many features with the growth plate. Biological factors identified at precise spatial locations in the growth plate include: PTHrP, Ihh, Ptc, Sox9, and type X collagen (Kronenberg 2003; Provot and Schipani 2005). Chemical gradients of these molecules are responsible for maintaining the graded morphology of the growth plate. These factors have also been localized to the developing tendon-to-bone insertion and may also impact development of a graded insertion (Bland and Ashhurst 1997; Fujioka, Wang et al. 1997; Bland and Ashhurst 2001; Chen, Macica et al. 2006; Galatz, Rothermich et al. 2007).

PTHrP was originally described for its role in regulating the growth plate in a negative feedback loop with Ihh (Figure 1.4) (Vortkamp, Lee et al. 1996; St-Jacques, Hammerschmidt et al. 1999; Broadus, Macica et al. 2007). Recently, PTHrP has been localized to tendon and ligament entheses during postnatal development (Chen, Macica et al. 2006; Chen, Macica et al. 2007). More specifically, it is localized to a group of fibroblast-like cells in the intermediate zone between the tendon proper and the transitional tissue that inserts into the underlying cortical bone (Chen, Macica et al. 2006). Furthermore, PTHrP has been generally localized to periosteal cells in addition to cells that will form the secondary ossification center of long bones (Chen, Macica et al. 2006). Elevated expression of PTHrP at tendon to bone insertions suggests that PTHrP may be important to maintain the mineralized interface during development. In the growth plate, PTHrP maintains chondrocyte proliferation and blocks maturation and
mineralization (Provot and Schipani 2005). PTHrP may have a similar function for enthesis development.

**Figure 1.6:** Spatially and temporally controlled expression of a number of transcription factors, growth factors, and transcription factors likely play important roles in enthesis development.

Scx is a transcription factor localized to mature tendon that is necessary for tenogenesis and is found in tendon progenitor cells (Cserjesi, Brown et al. 1995; Schweitzer, Chyung et al. 2001; Brent, Schweitzer et al. 2003; Murchison, Price et al. 2007). This cell population overlaps at bony eminences with the population of Sox9 positive cells that make up the developing cartilage rudiments (Blitz, Sharir et al. 2013; Sugimoto, Takimoto et al. 2013). Sox9 is a marker of a stable chondrocyte phenotype with important roles in endochondral bone formation (Huang, Chung et al. 2001; Akiyama, Chaboissier et al. 2002). Sox9 has additional roles in embryonic development, and together with Scx helps determine chondrogenic versus tenogenic cell lineage (Asou, Nifuji et al. 2002; Furumatsu, Shukunami et al. 2010). A distinct Sox9 positive-Scx positive pool of progenitor cells is responsible for forming tendon-to-bone attachment sites in a modular fashion (Blitz, Sharir et al. 2013; Sugimoto, Takimoto et al. 2013). In a study
investigating the development of the deltoid tuberosity, Blitz and coworkers demonstrated that Scx is necessary for the initiation phase of development (Blitz, Viukov et al. 2009). This insertion forms by endochondral ossification and is necessary to create a stable attachment point for the deltoid tendon. Scx expression in the tendon cells mediated BMP4 expression in cells near the insertion to initiate formation of the deltoid tuberosity. Subsequent growth of the insertion was dependent on muscle loading (Blitz, Viukov et al. 2009).

1.3.3. Mechanical factors required for insertion development

In order to probe the effects of muscle loading on the postnatal development of the tendon-to-bone attachment, it is useful to employ an animal model that can be combined with biological and genetic manipulations. By injecting botulinum toxin into the rotator cuff muscles of mice throughout postnatal development, effectively paralyzing the shoulder and eliminating muscle forces, it is possible to isolate the effects of loading on the development of the supraspinatus insertion. Using mice enables this model to be combined with genetic manipulation to investigate the role of specific biological molecules implicated in mechanotransduction pathways. Botulinum toxin injections are routinely used to induce localized and reversible muscle paralysis. Botulinum toxin chemically blocks the transmission of nerve impulses through neuromuscular junctions. To study the role of muscle loading on enthesis development, mice received botulinum toxin injections in one shoulder and saline injections in the contralateral shoulder beginning within 24 hours of birth (Thomopoulos, Kim et al. 2007; Kim, Galatz et al. 2009; Das, Rich et al. 2011). The saline group provided an internal
control for paired statistical comparisons. A third group of normal age matched mice was used as fully mobile controls.

This animal model displayed a similar phenotype to the human condition neonatal brachial plexus palsy. In order to ensure that there was no effect of botulinum toxin on shoulder development that wasn’t the direct result of muscle paralysis, a group of animals received a neurotomy of the upper trunk of the brachial plexus (Kim, Galatz et al.). The phenotype of the neurotomy group closely mimicked the botulinum toxin-injected group. Both groups showed a substantial decrease in muscle volume compared to controls. Decreases in muscle volume and mass correlated to decreases in muscle force generation in botulinum toxin injected shoulders compared to saline controls after 4 and 8 weeks of paralysis (Das, Rich et al. 2011).

The shoulder muscle paralysis induced in this animal model resulted in striking changes to tendon-to-bone insertion development (Figure 1.7). Unloading caused severe mineralization defects in the humeral head, including reduced overall volume and morphological changes. For example, the humeral head appeared flattened, similar to observations in children with neonatal brachial plexus palsy. This demonstrated that mechanical loading is critical for mineralization at the enthesis. The lower mineral density observed in micro computed tomography measurements in the unloaded groups can at least in part be attributed to an increase in osteoclast activity (Thomopoulos, Kim et al. 2007). When osteoclast activity was blocked using a bisphosphonate drug, there was a partial recovery of some bone mineralization measures (Tatara, Lipner et al. 2010). Bone volume was significantly recovered in a dose dependent manner.

Removal of muscle loading also affected the development of a fibrocartilaginous transition at the insertion (Figure 1.7). Based on histological analysis, little to no fibrocartilage was observed in the insertion after 8 weeks of paralysis (Thomopoulos, Kim et al. 2007).
Collagen fiber alignment, investigated using quantitative polarized light microscopy, indicated fibers were more disorganized in unloaded shoulders compared to saline controls. Impaired mineralization, disordered fiber alignment and a loss of fibrocartilage transitional tissue in the insertions likely contribute to the overall inferior mechanical properties of unloaded tendon insertions. Structural mechanical properties (e.g., maximum force, stiffness) and material mechanical properties (e.g., maximum stress, modulus) were decreased after 4 and 8 weeks of botulinum toxin injections (Figure 1.7).

Blitz et al. used genetically modified mice with muscular defects to examine the role of muscle loading on enthesis development (Blitz, Viukov et al. 2009). Consistent with the studies described above, this study demonstrated that while muscle loading was not required for initiation of enthesis formation, it was necessary for the subsequent growth and maturation of the enthesis.

Figure 1.7: Muscle paralysis dramatically impaired the development of the supraspinatus tendon-to-bone insertion in mice. (Top) A mature, compositionally graded insertion (“i”) is seen by 56 days post-natally in normal mice (scale bar = 200μm). In contrast, the enthesis in paralyzed shoulders appears disorganized, without a graded fibrocartilaginous transition between the supraspinatus tendon (“s”) and the humeral head bone (“h”).
1.4 Tendon-to-bone healing

Repair of ruptures at the tendon-to-bone insertion is a persistent problem in orthopaedic surgery. Rotator cuff tears are a common injury to the upper extremity and involve reattaching tendons to the humeral head (Iannotti 1994). ACL injuries are also very common and surgical reconstruction techniques use tendon grafts as ligament replacements that must heal in bone tunnels (Fu, Bennett et al. 1999). Tendon and ligament injuries can be classified as acute or chronic ruptures. Acute injuries are generally the result of extrinsic factors, while chronic injuries also involve intrinsic factors such as tissue degeneration and a predisposition toward injury. While acute tendon-to-bone ruptures usually have reasonable healing outcomes, the problem of healing in the case of a chronic injury is confounded by significant degeneration of the transitional tissue that extends to the tendon and underlying bone. Several studies have indicated that nearly all Achilles tendon ruptures have histological evidence of degeneration (Kannus and Jozsa 1991). In contrast to acute trauma of a healthy tendon, this type of injury can result from the compounded effects of aging and overuse, often from a lifetime of repetitive motion. Increased loading from overuse disrupts the tissue homeostasis, alters tissue composition, and results in functionally inferior tissue mechanical properties leading to injury.

1.4.1. Injury and repair result in a loss of the functionally graded tendon-to-bone interface

interface
Tendon-to-bone injuries are generally characterized by rupture of the tendon, requiring repair of the tissue to its original bony footprint. Tendon-to-bone healing can be roughly divided into three stages: inflammation, repair, and remodeling. The inflammation stage involves recruitment of vascular cells, such as erythrocytes and platelets, and immune cells, such as macrophages to the injury site to resorb necrotic tissues via phagocytosis. The inflammatory cells also recruit tendon fibroblasts for the repair phase, in which extracellular matrix (primarily collagen) is synthesized and deposited at the injury site. The remodeling phase begins approximately two months post-injury and is characterized by reduced cellularity and matrix synthesis, as the tissue becomes more fibrous and is then remodeled into scar-like tendon tissue (Wang 2006).

Studies in rabbits, goats, and rats have verified that repaired interfaces have inferior mechanical behavior, presumably because the functionally graded transition between tendon and bone is not regenerated (Figure 1.8) (Waggy 1994; St Pierre, Olson et al. 1995; Thomopoulos, Williams et al. 2003; Galatz, Sandell et al. 2006). The scar tissue that forms at the interface has a larger volume than the native tissue, but is functionally inferior. This may be explained by the dramatic decrease in the amount of underlying bone and the loss of collagen fiber organization (Ditsios, Boyer et al. 2003; Galatz, Rothermich et al. 2005; Thomopoulos, Matsuzaki et al. 2007; Cadet, Vorys et al. 2010). Additionally, the mineral gradient within the enthesis is lost (Wang, Su et al. 2013). These factors are compounded in the case of a chronic rupture, which is typically accompanied by significant degeneration prior to injury and increasing the difficulty of repair and the risk of re-rupture. While many factors contribute to the observed decrease in tissue functional behavior, the main distinction between a healing and a normal insertion is the near complete loss of transitional tissue in the enthesis.
Figure 1.8: The functionally graded transition between tendon and bone is not regenerated (hematoxylin and eosin stained images are shown under bright-field on the top row and under polarized light on the bottom row) (Silva, Thomopoulos et al. 2006).

1.5 Scope and Procedure of the Dissertation

As described above, tendon-to-bone repairs are plagued by poor healing. Inferior scar tissue replaces the complex enthesis transitional tissue resulting in decreased mechanical performance of the interface (Thomopoulos, Williams et al. 2003; Silva, Thomopoulos et al. 2006; Liu, Thomopoulos et al. 2012; Wang, Su et al. 2013). The goal of this dissertation is to study the natural developmental process of this tissue in order to inspire novel strategies to improve healing. A key feature of the enthesis is to modulate the stress environment at the mineral-tendon interface. The attachment of two dissimilar materials such as tendon and bone is a fundamental challenge because stresses are often amplified at the interface. The unique structure of the enthesis represents a good natural solution to this problem. In this dissertation,
the developmental course of mineralization leading to a functionally graded interface in the postnatal murine enthesis is described in Chapter 2. Chapter 3 expands on the description of mineralization patterns in the enthesis and presents estimates of the stress environment at the mineralized interface throughout development. Next, the physical and biological factors critical to enthesis development are explored. Chapter 4 investigates the role of muscle forces on enthesis structure and function throughout development using a murine model of localized paralysis. In Chapter 5, the role of Hedgehog signaling, a crucial regulator of endochondral mineralization is investigated in enthesis development and mineralization. Chapter 6 presents preliminary data describing the development of a murine enthesis injury model that could be used to probe biological factors critical to healing in animals as young as P7. This model was used to investigate the role of Ihh signaling in tendon healing. The central hypothesis of this thesis is that activated hedgehog signaling and muscle forces are necessary for the development of functionally graded fibrocartilage transitional tissue in the postnatal enthesis.

1.6.1 Specific Aims

Aim 1: Investigate the postnatal mineralization process that leads to a functionally graded mineralized fibrocartilage interface. During postnatal development, the interface transitions from a tendon-cartilage interface to a mineralized fibrocartilage interface critical for modulating the stress environment. Understanding patterns of mineralization will allow us to estimate changes in the local stress environment of the attachment throughout development. This data could be used to inform future tissue engineering approaches to improve tendon-to-bone healing.
**Hypothesis:** The micro-scale and nano-scale mineralization patterns in the developing insertion are initially guided by the cartilage template. The mineralization front of the secondary ossification center advances into the developing enthesis by 2 weeks after birth. Further advancement of the mineralization front leads to the formation of functional mineral gradient necessary for reducing peak stresses in the interface due to muscle loading.

**Study Design:** Mineralization will be investigated in a murine model throughout postnatal development. MicroCT and histology will be used to study gross mineralization patterns in the mouse supraspinatus enthesis. Raman spectroscopy, transmission electron microscopy, and x-ray diffraction and fluorescence will be used to investigate mineralization on the micro to nano scales. *In silico* modeling approaches will be used to examine the stress environment of the developing enthesis.

**Aim 2:** Examine the necessity of muscle forces for the postnatal development of a functional enthesis. Physical forces are known to be critical for guiding the development and homeostasis of musculoskeletal tissues.

**Hypothesis:** Muscle forces will be required throughout postnatal development to direct enthesis mineralization. Structural and compositional changes due to unloading in the postnatal enthesis will result in functional losses.
**Study Design:** Botulinium toxin will be used to locally reduce supraspinatus muscle forces throughout postnatal development. Enthesis structure and function will be evaluated using histology, Raman spectroscopy, X-ray diffraction, and uniaxial tensile testing.

**Aim 3:** Investigate the role of Ihh signaling in tendon enthesis development. The Ihh signaling pathway is a crucial regulator of endochondral mineralization. Additionally, expression levels of Ihh-related factors have been shown to be dependent on the mechanical environment.

**Hypothesis:** A population of Hh-responsive cells localized to the enthesis during late embryonic development is required to form mineralized fibrocartilage. Activated Ihh signaling is required for enthesis mineralization during postnatal development and levels of Ihh activity are regulated by the loading environment.

**Study Design:** Examine temporal and spatial patterns of Ihh activity throughout enthesis development using an inducible Gli1Cre reporter model. Next, Ihh signaling will be conditionally deleted from tendon progenitor cells. Finally, Ihh expression in the postnatal enthesis will be examined in the absence of muscle forces

**Aim 4:** Develop a fibrocartilage injury model that may be applied to early postnatal through adult mice. This model will be used to investigate healing differences between injuries sustained during early and late postnatal development. The model will be applied to investigate the role of the Hh-responsive cell population in tendon healing.
**Hypothesis:** The Hh responsive cell population will be recruited to the site of a fibrocartilage defect in early postnatal animals and these cells will participate in matrix remodeling. This cell population will no longer participate in the healing process of injuries sustained at later stages of development.

**Study Design:** Develop and characterize a fibrocartilage injury model using a simple needle punch defect in the supraspinatus tendon enthesis. Apply this model to the Gli1Cre$^{ERT2,:mTmG}$ mice to identify the role of the Hh responsive cell population during fibrocartilage healing.
Chapter 2: Mineral Distributions at the Developing Tendon Enthesis


Summary

A gradient of mineral content is believed to play an important role for dissipation of stress concentrations at mature fibrocartilaginous interfaces. This chapter investigates the development and the micro/nano-meter structure of this unique interface in order to provide novel insights for the improvement of repair strategies. This study monitored the development and mineralization of transitional tissue at the murine supraspinatus tendon enthesis, which begins to form postnatally and is morphologically mature by postnatal day 28. The micrometer-scale distribution of mineral across the developing enthesis was studied by X-ray microcomputed tomography and Raman microprobe spectroscopy. Analyzed regions were identified and further studied by histomorphometry. The nanometer-scale distribution of mineral and collagen fibrils at the developing interface was studied using transmission electron microscopy (TEM). A zone (~20μm) exhibiting a gradient in mineral relative to collagen was detected at the leading edge of the hard-soft tissue interface as early as postnatal day 7. Nanocharacterization by TEM suggested that this mineral gradient arose from intrinsic surface roughness on the scale of tens of nanometers at the mineralized front. Microcomputed tomography measurements indicated increases in bone mineral density with time. Raman spectroscopy measurements revealed that the mineral-to-collagen ratio on the mineralized side of the interface was constant.
throughout postnatal development. An increase in the carbonate concentration of the apatite mineral phase over time suggested possible matrix remodeling during postnatal development. Comparison of Raman-based observations of localized mineral content with histomorphological features indicated that development of the graded mineralized interface was linked to endochondral bone formation near the tendon insertion. These conserved and time-varying aspects of interface composition may have important implications for the growth and mechanical stability of the tendon-to-bone attachment throughout development. The biological and physical factors that give rise to the mineralization patterns observed here are discussed in the subsequent chapters.

2.1 Introduction

Tendons and ligaments attach to bone across transitional tissue interfaces that are several micrometers to millimeters in thickness. The interface, termed the “enthesis”, is classified as either fibrous (e.g., medial collateral ligament to tibia entheses) or fibrocartilaginous (e.g., supraspinatus tendon to humeral head entheses) (Benjamin, Kumai et al. 2002; Claudepierre and Voisin 2005; Doschak and Zernicke 2005). The fibrocartilaginous entheses contain a functionally graded transitional tissue, with variations in extracellular matrix structure and composition giving rise to variations in mechanical properties across the interface (Cooper and Misol 1970; Benjamin and Ralphs 1998; Benjamin and McGonagle 2001; Thomopoulos, Williams et al. 2003; Moffat, Sun et al. 2008). Tendon consists primarily of type I collagen with small amounts of decorin and biglycan. Bone consists of heavily mineralized type I collagen. Collagen fibers are well aligned in tendon. However, the collagen fibers become less organized
as they insert into the bone (Thomopoulos, Marquez et al. 2006). At the tendon enthesis, a fibrocartilaginous transitional zone is present that is rich in type II collagen and aggrecan produced by fibrochondrocytes, which have a rounder morphology compared to spindle-shaped tendon fibroblast cells and are phenotypically similar to chondrocytes. Within the transitional zone of the rat supraspinatus tendon enthesis, an increase in mineral relative to collagen has been observed through the transition from tendon to bone (Wopenka, Kent et al. 2008). These variations in structural and compositional properties result in graded mechanical behavior that contributes to an efficient transfer of muscle load from tendon to bone (Genin, Kent et al. 2009; Liu, Birman et al. 2011; Liu, Thomopoulos et al. 2012). It is believed that the gradient in mineral content is particularly important for limiting stress concentrations at the mineralized interface.

In an injury-and-repair scenario, the original graded transitional tissue of the fibrocartilaginous insertion is not recreated after the tendon is surgically reattached to bone. Surgical reattachment leads to a more abrupt interface of mechanically inferior and disorganized scar tissue (Thomopoulos, Hattersley et al. 2002; Thomopoulos, Williams et al. 2003). The loss of a gradual mineral transition likely contributes to the decreased mechanical performance of the load-bearing interface and results in frequent re-ruptures. For example, surgical repair of massive rotator cuff tears, which relies on tendon-to-bone healing for success, has a re-tear rate of up to 94% (Harryman, Mack et al. 1991; Galatz, Ball et al. 2004).

In contrast to the scar tissue that results from healing, developmental processes generate an effective fibrocartilaginous attachment. It remains unclear how the activities of cells coordinate during development to give rise to the complex graded structure of the enthesis. Histological studies have indicated that the enthesis develops post-natally in murine shoulders (Galatz, Rothermich et al. 2007). Whereas previous studies have examined mineral distributions
in the adult tendon enthesis (Wopenka, Kent et al. 2008), developmental patterns of gene and protein expression for the organic matrix components (Gao, Messner et al. 1996; Fujioka, Wang et al. 1997; Asou, Nifuji et al. 2002; Galatz, Rothermich et al. 2007), and the mineral content in the developing bovine ligament enthesis (Wang, Mitroo et al. 2006), localized mineralization patterns in the developing tendon enthesis have not yet been investigated.

The fibrocartilaginous enthesis has many similarities to the structure of the growth plate that is formed during endochondral ossification of bone (Benjamin, Kumai et al. 2002). Endochondral ossification is the process by which bone forms from a cartilaginous template (Mackie, Ahmed et al. 2008). Chondrocytes from the precursor template proliferate, synthesize extracellular matrix, and undergo hypertrophy to enlarge the tissue and eventually terminally differentiate, leaving behind a mineralized cartilage matrix. The mineralized cartilage template is invaded by vascular tissue that delivers bone cell precursors to remodel the mineralized matrix into bone. This process leads to specific gradients in cell type and matrix composition, and is tightly regulated by biological signals and influenced by applied loads. Blitz et al. reported similar events at the developing deltoid insertion into the humeral tuberosity (Blitz, Viukov et al. 2009).

The objective of the study presented in this chapter was to characterize the spatial distribution of mineral across the tendon-to-bone insertions of developing mouse shoulders. Specifically, our goal was to better understand the time sequence and stages of development of the graded region found at the interface between mineralized and non-mineralized fibrocartilage in the enthesis. We hypothesized that a region of graded mineral content would develop concurrently with the appearance of fibrocartilage in the insertion. Surprisingly, our results showed that a mineral gradient appeared near the enthesis much earlier in development and was
associated with the mineralization front. The micrometer- and nanometer-scale distributions of mineral across the attachment have important mechanical implications for the functional behavior of the tissue. Comparing the micrometer-scale patterns of mineralization to cell and matrix morphologies may provide insights into the developmental mechanisms controlling enthesis mineralization. Moreover, understanding the structure of the tendon-to-bone interface throughout maturation at these different spatial hierarchies can assist development of novel biomimetic materials and signaling molecules that can enhance clinical approaches for surgically repairing tendon-to-bone injuries.

2.2 Methods

2.2.1 Animal Model

The use of animals and our procedures for this study were approved by the animal studies committee at Washington University (Protocol Number: 20100091) and all efforts were made to minimize suffering. CD1 mice (Charles River Labs) were sacrificed in a CO2 chamber at five postnatal (P) time points (days): P7, P10, P14, P28, and P56. The time points were chosen to coincide with epiphyseal mineralization, which begins ~P3-P7 in the mouse shoulder, and the appearance of mature fibrocartilage at the insertion, which appears ~P21-P28 (Bland and Ashhurst 1997; Fujioka, Wang et al. 1997; Galatz, Rothermich et al. 2007). Five animals per time point were allocated for Raman analysis and subsequent frozen section histology, three animals per time point were scanned for X-ray microcomputed tomography and processed for paraffin histology, and two animals per time point were allocated for TEM analysis. The animal
sex and the choice of right or left shoulder were randomized for each time point and assay. Detailed descriptions of the specimen preparation procedures are described below.

2.2.2 Raman Microprobe Analysis

Raman spectroscopy was used to probe micrometer-scale variations in relative mineral concentration across the tendon-to-bone interface. This technique can be used to study unfixed, hydrated tissues, offering significant advantages over other micrometer-scale compositional analysis methods (Wopenka, Kent et al. 2008; Morris and Mandair 2011). Animals allocated for Raman analysis (N=5 per time point) were stored at -20 °C. After thawing, the humeral head and supraspinatus tendon were isolated, taking care to preserve the tendon-to-bone insertion. The samples were then embedded in optimal cutting temperature (OCT) embedding medium without further processing and re-frozen at -80 °C. 20 µm-thick sections of fresh un-decalcified tissue were cut in the coronal oblique plane on a cryostat. Sections were placed on glass slides and stored at -80 °C. A recent study indicates that multiple freeze-thaw cycles of bone tissues may alter some portions of the Raman spectrum (Dong, Yan et al. 2004; McElderry, Kole et al. 2011). However, for our evaluation of the degree of mineralization, we did not rely on the amide I and amide III bands, whose stability during freeze-thaw cycles was called into question in the prior study. Immediately prior to analysis, sections were washed three times with phosphate buffered saline (PBS) to thaw the samples and remove remaining embedding medium and any blood contamination from the bone marrow.

The Raman microprobe apparatus (HoloLab Series 5000 fiber-optically coupled Raman Microscope, Kaiser Optical Systems, Inc.) and analysis procedures have been described.
previously (Wopenka, Kent et al. 2008). Briefly, Raman spectra were collected using 10 mW (measured at the sample surface) of 532 nm laser excitation focused to an approximately 1 μm beam spot on the surface of the specimen using an 80x objective lens with a N.A. = 0.85. Lateral and depth resolution during sample analysis were on the order of a few micrometers. Although the 532 nm laser line used for this study allows for excellent signal-to-noise at relatively low laser power, blood contamination can present a problem, as it introduces a high fluorescence background under 532 nm excitation. Fortunately for our study, the tendon-to-bone insertion is relatively avascular, and any residual red blood cells from the bone marrow were removed by rinsing with PBS. The light scattered by the illuminated specimen was collected in a backscattered configuration through the same objective lens used to focus the laser onto the specimen. A 2048-channel CCD detector was used to concurrently detect the spectral range 100-4000 ∆cm⁻¹. Each spectrum used for analysis was the average of 32, 4-second spectral acquisitions. The laser spot was manually focused onto the surface of each sample using reflected light images as a guide to the surface topography.

The hydrated state and dramatically different material properties between tendon and bone made it impossible to create a polished surface that was flat enough for extensive 2D automated mapping. Instead, for each sample, spectra were acquired from at least 12 different regions along a roughly linear transect that spanned the tendon-to-bone interface. Each region of interest was manually selected to optimize the Raman signal. Example traverses are shown in Figure 2.1. The x-y stage position of the region of interest corresponding to each acquired spectrum was documented on the reflected light images acquired before each spectrum. The acquired Raman spectra were analyzed to interpret compositional properties of the tissue at each location. This study was primarily concerned with the degree of mineralization (i.e., mineral-to-
matrix ratio) and the spatial distribution of mineral in the tissue. In the non-mineralized tissue, no spectral differences were observed between fibrocartilage and tendon. The fully mineralized side of the insertion was defined where a plateau occurred in the spectroscopically determined relative mineral concentration in the tissue. To ensure that the traverses accurately captured the typical characteristics of the interface, three traverses were collected at different locations along the width of the insertion for several samples, and it was determined that the gradient region was consistent across all regions analyzed (data not shown). These pilot data indicated that the gradient was maintained along the width of the insertion and a single traverse was representative of the gradient.
Figure 2.1: Raman microprobe analysis of developing supraspinatus tendon insertions. Top row (A-C) shows a P7 insertion, middle row (D-F) a P14 insertion, and bottom row (G-I) a P28 insertion. Left column, A), D), G): 20 µm-thick sections stained with toluidine blue and according to von Kossa’s method (scale bar = 50 µm). Note that despite the sharp front of mineralization in these figures suggested by the von Kossa staining, a graded mineralization from is evident from the Raman scans. Middle column B), E), H): magnified view of square region of interest shown in images in the left column (scale bar = 10 µm). The relative mineral concentration determined by the ratio of the heights of the 960Δcm⁻¹ to 1003Δcm⁻¹ Raman peaks.
(corresponding to the ν1 P-O stretching band of hydroxylapatite and the aromatic ring stretching band of phenylalanine in collagen, respectively) is indicated by the color gradient of the overlaid points. SS: supraspinatus tendon and H: humeral head. Right column C), F), I): baseline-corrected Raman spectra corresponding to points along a traverse from tendon (no mineral - dark blue) to bone (high mineral - red). The mineral peak (960Δcm⁻¹) is indicated with a hollow arrow and the collagen peak (1003Δcm⁻¹) is indicated with a black-filled arrow.

2.2.3 Raman Spectral Analysis

Raman spectra were background corrected using the software package Grams32 (Galactic, Salem, NH) via a linear curve-fitting operation on approximately 10 operator-defined points. The relative mineral concentration at each region of interest was inferred from the ratio of the intensities of the peaks at 960Δcm⁻¹ and 1003Δcm⁻¹ for the ν₁ P-O stretching band of hydroxylapatite and the aromatic ring stretching band of phenylalanine residues in collagen, respectively. Many different reference peaks for collagen have been previously used, including the non-specific C-H stretching band (2940Δcm⁻¹) (Silva, Brodt et al. 2006; Wopenka, Kent et al. 2008), the amide I band (1667Δcm⁻¹) (McCreadie, Morris et al. 2006; Gourion-Arsiquaud, Burket et al. 2009), and the proline/hydroxyproline bands (855Δcm⁻¹ and 921Δcm⁻¹) (Kohn, Sahar et al. 2009). Here, the 1003 Δcm⁻¹ peak was chosen as a reference due to its proximity to the P-O stretch in hydroxylapatite at 960Δcm⁻¹. Fluorescence contributions to the background vary widely across the full spectral range and by selecting peaks in close proximity, their background intensities should be nearly identical. Additionally, the peak at 1003Δcm⁻¹ does not overlap other nearby peaks that might confound the measured intensity. In order to validate this
reference peak, additional analyses were performed using alternate collagen peaks (2940 $\Delta$ cm$^{-1}$ and 1667 $\Delta$ cm$^{-1}$) for reference (data not shown), and the overall trends in the data were unaffected. The analytical value of using the 1003 $\Delta$ cm$^{-1}$ peak is also supported by our previous work (Wopenka, Kent et al. 2008). It is important to recognize that, due to the nature of Raman back-scatter spectroscopy, the ratio of mineral signal strength divided by collagen signal strength does not indicate the absolute volumetric or mass ratio of mineral content to collagen content. However, changes in the spectral ratio have unambiguous meaning. Increases in this spectral ratio indeed do reflect increases in relative mineral concentration in the tissue but do not account for changes in the total amount of extracellular matrix.

To analyze changes in the properties of fully mineralized fibrocartilage near the interface as a function of age, spectra from the fully mineralized side of the insertion underwent further analysis for each sample. Three regions from each sample were identified as areas along the traverse where the mineral-to-matrix spectral ratio had reached a roughly constant maximum value. The traverses were ended before reaching the edge of the marrow cavity underlying the insertion tissue in order to exclude trabecular bone from this analysis. Therefore, the average spectra can be used to gauge the maximum relative mineral concentration of the enthesis fibrocartilage away from the interface. The three measurements from each tissue sample were averaged before statistical comparisons were made between time points. Spectra were background corrected, and peaks within the spectral region of interest were deconvolved from each other based on a mixed Gaussian-Lorentzian algorithm within the GRAMS32 software. The full width at half maximum of the 960$\Delta$cm$^{-1}$ peak (attributed to hydroxylapatite) was used as a measure of crystallographic atomic order, since narrower peaks suggest less structural variation in bond distances and angles. The carbonate content of the mineral crystallites was evaluated by
taking the ratio of the heights and the ratio of the areas of the 1070Δcm-1 peak (indicating carbonate substitution for phosphate) against the 960Δcm-1 peak. For spectra with lower signal:noise ratios, we previously determined that ratios of peak heights are more reproducible than that of peak areas (Wopenka, Kent et al. 2008). The 960Δcm-1: 1003Δcm-1 peak height ratio was evaluated to determine the maximum relative degree of mineralization of the tissue.

2.2.4 Histology and Image Analysis

After Raman analysis, sections were fixed in 10% neutral buffered formalin and stained using von Kossa’s method (to visualize the mineral) and toluidine blue (to visualize the cells and matrix). A custom MATLAB (2009a, MathWorks, Natick, MA) script was written using operator-defined control points to align the reflected-light images obtained during spectroscopic analysis with the tissue features visible in the corresponding histology image. This image correlation assured that the regions of interest analyzed by Raman spectroscopy would be associated with their respective histological features with an accuracy of a few micrometers. Several examples of the specific locations of the Raman analyses are overlaid onto histology images, which are presented together with their respective spectra in Figure 2.1. To compare the position of the mineralization front between age groups, the von Kossa- and toluidine blue-stained images were used to estimate the average distance between the stained mineral front and the tendon proper. The location of tendon tissue was approximated by the location of stained tendon fibroblasts, as distinguished by cell morphology and matrix relative to the fibrochondrocytes and proteoglycan-rich stained regions. The position of each acquired Raman-analyzed region of interest was determined relative to the von Kossa-stained mineral front and
the tendon boundary. The slope of the mineral gradient was calculated for each animal by plotting the Raman-measured mineral:matrix ratio versus the radial distance to the edge of the von Kossa-stained mineral front and performing a linear regression using a window of at least 6 sequential points. That window was rejected if the least squares fit was not statistically significant (p<0.05). The slope was then defined as the first-order term calculated from the window of points that resulted in the greatest r² statistic. This analysis resulted in an average r² statistic for all of the samples of 0.91 ± 0.08.

Additional 5 μm-thick sections from decalcified, paraffin-embedded specimens (N=3 per time point) were stained with toluidine blue and used to measure cell areas relative to extracellular matrix at each time point. Mineralized areas of the insertion were identified by close proximity to the bone side of the insertion and comparison to mineralized stained sections. Image analysis was performed using ImageJ (NIH, Bethesda, MD).

2.2.5 Microcomputed Tomography

Three freshly dissected samples from each time point were X-ray scanned for micro-computed tomography (μCT40; Scanco Medical AG, Switzerland). The X-ray tube settings were 55 kV and 145 μA and the integration time used was 99 ms. The resulting reconstructed isotropic voxel size was 30 μm (i.e., each voxel consisted of a volume of 2.7x10⁴ μm³). The humerus was potted in agarose and scanned with the muscle, tendon, and humeral head suspended in air. Using this method, the tendon was easily identified in the reconstructed images. By comparison to hydroxylapatite standards, bone mineral density was calculated from manually selected regions within the reconstructed three-dimensional x-ray data sets encompassing the
mineralized fibrocartilage within the tendon insertion. The analyzed regions were selected by identifying only the mineralized regions directly adjacent to the tendon and excluding the surrounding bone. The average total analysis volume for each sample was ~0.1mm³.

2.2.6 Transmission Electron Microscopy (TEM)

For TEM nano-characterization, the humerus with attached supraspinatus tendon was micro-dissected to isolate the insertion with a small piece of underlying bone attached (N=2 per time point). Samples were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, post fixed with osmium tetroxide, dehydrated in graded ethanol and embedded in Eponate 12 resin. Ultrathin sections (~70nm thickness) were sliced on a Leica EM UC6 ultramicrotome. Aqueous processing (and chemical fixation) of bone have been reported to induce structural modification to collagen fibrils as well as to induce some phase transformation, dissolution, and re-precipitation of bioapatite (Boothroyd 1964; Landis, Hauschka et al. 1977). Therefore, the boat of the diamond knife was filled with ethylene glycol in order to limit aqueous dissolution of the mineral phase. Sections were mounted on Cu TEM grids either uncoated or coated with amorphous carbon. Bright-field TEM images of unstained sections were collected using a Hitachi H 7500 transmission electron microscope operating at 80kV equipped with a CCD camera.

One sample from the P56 time point was nanocharacterized using a JEOL JEM-2100F field-emission scanning transmission electron microscope (for an instrument description, see (Alexander, Daulton et al. 2012)).Shortly after insertion into the scanning (S)-TEM instrument column, the specimen was subjected to a >1 hr beam shower to fix any hydrocarbons (and
prevent spot contamination) prior to exposing the specimen to a focused ~1 nm FWHM diameter probe. Specimens were imaged in STEM mode using the high-angle annular dark-field (HAADF) detector, which can measure electrons at high scattering angles (in our case, the range 36.8 ± 0.1 to 128.0 ± 0.4 mrad) where scattering is approximately incoherent. The high-angle scattering cross section for the volume probed by the electron beam is approximately proportional to the square of the mean atomic number in that volume. Therefore, high-angle scattering from heavy atoms will be more intense than from light atoms, producing an STEM-HAADF image with atomic z-contrast. Specimens were also spectrally imaged using a GIF Tridiem capable of electron energy loss spectroscopy (EELS). At each image pixel position a core-loss spectrum was collected over a 409.6 eV range starting at ~232 eV. The following conditions were used during collection of STEM-EELS spectral images: a collection angle of $2\beta = 22.66 \pm 0.06$ mrad, a 5 mm diameter spectrometer entrance aperture, and an energy dispersion of 0.2 eV/channel. Spectra were corrected for dark current and channel-to-channel gain variation of the GIF CCD detector array and collected in the diffraction mode of the transmission electron microscope (i.e., image coupling to the EELS spectrometer). The spectra were processed using Gatan Digital Micrograph by fitting a power-law background to each of the pre-edge regions. The background was subtracted from each edge signal, which was then integrated over a 45 eV wide window. Ratios of integrated EELS core-loss signal between two elements were converted to their corresponding atomic ratios using partial cross-sections that were calculated from theoretical Hartree-Slater models. Unlike maps of EELS core-loss signal, maps of relative elemental compositions are not influenced by variations in specimen thickness and electron diffraction (Egerton 1996).
2.2.7 Statistical Analysis

An analysis of variance (ANOVA) was applied to measurements of gradient slope, Raman peak height ratios, Raman peak width, bone mineral density, and matrix to cell area ratio, for the factor animal age. When the ANOVA was significant, a post hoc analysis was performed using Fisher’s least significant differences tests. Significance was defined as \( p < 0.05 \), and data are reported as the mean (error bars represent the standard deviation of the mean). Significant differences between time points are indicated above each bar.

2.3 Results

2.3.1 Microscale mineral gradients at the developing enthesis

Raman microprobe analysis demonstrated an increase in mineral content across the murine supraspinatus tendon-to-bone interface at all time points studied. Following Raman analysis, tissue sections were stained for histological analysis. The Raman measured regions of interest (documented by light microscopy images), were registered to and overlaid onto the subsequently collected histological images (Figure 2.1). Mineral was first detected by Raman at the edge of the von Kossa stained mineralization front. The \( \nu_1 \) P-O stretching band of hydroxylapatite was absent on the tendon side of the von Kossa stained mineralization front. The image at the interface produced by von Kossa staining was either transparent or opaque (with little gradation), implying an abrupt interface between mineralized and non-mineralized tissue. In contrast, the Raman data revealed a wider region of graded mineral-to-collagen ratio over a scale of \(~20 \mu\text{m}\). These seemingly contradictory results highlight the difference in sensitivity of the
two techniques to variations in mineral content (i.e., low sensitivity of von Kossa staining and high sensitivity of Raman spectroscopy). While von Kossa staining is excellent for producing a strong signal for a low volume fraction of mineral, it is relatively ineffective at distinguishing densely mineralized regions from sparsely mineralized regions.

The developmental origin of the mineral gradient was investigated by examining shoulders at P7 through P56. Raman traverses indicated a gradient in mineral concentration in the mineralizing cartilage nearest to the insertion as early as P7. Reference to histomorphological features demonstrated that the location of Raman detected mineral coincided with the leading edge of the secondary ossification center closest to the supraspinatus insertion (Figure 2.2). The observed increase in mineral concentration from the non-mineralized side to the mineralized side of the insertion occurred in narrow (~10 µm) regions of extra-cellular matrix located between hypertrophic chondrocytes. A similar result was observed for the P10 specimens. At all time points beyond P28, the morphology of the insertion was consistent with mature insertions; fibrochondrocytes were much smaller in size compared to earlier time points, and there was no evidence of epiphyseal cartilage between the mineral front and the tendon proper. At the P14 time point, the specimens exhibited either of the two morphologies described above.
Figure 2.2: Postnatal mineralization of the humeral head. Top row A-C): 20 µm-thick sections of toluidine blue- and von Kossa-stained mouse humeral heads with attached supraspinatus tendons at P7, P10, and P14 (scale bar = 500 µm). Bottom row D-F): Magnified views of the square regions of interest shown in the images of the top row (scale bar = 100 µm). Yellow arrows indicate the distance from the mineralization front to the tendon proper. SS: supraspinatus tendon, AC: articular cartilage, GP: growth plate. Top scale bars are 500µm and bottom scale bars are 100µm.

In order to quantify micrometer-scale patterns of mineralization in the gradient region, the magnitudes of the mineral gradients (i.e., the slopes of mineral concentration vs. spatial position) were determined for all the time points. The position of each analysis region relative to
the tangent to the mineral boundary (as determined by von Kossa staining) was determined. The rate of increase in mineral concentration with position along the insertion from the non-mineralized to the mineralized side did not vary within measurement error across the five post-natal time points investigated (Figure 2.3A and 2.3B). The width of the zone of increasing degree of mineralization (i.e., the gradient) also remained approximately constant at about 25μm from P7 to P56 (Fig. 2.3A). This width was calculated by dividing the maximum degree of relative mineralization for each sample by the gradient region slope (determined from a linear least squares fit of the mineral-to-matrix spectral ratio versus the absolute distance to the histological appearance of tendon).

**Figure 2.3:** Quantitative analysis of mineral gradients in the developing enthesis. A) The average mineral relative to collagen concentration as gauged by the ratio of the heights of the 960Δcm\(^{-1}\) to 1003Δcm\(^{-1}\) Raman peaks (corresponding to the \(\nu_1\) P-O stretching band of hydroxylapatite and the aromatic ring stretching band of phenylalanine in collagen, respectively) plotted vs. the
location relative to the tendon for the different time points in this study (P7, P10, P14, P28, and P56). The data plotted represent the mean of 5 separate measurements (i.e., from 5 mice) and the error bars represent the standard deviation of the mean. B) The slope of the data plotted in A (representing the magnitude of the gradient in mineral content) is shown. The slope was calculated from a linear least squares fit to the data from each animal individually (shown together in A) and the error bars represent the standard deviation of the slope fitting parameter across 5 animals per time point. The magnitude of the mineral gradient remains constant within measurement error throughout the postnatal development time points examined.

2.3.2 Characteristics of mineralized collagen at the insertion

To further characterize the accumulation of mineralized tissue at the insertion, three measures of mineral were used: 1) the ratio of collagen (1003Δcm⁻¹) to mineral (960Δcm⁻¹) Raman peak heights, 2) the matrix area fraction, as determined by histomorphometry, and 3) mineral density of bone, as determined by X-ray microcomputed tomography. The Raman-measured mineral-to-collagen spectral ratio from fully mineralized tissue underlying the insertion did not change with animal age (Figure 2.4a). This value is based on the mean of three spectra from ~1 μm in diameter regions of interest (~1 μm depth penetration) that spanned the mineralized tissue layer underlying the insertion. X-ray microcomputed tomography measurements of the density of the same region of mineralized tissue that underlies the supraspinatus tendon insertion indicated increasing bone mineral density over the course of postnatal development (Figure 2.4b and inset). This measurement represents the average measured intensity from ~30 μm isotropic voxels (corresponding volume 2.7x10⁴ μm³) within a
0.1 mm$^3$ volume encompassing the mineralized portion of the insertion. An example analysis region is shown in the inset to Figure 2.4b. While the ~1μm$^3$ volumes analyzed by Raman excludes cells, the larger volume analyzed by X-ray micro-computed tomography includes cells. In order to better interpret the different trends over time in mineral content between the two spatial scales, we quantified the histologically observed change in overall cell area by measuring cell areas as a fraction of total area (including cells and matrix) in regions corresponding to mineralized insertion transitional tissue. In mineralized regions, cell area decreased as postnatal time points advanced (Figure 2.4c).
Figure 2.4: Analysis of mineral content in the developing insertion. A) The average mineral relative to collagen concentration of fully mineralized fibrocartilage as gauged by the ratio of the heights of the 960 $\Delta \text{cm}^{-1}$ to 1003 $\Delta \text{cm}^{-1}$ Raman peaks for the different time points in this study (P7, P10, P14, P28, and P56). The average was taken over the 3 regions of interest from each traverse that were within the fully mineralized fibrocartilage closest to the bone side of the insertion. The data plotted represent the mean of 5 separate measurements (i.e., from 5 mice).
and the error bars represent the standard deviation of the mean. B) Bone mineral density (BMD) measured by X-ray microcomputed tomography increased throughout postnatal development in the mineralized region underlying the supraspinatus tendon (p<0.0001). The numbers above the bars indicate p<0.05 relative to the indicated time point. The inset shows a representative analysis region in black (scale = 1mm). C) Area fraction of matrix relative to cells, as measured from histological sections, increased throughout postnatal development in the mineralized areas of the insertion (p < 0.001). Significant differences between timepoints are indicated above each bar.

Raman spectroscopic analysis was used to investigate characteristics of the mineral crystallites within fully mineralized tissue adjacent to the mineral gradient region. Two parameters were measured based on the three analysis regions closest to the mineralized side of the insertion: 1) carbonate concentration of apatite mineral and 2) relative atomic order in the lattice of the mineral crystallites (i.e., crystallinity). Changes in these measures may be indicators of mineral age and remodeling (McCreadie, Morris et al. 2006; Pasteris, Wopenka et al. 2008; Gourion-Arsiquaud, Burket et al. 2009). The extent of carbonate substitution for phosphate was measured as the ratio of the heights of the 1070∆cm⁻¹ peak over the 960∆cm⁻¹ peak for carbonate and phosphate, respectively (Awonusi, Morris et al. 2007; Pasteris, Wopenka et al. 2008). Carbonate concentration increased significantly with animal age across the five developmental time-points investigated (Figure 2.5a). The change in peak height ratios of 0.11 (P7) to 0.13 (P56) with respective peak area ratios of 0.14 and 0.17 corresponds to an increase in carbonate concentration of ~5.0 wt.% to 5.8 wt.% (Pasteris, Wopenka et al. 2008). Relative atomic order within the lattice of the apatite nanocrystals, one aspect of crystallinity, was evaluated based on
the full width at half maximum of the $v_1$ phosphate band at $960\Delta cm^{-1}$. There were no statistically significant differences in crystallinity as a function of animal age (Figure 2.5b).

**Figure 2.5:** Raman analysis of the matrix mineral suggests remodeling. A) The average degree of carbonate substitution in apatite, as gauged by the ratio of the heights of the $1070 \Delta cm^{-1}$ to $960 \Delta cm^{-1}$ Raman peaks (corresponding to the $v_3$ PO$_4^{3-}$ and $v_1$ CO$_3^{2-}$ stretching bands of carbonate and the $v_1$ P-O stretching band of hydroxylapatite, respectively), within the fully mineralized insertion fibrocartilage increased with age ($p<0.001$). B) The atomic order in the apatite crystallites, as gauged by the peak width of $v_1$ P-O stretch at $960\Delta cm^{-1}$ (the more narrow the peak, the more crystalline), did not change over time outside of measurement error. For A) and B), the average was taken over the 3 regions of interest from each traverse within the fully mineralized fibrocartilage close to the bone side of the insertion. The data plotted represent the mean of 5 separate measurements (i.e., from 5 mice) and the error bars represent the standard
deviation of the mean. Significant differences (p < 0.05) between time points are indicated above each bar in A).

2.3.3 Nanoscale characterization of the mineralized interface

In order to further investigate the gradient in relative mineral concentration determined by Raman spectroscopy (Figure 2.1), the nanostructure of the interface was characterized by TEM. Bright-field TEM images (Figure 2.6) exhibit regions of dark image contrast. Similar regions in a P56 specimen were found to be rich in calcium, oxygen and phosphorus (i.e. mineral) by STEM-EELS (Figure 2.7), and this likely holds true for the other time points examined. In bright-field TEM images of the mineralized interface, clusters of mineral (i.e., regions of dark contrast) were observed that increased in size and density along the insertion (Figure 2.6). Before P14, the matrix appeared disorganized. It contained roughly oval, dendrite-like clusters of mineral approximately 1 μm in diameter, which increased in density across the mineralized interface from tendon to bone. At P14, similar large clusters of mineral were observed on the humeral head side of the interface, but some appear to be patterned by the increasingly ordered larger collagen fibrils. By P28, the mineralization morphology appeared to be driven by the structure of larger, aligned collagen fibrils at the edge of the interface. Furthermore, elemental mapping indicated that in regions near the interface, mineral was confined to discrete patches associated with the collagen fibrils. There was no evidence of homogenously dispersed mineral crystals or mineral clusters in collagen-rich regions.
Figure 2.6: TEM images of the mineralized interface in the supraspinatus tendon enthesis. Bright-field images for various postnatal development times: A) P10, B) P14, C) P28, D) P56 (scale bar = 2μm). Dark areas indicate mineral (e.g., see Figure 2.7). The white circle in D) shows the approximate probe diameter of the laser used for Raman spectroscopy in this study.
**Figure 2.7:** Mineralized interface observed at the supraspinatus tendon-to-bone insertion 56 days after birth. The interface was not smooth and planar but rather exhibited significant “surface roughness” at the length scale of tens of nanometers. 

A) STEM- high angle annular dark field (HAADF) image. Brighter STEM-HAADF image intensity in regions of uniform thickness corresponds to higher mean atomic number (i.e., locations of mineral relative to collagen). B) STEM-electron energy loss spectroscopy (EELS) spectral image map of C) (in atomic (at) %
shown by green intensity) as well as summed Ca, O, and P (shown in at% shown by red intensity). Brighter color indicates higher concentrations. STEM-EELS mapped elemental composition (in at%) for C) carbon, D) calcium, E) oxygen, and F) phosphorus. While all tissues contain calcium, oxygen, and phosphorus to varying amounts, the major sources of these signals were spatially well correlated to one another as well as correlated to the STEM-HAADF intensity and are indicative of apatite. The gray-scale ranges for the STEM-EELS maps were not normalized between 0 at% and 100 at% but rather were independently adjusted to enhance visual clarity (consequently they are not directly comparable).

2.4 Discussion

This study investigated the development of the mineralized interface at the tendon-to-bone insertion. The aim of this study was to understand the developmental origin of the mineral gradient identified at the mature tendon-to-bone interface. This region is believed to play a critical role in the dissipation of stresses at the tendon-to-bone interface, thereby preventing ruptures at the interface (Genin, Kent et al. 2009; Liu, Thomopoulos et al. 2012). Previous analyses of this region have indicated an increase in mineral content near the insertion as the humeral head mineralizes during postnatal development (Wang, Mitroo et al. 2006; Thomopoulos, Kim et al. 2007). Microcomputed tomographic measurements in the current study similarly showed mineral density increased throughout postnatal development. At a higher spatial resolution, Raman microprobe analysis revealed the presence of a mineral gradient near the insertion early in postnatal development—a gradient that persisted through P56. Raman spectroscopic analysis also showed a relatively constant mineral-to-collagen ratio among the
fully mineralized regions at all time points studied. Further investigation of the mineralized interface by TEM at nanoscale resolution demonstrated a high density of mineral clusters that were discontinuous and exhibited a finger-like morphology that protruded perpendicular to the region of continuous mineralization. Collagen fibrils appeared to extend continuously from the tendon region, through the discontinuous mineralized front, into the heavily mineralized region.

We identified the gradient region using Raman analysis and compared its location to histological features in order to characterize the formation of this interface. Fibrocartilage tissue, a critical component of the transitional tissue between tendon and bone, is not histologically evident at the insertion until 3-4 weeks after birth (Galatz, Rothermich et al. 2007). In contrast to this timeframe, a gradient in mineral content appears near the insertion as early as P7. The current study further indicated that, instead of co-developing with fibrocartilage, the mineral gradient was intrinsic to mineralization fronts associated with endochondral ossification. This association is consistent with the observation that the mature enthesis is similar to the developing growth plate and has characteristics of an arrested growth front (Benjamin, Kumai et al. 2002; Blitz, Viukov et al. 2009). In the humeral head, the secondary ossification center drives mineralization of the cartilaginous bone template over the first two weeks of postnatal development. The mineralization front gradually expands outward as chondrocytes of the cartilage template are terminally differentiated and the matrix mineralizes (Mackie, Ahmed et al. 2008). Our results indicate that the mineralization front is adjacent to the developing tendon by P14. Prior to this time, the tendon inserts into the epiphyseal cartilage of the humeral head, which is not yet mineralized. Surprisingly, even though the size of the humeral head increases, the slope of the mineral gradient does not change significantly between P7 and P56 (as illustrated by the slope of the Raman data in Fig. 3A, which is plotted in Fig. 3B). This suggests that the
mineral gradient is a consistent feature of the mineralizing growth front in mice, agreeing with the interpretation that it arises from an intrinsic tens-of-nanometer scale surface roughness of the interface. Gradients in mineralization levels have also been observed across remodeling osteons, periosteal growth fronts, and cartilage-bone interfaces in several different animal models (Gupta, Schratte et al. 2005; Gourion-Arsiquaud, Burket et al. 2009; Donnelly, Boskey et al. 2010; Burket, Gourion-Arsiquaud et al. 2011). Our previous observations in rats indicated a wider gradient (Wopenka, Kent et al. 2008). Further analyses are necessary to explore how the gradient region may scale in larger organisms. Developmentally, it is probable that enthesis mineralization also coincides with mineralization of the proximal epiphysis in larger organisms.

In the present study, throughout the time course investigated, the mineral gradient was maintained as the organic matrix was remodeled from epiphyseal cartilage with disorganized type II collagen fibers to fibrocartilage with more aligned and increased amounts of type I collagen (Galatz, Rothermich et al. 2007). The zone of gradation shifted radially over time from the center of the humeral head in the early post-natal period to the perimeter of the humeral head by P14 (Fig. 2). The ~25 μm width of the gradient region in the mouse supraspinatus enthesis is similar to the diameter of the large hypertrophic chondrocytes present early in development (Farnum, Tinsley et al. 2008). At the early developmental time points P7 and P10, the gradient region was often located between cells (but not always radially outward from cells), resulting in mineral on one side of the cell only (Figure 2.1B). This observation indicates two possible cell-mediated mineralization mechanisms to produce a graded interface: 1) polarized cells located directly at the interface control mineralization, so that mineralization can occur on one side of the cell but not the other, or 2) matrix mineralization is driven by cells located away from the interface, and the matrix composition itself determines where and how much mineral is
deposited. The latter could occur through control of mass transport of Ca and the concentration of mineralization inhibitors and nucleators. An important question is how this process extends to larger organisms. It is unknown how the mineral gradient scales with organism size. Wopenka et al. found a slightly wider gradient region (~100μm) in rats [10] compared to 25μm in this study, but it is unknown how and if these results scale for larger organisms. We hope to pursue this issue in further work. Hypertrophic cell size varies with the longitudinal growth rate; a function of anatomic location and species during development (Breur, VanEnkevort et al. 1991), but cell size in mature fibrocartilage is approximately constant across species. The latter observation implies that if the gradient width also scales with size, the gradient zone may be wider than one cell diameter in larger organisms. The relationship between the dimensions of the gradient region and cell size also has important implications for the local stress environment within the joint, as discussed below.

The maximum relative mineral concentration of the mineralized tissue adjacent to the gradient region (measured by Raman at a volumetric scale of several μm$^3$ at three spots within the fully mineralized enthesis fibrocartilage) did not vary throughout postnatal development. This result is consistent with other reports from murine calvaria and cortical bone (Tarnowski, Ignelzi et al. 2002; Gamsjaeger, Masic et al. 2010). Bone mineral density of the mineralized tissue at the insertion (measured at a volumetric scale of ~0.1 mm$^3$ by microcomputed tomography), however, increased with age. The area fraction of extracellular matrix relative to cell area (measured at an aerial scale of ~ 1 μm$^2$ by histomorphometry) increased with time. At early time points, the humeral head tissue consisted of densely packed large chondrocytes separated by thin regions of highly mineralized matrix. These features are not resolvable at the resolution of the micro-computed tomography measurements. As the insertion matured, the cell
area fraction decreased relative to the amount of mineralized matrix present, resulting in a higher bulk mineral density. Apparent increases in mineral mass are likely due to structural changes (i.e., increased matrix to cell ratio) or changes in the total amount of mineralized matrix present rather than increases in the mineral-to-collagen ratio within the extracellular matrix.

The carbonate content of the apatite crystals that make up the mineralized component increased with animal age. This finding is consistent with other reports (Tarnowski, Ignelzi et al. 2002; Miller, Little et al. 2007; Gamsjaeger, Masic et al. 2010; Burket, Gourion-Arsiquaud et al. 2011), but we did not observe a statistically significant corresponding change in mineral crystallinity. These data suggest that, although the gradient in relative mineral-to-collagen concentration is held approximately constant, the composition of the mineral phase changes throughout development as the animal approaches skeletal maturity. One possible explanation for the change in composition is that, during development, mineral is first deposited on a cartilaginous template, then it is subsequently resorbed and remodeled into mature bone or mineralized fibrocartilage (McCreadie, Morris et al. 2006). The observed variations in composition and structure of the mineral phase may play an important role in shaping the evolving mechanical environment at the enthesis.

Nano/micro-characterization by TEM revealed that the morphology of mineralization at the tendon enthesis varied throughout development; although a gradual increase in the mineral-to-collagen content was observed from tendon to bone, consistent with the Raman spectroscopic observations, there were also spatial and temporal variations. The organization of the organic component of the matrix progressed throughout development from a more disorganized matrix characteristic of cartilage (Keene and Tufa 2010; Amizuka, Hasegawa et al. 2012) to a more ordered matrix with well-aligned fibrils. During development of the enthesis, the morphology of
the mineralization front changed from large (~1 μm) dendritic patches of mineral within the cartilage-like matrix (Fig. 6A, P10) through an intermediate stage with some dendritic-like mineralized patches and also patches with a morphology that appeared to match the sparsely organized collagen fiber patterns (Fig. 2.6B, P14) to a morphology in which mineral appeared to be controlled by well-aligned collagen fibrils (Fig. 2.6D, P56). Furthermore, significant surface roughness at the tendon-to-bone interface was observed at the nanometer-scale (Figure 2.7) and this surface roughness could account for the micrometer-scale gradient in mineral to collagen content observed by Raman spectroscopy. Our multi-scale imaging results indicate that markedly different nanometer-scale morphologies may appear as similar mineralization patterns when observed at the micrometer-scale. These results highlight the importance of studying this system across multiple length scales.

The unique morphology and mineral distribution at multiple hierarchical levels have important implications for the mechanics of the tendon-to-bone attachment. A gradient in mineral content at the interface leads to a more gradual increase in tissue stiffness (Thomopoulos, Williams et al. 2003; Hauch, Oyen et al. 2009; Donnelly, Chen et al. 2010; Burket, Gourion-Arsiquaud et al. 2011), ultimately modulating the strains experienced by cells encapsulated in the matrix. Mechanical cues are critical to the modulation of skeletal patterns and the advancement of growth fronts (Villemure and Stokes 2009; Nowlan, Sharpe et al. 2010). The morphology of the tendon-to-bone interface during early postnatal development is very different from that in the mature insertion, as illustrated in Figure 2.6; however, a mineral gradient is present in both cases. It is possible that the mineral gradient provides different mechanical functions over the time course of murine development. Early in development, the high cell-to-matrix ratio coupled with localized high levels of mineral may lead to enhanced
local stresses around the compliant chondrocytes. At this early phase of development, these stresses may serve a mechanotransduction role for the initiation of mineralization. In contrast, growth and maturation produce a decrease in cell-to-matrix ratio, in which the mineral gradient may serve to minimize micrometer-scale stress concentrations at the interface. This concept will be explored in the next chapter.

2.5 Conclusion

To summarize, we observed a gradient in mineral-to-matrix ratio across 20 μm at the mineralization front nearest to the supraspinatus tendon insertion throughout postnatal development. Although mice become much larger over the course of development, the size of the region over which this gradual increase occurred was constant from P7 onwards. This constancy in the width of the gradient region occurred despite temporal variations in matrix structure (evaluated by TEM), cell area fraction relative to matrix (evaluated by optical microscopy), and composition of the mineral crystallites (evaluated by Raman spectroscopy). The mineral concentration of fully mineralized collagen was shown to be constant throughout development. The tissue-level volume fraction of mineral increased throughout development, likely due to a reduction in the volume fraction of cells rather than an increase in mineral density of the extracellular matrix. We believe that these conserved and time-varying aspects of the enthesis have important mechanical implications for the way that the tendon-to-bone attachment grows and maintains stability throughout development.

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Chapter 3 - Stress Amplification During Development of the Tendon-to-Bone Attachment

This chapter is reproduced with permission from Liu, Y.X, Schwartz, A.G., Birman, V., Thomopoulos, S., and Genin, G.M. Stress amplification during development of the tendon-to-bone attachment. Biomech Model Mechanobiol. 2013 Dec 27. PMID: 24370852 (Liu, Schwartz et al. 2013). Y.X. Liu carried out the modeling experiments and A.G. Schwartz provided the morphological data and muscle force estimates and helped design the model.

Summary

Mechanical stress is necessary to sustain the mineral content of bone in adults. However, in a developing neonatal mouse, the mineralization of soft tissues progresses despite greatly reduced average mechanical stresses. In adults, these reduced loads would likely lead to bone loss. Although biochemical factors may partly explain these different responses, it is unclear how mineralization is initiated in low load environments. This chapter presents morphometric data based on the animal model presented in the previous chapter and initial modeling supporting a hypothesis that structural and compositional factors across several length scales amplify stresses in the developing enthesis. We suggest that these stresses are of a level adequate to contribute to mechanical signaling for initiation of mineralization at the developing tendon enthesis. As noted in the previous chapter, a mineral gradient is evident across the insertion from the onset of mineralization. This grading maintains a constant size from early postnatal time points to adulthood. At the tissue level, this grading contributes to reduced stresses in an adult animal and
to a minor elevation of stresses in a neonatal animal. At the cellular level, stress concentrations around mineralizing chondrocytes are enhanced in neonatal animals compared with adult animals. The enhancement of stresses around cells at the mineralization front at early time points may serve to amplify and transduce low loads in order to initiate mineralization.

3.1 Introduction

This chapter examines mechanical loading of the developing enthesis structure over time, offering an understanding of mechanical factors that may be important for the development, homeostasis, and healing of a functional enthesis. Results from the current study may also offer guidance for tissue-engineered repair of tendon/ligament-to-bone enthesis injuries (Smith, Xia et al. 2012).

Mechanical loading affects the structure and function of mature tissues via well-understood cell-mediated responses that are reviewed elsewhere (Thomopoulos, Das et al. 2011) and described in upcoming chapters. Skeletally mature bone adapts both its mass and its architecture to external mechanical loads (Tanck, Hannink et al. 2006). Bone density increases with increasing mechanical loading due to physical activity (Bailey, McKay et al. 1999) and decreases with reduced mechanical loading under microgravity conditions (Collet, Uebelhart et al. 1997). Likewise, tendon strength is reduced when the muscle is immobilized (Amiel, Woo et al. 1982; Woo, Gomez et al. 1982). In the healing process, a low level of controlled force is more beneficial than the complete removal of a mechanical load in the healing of the rotator cuff (Galatz, Charlton et al. 2009). Decreasing fetal movement and/or muscle loading leads to dramatic defects in skeletal development (Nowlan, Sharpe et al. 2010). A reduced load impairs
the development of the tendon enthesis, reducing its modulus, strength, and mineral content (Thomopoulos, Genin et al. 2010). The responsiveness of mature, healing, and developing bones, tendons, and their attachments to the mechanical environment suggests that mechanical factors play an important role for the formation and maintenance of these tissues.

How, then, does mineralized tissue form in a developing animal, especially at early postnatal time points, when stresses applied by muscles are very low? The previous chapter characterized the development of a mineralized enthesis in a mouse model (Schwartz, Pasteris et al. 2012). Many of the expected elements were observed. The enthesis developed following a course similar to what has been described by others (Benjamin, Toumi et al. 2006; Blitz, Viukov et al. 2009). Endochondral ossification at growth plates followed a well-described sequence: reserve chondrocytes proliferated and then became hypertrophic, altering the extracellular matrix and facilitating mineralization and were eventually replaced by osteoblasts and a fully mineralized collagen matrix. At the developing rotator cuff insertions into the humeral head, we observed the expected growth of bone as the animals aged.

However, we also observed a surprising result: a mineral gradient was seen at the mineralization front from the onset of mineralization at early postnatal time points through adulthood. The size of this graded region was nearly constant at all time points. In the mature enthesis, this graded region serves to reduce stress concentration and improve load transfer (Genin, Kent et al. 2009; Liu, Thomopoulos et al. 2012). What role does this gradient play at early time points when muscle loads are relatively low? We modeled stresses in the developing enthesis to gain insight into these questions. We hypothesized that the graded region serves a different role at earlier time points, namely that it combines with other morphological changes to instead elevate local stresses at early time points. We argue that, in addition to the effects of the
many soluble factors present during development, these stress concentrations can serve to elevate stresses in the matrix surrounding cells and thereby contribute to the initiation of mineral accumulation.

In this chapter, we first describe the experimental methods used to characterize the morphology of the rotator cuff tendon enthesis in a mouse model at time points throughout postnatal development, from early postnatal (as early as 7 days), to adult (56 days). We then present the morphologies observed throughout development and first-order models to explore the mechanical consequences of these morphologies. We conclude by discussing the potential role of these elevated stresses in the transduction of mineralization cues.

3.2 Methods

3.2.1 Animal model, histology, and morphometry

The use of animals for this study was approved by the animal studies committee at Washington University. Fifteen CD1 mice (Charles River Labs) were used in this study. Mice were killed in a CO₂ chamber at 7, 10, 14, 28, and 56 days after birth (denoted as P7, P10, P14, P28, and P56, respectively; N = 3 per time point). The humerus and supraspinatus tendon with muscle attached were dissected free of all other tissues.

3.2.1.1 Micro-computed tomography and muscle forces

The isolated humerus and supraspinatus muscle complexes were fixed in 4% paraformaldehyde and dehydrated to 70% ethanol. Two dimensional sagittal plane images were acquired using a micro-computed tomography scanner at tube settings of 55kV and 145µA with
a 25 mm diameter tube resulting in an ∼20 μm isometric resolution (μCT 40; Scanco Medical, Basserdorf, Switzerland). A soft tissue threshold was used throughout to distinguish unmineralized and mineralized tissue from background due to the low level of mineralization found in the humeral head of the 7–14 day time points. Humeral head volume, supraspinatus muscle volume, and supraspinatus muscle length were calculated as previously described (Thomopoulos, Kim et al. 2007). A cross-sectional area of the tendon was determined by averaging the area of a 0.8 mm² region of interest centered about the minimum area of the tendon.

The method of Gokhin et al. (Gokhin, Ward et al. 2008) was used to approximate the isometric tension generated by the supraspinatus muscle at each time point. In that study, the muscle fiber cross-sectional area and the isometric stress generated by the murine tibialis anterior at various postnatal time points were measured. Isometric tension was calculated by multiplying isometric stress by the physiological cross-sectional area of the muscle (PCSA). Consistent with Gokhin et al. (Gokhin, Ward et al. 2008), we used an adjusted PCSA that accounts for muscle fiber packing:

\[
\text{PCSA} = \left( \frac{V \cos \theta}{L_f} \right) X_{\text{csa}}
\]

\( V \) is the muscle volume determined from μCT; \( \theta \) is the fiber pennation angle (11.7°) (Burkholder et al. 1994); \( L_f \) is the muscle fiber length, calculated as 0.6 times the μCT measured muscle length; and \( X_{\text{csa}} \) represents the age-appropriate value of the cross-sectional area fraction of contractile material, as estimated by Gokhin et al. (Gokhin, Ward et al. 2008).
3.2.1.2 Histomorphometry

The shoulder specimens used for \( \mu \)CT were rehydrated and decalcified using 14% EDTA for two weeks. Samples were then dehydrated, embedded in paraffin, and sectioned to 5 \( \mu \)m in the coronal plane. Tissue sections were stained with toluidine blue to estimate extracellular matrix area fraction. The humeral head diameter was determined by calibrating ImageJ to a scale bar to measure the widest diameter of the humeral head on the section. Tendon length was measured using ImageJ from the insertion to the beginning of muscle (i.e., where muscle fibers were identified).

The volume fraction ratio of cells and matrix was a central morphometric parameter needed both to evaluate the cell-level stress concentrations and the homogenized moduli for the gradient and unmineralized regions. To calculate the approximate matrix area fraction, we analyzed images using ImageJ software. A rectangle (\( \sim 0.01\text{mm}^2 \)) was drawn over the matrix region of interest. Cells were identified by manual inspection, and regions of nuclear staining within cells were excluded to enable separation of cells from an extracellular matrix by an intensity threshold. The area fractions of cells was then used to estimate the volume fraction of cells using Delesse’s principle, which states that the mean value of the area fraction estimated from a planar section is equal to the volume fraction in three dimensions (Delesse 1847). However, the histology sample slices have finite thickness and, because the projected areas of cells are different at different planes, inaccuracies will be introduced when estimating area fraction. We used the largest of the cross-sectional areas in each toluidine blue stained histology slice, because there we could see the boundary between the brighter, more transparent cells and the dark purple matrix. With this approach, the true area fraction was likely slightly
overestimated at each slice. Therefore, a correction factor \( k \) was introduced in the area fraction by the following formula (Chayes 1956):

\[
k = \frac{4R}{4R + 3t},
\]

where \( R \) is the average radius of cells and \( t = 5 \, \mu m \) is the thickness of the histology sections. We used the average cell diameter observed from 2D sections to represent the 3D size of the spherical cell. The true mean radius of a sphere, by taking all the transection planes, is \( \pi R^2/4 \), and furthermore, the tendency of the largest area in a slice observed by using toluidine blue staining method decreased the discrepancy of the 2D observed size of the cells and the real cell size.

The details for calculating the slope of the graded mineralized region and the distance from the tendon to the start of mineralization were described in the previous chapter (Schwartz, Pasteris et al. 2012). Briefly, the length of the gradient region was calculated by dividing the gradient slope by the average mineral content of the bone underlying the insertion and the data are listed here for reference. The cortical bone outer radius was calculated by subtracting the distance from the tendon to the start of mineralization and length of the gradient from the measured humeral head radius.

### 3.2.2 Modeling

#### 3.2.2.1 Overview of multiscale modeling

We applied our cross-scale morphological data of the developmental enthesis to model how the stress environment in the vicinity of the cell-matrix interface changes over time in the
mineralized region. At the tissue level, an idealized representation of was studied to estimate the amplification of stresses at points proximal to the bone relative to the stresses in the tendons. At the cellular level, a periodic unit cell model was used to estimate local stress concentrations in the direct vicinity of chondrocytes in the mineralized region. The overall amplification of stress near cells was obtained by combining estimates of the age-appropriate muscle stresses with the estimated concentrations of stress from both tissue-level and cell-level effects.

### 3.2.2.2 Tissue-level stress concentrations

The enthesis is a hierarchical structure and is believed to present toughening mechanisms over several of these hierarchies Liu et al. (Liu, Birman et al. 2011). The first level studied was the enthesis as a whole (tissue level). Throughout development, four regions present: (1) the tendon, (2) the unmineralized fibrocartilage region, (3) the graded mineralized fibrocartilage region, and (4) the bone, with the widths of the unmineralized and mineralized regions in the enthesis much smaller than those of the tendon and the humeral head. The graded region maintains a constant size throughout development (Schwartz, Pasteris et al. 2012). To determine what effect this gradient has on the tissue-level scale stress field near the mineralization front, we studied a simplified one dimensional axisymmetric concentric ring model (Figure 3.1).

This representation approximates the structure of the entheses of the rotator cuff of the humeral head when viewed in a sagittal plane. The rotator cuff tendons insert radially into the humeral head and form a nearly complete ring around the approximately spherical humeral head (Liu, Thomopoulos et al. 2012). The concentric ring model was loaded by uniform radial tensile stress to estimate the tissue-level stress concentrations in the structure. The magnitude of this stress was estimated based upon the above measurements and upon the assumption that peak
muscle stress was approximately independent of the overall stiffness of the insertion over the age range studied. Further details of the stress analysis are omitted here and may be found in the Appendix of Liu et al. (Liu, Schwartz et al. 2013).

The core of the concentric ring model representing cortical bone was modeled with linear elastic, isotropic material properties $E_{\text{bone}} = 20\text{GPa}$ and $\nu_{\text{bone}} = 0.3$ (Kaplan, Hayes et al. 1994). The outermost ring, representing tendon, was modeled as transversely isotropic, with longitudinal modulus $E_1 \approx 460\text{MPa}$, transverse modulus $E_2 \approx 2.4\text{MPa}$, Poisson’s ratio $\nu_{12} \approx 1$, Poisson’s ratio in the transverse plane $\nu_{23} = 0.3$, and $G_{12} \approx 7.1 \text{MPa}$ (Maganaris and Paul 1999; Weiss, Gardiner et al. 2002; Lynch, Johannessen et al. 2003; Stabile, Pfaeffle et al. 2004; Yin and Elliott 2004). The shear modulus was estimated from $G_{12} = 3\beta E_1 (R/L)^2$, where $\beta = 1.07$ and the aspect ratio of collagen fibers $(R/L) = 1 : 20$ (Genin, Kent et al. 2009). Longitudinal and transverse moduli for the gradient and unmineralized regions at the enthesis were estimated by solving a boundary value problem involving the axisymmetric unit cell model as described below.

3.2.2.3 Cell-level stress concentrations

We studied an axisymmetric unit cell model of a chondrocyte at the tendon enthesis to estimate how age-related changes to cell size and shape affected the stress field near these cells. We additionally applied these models to estimate homogenized mechanical moduli of tissue in the graded region. As described below, our observations at all time points supported a model of the cells as spherical. At early time points, we observed dense hypertrophic chondrocytes with a roughly spherical shape at the mineralized region of the enthesis. Here, the average radius of
hypertrophic chondrocytes was comparable with the length of the gradient region. Over the course of development, the enthesis cells became dramatically smaller.

In the axisymmetric unit cell model, the center of the spherical cell was coincident with the center of a cylinder, with the height of the cylinder two times the radius of the cylinder (schematic in Figure 3.1). The radius was determined from experimental data. Symmetrical boundary conditions were applied at the bottom surface of the cylinder, with zero shear traction on the lower surface and displacement prohibited along the height of the cylinder ($z$-direction). The top surface was loaded by a uniform displacement in the $z$-direction, which yielded a uniform mean strain $\varepsilon_z = 0.01$, and was traction-free in the azimuthal and radial directions. The circumferential surface of the cylinder was constrained so that it had uniform displacement in the radial direction and was traction-free in the azimuthal and $z$ directions. The applied stress $\sigma_z$ was calculated as the total reaction force in the $z$ direction at the top surface divided by the cross-sectional area of the cylinder, and the stress concentration factor was defined as the ratio of the maximum first principal stress to $\sigma$. 
Figure 3.1: Schematic of the modeling approach to estimate the stress environment of developing tendon enthesis at the rotator cuff of a mouse. The rotator cuff to humeral head insertion is modeled as concentric rings. Because the size of the enthesis (mineralized region and unmineralized region) is much smaller than that of the tendon and bone, and the size of the cells are comparable with the length of gradient region, an axisymmetric unit cell model (a spherical cell in the center of cylindrical extracellular matrix) was used to estimate the stress concentration at cell and matrix interface.

Constitutive models

Each unit cell spanned the region between the bone and the mineralization front (the mineralized region) and the region between the mineralization front and the tendon (the unmineralized region) at the enthesis and contained a single chondrocyte. Following a review of the literature, we assumed the chondrocyte to be isotropic with Young’s modulus $E_{\text{cell}} = 350$ kPa and Poisson’s ratio $\nu_{\text{cell}} = 0.43$ (Jones, Ting-Beall et al. 1999; Alexopoulos, Williams et al. 2005; Kim, Guilak et al. 2010). In the unmineralized region, the extracellular matrix was assigned the same material properties as a tendon.

The mineralized region was graded, with the accumulation of mineral beginning at the mineralization front and increasing until bone was reached at the base of the unit cell. In the mineralized region, the extracellular matrix properties were thus varied continuously, from no mineral at the mineralization front to fully mineralized at the bone side. The volume fraction of mineral increased linearly from the mineralization front to the bone. However, the material properties of partially mineralized collagen do not vary linearly with mineral volume fraction. In our recent study, we developed models predicting the material properties of mineralized collagen.
tissue with a variation of volume fraction, based on the nanoscopic details of the accumulation of mineral on collagen fibrils (Alexander, Daulton et al. 2012; Liu, Thomopoulos et al. 2014) using linear, multiphase homogenization theory, c.f. (Genin, Kent et al. 2009). Here, we followed a model from our earlier work, in which mineral first deposited within the gap channels of the periodic collagen fibrils structure and then accumulated randomly in the extra-fibrillar regions (Liu, Thomopoulos et al. 2014). The results for moduli were derived in our earlier work from the Monte Carlo finite element simulation and interpolated with a cubic spline function (see (Liu, Schwartz et al. 2013)). The extracellular matrix properties were assumed to be transversely isotropic, with the longitudinal direction in parallel with the \( z \)-direction of the unit cell model. At earlier time points, the collagen fibers in the enthesis as a whole are more disorganized than a mature structure (the results of measurement of collagen fiber orientation throughout development are not shown). However, local organization over a length scale associated with our finite element model is not known. As a first-order approximation, we ignored those effects.

Interpretation of cell-level data to estimate constitutive properties for the tissue-level model

The homogenized longitudinal modulus was obtained from unit cell models as the calculated applied stress \( \sigma_z \) divided by the nominal strain \( \varepsilon_z \) resulting from displacement boundary conditions applied to the unit cell. To obtain the homogenized transverse modulus, the circumferential surface was loaded by a uniform displacement in the \( r \)-direction, which yielded a uniform nominal radial strain \( \varepsilon_r = 0.01 \), while the model was traction free in the azimuthal and \( z \)-direction. The top and bottom surfaces of the cylinder were constrained to have uniform displacement in the \( z \)-direction and were traction-free in the azimuthal and radial directions. The applied stress \( \sigma_r \) was calculated as the total reaction force in the \( r \) direction at the circumferential
surface divided by the circumferential surface area of the cylinder. $\nu_{r\theta} = 0.3$ was assumed for the unit cell structure and the effective transverse modulus was equal to $\sigma_r (1 - \nu_{r\theta}) / r$ because of the Poisson effect in the transverse plane. Poisson’s ratio $\nu_{zr}$ or $\nu_{z\theta}$ was assumed to be 0.1, which satisfied positive definite constraint of the stiffness tensor. The homogenized moduli depended on the volume fraction of cells. The results are shown in the Appendix of Liu et. al. (Liu, Schwartz et al. 2013).

*Finite element models*

Finite element analysis software COMSOL (COMSOL, Inc) was used to estimate cell-level stresses at the cell and extracellular matrix interface. The linear elastic axisymmetric model was described above. A fine mesh with triangular elements was used, and elements were further divided at the equator of the cell-matrix interface characterized by stress concentration. Adequate convergence was achieved by using approximately 75,000 degrees of freedom.

**3.2.2.4 Development of cell-level stresses in the mineralized region**

Combining tissue-level (concentric ring model) and cell level (axisymmetric unit cell model) stresses with the applied muscle stress yields an estimate of the stress level around cells in the gradient region. The variance of the volume fraction of cells at the gradient and the unmineralized regions introduced bounds on the total stress concentration effects at different time points. Another uncertainty came from estimates of the peak muscle stress. The combined cell-level stress value at the cell-matrix interface in the mineralized region at different time points was calculated and normalized to the average value at 56 days.
3.3 Results and discussion

3.3.1 Over the course of postnatal development the humeral head grew and cells shrank but the mineral gradient stayed the same size

Table 3.1 shows the length of supraspinatus tendon, the distance from the mineralization front to the tendon, the length of the gradient region in the enthesis, the cortical bone outer radius, and the humeral head diameters (the summation of the later three) at P7, P10, P14, P28 and P56, as measured from histology sections. The data show that, during development, both the tendon and the humeral head grew, the size of the gradient region remained constant (with an average length ~20μm), the unmineralized region shrank, and the size of the enthesis was small relative to that of tendon and bone (schematic, Figure 3.2).

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>SS length (mm)</th>
<th>Humeral head diameter (mm)</th>
<th>Distance from SS to mineralization front (μm)</th>
<th>Mineral gradient length (μm)</th>
<th>Cortical bone outer radius (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>1.07 ± 0.25</td>
<td>1.73 ± 0.15</td>
<td>96.2 ± 11.8</td>
<td>23.8 ± 7.20</td>
<td>836</td>
</tr>
<tr>
<td>10</td>
<td>1.21 ± 0.04</td>
<td>2.04 ± 0.05</td>
<td>65.8 ± 13.6</td>
<td>18.6 ± 5.96</td>
<td>1,000</td>
</tr>
<tr>
<td>14</td>
<td>1.39 ± 0.16</td>
<td>2.11 ± 0.05</td>
<td>22.9 ± 4.4</td>
<td>22.6 ± 10.7</td>
<td>1,100</td>
</tr>
<tr>
<td>28</td>
<td>1.44 ± 0.29</td>
<td>2.27 ± 0.15</td>
<td>10.4 ± 3.4</td>
<td>25.9 ± 11.2</td>
<td>1,240</td>
</tr>
<tr>
<td>56</td>
<td>1.34 ± 0.23</td>
<td>2.27 ± 0.14</td>
<td>11.1 ± 5.1</td>
<td>19.1 ± 9.98</td>
<td>1,210</td>
</tr>
</tbody>
</table>
Table 3.1: Morphometric data for the surraspinatus tendon, enthesis, and humeral head during postnatal development. ± indicates standard deviation (N = 3). The cortical bone outer radius was calculated by subtracting the “distance from the tendon to the start of mineralization” and “gradient length” from the humeral radius, mean values are used, no standard deviation SS: supraspinatus tendon, HH: humeral head.

Figure 3.2: Schematic of the developing tendon enthesis at the rotator cuff of a mouse. The graded mineralized region maintained a relatively constant thickness over time, while the outer radius of the cortical bone (humeral head) and tendon length grew. The unmineralized “fibrocartilage” disappeared with age. Rings are drawn to scale.

The volume fraction ratio of cells and matrix was a central morphometric parameter needed both to evaluate the cell-level stress concentrations and the homogenized moduli for the
gradient and unmineralized regions. Representative histology sections at P7, P10, P14, and P28 (Figure 3.3) show that the fraction of cells relative to extracellular matrix decreased throughout development (enthesis structure at P56 (not shown) was similar to that at P28). From the histology sections, the area fraction of cells was estimated (Figures 3.4A, 3.4B). The area fraction was then used to estimate the volume fraction of cells using Delesse’s principle. The cells shrank over time (Figure 3.4C).

Figure 3.3: Histologic tissue sections of the tendon enthesis at the rotator cuff of a mouse, showing a steady decline in the size and volume fraction of chondrocytes with age. Time points: A) P7, B) P10, C) P14, D) P28. The black/dark areas indicate mineral, the cell nuclei are stained
with dark blue, hypertrophic chondrocytes appear as white circles (see arrows in A), B), and C), and the purplish/pinkish areas indicate the presence of proteoglycans, which are characteristic of cartilage. Note that the cells remain spherical throughout and start to organize into columns at later time points. Although Raman spectroscopic analysis clearly indicates a gradient in mineral at all time points (Wopenka, Kent et al. 2008; Schwartz, Pasteris et al. 2012), the graded transition zone is not visible by von Kossa staining at any time point, as this staining yields an opaque band with even a small level of mineralization. (5 μm thick sections, von Kossa and Toluidine blue staining, scale bar = 100 μm, arrows: hypertrophic chondrocytes).

**Figure 3.4**: Development of the volume fraction of cells ($\phi_{cell}$) at A) mineralized fibrocartilage B) unmineralized fibrocartilage. The range of data indicates standard deviation ($N = 3$). C) Mean radius of cells as a function of age. Both the volume fraction and size of cells decrease with age. The fraction of cells decreases rapidly after 14 days in the mineralized region. The volume fraction is much larger in the mineralized region than in the unmineralized region at early development.

**3.3.2 Muscle stresses increase steadily from birth through adulthood**
The peak muscle stresses that were used as inputs to the stress concentration models were estimated as a function of age using micro-computed tomography data (Figure 3.5). As expected, muscle volume increased through development, and consequently, muscle stresses increased steadily as well. The stress ratio between P56 (maturity) and P7 was approximately 15.

![Graph showing muscle stress at tendon](image)

**Figure 3.5**: Estimated peak muscle stresses as a function of age. Peak muscle stresses increase with age, with the stress ratio between the P56 (maturity) and P7 (early postnatal) about 14.8. The range of data indicates standard deviation (N = 3).

### 3.3.3 Cell-level stress concentrations decrease with age

First-order models were used to estimate how observed morphologies affected the stress field in the vicinity of cells. Collagen is expected to have nonlinear viscoelastic behavior (Pryse, Nekouzadeh et al. 2003; Nekouzadeh, Pryse et al. 2007), and cell mechanics are expected to change with age (Qiu, Zhu et al. 2010), ECM stiffness (Marquez, Elson et al. 2010), and cell volume fraction (Marquez, Genin et al. 2005; Marquez, Genin et al. 2006). However, because
accurate, calibrated models are not available these effects could not be considered and simple linear theories were used.

Within this framework, the stress concentration factor depended on the volume fraction of cells, with a broad range demonstrated for the axisymmetric unit cell model (Figure 3.6). The cell volume fraction decreased from 60 to 10% in the mineralized region throughout development (Figure 3.4A); this resulted in a cell-level SCF decrease from 28 to 5.5 (Figure 3.6). The SCF and the corresponding first principal stress contour in the gradient region at different time points are shown in Figure 3.7. The cell-level SCF was much higher at early time points (P7 and P10) than at later time points, and the highest stress regions around the cell were larger at early time points.

![Figure 3.6](image)

**Figure 3.6:** As the volume fraction and the size of cells decrease over time, the SCF decreases at the cell-matrix interface. The SCF was determined numerically using finite element simulations; a schematic axisymmetric unit cell model with mesh is shown.
Figure 3.7: The stress concentration at the cell-matrix interface decreased as a function of age. The stress field remained qualitatively similar over time, as shown in the contour of peak principal stresses.

3.3.4 Tissue-level stress concentrations decrease slightly with age

The tissue-level SCF for the concentric ring model is shown in Figure 3.8. The constant gradient size and decreasing unmineralized region size over time (with the stiffness adjusted for the volume fraction of cells) led to a slight decrease in macroscopic stress at the tendon enthesis from P7 to P56.
Figure 3.8: The effect of the mineral gradient region on the macroscopic stress concentration at the tendon enthesis is shown as a function of age. The gradient region did not change over time and the stiffness was adjusted for the volume fraction of cells using the results of the finite element analyses shown in Figure 3.7.

3.3.5 Morphological factors combine to stress cells at adult levels during development

The peak principal stress at the equator of cells in the gradient region was estimated at each time point. This involved, first, estimating the combined stress concentration factor that accounts for amplification of stresses at both the cell- and tissue-level, and, second, scaling the applied muscle stresses by this combined stress concentration factor (Figure 3.9). The estimated peak principal stress did not change significantly from P10 onwards; only the difference between P7 and P56 was significant statistically, with the average value at P7 0.23 times of that at P56. At all time points, the peak principal stress was not different statistically from that at young adulthood (P28).
Figure 3.9: The peak principal stress surrounding cells during early development are elevated to near adult physiological levels through stress concentrations. The estimated peak principal stress did not change significantly from P10 onwards; only the difference between P7 and P56 was significant statistically, with the average value at P7 0.23 times of that at P56. At all time points, the peak principal stress was not different statistically from that at young adulthood (P28).

Why might these morphologically elevated stresses be important? Failure to provide the appropriate local stress stimuli to cells during early enthesis development leads to severe defects in enthesis mineralization, fibrocartilage formation, and collagen organization (Thomopoulos, Kim et al. 2007; Blitz, Viukov et al. 2009). Experimental results from the last chapter show that local mineral content in developing tissues is the same as in mature tissues, both at the mineralized region of the enthesis and at the bone, although the tissue-level volume fraction is lower because of the larger volume fraction of chondrocytes at early time points (Schwartz, Pasteris et al. 2012). Our work suggests that the local stresses at early time points are also elevated to near adult physiological levels, presumably to stimulate chondrocyte mineralization.
While the enthesis serves to reduce the tissue-level level of stress concentration caused by directly connecting two dissimilar materials (i.e., tendon to bone) in adulthood, its role might be different in development. The highly localized stress around cells may serve to amplify the low muscle loads present during early tendon enthesis development. One possible effect of the highly localized stress around the cells may be to stimulate the enlargement of the chondrocytes, inducing a phenotypic change into hypertrophic chondrocytes accompanied by extracellular mineralization (Coe, Summers et al. 1992; Hall 2005). Mechanical stress might play a role in the proliferative to hypertrophic transition (Stokes 2002; Stokes, Mente et al. 2002), and our observations are consistent with this. Although the load-bearing capability is low because of the immaturity of tissues in the early time points of development, removing all muscle load is clearly detrimental to development (Blitz, Viukov et al. 2009; Thomopoulos, Genin et al. 2010).

These results suggest that amplified local stresses around cells in the mineralized region are present during development and support our hypothesis that mechanical factors across several length scales amplify stresses. The contribution of this work is to show that even in early development, stresses around cells in the mineralized region can reach levels associated with early adulthood. We suggest that these stresses are of a level adequate to contribute to mechanical signaling during mineralization.

### 3.3.6 Other interpretations

The mechanical factors we describe are likely just one of many possible explanations for the ability of cells to mineralize tissue at early time points. Early in development, the signaling factors surrounding cells at the enthesis differ from factors present in adulthood. One possibility
is that all mineralization around hypertrophic chondrocytes is triggered by such soluble factors through a pathway that is completely independent of mechanical stresses, but this is not likely due to the observation that immobilization leads to development of a highly abnormal insertion of tendon to bone (Thomopoulos, Genin et al. 2010). This idea will be further explored in a subsequent chapter for one soluble factor important for mineralization, Indian hedgehog.

An important potential role of mechanical factors in signal transduction relates to the ways that gradients and associated inhomogeneities in the stress field distort cells, particularly at early time points. Studies conducted in vitro indicate a strong relationship between cell shape and mechano-sensitivity, and there are typically a number of molecular mechanisms beyond shape that drive this response (e.g., level of attachment to a substrate, stiffness of substrate, etc.) (Adams, Pallante et al. 1989). Although hypertrophic chondrocytes and cells within the gradient region of the enthesis do in fact retain a spherical geometry (Guilak, Ratcliffe et al. 1995), subtle, stress induced deformation of cell shape is an important factor to consider. However, we note that these changes might be small because the stress around these cells has a large hydrostatic component due to the azimuthal constraint associated with the spherical shape of the humeral head.

Another interesting mechanical factor is that the pericellular matrix surrounding chondrocytes in cartilage is highly inhomogeneous (Guilak, Ratcliffe et al. 1995). Although the dominant factor of mineralization was included in our first-order approximations to local stress concentrations around cells, potential second order contributions of the pericellular matrix are an important consideration for future work.
3.3.7 Caveats

Mechanical factors described in this work might play an important role during the development of the tendon-to-bone attachment. Note, however, that this work is preliminary, with idealized models: (1) the geometric models ignored the spacing and size of the cells in the fully mineralized region; (2) due to the lack of experimental data, collagen fiber angular distribution was ignored in estimating the stiffness of the extracellular matrix at the enthesis. Despite the idealized nature of the modeling, stress analysis results qualitatively show that local stresses around cells are elevated in the regions where mineralization occurs. The highly localized stress level at the cell appears at early postnatal time points, suggesting that mechanical cues may be important for the initial deposition and later accumulation of mineral. This is supported by experimental evidence demonstrating that muscle loads are necessary for mineralization at the developing tendon-to-bone insertion. Elevated local stress, then, may be crucial for early development and, likewise, for the early stages of postsurgical healing.

Although physiological muscle forces are typically cyclical in nature, the model assumed static muscle loads. Cells respond to both peak and time-averaged muscle loads in vivo. Morphological data and finite element analyses of developing chicks (Nekouzadeh et al. 2008) showed that high cycles of stress precede ossification of cartilage. Furthermore, analysis of biophysical stimuli in a “muscleless” mouse model showed that passive cyclical movements (e.g., via movements of the mother) can compensate for the lack of direct skeletal muscle loading (Nowlan et al. 2012). Future studies will therefore expand the current model to include physiologically relevant cyclical loads.

Many questions remain and warrant further study. At the enthesis, there is a large volume fraction of cells in the unmineralized region, although the fraction of cells is smaller than that in
the mineralized region. Furthermore, how is mineral retained away from these regions, after the front of mineralization has passed? At the nanoscale, where and how does mineral first form on/in the collagen fibrils? How is a gradient in the mineral content achieved from the mineralization front to the bone? It is clear that biochemical and genetic factors also play important roles in both enthesis development (Blitz, Viukov et al. 2009; Thomopoulos, Genin et al. 2010) and mineralization during development (Coe, Summers et al. 1992; Yagami, Suh et al. 1999; Liu, Lavine et al. 2007); these factors likely interact with the mechanical factors described in the current chapter to regulate enthesis development. In the following chapters, we will explore the link between the loading environment and the biological factors that modulate mineralization processes.

3.4 Conclusions

In a mouse, the approximately linear gradation in mineral content at the supraspinatus enthesis stays a constant size from early postnatal time points through adulthood, while the humeral head grows substantially. The morphology of the enthesis in the adult mouse is believed to serve the role of reducing stress concentrations, but the analyses presented here suggest a different role at early time points. Effects of the mineralization gradient at the tissue level may combine with effects of cell volume fraction at the cell level to compensate for extremely low muscle stresses present at earlier time points. This leads to an elevation of stresses at the cell-matrix interface in juveniles to levels similar to those estimated for adults. In the next chapter, we will consider enthesis development in the absence of muscle forces, highlighting the critical role that mechanical loading plays in enthesis development. These observations have important
implications for postsurgical physical therapy regiments for patients who receive engineered tissue scaffolds to guide enthesis redevelopment. Stress concentrations at the cell level, which are favorable in the early time points of enthesis development, may also be favorable for enthesis tissue engineering.

3.5 Acknowledgments

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Chapter 4 – Muscle Loading is Necessary for the Formation of a Functional Tendon Enthesis


Summary

As demonstrated in the previous chapter from a modeling perspective, muscle forces likely play an important role in stimulating mineralization at the developing enthesis. Eliminating muscle forces, e.g., through paralysis, has been shown in numerous studies to lead to bone and joint deformities. In the mouse, neonatal Botulinum toxin (BtxA)-induced paralysis resulted in defects in mineral accumulation and fibrocartilage formation at the tendon enthesis, presumably impairing the function of the tissue. The objectives of the current study was to investigate the functional consequences of muscle unloading using BtxA on the developing supraspinatus tendon enthesis and to characterize compositional changes in the mineralized fibrocartilage that might contribute to decreased mechanical behavior of the interface. We found that the strength, modulus, and toughness of the enthesis were decreased in the BtxA-unloaded group compared to controls, indicating a decrease in tissue quality. Polarized-light microscopy, Raman microprobe analysis, and X-ray diffraction analysis were used to determine changes in the collagen fiber alignment and mineral characteristics of the tendon enthesis due to BtxA-unloading. Collagen fiber alignment was significantly reduced in BtxA-unloaded shoulders. The
mineral-to-matrix ratio in mineralized fibrocartilage was not affected by loading. However, the Raman-measured crystallographic atomic order of the hydroxylapatite phase (a measure of crystallinity) was reduced and the amount of carbonate (substituting for phosphate) in the hydroxylapatite crystals was increased. XRD measurements indicated changes in the strain environment of the mineral crystals and some differences in crystal orientation and size. Taken together, these nanometer through micrometer-scale structural and compositional changes partly explain the observed decreases in the mechanical functionality of the tendon enthesis in the absence of muscle loading.

4.1 Introduction

Musculoskeletal tissues are highly sensitive to their mechanical environments. Mechanical forces not only maintain skeletal tissue homeostasis, they also guide tissue development. Disruptions in the loading environment during development often lead to pathological conditions. For example, in neonatal brachial plexus palsy (NBPP), damage to the brachial plexus during birth leads to shoulder paralysis and results in a number of shoulder pathologies in children, including bony defects, a decreased range of motion, and ultimately a loss of limb functionality in the most severe cases (Moukoko, Ezaki et al. 2004; Kirkos, Kyrkos et al. 2005; Mehta, Blackwell et al. 2006). This condition affects approximately 1 in 100 births, making it one of the most common pediatric shoulder disorders (Hoeksma, Ter Steeg et al. 2003).

We recently developed an animal model of this condition. Botulinum toxin A (BtxA) injections into the supraspinatus muscles of neonatal mice led to reversible paralysis that closely
mimicked the developmental pathologies characteristic of NBPP (Thomopoulos, Kim et al. 2007; Kim, Galatz et al. 2009; Das, Rich et al. 2011). This model was validated by comparison to mice that underwent neurotomy of the upper trunk of the brachial plexus at birth (Kim, Galatz et al.). Mouse shoulders that were paralyzed at birth exhibited internal rotation contractures due to the muscle imbalance across the shoulder, as seen in the clinical condition (Hoeksma, Ter Steeg et al. 2003; Kirkos, Kyrkos et al. 2005). This muscle imbalance ultimately led to bone loss and humeral head deformities. Previous studies using this animal model also showed that unloading disrupted the development of the tendon enthesis, a tissue critical for transferring loads from muscle to bone (Kim, Galatz et al.; Thomopoulos, Kim et al. 2007). Notably, unloading led to a loss of fibrocartilage transitional tissue and a loss of underlying bone at the tendon-to-bone attachment (Thomopoulos, Kim et al. 2007).

The gross morphological and bone architectural consequences of upper limb paralysis throughout postnatal development have been previously described (Kim, Galatz et al.; Thomopoulos, Kim et al. 2007; Kim, Galatz et al. 2009). However, the structural and functional consequences of reduced loading on the tendon enthesis have not yet been investigated. We hypothesized that a loss of bone and fibrocartilage would have a detrimental effect on the ability of the enthesis to transfer forces from tendon to bone. We propose that this loss of mechanical function would be due to disruption of collagen fiber structural organization and due to alterations in the composition of the mineralized interface. In this study, we investigated the functional consequences of muscle unloading throughout post-natal development of the tendon-to-bone attachment. Furthermore, we characterized micrometer-scale structural and compositional changes that might contribute to changes in mechanical behavior.
4.2 Methods

4.2.1 Animal Model

The use of animals for this study was approved by the Animal Studies Committee at Washington University. 70 CD1 mice of mixed genders, obtained from Charles River Labs (Wilmington, MA), were divided into two groups, 1) experimental and 2) normal age-matched control animals, consistent with previous studies (Thomopoulos, Kim et al. 2007; Kim, Galatz et al. 2009; Das, Rich et al. 2011). Experimental mice received injections of BtxA (BOTOX; Allergan, Irvine, CA) in the left supraspinatus muscle and injections of saline in the right supraspinatus muscle beginning at birth. Specifically, 0.2 U of BtxA dissolved in 10 μL of saline solution was injected twice per week into the left shoulders for the first two weeks after birth (the frequency of injection was necessary to maintain paralysis). Injections of 0.15 U in 10 μL of saline were then administered twice per week until the mice were 4 weeks old, and then once per week until euthanasia. 10 μL of saline solution was injected concurrently with BtxA into the right supraspinatus muscles as an internal control. Previous experiments comparing paired normal and saline-injected shoulders indicated no differences in shoulder development that could be attributed to the saline injections (Thomopoulos, Kim et al. 2007). However, previous studies using this model have noted differences between saline-injected shoulders of BtxA animals and age-matched controls (Thomopoulos, Kim et al. 2007; Kim, Galatz et al. 2009). Mice were euthanized by carbon dioxide narcosis at two timepoints: postnatal days 28 (P28) and 56 (P56). One shoulder was used from each normal animal at each time point and both shoulders were used from each BtxA experimental animal, resulting in three groups of samples from each of two time points. Five shoulders per group were allocated for analysis of collagen fiber alignment and
processed immediately as described below. Five animals per time point from the experimental group were allocated to Raman microprobe analysis and stored at -20 °C until processing, as described below. Data from Schwartz et al. (Schwartz, Pasteris et al. 2012) were used for normal age-matched comparisons for the Raman microprobe analyses. The remaining 40 animals (20 normal and 20 experimental) were stored at -20 °C until processing for biomechanical analysis, as described below.

4.2.2 Tendon cross-sectional area

Eight shoulders from each of the six groups were used to determine tendon cross-sectional area. Mice were thawed and dissected to isolate the humerus, supraspinatus tendon, and muscle. Micro-computed tomography (μCT40; Scanco Medical AG, Switzerland) was used to determine tendon cross-sectional area. Samples were stored in saline-soaked gauze until scanning. Immediately prior to scanning, the distal humerus was potted in agarose in a plastic tube and the tube was inverted within a sample holder. In order to limit background signal in the measurement, the tendon and muscle were suspended in air, aligned with the long axis of the humerus. Scans were performed at X-ray tube settings of 55 kV and 145 μA with a 99 ms integration time. These parameters resulted in 30 μm isometric resolution. The measurement time was <7 minutes and the total time in the scanner was <20 minutes. No appreciable dehydration was seen in any specimen after scanning. The scanned region was approximately 2 mm in the axial direction and included a small amount of the humeral head and supraspinatus muscle. The cross-sectional areas of sagittal slices through the tendon were estimated using thresholding and the minimum cross-sectional area was recorded.
4.2.3 Biomechanical testing

Uniaxial tensile testing was used to evaluate the biomechanical properties of the tendon entheses. After micro-CT analysis, the humerus was potted in epoxy up to the humeral head to stabilize the bone. A paperclip was embedded in additional epoxy over the articular surface of the humeral head away from the tendon insertion to prevent fracture of the growth plate. Tendons were wrapped in saline-soaked gauze throughout potting to maintain hydration. Tendons were sprayed with Verhoeff’s tissue stain to generate a random pattern of speckles that was used to optically determine strain. Each tendon was secured between two layers of thin paper with a drop of cyanoacrylate adhesive before the sample was mounted in custom aluminum grips. Samples were tested in a 37 °C saline bath on an Instron Electropuls E1000 (Instron Corp., Canton, MA) fitted with a 30 N load cell. The tendon was imaged continuously throughout the testing protocol using either an Olympus DP70 (1360 x 1024 pixels) or an Illunis VMV-8M (3296 x 2472 pixels) CCD camera at ~3 fps. The tendon gauge length was determined optically by comparison to an internal standard on the top grip and was approximately 2 mm for all groups.

The testing protocol (WaveMatrix software, Instron Corp., Canton, MA) consisted first of a 50 mN (P28) or 100 mN (P56) preload followed by 5 cycles of preconditioning using a triangle waveform with a peak load of 50 mN. A lower preload was used for the P28 samples due to the very low observed maximum load (~0.3 N) in the P28 BtxA unloaded samples. Next, a stress-relaxation test was performed with a strain rate of 100 %/s to a maximum strain of 3 % followed by 5 minutes of relaxation. Tendons were returned to the baseline state and held isometrically for 5 minutes before loading to failure at 0.5 %/s. Strain was determined optically for the linear region by tracking the speckled stain pattern within two regions, one at the top of the tendon
including the grip and one on the bone near the insertion using a custom texture correlation algorithm written in MATLAB (Mathworks, Natick, MA). The cross-sectional area used for normalization of the biomechanical properties was estimated by assuming an elliptical cross-section with the width of the tendon observed in the video as the major axis and the average thickness for each group determined from the microCT analysis as the minor axis.

Estimates of the stiffness/modulus, yield strength/stress, yield strain, resilience, and toughness were made from the load-displacement and stress-strain curves of each uniaxial tensile failure test. The stiffness/elastic modulus of each sample was determined from the linear portion of each load-displacement/stress-strain curve. The linear portion was identified by adjusting a window of points within the load-deformation or stress-strain so as to maximize the $R^2$ value for a linear least squares regression of the data in the window. The yield stress was defined as the stress value when the tangent modulus decreased to 50% of the maximum value in the load-to-failure tests. The yield strain was defined as the strain value corresponding to the yield stress. The energy to yield, a measure of resilience, was defined as the total area under the stress-strain plot beginning from the point at which the tendon reached a load of 0.05 N and continuing until the yield stress was attained. The toughness was defined as the total area under the stress-strain curve beginning at 0.05 N and continuing until this baseline value of stress was again attained. The ultimate force and stress were defined as the peak values on the force-displacement and stress-strain curves respectively. Samples that failed either at the grip or the growth plate were excluded from analysis of failure properties.

Estimates of the fully relaxed modulus of the samples were made from stress-relaxation data. These data, however, were extremely noisy for the low loads sustained by the P28 tendons, so this data was excluded from analysis. For the P56 samples, the fully relaxed state was defined
as the state achieved after 4.5 minutes of viscoelastic relaxation in isometric conditions. Percent relaxation was defined as the percent change in load from the maximum value of force at the beginning of the stress-relaxation test to the average load measured over the last 30 seconds of the 5 minute isometric test.

4.2.4 Collagen fiber alignment

Quantitative polarized light microscopy was used to analyze the structure of collagen fibrils within the enthesis fibrocartilage. Details of this technique are described in Thomopoulos et al. (Thomopoulos, Marquez et al. 2006). Tendon insertions from each of the six groups previously mentioned were dissected free of all surrounding tissues, leaving only a small amount of muscle and bone, and then processed for histology. Dissected muscle-tendon-bone complexes were immediately fixed in 4% paraformaldehyde with the tendon forming a 180° angle with the humerus (equivalent to 90° of abduction in the shoulder joint), dehydrated, embedded in paraffin, and sectioned at 5μm in the transverse plane. One section per tendon was manually selected to control for orientation and stained in 0.1 % Picrosirius Red solution, destained in 1 % acetic acid, counterstained with hematoxylin, and differentiated in 1 % HCl in 70 % ethanol. The distribution of collagen fiber angles was evaluated for a region of each tissue section encompassing both mineralized and non-mineralized fibrocartilage within the tendon enthesis. Each section was imaged through crossed polarizers at 10 different polarization angles using a 40x objective lens with and without a compensator (λ/4 wave plate oriented NW-SE). The extinction angle was calculated for groups of pixels (5 μm x 5 μm) within a user defined region of interest in the insertion fibrocartilage using custom MATLAB (Mathworks, Natick, MA) scripts.
(Thomopoulos, Williams et al. 2003; Thomopoulos, Marquez et al. 2006). The angular deviation of the distribution of angles was calculated for each sample using circular statistics (Thomopoulos, Marquez et al. 2006).

### 4.2.5 Raman microprobe analysis

Raman microprobe analysis is a useful technique for investigating mineralization at the tendon enthesis because it can be used on unfixed, hydrated tissues with minimal processing that might disrupt the interface. Five shoulders per group were thawed and dissected as described above for the polarized light analysis. Shoulders were directly embedded in Optimal cutting temperature (OCT) medium and sectioned to 20 μm on a cryostat. Immediately before analysis, one tissue section per sample was thawed and washed twice with phosphate buffered saline.

The Raman microprobe apparatus (HoloLab Series 5000 fiber-optically coupled Raman Microscope, Kaiser Optical Systems, Inc.) has been described previously (Wopenka, Kent et al. 2008). Spectra were acquired using 10 mW of a 532 nm laser focused using an 80x objective (N.A. = 0.85) to a ~1 μm beam spot on the surface of the sample. The scattered light was collected for 32, 4 second acquisitions in a backscattered configuration through the objective lens to a 2048-channel CCD detector. This allowed for concurrent detection of the spectral range 100-4000 Δcm\(^{-1}\). Raman spectra were collected from at least three (~1 μm diameter) regions within fully mineralized regions of enthesis fibrocartilage for each tissue section. The Raman signal was optimized at each spot via manual focusing and care was taken to avoid cells and vascular tissue that might affect the spectral signal.
Spectra were analyzed as previously described (Wopenka, Kent et al. 2008; Genin, Kent et al. 2009; Schwartz, Pasteris et al. 2012). Briefly, spectra were background corrected to eliminate the effects of fluorescence, and peaks within the spectral range 700-1200 Δcm⁻¹ were deconvolved with a mixed Gaussian-Lorentzian peak fitting algorithm in the Grams32 software package (Galactic, Salem, NH). The relative mineral concentration of the mineralized matrix within the enthesis fibrocartilage was inferred from the ratio of the peak intensities for the ν₁ P-O stretching band of hydroxylapatite (960 Δcm⁻¹) and the aromatic ring stretching band of phenylalanine residues in collagen (1003 Δcm⁻¹). This measure does not indicate the absolute amount of either mineral or matrix present, but provides useful information about the relative concentration, which can be compared between groups.

Two other spectral signatures were used to characterize the carbonated hydroxylapatite within the mineralized fibrocartilage. First, the crystallographic atomic order of the hydroxylapatite crystals (i.e., one aspect of crystallinity) was measured by the full width at half maximum (FWHM) of the 960 Δcm⁻¹ peak. A narrower peak is indicative of increased crystallographic order, or less variation in bond angles and distances. Second, biological hydroxylapatite is characterized by carbonate substitutions for phosphate (Pasteris, Wopenka et al. 2008). Therefore, the carbonate content of the mineral within the enthesis fibrocartilage was evaluated by taking the ratio of the heights of the 1070 Δcm⁻¹ peak, indicative of carbonate substitution for phosphate, and the 960 Δcm⁻¹ peak. Our past studies have shown more reproducible results from ratios of peak heights, in this particular case, compared to the standard ratio of peak areas (Wopenka, Kent et al. 2008). Following Raman microprobe analysis, sections were fixed in 10% neutral buffered formalin and stained using von Kossa’s method (for mineral)
and toluidine blue (for cells and organic matrix) so that the Raman-analyzed regions could be interpreted histologically.

4.2.6 X-ray diffraction and x-ray fluorescence measurements

4.2.6.1 Sample preparation and data collection:

X-ray fluorescence (XRF) and diffraction (XRD) experiments were performed at the Advanced Photon Source (APS) at Argonne National Lab using beamline 2I-D-D. 20 μm frozen tissue sections from the same 5 tissue blocks per group used for Raman analysis were mounted on silicon nitride windows (Silson Ltd., Northampton, England). Immediately prior to analysis, samples were defrosted and air dried. The silicon nitride mounted samples were attached to the sample holder using adhesive and placed normal to the incident x-ray beam at the focal plane of the x-ray zone plate. The enthesis was identified by preliminary optical mapping followed by X-ray fluorescence mapping of regions of mineralized fibrocartilage near the bone-tendon interface using a 250 nm x 250 nm X-ray beam. The XRF signal was collected with a Vortex-EX silicon drift detector (SII Nanotechnology USA, Northridge, CA) positioned as close to the specimen as possible and aligned to collect x-rays emerging nearly parallel to the front surface of the specimen and in the horizontal plane (i.e., the plane of the storage ring) for the best signal-to-noise ratio. For each sample, XRF mapping was performed for a region perpendicular to the mineral front encompassing the mineral gradient. Mineral phase compositional data was collected every 200 nm along 3 lines approximately 25-30 μm long and spaced by 1 μm. Following this preliminary mapping, 12 spots within the fully mineralized MFC region were analyzed by XRD. The 12 analysis regions were arranged in 2 rows of 6 parallel to the mineral
interface and spaced by 1 μm. XRF measurements at each spot were followed by collection of wide-angle x-ray scattering (WAXS) peaks from the hydroxylapatite (HAP) mineral using a MAR165 CCD detector (Rayonix, Evanston, IL) placed behind the specimen. The acquisition time for each diffraction measurement was approximately 2 minutes.

4.2.6.2 Data Analysis:

For each XRF measurement, the Ca signal was normalized to the incident X-ray flux to obtain a relative measure of localized mineral content. This measurement is also dependent on section thickness so it is more useful for comparisons within each specimen rather than between specimens. Analysis regions with a low Ca content compared to the other regions of the same sample were excluded from further analysis. The acquired diffraction patterns for each region of interest were analyzed using custom software previously developed by Dr. Jon Almer (APS, Sector 1) and modified by Dr. Alix Deymier-Black for this application (Deymier-Black, Singhal et al. 2013). The diffraction rings were fitted and measures of angular intensity, crystallite size, crystal strain, and root mean square strain ($\varepsilon_{\text{RMS}}$) were averaged over the 12 mapped spots for each specimen. Angular intensity was determined by fitting the peak intensity of the HAP(002) ring as a function of azimuthal angle ($\eta$, see Figure 4.5A) and calculating the FWHM. A wider distribution indicates less alignment of the mineral crystals. Crystal lattice strain was determined from the deviation from circularity of the HAP(002), HAP(211) and HAP(004) diffraction rings (Almer and Stock 2007; Deymier-Black, Almer et al. 2010; Deymier-Black, Singhal et al. 2013). Crystallite size and $\varepsilon_{\text{RMS}}$ were calculated from the analysis of peak broadening as described in Deymier-Black et al. (Deymier-Black, Singhal et al. 2013). Briefly, instrumental broadening was removed by comparison to a ceria standard and the intensity of the HAP(002) and HAP(004) peaks were fitted using a pseudo-Voigt function. A modified version of Scherrer’s equation was
used to convert the experimentally determined peak widths into estimates of crystal size and $\varepsilon_{\text{RMS}}$. The $\varepsilon_{\text{RMS}}$ is a measure of the variation of strains within the analysis volume.

4.2.7 Statistical analysis

Normal, saline, and BtxA groups at P28 and P56 were compared using a two-way analysis of variance (ANOVA) to determine effects of and interactions between treatment groups and animal age. When the effect of experimental group reached significance ($p<0.05$), Fisher’s least significant difference tests with a Bonferroni correction were used to evaluate post hoc differences for six predetermined comparisons ($\alpha = 0.05/6 = 0.0083$). The comparisons used were P28 normal vs. P28 BtxA, P28 saline vs. P28 BtxA, P28 normal vs. P28 saline, P56 normal vs. P56 BtxA, P56 saline vs. P56 BtxA, and P56 normal vs. P56 saline. A paired t-test was used for the XRD analysis since only BtxA and saline groups were analyzed. All data are reported as mean ± standard deviation.

4.3 Results

4.3.1 Effects of paralysis on morphology and mechanics

Local muscle paralysis was evaluated by observing shoulder motion during cage activity. BtxA animals had decreased abduction and external rotation, consistent with previous studies (Thomopoulos, Kim et al. 2007; Kim, Galatz et al. 2009; Das, Rich et al. 2011). Micro-computed tomography measurements indicated that paralysis did not significantly reduce supraspinatus
tendon cross-sectional areas relative to normal age-matched or saline contralateral controls at both P28 and P56 (Table 4.1). Tendons significantly increased in size from P28 to P56.

Table 4.1: Cross-sectional area and biomechanical properties of supraspinatus tendon entheses subjected to BtxA muscle unloading at postnatal days 28 and 56. * Indicates p<0.05 relative to normal age-matched animals, # indicates p<0.05 relative to saline injected age-matched animals.

<table>
<thead>
<tr>
<th>Group</th>
<th>Area (mm$^2$)</th>
<th>Yield Stress (MPa)</th>
<th>Energy to Yield (MPa)</th>
<th>Yield Strain</th>
<th>Toughness (MPa)</th>
<th>% Relax</th>
</tr>
</thead>
<tbody>
<tr>
<td>P28 Normal</td>
<td>0.24 ± 0.06</td>
<td>9.27 ± 3.48*</td>
<td>0.84 ± 0.45*</td>
<td>0.18 ± 0.03</td>
<td>4.02 ± 2.80</td>
<td></td>
</tr>
<tr>
<td>P28 Saline</td>
<td>0.19 ± 0.05</td>
<td>5.25 ± 1.80*</td>
<td>0.49 ± 0.19*</td>
<td>0.22 ± 0.07</td>
<td>2.02 ± 0.69</td>
<td>*</td>
</tr>
<tr>
<td>P28 BtxA</td>
<td>0.18 ± 0.05</td>
<td>1.82 ± 1.08*#</td>
<td>0.15 ± 0.13*#</td>
<td>0.14 ± 0.06</td>
<td>0.68 ± 0.41*</td>
<td>#</td>
</tr>
<tr>
<td>P56 Normal</td>
<td>0.28 ± 0.07</td>
<td>9.15 ± 2.79</td>
<td>0.74 ± 0.33</td>
<td>0.21 ± 0.07</td>
<td>3.43 ± 1.81</td>
<td>51.1 ± 7.6</td>
</tr>
<tr>
<td>P56 Saline</td>
<td>0.26 ± 0.03</td>
<td>8.56 ± 2.32</td>
<td>0.63 ± 0.21</td>
<td>0.17 ± 0.05</td>
<td>2.02 ± 0.61</td>
<td>51.8 ± 12.9</td>
</tr>
<tr>
<td>P56 BtxA</td>
<td>0.24 ± 0.04</td>
<td>4.03 ± 1.16*#</td>
<td>0.22 ± 0.09*#</td>
<td>0.13 ± 0.04</td>
<td>1.39 ± 0.80</td>
<td>61.8 ± 8.7</td>
</tr>
</tbody>
</table>

* p<0.05 relative to age-matched normal, *p<0.05 relative to age-matched saline, # significant effect of age

The metrics of mechanical performance estimated for tendon entheses were all significantly lower in the BtxA group compared to controls. In the absence of gripping failures, the tissue consistently failed at the tendon-to-bone interface in all groups, and all groups exhibited force-displacement that were qualitatively similar at low strains (Figure 4.1A). Maximum force was reduced at P28 and P56 in the BtxA group compared to normal controls and saline controls at P56 (Figure 4.1B). Significant differences were also detected between saline and normal controls at P28 and P56. Tendon stiffness was decreased relative to saline and
normal controls after 56 days of unloading (Figure 4.1C). At P28, both saline control and BtxA groups had lower stiffness than normal controls. Maximum force and stiffness both increased with increasing animal age.

![Graph showing force displacement curves](image)

**Figure 4.1:** Supraspinatus tendon enthesis structural properties were compromised by BtxA-muscle unloading throughout postnatal development. A) Representative force displacement curves. B) Maximum force was decreased in the BtxA-unloaded group compared to normal age-matched controls at P28 and P56 and saline contralateral controls at P56. Maximum force was increased in P56 animals compared to P28 animals. C) Tendon enthesis stiffness was reduced in the BtxA-unloaded group compared to normal controls at both P28 and P56 and relative to saline contralateral controls at P56. Stiffness increased with increasing animal age. [* p<0.05].

At P56, ultimate stress was reduced in the BtxA group compared to controls (Figure 4.2A). At P28, strength was also reduced in both the saline and BtxA groups compared to the normal group. The elastic modulus was significantly decreased in the BtxA group relative to normal and saline controls after 28 days of unloading but no significant differences were
detected at P56 among the three groups (Figure 4.2B). Yield stress was significantly reduced after both 28 and 56 days of unloading compared to saline and normal controls (Table 4.1). The resilience (energy to yield) was also decreased at P28 and P56 in the BtxA group relative to saline and normal controls. The toughness was decreased relative to normal controls at P28. At P28, yield stress, energy to yield, and toughness were also reduced in saline controls compared to normal age-matched animals. The percent relaxation associated with viscoelastic relaxation at P56 was not significantly affected in BtxA tendons.

**Figure 4.2:** Supraspinatus tendon enthesis mechanical property values were compromised by muscle unloading with BtxA throughout postnatal development. A) Tendon enthesis strength was reduced in the BtxA-unloaded group relative to normal controls at P28 and P56 and relative to saline controls at P56. Strength increased with increasing animal age. B) The elastic modulus was reduced in the BtxA-unloaded group relative to normal and saline controls at P28 and recovered by P56. [* p<0.05].
4.3.2 Effect of paralysis on collagen fiber alignment

Representative analysis regions for polarized light analysis of collagen fiber alignment are shown in Figures 4.3E-G. There was a statistically significant increase in the angular deviation of the distribution of collagen fiber angles (i.e., the fiber distribution was less organized) within the enthesis fibrocartilage in BtxA samples compared to normal aged-matched and saline contralateral controls at P28 and P56 (Figure 4.3A-D).
Figure 4.3: Tendon enthesis collagen fiber alignment was affected by muscle unloading throughout postnatal development. A) The angular deviation of collagen fiber angles within the enthesis of BtxA-unloaded shoulders was increased (i.e., the fibers were less organized) at P28
and P56 BtxA relative to loaded controls. Angular deviation decreased with increasing animal age. [* p<0.05]. B-D) Representative histograms of the collagen fiber angles, as determined by polarized light microscopy for P56 entheses (B: normal, C: saline, and D: BtxA–unloaded). E-G) Representative picrosirius red stained sections are shown, as viewed under polarized light. Local collagen fiber mean angles are indicated by the colored dots (E: normal, F: saline, and G: BtxA-unloaded, corresponding to the respective histograms shown in B, D, and E). [Scale bar = 50 μm].

### 4.3.3 Effect of paralysis on mineralized fibrocartilage

Representative mineralized tissue sections from normal, saline and BtxA entheses at P56 are shown in Figures 4.4A-C respectively. The Raman microprobe measured mineral-to-matrix ratio averaged from three ~1 μm analysis spots per sample was not significantly changed by BtxA unloading (Figure 4.4D). Carbonate substitution for phosphate, determined by comparing the 960 Δcm⁻¹ peak for phosphate to the 1070 Δcm⁻¹ peak for B-type carbonate substitution for phosphate (Antonakos, Liarokapis et al. 2007; Pasteris, Wopenka et al. 2008), was increased in entheses unloaded using BtxA for 56 days compared to normal controls (Figure 4.4E). The crystallinity of the mineral within mineralized fibrocartilage, averaged over the sampling volume, was significantly reduced in BtxA-unloaded entheses at P56 compared to normal and saline controls (i.e., FWHM was increased, Figure 4.4F).
Figure 4.4: Raman microprobe analysis of mineralized fibrocartilage within the enthesis of BtxA-unloaded shoulders. A-C) Example von Kossa and Toluidine blue stained mineralized sections from A) Normal, B) Saline, and C) BtxA shoulders. [Scale bar = 50 μm]. D) The relative mineral content determined by the ratio of the 960 Δcm⁻¹ peak (phosphate ν₁) representing mineral and the 1003 Δcm⁻¹ peak (phenyl group) representing collagen was not significantly changed by muscle unloading. E) The amount of carbonate substituted for phosphate in the hydroxylapatite mineral phase was increased at P56 by BtxA unloading relative to normal controls. Carbonate substitution increased with increasing animal age. F) The crystallinity (i.e., relative atomic order) of the hydroxylapatite mineral was decreased (i.e., FWHM was increased) at P56 in BtxA-unloaded mineralized fibrocartilage compared to loaded controls. [* indicates p<0.05].
Consistent with the Raman measurements, X-ray fluorescence measurements did not indicate any significant differences in the calcium content between BtxA unloaded entheses and contralateral controls (data not shown). X-ray diffraction analysis indicated that the size of the hydroxylapatite (HAP) mineral crystals was decreased in BtxA unloaded MFC; this difference was statistically significant at P28 but not at P56 (Figure 4.5A). The FWHM of the HAP(002) intensity as a function of the azimuthal angle, a measure of crystal texture or alignment of the c-axis of the mineral crystallites within the X-ray analysis volume, was significantly decreased in unloaded samples at P56 but not at P28. This suggests that within each 200 nm diameter x 20 μm analysis volume, the mineral crystallites were more uniformly aligned in unloaded shoulders compared to controls. εRMS, the width of the distribution of strain environments within the analysis volume, was significantly increased at P56, with a trend toward increased at P28 (p = 0.07). This suggests that the crystal strain environment was more variable in unloaded samples compared to controls. The peak HAP crystal lattice strain in the HAP(002) was significantly increased at P28 but not at P56 (data not shown).
Figure 4.5: Wide angle X-ray diffraction analysis of BtxA unloaded entheses. A) A representative example of a HAP diffraction pattern is shown. The HAP(002), HAP(211), and HAP(004) peaks are indicated by the labels. B) The size of the HAP mineral crystals in the enthesis fibrocartilage was decreased in BtxA unloaded animals at P28. C) The relative HAP mineral crystal orientation distribution was determined by the FWHM of the angular intensity of the HAP(002) diffraction ring. The crystal orientation distribution was narrower in BtxA unloaded animals at P56 compared to controls. D) The RMS strain ($\varepsilon_{RMS}$), a measure of the distribution of the HAP crystal strain environments within the X-ray analysis volume, was increased in BtxA unloaded animals compared to controls. * indicates $p < 0.05$. 

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4.4 Discussion

In this study, we investigated the effect of muscle unloading on the postnatal development of the tendon enthesis and found diminished biomechanical function in BtxA-unloaded tissues. An important distinction must be made between the concepts of material and structure when interpreting biomechanical results. We define material effects as those due to local changes to tissue, including its composition and hierarchical structure, at a specific point along the tendon-to-bone insertion site. We define structural effects as those due to changes in the spatial distribution of material (e.g., cross-sectional area, the spatial distribution of material with different mechanical properties). Clear effects of BtxA-induced unloading were observed in the biomechanical tests performed, and these likely involved both material and structural effects.

The simplest gross structural metric studied was tendon size, and this was maintained relative to saline contralateral controls but not relative to normal age-matched controls following BtxA treatment. The constant size of the BtxA tendons relative to contralateral saline controls is consistent with the results of Eliasson et al., who reported that tendons continue to increase in size normally in the absence of loading during development (Eliasson, Fahlgren et al. 2007). Relative to normal controls however, we observed decreases in tendon size due to BtxA-unloading, similar to studies using other unloading models in mature animals (Johnson, Tsao et al. 2005; Foutz, Ratterman et al. 2007; Sun, Thoreson et al. 2010). These results suggest that developing tendons may respond differently to unloading compared to mature tendons.

BtxA-unloading resulted in tendon-to-bone entheses with inferior mechanical properties. This suggests that the material that comprises the developing tendon enthesis tissue must be
altered by BtxA to produce an attachment with diminished mechanical function. To explore potential tissue changes that might contribute to the observed biomechanical changes, we measured collagen fiber alignment and characteristics of the mineralized fibrocartilage matrix within the enthesis. We observed a small, but significant, increase in the angular deviation of collagen fibrils (6°-8°), i.e., the absence of muscle loading resulted in a less organized extracellular matrix. This is consistent with observations in vitro, in which cells within mechanically stimulated tissue constructs deposit matrix proteins predominantly in the direction of loading (Thomopoulos, Fomovsky et al. 2005). Clear structure-function relationships between fiber alignment and tensile properties in collagenous tissues are long-established in the literature (Sacks 2003; Lokshin and Lanir 2009), and models for this relationship allow quantitative estimation of the degree to which the disorganization of collagen contributes to the lower moduli observed in the entheses of the BtxA-treated group. The essence of this calculation is averaging an estimate of the homogenized moduli of cross-linked, aligned fibers over the measured fiber orientation distribution. Using a simple formulation and that we published elsewhere (Thomopoulos, Marquez et al. 2006; Genin, Kent et al. 2009; Liu, Thomopoulos et al. 2012; Miller, Connizzo et al. 2012) and the unit cell properties that we used to model adult tendon (Genin, Kent et al. 2009), the decrease in collagen organization caused by BtxA-unloading could account for an approximately 10% reduction in elastic modulus. This is far less than the observed 75% and 42% decreases in elastic modulus with BtxA at P28 and P56, respectively, compared to normal controls. Thus, although alignment is known to correlate positively with tensile strength and stiffness (in the direction of fiber alignment) and with tissue anisotropy (Pins, Christiansen et al. 1997; Thomopoulos, Fomovsky et al. 2005), the reduction in collagen fiber alignment observed here accounts for only a small fraction of the reduced elastic modulus of the tissue.
Other compositional factors must therefore be involved. This conclusion is consistent with observations of Juncosa-Melvin et al. who demonstrated in vitro that mechanical stimulation up-regulates production of extracellular matrix genes, which also correlate with increased stiffness (Juncosa-Melvin, Matlin et al. 2007). Factors such as this may affect the tissue as a material and therefore affect its mechanical response.

Failure loads are determined by the interplay between structural effects such as the gross shape and distribution of tissue types within the enthesis, and material effects such as local failure loads. In nearly all of the samples, failure occurred at a location within the tendon-bone interface and not in the tendon midsubstance. The mechanics of the tissue within the enthesis is dominated by collagen for low mineral concentrations, and the mineral phase for higher mineral concentrations (Genin, Kent et al. 2009); as with many composite materials and tissues, the transition point is the mineral volume fraction associated with percolation (Milton 2002; Genin and Birman 2009). This critical mineral concentration is a strong function of the shape and organization of the mineral phase (e.g., (Marquez, Genin et al. 2005; Marquez, Genin et al. 2005)). We assayed the relative mineral concentration of the mineralized matrix within the enthesis using Raman microprobe analysis. We observed no statistically significant differences in the intensity ratio of the 960 Δcm⁻¹ peak for mineral and the 1003 Δcm⁻¹ peak for collagen. Raman measurements cannot accurately describe the absolute volume or mass of material present. Rather, this measure describes changes in the concentration of mineral, assuming that the entire tissue volume consists of only mineral and collagen. Previous analyses using X-ray micro-computed tomography have demonstrated decreases in bone volume when muscle forces are removed using BtxA (Warner, Sanford et al. 2006; Grimston, Silva et al. 2007; Poliachik, Bain et al. 2010). This is also true of the developing murine tendon enthesis (Thomopoulos, Kim
et al. 2007; Kim, Galatz et al. 2009). Our results suggest that the local mineral-to-collagen ratio within the extra-cellular matrix is not affected strongly by BtxA, in contrast to the total amount of mineralized tissue, which is decreased due to BtxA-unloading.

We probed the characteristics of the hydroxylapatite crystals within the mineralized fibrocartilage extracellular matrix using both XRD and Raman analysis. These two complimentary techniques provide information about the nano- and micro-scale environment of the HAP mineral crystals. The differences observed in the properties of the mineral crystals between BtxA unloaded and normal controls suggest that the mineralization process is disrupted in entheses that develop in the absence of muscle forces. The observed mineralization differences could indicate changes in both the pattern of mineral deposition and changes in the natural mineral evolution process that occurs over time. Biominerals are not static, purely structural elements. The mineral phase within bone plays important roles in cellular metabolism by regulating the concentration of ions such as phosphate, calcium, and trace elements. This suggests that mineral crystals evolve in response to the changing chemical environment of the bones. Within osteons, the properties of newly deposited mineral crystals are quite different than mineral that has been the matrix for a longer period of time, suggesting that a mineral evolution process naturally occurs with time (Gourion-Arsiquaud, Burket et al. 2009; Burket, Gourion-Arsiquaud et al. 2011).

From the WAXS patterns, we observed that the average c-axis length of the mineral crystals was increased to P56 levels at P28 in BtxA unloaded samples. The larger crystal size may be indicative of mineral preferentially depositing on the exterior of collagen fibrils where growth is less restricted than in the gap channels. Owing to the fact that a few very large crystals may dominate the grain size estimate because this measurement is inherently weighted by the
volume fraction of each crystal, a larger crystal size could indicate either mineral both on the exterior and interior of collagen fibrils or only the exterior. One potential explanation for an altered mineral location relative to the collagen might be changes in the organic matrix composition. Fibrocartilage development is impaired in BtxA unloaded entheses (Thomopoulos, Kim et al. 2007), so the attachment matrix may be more tendon-like. Native tendon is highly resistant to mineralization, probably due to a high concentration of mineralization inhibitors within the matrix. It is possible that this type extracellular matrix might favor mineralization on the exterior of collagen fibrils in contrast to a more fibrocartilage or bone-like matrix that favors mineral deposition within collagen fibrils. Thus, in the P28 control animals, crystals within the mineralized fibrocartilage may be smaller because mineralization is occurring both inside and outside the fibrils while in the BtxA unloaded group, larger mineral grains may be preferentially deposited on the exterior of collagen fibrils.

Alternatively, it could be that the mineral evolution process after mineral is deposited within the matrix is altered or accelerated by BtxA unloading. In support of this, the FWHM of the intensity of the HAP(002) diffraction ring as a function of azimuthal angle was reduced only in older P56 BtxA unloaded shoulders. This result is surprising in light of the increased disorder of the collagen fiber alignment. However, this inconsistency may be a result of the difference in length scale of the two techniques: collagen fiber alignment was based on a distribution of angles averaged over a large area of the insertion while HAP alignment distribution was determined within each much smaller X-ray analysis volume. The increase in the relative alignment of the c-axes of the HAP crystals could also be due to changes in the location of the mineral relative to the collagen fibrils. If the mineral is largely extra-fibrillar, it may be less aligned with the collagen fibrils than mineral crystals that reside within the ordered collagen fibril structure. This
result might support the idea that the mineral evolution process is altered or accelerated in BtxA unloaded entheses. The initial rapidly deposited mineral may be gradually rearranged and patterned by the collagen fibers over time. P56 mice are not yet skeletally mature so mineral from these animals is expected to be somewhat immature or more recently deposited. In further support of this idea that mineral evolution might be accelerated in BtxA unloaded entheses, the HAP crystal RMS strain, representing the distribution of strain environments within the total X-ray analysis volume, is increased in BtxA unloaded shoulders. This might indicate a more energetically favorable environment for mineral crystal evolution in BtxA unloaded shoulders.

Furthermore, from the Raman measurements, we found that for the BtxA group the carbonate concentration was increased with respect to self-comparison between P28 and P56, as well as comparison to the normal and saline groups at P56. Crystallinity of the BtxA mineral crystallites was decreased with respect to the normal and saline groups by P56. Although the exact role of carbonate incorporation in matrix remodeling is unclear, several reports have shown decreased carbonate content and/or crystallinity in mineralized tissue from younger animals that has yet to undergo remodeling (Miller, Little et al. 2007; Gourion-Arsiquaud, Burket et al. 2009; Gamsjaeger, Masic et al. 2010; Burket, Gourion-Arsiquaud et al. 2011; Schwartz, Pasteris et al. 2012). The BtxA-unloaded group had a carbonate concentration characteristic of much older mineralized matrix that had likely been remodeled, yet it was less crystalline. This also supports the idea presented above mineral evolution may be accelerated or altered in unloaded limbs. The typical effect of aging in bone, however, is the co-varying increase in both carbonate concentration and crystallinity (Akkus, Polyakova-Akkus et al. 2003; Akkus, Adar et al. 2004; Burket, Gourion-Arsiquaud et al. 2011). The present changes in mineral composition and structure might also be the result of changes in organization and composition of the organic
matrix components. Furthermore, previous results indicated increased osteoclast activity in BtxA unloaded compared to loaded shoulders which resulted in a higher rate of bone resorption (Warner, Sanford et al. 2006; Thomopoulos, Kim et al. 2007; Aliprantis, Stolina et al. 2012). This could amount to disruptions in the balance of mineralized matrix deposition versus resorption, leading to lower net amounts of bone accumulation and a less mature mineralized matrix. In summary, the observed changes in mineral characteristics are likely due to both altered cellular activity and organic matrix composition.

The mechanical consequence of decreased atomic order and increased carbonate content of the mineral crystallites within the enthesis fibrocartilage is unclear. The boundary between mineralized and non-mineralized fibrocartilage is characterized by a narrow zone of increasing mineral content that may dissipate stress concentrations arising at the interface (Wopenka, Kent et al. 2008; Genin, Kent et al. 2009; Liu, Thomopoulos et al. 2012; Schwartz, Pasteris et al. 2012). Carbonate content has been observed to regulate crystal size, with higher carbonate content leading to smaller crystals with elongated aspect ratios (Liao, Watari et al. 2007; Xiao, Liu et al. 2008). However, we did not observe any differences in the crystal grain size between BtxA unloaded and saline controls at P56. Additionally, changes in the carbonate content and crystallinity have been correlated with inferior bone biomechanical properties (Akkus, Adar et al. 2004; Yerramshetty and Akkus 2008; Donnelly, Boskey et al. 2010; Burket, Gourion-Arsiquaud et al. 2011). The failure mode of the samples tested in the current study was invariably the tendon-bone interface if there was not a readily identified gripping problem. However, it is not known whether the failure occurred within the unmineralized fibrocartilage, within the fully mineralized fibrocartilage, or within the mineral gradient between the two. Changes to the mineral aspect ratio are expected to shift the mineral volume fraction needed for percolation, and
hence have a strong effect on the mechanics and stress fields within tissue at the enthesis (Genin, Kent et al. 2009). Additionally, changing the location of the mineral relative to the collagen fibrils affects the mechanical behavior of the tissue (Liu, Thomopoulos et al. 2014). These material effects could lead at the structural level to macroscopic stress concentrations that reduce strength and toughness of the enthesis.

There were a number of limitations in this study. BtxA injections lead to a small decrease in animal body weight, although this decrease was not statistically significant in a previous report (Thomopoulos, Kim et al. 2007). The current and prior studies using this animal model have not controlled for animal gender or litter size. These factors contribute to variation in body weights and may also contribute to variations in the functional outcomes measured. Differences in animal sizes may partly explain the observed differences in some outcomes between the normal and saline controls. Additionally, there may be some direct or indirect systemic effects of BtxA, as the injected pups were generally more lethargic and less active. Due to various factors contributing to variation in the animal model, it is possible that some of the outcomes may be underpowered, although statistically significant differences were detected with N=5 for most outcomes.

4.5 Conclusions

In conclusion, the results presented here indicate that muscle forces are necessary for the development of a functional tendon enthesis. Biomechanical behavior was significantly affected after only four weeks of BtxA-unloading during postnatal development. Mechanical property changes were due to a small degree to changes in the organization of collagen fibers. Changes to
the mineral composition due to BtxA-unloading likely had deleterious effects on both the material behavior of the tissue and the structural response of the enthesis as a whole. These results have important implications for patients of NBPP. Understanding the developmental time course of loss of enthesis function will lead to a more informed strategy for treatment. Future studies will determine whether these detrimental functional changes to the enthesis are reversible upon return of muscle loading, as this condition spontaneously recovers in a sub-population of patients with NBPP (Anand and Birch 2002). Understanding the time course of recovery may inform the timing of surgical repair treatments.

4.6 Acknowledgements

Justin Lipner assisted with the mechanical testing. Bill Coleman and Marlene Scott prepared the paraffin sections for collagen fiber alignment analysis. Jill Pasteris assisted with the analysis of the Raman measurements. Alix Black, Zhonghou Cai, and Jon Almer assisted with the X-ray experiments performed at the APS at Argonne National Lab. Guy Genin performed the estimates of stiffness due to changes in collagen fiber orientation.
Chapter 5: Enthesis fibrocartilage cells originate from a population of Hedgehog responsive cells modulated by the loading environment

Summary:

As described in the previous chapters, fibrocartilagenous entheses develop postnatally concurrent with epiphyseal mineralization, and muscle loading is necessary for the formation of a functional enthesis. The biological factors that modulate this developmental processes, however, are less well understood. This chapter investigates the role Indian hedgehog (Ihh), a known mechano-responsive signaling molecule necessary for endochondral bone formation, for enthesis development. We discovered a unique population of Hedgehog-active cells in the enthesis beginning in the late embryonic stages of development that were distinct from tenocytes and epiphyseal chondrocytes. Lineage tracing experiments revealed that this cell population remained at the mature enthesis, populating the mineralized fibrocartilage (MFC), even at skeletally mature time points. Ablation of this cell population during the first week of postnatal development resulted in a loss of mineralized fibrocartilage. Surprisingly, very little tissue remodeling was observed 5 weeks after cell ablation. Conditional deletion of Smoothened, a molecule necessary for Ihh-responsiveness, from the developing tendon and enthesis resulted in significantly reduced fibrocartilage mineralization and decreased biomechanical function, without a significant decrease in the total amount of fibrocartilage. Using the BtxA unloading model described in the previous chapter, we further demonstrated that hedgehog (Hh) activity in this cell population was modulated by the loading environment. Taken together, these results
demonstrate that load-modulated Ihh signaling within developing fibrocartilage cells is required for enthesis mineralization.

5.1 Introduction

The Indian hedgehog (Ihh) signaling pathway has been well characterized in developing bone and is known to play a critical role in endochondral bone formation (Kronenberg 2003; Long and Ornitz 2013). Ihh activity directs the rate of endochondral mineralization in response to the mechanical forces sustained by developing bones (Nowlan, Prendergast et al. 2008). Ihh regulates patterns of chondrocyte differentiation and mineralization through a negative feedback loop with Parathyroid hormone related protein (PTHrP). Ihh, which is expressed by prehypertrophic and hypertrophic chondrocytes, induces expression of PTHrP in proliferating chondrocytes away from the growth front, which in turn represses Ihh expression. Ihh acts in a paracrine fashion on cells through the cell surface receptors Patched (Ptch) and Smoothened (Smo). In the absence of Ihh, Ptch represses Smo, which allows members of the glioma-associated oncogene homolog (Gli) family within the cell to be processed into a transcriptional repressors. In the presence of the Ihh ligand, Ihh binds to Ptch, removing repression of Smo, which translocates into the primary cilia and stabilizes Gli proteins into transcriptional activators for Hh target genes such as Ptch and Gli1 (Ingham and McMahon 2001; Lai and Mitchell 2005; Wang, Zhou et al. 2009).

As described in chapter 2, the development of fibrocartilage in the enthesis occurs postnatally, concurrently and in close proximity to epiphyseal mineralization of the humeral head (Schwartz, Pasteris et al. 2012). Fibrocartilage is not evident at the enthesis of mouse rotator
cuffs until 2-3 weeks after birth (Galatz, Rothermich et al. 2007). Recently, factors associated with the Hh pathway have been localized to various entheses. PTHrP is widely expressed in numerous fibrous entheses and deletion of PTHrP results in bony outgrowths (Wang, VanHouten et al. 2013). Additionally, expression levels of this molecule depend on the loading environment (Chen, Macica et al. 2007; Wang, VanHouten et al. 2013). Expression of Gli1, a transcriptional effector and a direct target gene of all Hh proteins, has also been identified in fibrocartilagenous entheses (Blitz, Viukov et al. 2009; Liu, Aschbacher-Smith et al. 2012). Based on these findings, we hypothesized that Ihh signaling would be necessary for enthesis mineralization.

In this chapter, we identify potential Hh-responsive cells and their progenies in the late embryonic and postnatal enthesis using a lineage tracing approach. The Gli1-Cre\textsuperscript{ERT2} knock-in mouse that expresses tamoxifen (TAM)-inducible Cre (Cre\textsuperscript{ERT2}) from the endogenous Gli1 locus was crossed with reporter mice and activated at various timepoints. Next, we demonstrate that Ihh signaling and the size of this cell population is modulated by the loading environment. Cell ablation experiments then demonstrate that this precursor cell population is necessary for the development, mineralization, and maintenance of fibrocartilage in the enthesis. Finally, we demonstrate that activated Hh signaling in this cell population is required for proper mineralization and biomechanical properties of the enthesis.

5.2 Methods

5.2.1 Animal models

The use of animals for this study was approved by the animal studies committee at Washington University. For lineage tracing experiments, tamoxifen inducible Gli1-Cre\textsuperscript{ERT2} mice
(Ahn and Joyner 2004) were crossed with Rosa26 – mTmG (mTmG) mice (Jackson Laboratory, Bar Harbor, ME). TAM was dissolved in corn oil and 100-200 μg/g body weight was injected sub-cutaneously between the shoulder blades at the time points indicated. At least 3 animals were used for each labeling experiment. For comparison, Col2-Cre\textsuperscript{TM}:mT/mG mice were also used (Hilton, Tu et al. 2007). For cell ablation experiments, Rosa-DTA (DTA) mice were crossed with Gli1Cre\textsuperscript{ERT2} animals (Voehringer, Liang et al. 2008). These animals received 200 μg/g body weight TAM on P6 (N=5 per time point). ScxCre mice (Blitz, Viukov et al. 2009) were crossed with Smo\textsuperscript{fl/fl} (Long, Zhang et al. 2001) mice to investigate the conditional knockdown of Ihh signaling.

5.2.2 Histology

Following euthanasia, the humerus, supraspinatus tendon, and muscle were dissected free of all other tissues, fixed in 4% paraformaldehyde, decalcified with 14% EDTA, embedded in OCT medium, and sectioned on a cryostat. Sections were washed 2x with PBS and mounted with aqueous mounting medium. Sections used for cell counting were mounted with aqueous medium containing DAPI. Following fluorescent imaging, sections were stained with Toluidine blue. Mineralized frozen sections were cut using cryofilm (Section Lab, Kanangawa, Japan) and stained by Von Kossa’s method followed by Toluidine blue.

5.2.3 Botulinium toxin unloading model

BtxA was injected according to previously published protocols (Thomopoulos, Kim et al. 2007; Schwartz, Lipner et al. 2013). Briefly, Gli1Cre\textsuperscript{ERT2};mTmG animals were injected with
0.2U in 10μL sterile saline 2x per week until sacrifice and the contralateral limb was injected concurrently with the same volume of saline. 5-6 animals were used for each time point of each assay. These animals also received injections of TAM 2-3 days prior to sacrifice to label Hh active cells.

5.2.4 Laser capture microdissection and RT-qPCR

The murine enthesis is a very small complex tissue that has structural and compositional properties that are distinct from both the adjacent tendon and bone. Laser capture microdissection was used to isolate cells from this tissue to study localized gene expression levels within the enthesis fibrocartilage without contamination from the adjacent tendon and bone. Freshly dissected humerus-supraspinatus tendon complexes were frozen in OCT medium under RNAs-free conditions using dry ice. 5-6 30 μm sections were cut using laser capture microscopy (LCM) tape (Section Lab, Kanangawa, Japan) on a cryostat. The tape sections were then mounted on plastic slides containing a hole for laser capture micro-dissection using a LMD7000 system (Leica Microsystems). Sections were briefly thawed, then micro-dissected and deposited into caps filled with lysis buffer. The Norgen total RNA micro kit (Thorold, Ontario, Canada) with additional proteinase K digestion and DNase I treatment was used to extract RNA. Each RNA sample was converted to cDNA using the Superscript VILO cDNA synthesis kit (Life Technologies, Carlsbad, CA). RNA was quantified using the Agilent RNA 6000 pico kit with the Agilent Bioanalyzer (Santa Clara, CA). As is typical for LCM experiments, RNA yields were relatively low (0.2-1 ng/μL), with low RNA integrity numbers’s (1.5-3). However, there were no significant differences between groups for RNA yield or integrity, so the overall trends in the
data are likely representative. Real time PCR was performed using TaqMan chemistry on a StepOnePlus Real-Time PCR System (Applied Biosystems, CA). Primers for Smo (Mm01162710_m1), Ptch (Mm00436026_m1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Mm99999915_g1) were purchased from Life Technologies (Carlsbad, CA). PCR reactions were performed in duplicate and a third reaction was performed for samples where the first two cycle numbers were inconsistent. All results are expressed as fold change relative to the housekeeping gene GAPDH.

5.2.4 Micro-computed tomography analysis

Micro-computed tomography (microCT) (μCT40; Scanco Medical AG, Switzerland) was used to determine the volume of MFC, humeral head volume, humeral head trabecular architecture, and tendon cross-sectional area. Supraspinatus muscle-tendon-bone samples were fixed in 4% paraformaldehyde prior to scanning. The distal humerus was potted in agarose in a plastic tube that was inverted within the sample holder. This suspended the supraspinatus tendon and muscle in air aligned with the long axis of the humerus to optimize soft tissue contrast. Scans were performed at X-ray tube settings of 45 kV and 177 μA with a 200 ms integration time, resulting in a 20 μm voxel size.

The humeral head volume was estimated from all of the slices proximal to the distal end of the anatomic neck of the humerus. Trabecular architecture was measured from all slices proximal to the epiphysseal plate. Fibrocartilage volume was measured by manually drawing regions of interest around the dense mineralized areas adjacent to the supraspinatus tendon. Tendon cross-sectional area was determined from the axial slice with the minimum tendon area.
5.2.5 X-ray diffraction and fluorescence mapping

X-ray fluorescence (XRF) and diffraction (XRD) experiments were performed at the APS (Argonne National Lab) using beamline 2I-D-D as described in Chapter 4. Fresh frozen tendon insertions from ScxCre;Smo^{fl/fl} animals and littermate controls (N=3 per group) were isolated, embedded in optimal cutting temperature (OCT) embedding medium, frozen at -80 °C, and sectioned to 20μm on a cryostat. Frozen tissue sections were mounted on silicon nitride windows (Silson Ltd., Northampton, Eng.). Immediately prior to analysis, samples were defrosted and air dried. The OCT was not removed in order to minimize section adhesion to the silicon nitride windows and it was not detectable in the wide-angle X-ray scattering (WAXS) diffraction patterns. The silicon nitride mounted samples were attached to the sample holder using adhesive and placed normal to the incident x-ray beam at the focal plane of the X-ray zone plate. The insertion site was identified by preliminary optical mapping followed by X-ray fluorescence mapping of regions of mineralized fibrocartilage near the bone-tendon interface using a 250 nm x 250 nm X-ray beam. The XRF signal was collected with a Vortex-EX silicon drift detector (SII Nanotechnology USA, Northridge, CA) positioned as close to the specimen as possible and aligned to collect X-rays emerging nearly parallel to the front surface of the specimen and in the horizontal plane (i.e., the plane of the storage ring) for the best signal-to-noise ratio. Following preliminary mapping, 20 spots were analyzed within the fully mineralized MFC region arranged in 2 rows of 10 parallel to the mineral interface and spaced by 1 μm. XRF measurements at each spot were followed by collection of WAXS diffraction peaks from the mineral using a MAR165 CCD detector (Rayonix, Evanston, IL) placed behind the specimen. The acquisition time for
each diffraction measurement was approximately 2 minutes. The data acquired was analyzed as described in Chapter 4. Diffraction rings were fitted and angular intensity, crystallite size, and residual strain were averaged over the 20 mapped spots for each specimen.

5.2.6 Mechanical testing

Supraspinatus tendons from 12 ScxCre;Smo$^{fl/fl}$ animals per group (cKO and control) were tested according to our previously published methods (Schwartz, Lipner et al. 2013). Limbs were dissected to isolate the supraspinatus tendon, muscle, and humerus, and scanned with uCT to determine tendon cross-sectional area ($\mu$CT40; Scanco Medical AG, Switzerland). The cross-sectional area was defined as the minimum tendon area determined from the series of axial slices spanning the tendon. Next, the humerus was potted in 1 minute epoxy with an embedded paperclip to secure the proximal growth plate. The tendon was secured between single layers of paper using superglue and then mounted in custom grips in a 37 °C saline bath on an Instron Electropuls E1000 (Instron Corp., Canton, MA) fitted with a 5 N load cell. The gauge length was determined optically via a calibration standard on the top grip and was approximately 2 mm for all specimens. Tendons were subjected to 5 cycles of preconditioning (3% strain) before loading to failure in uniaxial tension at 0.5% strain/s (WaveMatrix software, Instron Corp., Canton, MA). Samples that failed either at the grip or the growth plate were excluded from all analyses.

The stiffness and ultimate force were defined as the slope of the linear region and maximum value of the load-displacement curve as previously described (Schwartz, Lipner et al. 2013). Each load-displacement curve was normalized by the cross-sectional area and the initial tendon length to obtain the corresponding stress-strain curve. The modulus, yield strength/stress,
yield strain, resilience, and toughness were determined from the stress-strain curve of each load-to-failure test. The ultimate stress was defined as the maximum value on the stress-strain curve. The toughness was defined as the total area under the stress-strain curve beginning at 0.05 N and continuing until this baseline value of stress was again attained. Although all samples failed at the bony insertion site, this measure was highly variable due to the inconsistent post-failure behavior of the tendon samples. The yield stress was defined as the stress value when the tangent modulus decreased to 50% of the maximum value in the load-to-failure tests. The yield strain and energy to yield were defined as the strain value and total area under the stress-strain plot up to the yield stress.

5.2.7 Statistical methods

For the BtxA unloading model, BtxA shoulders were compared to contralateral saline shoulders using a paired t-test (N=5-6 per group). DTA;Gli1CreERT2 animals were compared to age-matched littermate controls using an ANOVA. ScxCre;Smoflox animals were compared to littermate controls using an ANCOVA analysis to account for the effects of genotype and animal age using animal sex as a covariate in the analysis. 6 males and 6 females of each genotype were tested mechanically and analyzed for microCT. 5 animals per group were used for histomorphometric analysis. Significance was defined as p < 0.05 and all data are reported as mean ± standard deviation.

5.3 Results
5.3.1 Enthesis fibrocartilage cells are derived from a unique population of hedgehog responsive cells

In order to track the temporal and spatial distribution of Hh activity in the developing enthesis, Gli1-Cre<sup>ERT2</sup>;mTmG mice were injected with TAM at distinct time points throughout postnatal development (Figure 5.1). All cells from these mice display red membrane fluorescence (mTomato) and in Hh-responsive (Gli1-expressing) cells the red fluorescence is replaced by green. Hh activity was detected in the postnatal supraspinatus enthesis consistent with previous reports for other entheses (Wang, Mitroo et al. 2006; Blitz, Viukov et al. 2009; Liu, Aschbacher-Smith et al. 2012). Expression of Gli1 was first observed at the immature tendon-to-bone interface at approximately E16.5 (Figures 5.1A and 5.1B). At birth, positive cells were seen in a narrow zone between tendon and the epiphyseal cartilage (Figure 5.1C). These cells had a round morphology characteristic of chondrocytes (distinct from spindle-shaped tendon fibroblasts). However, these cells were not labeled in a parallel experiment with Col2-Cre<sup>TM</sup>; mTmG mice, thus representing a distinct population from the epiphyseal chondrocytes of the neonatal humeral head (data not shown). At this neonatal stage, the humeral head was completely unmineralized with the only source of Ihh arising from the primary ossification center of the humerus (Vortkamp, Pathi et al. 1998). Furthermore, although a few Gli1-positive cells were observed in the perichondrium, the number of Gli1 positive cells at the enthesis was much larger.
Figure 5.1: The enthesis is derived from a unique population of Hh-responsive cells. Gli1Cre<sup>ERT2</sup>;mTmG animals were labeled with TM on the day indicated in green and animals were euthanized on the day indicated in white. Scale is 100 μm.

At P8, the secondary ossification center was evident in the humeral head and two distinct populations of Gli1 expressing cells were observed near the developing enthesis (Figure 5.1E). One population was more abundant, corresponding to the population observed at birth at the tendon-epiphyseal cartilage interface. A second distinct population was separated from the first by a band of non-mineralized epiphyseal chondrocytes, corresponded to the larger hypertrophic
chondrocytes adjacent to the mineralization front of the secondary ossification center (Figure 5.1E). By P14 (Figure 5.1F), a single Gli1-expressing cell population was observed in the enthesis spanning the developing fibrocartilage. Gli1 expression persisted throughout the thickness of the fibrocartilage zone through P28 (Figure 5.1G). By P56 (Figure 5.1H), the Gli1-expressing cell population was limited to non-mineralized areas.

In order to determine the origin of the fibrocartilage cell population, the lineage of Gli1-expressing cells was traced. Cells identified as Gli1-positive at P6 (Figure 5.1E) spanned the entire region of fibrocartilage and the underlying bone by P56 (i.e., all cells in mature fibrocartilage and underlying bone were derived from Gli1 positive cells) (Figure 5.1I). Cells identified as Gli1-positive when labeled at P13 (Figure 5.1F) were restricted to just fibrocartilage cells at P56 (Figure 5.1J), with a narrow gap evident between the cells, underlying bone, and marrow cavity. This gap progressively increased in width as cells were labeled at later developmental stages (Figure 5.1I-L, P6 through P42). This suggests that the Hh-responsive cell population shifts toward the tendon side of the enthesis as the animal matures. Notably, the original Hh-expressing cells remain in the enthesis but are no longer Hedgehog responsive.

### 5.3.2 Hh-responsive cells in the enthesis do not rapidly proliferate

The lineage tracing experiments described above suggest that a distinct cell population, which begins to differentiate early in perinatal development, is responsible for populating and maintaining the mature enthesis fibrocartilage. In order to understand how a small initial cell population (Figure 5.1A-C) could populate the entire fibrocartilage region of the mature enthesis, we performed cell proliferation experiments and measured cell density as a function of animal
age. Using injections of EdU, a small molecule that binds to DNA during the S-phase of the cell cycle, we selectively labeled rapidly proliferating cells \textit{in vivo} and compared this to the Hh-responsive cell population in the enthesis throughout postnatal development. We observed very few rapidly proliferating cells at the enthesis in the early postnatal period. Labeling cells with TAM on P3 and EdU on P5 resulted in only a few proliferating cells near the edges of the Hh-responsive cell population (Figure 5.2A). By P14 and at later stages, no rapidly proliferating cells were observed to overlap with the Hh-responsive cell population (Figure 5.2B and 5.2C). In contrast, proliferating cells were observed in the proliferating zone of the growth plate (Figure 5.2D-5.2F) and in the articular cartilage (data not shown). These results suggest that the Hh-responsive cell population that populates the enthesis proliferates slower than other types of chondrocytes. Another mechanism of tissue growth besides proliferation is an increase in extracellular matrix volume accompanied by a decrease in cell density. In support of this idea, we estimated cell density as a function of animal age for the enthesis (Figure 5.2G). Cell density was defined as the total number of DAPI stained nuclei at the enthesis (i.e., the region marked by the Hh-responsive cell population, including the cells within the mineralized fibrocartilage which were not Hh-responsive at the later postnatal time points). Cell density at the enthesis decreased nearly 3-fold between birth and P42.
Figure 5.2: The Hh-responsive cell population at the enthesis does not proliferate rapidly. Cells from the supraspinatus enthesis A-C) and growth plate D-F) were activated with TAM (red fluorescence) 2 days prior to sacrifice and EdU (green fluorescence) 2-4 hours prior to sacrifice on P5 A) and D), P14 B) and E), and P42 C) and F). The yellow arrow in A) points a few EdU positive cells at the P5 enthesis. The yellow arrows in D-F) indicate proliferating (EdU positive)
cells in the growth plate. Labels indicate t: tendon, i: insertion, 2°: secondary ossification center of the humeral head. Scale bars = 100 μm. (G) Cell density at the enthesis decreases a function of animal age.

5.3.3 Reduced muscle loading increases the Hh-responsive cell population and Hh signaling at the enthesis

Since the Ihh-expressing hypertrophic cell population in the growth plate is known to be modulated by the loading environment (Wu, Zhang et al. 2001; Nowlan, Prendergast et al. 2008), we next examined the effect of muscle forces during postnatal development on the population of Ihh responsive cells in the enthesis. One limb of Gli1CreERT2;mTmG animals was continuously injected with BtxA beginning on P1 and the contralateral limb received simultaneous saline injections. Hh-responsive cells were measured by counting the number of Gli1 positive (green) cells compared to the total number of enthesis cells (DAPI stained) at each time point. BtxA unloaded limbs had a significantly increased number of Hh-responsive cells in the enthesis relative to controls at all time points investigated, which ranged from P28 to P56 (Figure 5.3). Gene expression analysis was then used to measure the relative expression level of genes involved in the hedgehog signaling pathway in BtxA unloaded limbs and contralateral controls. Smo and Ptc, both membrane receptors necessary for Hh signaling, were chosen as target genes. Up-regulation of Ptc expression, like Gli1, is an indicator of activated Hh signaling (Vortkamp, Lee et al. 1996). RNA was extracted from laser micro-dissected regions of the enthesis encompassing both mineralized and non-mineralized fibrocartilage. Expression levels of Smo and Ptc were not significantly affected by unloading at P7 but Smo expression was
significantly up-regulated at P21 and P42 in BtxA unloaded entheses relative to contralateral controls. Ptch expression was significantly up-regulated at P56 and showed a trend toward up-regulation at P21 ($p=.07$). Taken together, these results suggest that muscle forces are critical at later stages of postnatal enthesis to modulate Hh signaling and promote maturation of the enthesis.

**Figure 5.3:** BtxA unloading increases the Hh-responsive cell population in the postnatal enthesis. Supraspinatus entheses from paired control Gli1Cre$^{ERT2}$;mTmG shoulders A), B) and
BtxA unloaded shoulders C), D). Animals were injected with TAM 2-3 days prior to sacrifice on P28 A), C) or P42 B), D) to label the Hh responsive cell population (green cells). The fraction of Hh responsive cells is quantified in E). RT-qPCR gene expression analysis of BtxA unloaded and control shoulders for Smo and Ptch is shown relative to GAPDH in F) and G), respectively. BtxA unloaded shoulders were compared to contralateral controls using paired t-tests. Scale bar = 200 μm.

5.3.4 Ablating the Hh-responsive cell population inhibits fibrocartilage development

To determine if the Hh-responsive cell population we identified is required for fibrocartilage formation at the enthesis, Gli1CreERT2 animals (Ahn and Joyner 2004) were crossed with DTA mice (Voehringer, Liang et al. 2008). In these mice, Cre expressing cells produce diphtheria toxin, ablating the cell that produces it. The timing of cell ablation was controlled by using an inducible Gli1Cre model. Since activation of the DTA;Gli1CreERT2 mouse would lead to ablation of all Hh-active cells, not only those at the enthesis, mice were injected with TAM on P6 (see Figure 5.1D for an example of the enthesis cells that would be ablated at this time point) to selectively ablate enthesis cells while minimizing damage to the development of other organs. P6 labeled DTA;Gli1CreERT2 mice were viable past P42, although they were significantly smaller than TAM injected littermate controls by P14 (Table 5.1). Cells populating the region of enthesis fibrocartilage appeared dead, with absent nuclei at P42 indicated by a lack of DAPI staining (Figure 5.4A and 5.4D). This is consistent with the presence of ablated cells, whose nuclear membranes have broken down.
Safranin-O staining of decalcified sections was less intense in DTA;Gli1Cre\textsuperscript{ERT2} mice at P42 compared to littermate controls (Figures 5.4B and 5.4D, red stain). This indicates that enthesis proteoglycan content was reduced in DTA;Gli1Cre\textsuperscript{ERT2} mice, supporting the idea that the Hh-responsive cell population is responsible for formation and maintenance of the enthesis fibrocartilage.
Figure 5.4: Ablation of the Hh-responsive cell population resulted in a loss of enthesis fibrocartilage. A), D) DAPI stained 6 week control and DTA;Gli1CreERT2 sections demonstrate a loss of nuclei (yellow arrows) in the enthesis 5 weeks post cell ablation. B), E) Safranin-O staining indicated a loss of enthesis proteoglycan in ablated entheses relative to control. C), F) Von Kossa staining demonstrated a decrease in mineralized fibrocartilage in DTA;Gli1CreERT2 compared to control. Scale = 200 μm. G) MicroCT analysis demonstrates mineralized fibrocartilage volume relative to the humeral head volume (MFC.V/(Humeral Head.V) was decreased in DTA;Gli1CreERT2 compared to control. An example MFC volume (green area) from a control animal is shown in H) and I). Scale is 1 mm.

We observed very little remodeling of the enthesis fibrocartilage even 5 weeks after cell ablation. No evidence of vascular invasion or matrix resorption was observed. These results are consistent with the observation that Hh-responsive cells in the enthesis do not rapidly proliferate (Figure 5.2). In contrast, mineralized fibrocartilage in the growth plate was rapidly resorbed and remodeled after ablation of the hypertrophic chondrocytes. Chondrocytes in the growth plate proliferate, activate Hh signaling, undergo hypertrophy, mineralize their matrix, and then undergo apoptosis. Therefore, it is expected that the Gli1-positive cells in the growth plate that were ablated at P6 would be replenished by proliferative chondrocytes that became Gli-positive at a later time point. Therefore, the Gli1-positive cell population associated with the growth plate should be less affected by Gli-1-specific cell ablation than the enthesis population, because these growth plate cells terminally differentiate and undergo apoptosis naturally. Von Kossa staining indicated a decreased amount of mineralized fibrocartilage (MFC) in DTA-Gli1CreERT2 mice compared to littermate controls (Figures 5.4C and 5.4F). These mice were smaller than littermate
controls at P42 and had smaller, poorly mineralized humeral heads, indicative of aberrant overall mineralization (Table 5.1). Despite this, the average mineral density of the MFC was not changed (Table 5.1). The volume of MFC was estimated using microCT and normalized to the humeral head volume to scale for the overall size and mineralization defects observed in these mice. The normalized MFC volume was significantly reduced in DTA-Gli1CreERT2 animals compared to littermate controls (Figure 5.4G). This indicates that the mineralization defect in the enthesis is more severe than in the bone.

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<th>Body Wt. (g)</th>
<th>Insertion BMD (mg HA/cm³)</th>
<th>Humeral Head TV (cm³)</th>
<th>Humeral Head BV (cm³)</th>
<th>Humeral Head BV/TV</th>
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Table 5.1: MicroCT analysis of P42 DTA;Gli1CreERT2 mice. Mean values are shown in bold with the standard deviation below. * indicates $p < 0.05$, # indicates $p = 0.057$.

5.3.5 Reduced Ihh signaling decreases fibrocartilage mineralization

To investigate the necessity of Hh signaling for enthesis development, Smo^{fl/fl} mice (Long, Zhang et al. 2001) were crossed with with ScxCre mice (Blitz, Viukov et al. 2009). Deletion of Smo, necessary for Hh signaling, was targeted to tendon and enthesis progenitor cells using the ScxCre mouse model. Recent studies identified a fetal progenitor population of cells positive for both Scx and Sox9 cells early at the junction of developing tendon and as-yet
unmineralized bone (Blitz, Sharir et al. 2013; Sugimoto, Takimoto et al. 2013). Using a reporter mouse, we verified that ScxCre targeted all of the enthesis and tendon cells (data not shown). There was a severe mineralization defect in the MFC of Smo<sup>fl/fl</sup>;ScxCre (cKO) mice. Staining the mineral using von Kossa’s method showed that entheses from Smo<sup>fl/fl</sup>;ScxCre animals had severely disrupted mineralization relative to controls (Figure 5.5A-B). Histomorphometric analysis of the fibrocartilage thickness revealed that the zone of mineralized fibrocartilage was thinner in Smo<sup>fl/fl</sup>;ScxCre animals while the zone of non-mineralized fibrocartilage was thicker (Figure 5.5C). The overall thickness of the entire fibrocartilage was not significantly different between the two groups. This result suggests that deletion of Smo results in a defect in mineralization of fibrocartilage cells but no change in the amount of fibrocartilage at the enthesis. MicroCT analysis revealed that both the fibrocartilage volume and mineral density were significantly reduced in Smo<sup>fl/fl</sup>;ScxCre animals compared to littermate controls (Smo<sup>fl/fl</sup>) (Figure 5.5D-E). In contrast, there were no observed differences in the trabecular bone parameters within the humeral head, suggesting that the mineralization defect was localized to the entheses (Table 5.2). This result is consistent with expression patterns for the ScxCre mouse model, which targets fibrocartilagenous attachments but not the remainder of the epiphysis. XRD analysis of the MFC indicated that the size of the hydroxylapatite crystals was reduced in cKO animals compared to controls (Figure 5.5F). The coefficient of variation of the crystal grain sizes among the 20 analysis spots within each sample was narrower in cKO animals relative to controls (Table 5.2). No significant differences in the mineral crystal orientation (FWHM of azimuthal intensity) or mineral ε<sub>RMS</sub> were observed (Table 5.2). Previous results have demonstrated that Ihh regulates the transition from round, resting/early proliferative chondrocytes to stacked columnar chondrocytes in the growth plate (Kobayashi, Soegiarto et al.
2005; Hilton, Tu et al. 2007). In the enthesis, toluidine blue staining of decalcified sections revealed that the fibrochondrocytes in the mineralized fibrocartilage region of cKO animals were significantly larger than cells in this region from control animals (data not shown).

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</table>

**Table 5.2**: Mineralization patterns in ScxCre; Smo^{fl/fl} cKO animals. MicroCT analysis indicated no differences in trabecular architecture in cKO animals. Average values are shown in bold with the standard deviation below. * indicates $p < 0.05$ relative to controls.

Uniaxial tensile testing was used to evaluate the functional changes caused by reduced Hh signaling during development. The tendon cross-sectional area was not significantly altered when Hh signaling was deleted in tendon progenitor cells (Figure 5.5I). Tendon enthesis strength and stiffness, however, were significantly reduced in Smo^{fl/fl};ScxCre animals compared to controls (Figure 5.5G-H). Additionally, the normalized material properties maximum stress, yield stress, yield strain, and the energy to yield (modulus of resilience) were significantly reduced in conditional knock-out animals compared to controls (Table 5.3). Together, these
results suggest that reducing Hh signaling in tendon and enthesis progenitors throughout development results in reduced mechanical properties of the enthesis.

**Figure 5.5**: Hh signaling is necessary for enthesis fibrocartilage mineralization. A), B) Von Kossa staining in control and ScxCre;Smo<sup>fl/fl</sup> entheses demonstrated aberrant mineralization in cKO animals. T: tendon, MFC: mineralized fibrocartilage, B: bone. Scale bar = 100 μm. C)
Fibrocartilage (FC) thickness was increased and mineralized fibrocartilage (MFC) was decreased in ScxCre;Smo\textsuperscript{fl/fl} entheses compared to control entheses. D), E) MicroCT quantification of mineralized fibrocartilage volume and mineral density demonstrated a mineralization defect in ScxCre;Smo\textsuperscript{fl/fl} mice compared to control mice. F) Hydroxylapatite crystal size, as measured by XRD, was reduced in ScxCre;Smo\textsuperscript{fl/fl} mice compared to controls mice. G), H) Uniaxial tensile testing revealed that enthesis function was impaired in ScxCre;Smo\textsuperscript{fl/fl} mice compared to control mice. Specifically, the ultimate strength and stiffness of the supraspinatus enthesis were reduced. I) Tendon cross-sectional area was unchanged in ScxCre;Smo\textsuperscript{fl/fl} mice compared to control mice.

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Table 5.3: Biomechanical properties of ScxCre;Smo\textsuperscript{fl/fl} mice. Mean values are shown in bold with the standard deviation below. * indicates $p < 0.05$ relative to Smo\textsuperscript{fl/fl} controls.

5.4 Discussion

A unique population of Gli1-positive cells, distinct from tenocytes and epiphyseal chondrocytes, exists at the enthesis in the perinatal period. Unlike the Gli1-expressing zone of chondrocytes adjacent to the mineralization front in the growth plate, this cell population appears well before mineralization begins at the enthesis. Additionally, as demonstrated by lineage
tracing experiments, this cell population does not undergo apoptosis like growth plate hypertrophic chondrocytes. Instead, these cells and their progeny remain at the enthesis throughout postnatal development and populate the entire fibrocartilage between tendon and bone. This process is summarized in Figure 5.6. The mechanisms of enthesis fibrocartilage formation and mineralization are therefore likely different than those for endochondral bone formation. As mineralization is established at the enthesis, instead of undergoing hypertrophy and apoptosis like the chondrocytes of the growth plate, enthesis fibrochondrocytes in mineralized fibrocartilage lose their Hh-responsiveness and remain viable. The population of Hh-responsive cells then shifts toward the non-mineralized side of the enthesis. These observations suggest that a loss of Hh signaling in mineralized fibrocartilage has a protective effect on the fibrochondrocyte lineage in the mineralized matrix. This is in contrast to the Hh-responsive hypertrophic cells of the growth plate, which undergo apoptosis followed by remodeling of the matrix into bone. This model of enthesis formation is supported by our results using DTA-Gli1CreERT2 mice; the fibrocartilage in these mice remained acellular and the matrix was not remodeled 5 weeks after cell ablation. This tissue therefore lacks the pro-angiogenic and matrix degradation signals necessary to promote tissue remodeling and is likely a factor in the poor healing response seen at adult tendon-to-bone interfaces.
Figure 5.6: Schematic illustrating the Hh-active cell populations throughout enthesis development.

The source of the Ihh ligand during enthesis development and maturation remains unclear. Ihh is known to act in a paracrine fashion in developing bones. The sources of the Ihh ligand in this case are the hypertrophic cells in the primary ossification center (Vortkamp, Lee et al. 1996; Vortkamp, Pathi et al. 1998; Long, Zhang et al. 2001). The cells that respond to this Ihh are found in the perichondrium and to a lesser extent in the growth plate proliferating zone. Our results suggest a somewhat different scenario for Ihh regulation at the developing enthesis (See schematic in Figure 5.7). The Hh-responsive cells at the developing enthesis are likely even more responsive than the perichondrial and proliferating chondrocytes in the neonatal humerus. After the first week of postnatal development, the secondary ossification center is present in the humeral epiphysis but the mineralization front has not reached the Hh-responsive cell population at the enthesis. At this stage, the source of Ihh at the enthesis is likely from the mineralization front of the secondary ossification center (Vortkamp, Pathi et al. 1998). By P21, the fibrocartilage zone is mineralized, yet most of the cells in the mineralized and non-mineralized zones remain Hh-responsive. At this point, it is unclear what the source of Ihh might be, but it is
possible that it is still stabilized in the mineralizing extracellular matrix by heparin sulfate proteoglycans (Jochmann, Bachvarova et al. 2013; Whalen, Malinauskas et al. 2013). As enthesis mineralization proceeds, cells in the MFC are no longer Hh-responsive. It is possible that the extracellular matrix-stabilized Ihh has degraded at this point and the Hh-responsive cell population shifts toward the non-mineralized fibrocartilage. It is possible that these cells then begin to produce Ihh.

Figure 5.7: Schematic of Ihh-related gene expression patterns in the postnatal enthesis.

Our results further suggest that the down-regulation of Hh signaling that occurs after enthesis mineralization is modulated by the loading environment. When muscle loading was removed at birth, there was an increase in the number of Hh-responsive cells by P28. This result
compliments previous studies that have shown that Ihh expression is either upregulated or
downregulated respectively in response to increased or decreased cyclic compressive loading
(Wu, Zhang et al. 2001; Ng, Chiu et al. 2006; Chen, Sorensen et al. 2009; Shao, Wang et al.
2012). Here, decreased cyclic tensile loads (less compressive) led to increased Ihh signaling.
This was accompanied by a mineralization defect in the humeral head and increased osteoclast
activity, which suggests increased resorption and remodeling of endosteal surface of the
mineralized fibrocartilage (Thomopoulos, Kim et al. 2007). These results support the idea that
decreased Hh signaling in the MFC has a protective effect on the fibrocartilage by preventing its
remodeling into bone. The unloading experiments in the current study demonstrate that muscle
forces play a critical role in reducing Ihh expression in MFC after mineralization, preventing
tissue degradation and remodeling into bone.

Hh signaling also plays an important role during the earlier stages of postnatal enthesis
development. Mineralization of the enthesis occurs between P7 and P14; the cell ablation
experiments, performed within this time frame, demonstrated that the Hh-responsive cell
population is critical for normal mineralization. Mineralization during this time period may be
driven by biochemical signals and may be less influenced by muscle loads, evidenced by no
changes in gene expression due to unloading at P7. This is consistent with the findings of Blitz et
al., who showed that the initiation of bone ridge formation required only biochemical signals
from the tendon, while the subsequent growth phase required muscle forces (Blitz, Viukov et al.
2009). Furthermore, reduced Hh signaling in cells from tenocyte and fibrochondrocyte lineages
led to severely disrupted fibrocartilage mineralization. Conversely, the total width of the
fibrocartilage transitional zone identified by the presence of a rounded cell morphology was not
significantly reduced in size in animals lacking Hh signaling. This suggests that cell
differentiation from enthesis precursor cells to a rounded fibrochondrocyte cell phenotype is less affected by Hh signaling. Instead, this process may be modulated instead by another pathway such as BMP signaling. Blitz et al. showed that Scleraxis expression in tendon cells regulates BMP4, leading to bone ridge formation at tendon attachment sites (Blitz, Viukov et al. 2009). BMP signaling has also been shown to induce Ihh signaling in chondrocytes (Grimsrud, Romano et al. 2001; Minina, Wenzel et al. 2001). Based on these findings, it is probable that BMP signaling drives fibrocartilage development while Hh signaling is subsequently required for enthesis mineralization.

In contrast to the large mineralization defect observed in the enthesis of the Smo°/°;ScxCre mice, no mineralization defect was observed in the trabecular bone of the humeral head. This implies that the loss of fibrocartilage volume is not due to decreased thickness in the layer of trabecular bone underlying the mineralized fibrocartilage. We did observe a small but significant decrease in humeral length in these mice. This phenotype may be caused by the ScxCre model targeting the cell populations that lead to the bony prominences at the ends of bones which contribute to overall bone length (Blitz, Sharir et al. 2013). These prominences are often attachment sites for tendons and ligaments. Lending further support to defective fibrocartilage mineralization in this model, we observed that the size of the mineral crystals is reduced as well as their size distribution. This is consistent with a more immature mineralized matrix in which hydroxyapatite has nucleated on the collagen template but crystal growth has been limited. The reduced biomechanical function of the enthesis suggests that the zone of mineralized fibrocartilage is crucial for modulating the transfer of muscle loads from tendon to bone.
Due to the early postnatal time points investigated in this study, using an oral gavage method of TAM administration was not possible. In some of the lineage tracing animals, a small amount of corn oil was observed in the scruffs at the time of dissection. This suggests that the timing of some of the labeling experiments may not be precise and the TAM administration may have lasted longer than a few days. This is less of an issue in the very early time points, as the TAM was scaled by animal body weight, resulting in very small volumes of drug compared to adult animals. Furthermore, the population of cells labeled in the P7→P56 experiments did not overlap with the cells labeled in the P56 acute experiments, suggesting that the lineage tracing results were indeed accurate.

5.5 Conclusions

In summary, a unique population of cells was identified in the fibrocartilagenous enthesis that were Hh-responsive in the perinatal period and fully populated the mature enthesis fibrocartilage. Hh signaling in this cell population was regulated by muscle loading. Ablation of this population resulted in a cell-free enthesis weeks after cell death, suggesting that this tissue is resistant to remodeling and a loss of this fibrocartilage cell population due to injury is likely permanently detrimental to the enthesis. Knock-down of Hh signaling in these cells affected fibrocartilage mineralization but not differentiation of fibrochondrocytes. These results suggest that this cell population and Hh signaling might play a critical role in fibrocartilage healing after injury. Preliminary results tracking this cell population in a tendon defect model will be presented in the next chapter.
5.6 Acknowledgements

Alix Black, Zhongzhou Cai, and Jon Almer assisted with the XRD/XRF experiments performed at the APS at Argonne national lab. Crystal Idleburg assisted with histology.
Chapter 6: The role of hedgehog signaling at the healing enthesis in neonatal and adult mice

Summary

The previous chapter illustrated the important function of Ihh signaling during enthesis development. In this chapter, we aim to elucidate the involvement of Ihh signaling in tendon-to-bone healing. A new surgical model was developed to produce fibrocartilage injuries at the enthesis. This model will enable investigations into the biological mechanisms involved in fibrocartilage healing by utilizing the abundantly available varieties of genetically modified mice. As tendon-to-bone healing in adults heals via scar, without regeneration of a fibrocartilage enthesis, understanding what genes are mis-regulated during the healing process may lead to new therapies for enhanced repair. Using a simple needle-puncture fibrocartilage defect, early postnatal mice and mature mice were injured in order to compare potentially differential healing patterns that may occur with aging. Preliminary results using this model suggest that younger animals resolve fibrocartilage defects more quickly than adults. Application of this model to Gli1Cre\textsuperscript{ERT2};mTmG mice showed that the Hh-responsive cell population identified in the previous chapter appears to be recruited to the defect site in young animals. However, once the cells differentiated into mature mineralized fibrochondocytes in adults, they were no longer able to actively participate in the healing/remodeling process. Preliminary experiments investigating the role of Hh signaling in tissue remodeling after injury support previously reported results that showed up-regulation of Hh leading to ectopic calcifications in the tendon. Further studies with
this model could enhance our understanding of the role of Hh signaling and other pathways critical to fibrocartilage healing.

6.1 Introduction

Enthesis fibrocartilage, like cartilage at other locations, does not readily regenerate or heal after an injury and is prone to age-related degeneration (Steinert, Ghivizzani et al. 2007; Killian, Cavinatto et al. 2012). Many musculoskeletal injuries require surgical repair of tendon to bone. Examples of this include rotator cuff tears, where the tendons often fail near their bony attachment sites, and anterior cruciate ligament (ACL) reconstructions, which require tendon graft healing in a bone tunnel. Fibrocartilage is not recreated at the healing tendon-to-bone attachment site; rather, the insertion is replaced by mechanically inferior scar tissue (Aoki, Oguma et al. 2001; Thomopoulos, Williams et al. 2003; Galatz, Sandell et al. 2006; Lui, Zhang et al. 2010). This leads to repair failure rates as high as 94% for massive rotator cuff tears, and loosening of grafts after ACL reconstruction (Galatz, Ball et al. 2004). In light of these clinical results, the objective of the current study was to develop a model system with which to study healing of fibrocartilagenous attachments, with the hope of inspiring novel therapeutics that might improve tendon-to-bone healing outcomes.

Previous animal models of tendon injury and repair have included rats and other larger animals (Aoki, Oguma et al. 2001; Soslowsky, Thomopoulos et al. 2002; Ditsios, Leversedge et al. 2003; Thomopoulos, Zampiakis et al. 2008; Edelstein, Thomas et al. 2011). While these models are useful for recreating surgical repair scenarios, the use of larger animals precludes the use of most genetic manipulations available in mice. Currently available mouse tendon injury
models include patellar tendon defects (Dyment, Kazemi et al. 2012; Lui, Cheuk et al. 2012) and Achilles tendon transections (Ansorge, Hsu et al. 2012). Patellar tendon models are clinically relevant, as they mimic the tendon harvest procedure used in ACL surgeries. Achilles tendon transections have been applied to neonatal animals (Ansorge, Hsu et al. 2012), but this model leads to damage of the tendon midsubstance rather than the enthesis fibrocartilage. Mouse shoulders, particularly from early postnatal animals, have the limitation of being too small to accurately and reproducibly repair. In light of these considerations, a simple punch needle defect was employed in this study to simplify the surgical technique while still creating a reproducible fibrocartilage defect in the murine supraspinatus.

Skin and tendon develop during fetal time points, and wound healing of these tissues in embryos occurs via regeneration rather than the scar-mediated process seen in adults (Yokoyama 2008). In contrast, as shown in previous chapters, enthesis fibrocartilage in mice develops and mineralizes during the first several weeks after birth (Galatz, Rothermich et al. 2007; Schwartz, Pasteris et al. 2012). Due to this, we hypothesized that fibrocartilage defects in early postnatal mice would heal via regeneration, recapitulating development, compared to adult mice, which would heal via scar. In support of this hypothesis, a recent mouse femur fracture model demonstrated that early postnatal animals were able to heal large displaced fractures that would have resulted in deformities and non-unions in adult mice (Dr. Elazar Zelzer, personal communication). Furthermore, a separate study investigating tendon midsubstance defects in early postnatal achilles tendons found evidence of differential healing between younger and older animals (Ansorge, Hsu et al. 2012).

Despite observed differences in injury responses, the molecular mechanisms controlling differential healing in fetal, neonatal, and adult healing remain unknown. As described in the last
chapter, the Ihh pathway is critical for enthesis mineralization and subsequent development of tissue mechanical properties. The role of this pathway in healing fibrocartilage, however, has yet to be determined. In this study, we examined the role of Ihh signaling in enthesis healing in early postnatal and adult mice using the previously described Gli1Cre<sup>ERT2</sup>;mTmG model to trace Hh responsive cell populations throughout the healing process.

6.2 Methods

6.2.1 Animal Model of Enthesis Injury

The use and handling of animals was approved by the animal studies committee at Washington University. Gli1Cre<sup>ERT2</sup> mice crossed with the Rosa-mTmG Cre reporter were chosen in order to investigate the role of Hh signaling in fibrocartilage healing. At either 1 week (early postnatal group), or 6 weeks (adult group), animals underwent a unilateral fibrocartilage injury. Animals were anesthetized using inhaled Isofluorane in oxygen. The right forelimb was externally rotated and secured with tape. A small skin incision was made in the coronal plane over the right shoulder. The acromial branch vessel was cauterized and used to locate the supraspinatus attachment. A 28 G needle was manually inserted into the humeral head to make a punch defect into the supraspinatus enthesis. The needle was inserted deep enough to completely bisect the mineralized fibrocartilage into the marrow cavity. The wound was closed using a 6.0 proline suture and animals were allowed to heal for 1, 3, or 6 weeks.

Animals received a 200 µg/g body weight injection of tamoxifen (TAM) either on P4 to label the Hh active cell population that would form the mineralized fibrocartilage, or 3, 7 or 14 days post injury to label cells actively expressing Hh during the healing process (Figure 6.1). 2-5
animals were used for each injection time point. Animals were euthanized using carbon dioxide 1, 3 or 6 weeks following the injury. The supraspinatus muscle-tendon-bone complex was dissected free of all other tissues and fixed overnight in 4% paraformaldehyde. The next day, samples were washed with PBS and dehydrated to 70% ethanol.

**Figure 6.1**: Schematic of the study design. Animals were injured at 1 week, top panels (early postnatal injury) or 6 weeks of age, bottom panels (adult injury). TAM (green) was injected on P4 to label the Hh-responsive cell population that forms the MFC or 3, 7, or 14 days after the injury to label Hh activity during the healing process. Animals were euthanized 1 or 3 weeks after the injury.
6.2.2 MicroCT Analysis of Injured Entheses

The distal humerus was potted in agarose inside the bottom of a conical tube. The conical tube was inverted within the analysis tube in order to suspend the supraspinatus tendon and muscle in air, optimizing soft tissue contrast. Samples were scanned using Micro-computed tomography as described in previous chapters (microCT) (μCT40; Scanco Medical AG, Switzerland) to visualize the defect to the mineralized fibrocartilage and to determine the scar volume. Scans were performed at X-ray tube settings of 45 kV and 177 μA with a 200 ms integration time, resulting in a 20 μm voxel size. Scans were analyzed using two different thresholds to visualize the soft tissue (used to determine scar volume) and the mineralized tissue. Visual inspection of three dimensional reconstructions was used to evaluate bony defects. An example defect from a P42 animal sacrificed 1 day post injury is shown in Figure 6.2.

Figure 6.2: Example of the needle punch defect in an adult (P42) animal euthanized 1 day post injury. A) MicroCT superior-lateral view demonstrates that the needle defect can be visualized
post injury. (B) A coronal cut-plane view demonstrates that the injury is localized to the fibrocartilage and the needle penetrates through the mineralized fibrocartilage. Scale = 1 mm.

6.2.3 Histological Analysis

After microCT scanning, samples were rehydrated, washed with PBS, soaked in sucrose solution, embedded in OCT medium, and sectioned on a cryostat. 10 μm sections were thawed, washed with PBS to remove remaining OCT, and mounted with aqueous mounting medium containing DAPI. Sections were imaged using an Olympus IX51 microscope in epifluorescence mode.

6.3 Results

6.3.1 Differential healing in early postnatal injuries compared to adults

To investigate differences in healing between the early postnatal and adult groups, microCT was used to analyze differences in the scar volume and the extent of damage to the mineralized fibrocartilage. Scar volume was estimated by measuring the soft tissue volume 0.5 mm proximally and distally to the top of the humeral head and normalizing this to the same volume obtained from the uninjured control limb. Young animals had a trend toward decreased normalized scar volume (1.3 ± 0.6, N=12) compared to adult animals (1.9 ± 0.8, N=18) (p = 0.07). These measures had considerable variation due to the preliminary nature of this study and were pooled across all of the healing durations investigated.
The presence or absence of a bony fibrocartilage defect was visually graded (Yes/No) from the microCT reconstructions of each sample relative to the contralateral uninjured control. We observed an increased percentage of samples that did not exhibit an easily visualized bony defect in the younger animals (N=12) after 3 weeks of healing compared to the adult group (N=15) (Figure 6.3A). Additionally, for the adult group, the number of healed bony defects increased with increasing healing time (Figure 6.3B).

Figure 6.3: Gross morphologic evaluation of bony defects post fibrocartilage injury. A) The fraction of mineralized fibrocartilage defects healed in early postnatal animals compared to adults. (* \( p = 0.07 \) based on a chi-squared test). B) The number of healed bony defects increased with increasing healing time in the adult group.
6.3.2 Hedgehog-responsive cells were localized to enthesis defects in young animals

Utilizing the Gli1Cre\textsuperscript{ERT2};Rosa-mTmG mouse as a reporter of Hh activity at the enthesis, we labeled cells during the first week of development to identify the Hh-responsive cell population, discussed in the previous chapter, that forms the mineralized fibrocartilage. In young animals, this cell population that is actively involved in fibrocartilage development and mineralization is also actively involved with healing. The defect is indicated by the yellow arrow in Figure 6.4A, with the contralateral (uninjured) shoulder shown in 6.4E. Figure 6.4F shows the epifluorescence image of the contralateral limb demonstrating the normal extent of Ihh-responsive cell labeling for this animal in green. In the injured limb, shown in Figure 6.4B, the injury is marked by a large area lacking DAPI staining (yellow arrow). Based on our previous observations in Chapter 5 using the diphtheria toxin cell ablation model, this suggests that the injury trauma likely causes cell death. Fewer green cells are observed in the injured enthesis compared to the control, but a group of Hh-responsive cells can be seen lining the edge of the defect. These cells may be participating in the healing process. This is further supported by the potentially proliferative pattern of cells shown in Figure 6.4D. One caveat to this experiment is that the TAM was administered only three days prior to the injury in the younger animals and our previous observations suggest that the residence time of TAM in the injection may be longer than three days. Because of this, it is not possible to conclusively determine whether these cells were Hh-responsive prior to injury or became Hh-responsive soon after the injury. Regardless, this preliminary data suggests that Hh-responsive cells in young animals are likely also involved in healing fibrocartilage defects as well as fibrocartilage development.
Figure 6.4: Hh-responsive cells labeled prior to injury were localized to the site of a fibrocartilage at P28 for an injury sustained P1. A)-D) Injured shoulder, the yellow arrows indicate the defect site. E)-G) Contralateral control (uninjured) shoulder. A) and E) MicroCT images were used to visualize the bony defect (indicated by the yellow arrow in A), scale bar = 1 mm. B), D), F) Epifluorescence images of the supraspinatus enthesis. Green cells are Hh-responsive, all other cells are red, and the nuclei are stained with DAPI (blue). Hh responsive cells (green) lined the edge of the defect (dark area) in B). A group of potentially proliferative cells (green) at the edge of the defect (dark area) is shown in D). F) Epifluorescence image of the normal control limb. Scale bar = 100 µm. C), G) Toluidine blue stained images corresponding to the respective images in B) and F). Scale bar = 100 µm.
6.3.3 The early Hh-responsive cell population does not participate in adult fibrocartilage injuries

We investigated the role of the early Hh-responsive cell population on fibrocartilage healing in adult mice. Gli1Cre^{ERT2};Rosa-mTmG animals were injected with TAM on P5 and sustained a fibrocartilage injury on P42. Animals were euthanized one week after injury (Figure 6.5A-D) or 3 weeks after injury (Figure 6.5E-F). In both cases, scar formation was evident at the defect site. After only 1 week of healing, fragments of green cells or matrix were observed in the defect site (yellow arrow, Figure 6.5A). At this stage of healing, the damaged matrix was likely still being resorbed, as the observed tissue was a mix of non-green labeled cells with a fibroblast-like morphology. After 3 weeks of healing, a mass of scar with numerous fibroblastic cells was observed at the defect site, but fragments of green cells/matrix were no longer observed (Figure 6.5E-F, yellow arrow). This is consistent with the well-described scar-mediated healing in tendon and other fibrous tissues. A control shoulder demonstrating the normal pattern of the early Hh-responsive cell population is shown in Figure 6.5C-D.
**Figure 6.5:** Evaluation of the response of the Hedgehog responsive cell population (green) in adult fibrocartilage injury. Gli1Cre$^{ERT2}$:mTmG animals received TAM on P5 and a fibrocartilage injury on P42. Animals were euthanized after 1 week of healing (A-D) or 3 weeks of healing (E-F). The yellow arrows indicate the scar developing within the injury site. A) After one week of healing, the damaged fibrocartilage is being resorbed and there are a few green cells evident in the scar. B) The toluidine blue stained region corresponding to A). E) After 3 weeks of healing, the fibrocartilage is being replaced by fibrous tissue and no green cells are evident in the fibroblastic scar (yellow arrow). F) The toluidine blue stained region corresponding to E). Scale is 100 μm.
6.3.4 *Hedgehog activity in the healing enthesis*

To examine the involvement of Hh signaling in the fibrocartilage healing process, we again utilized the Gli1Cre\textsuperscript{ERT2};mTmG mouse to provide a temporally controlled read-out of Hh-responsive cells. Animals were injured on P7 or P42 and TAM was injected to label cells 3, 7 or 14 days after the injury. In an animal injured on P7 and labeled with TAM on P14, we observed labeling of most of the remaining fibrocartilage (Figure 6.6B), consistent with previous results. There were also additional Hh positive cells extending toward the tendon side of the defect and a few possibly positive fibroblasts near the bone side of the defect. These results suggest that early damage to the fibrocartilage induces additional Hh expression near the tendon.
Figure 6.6: Hh activity during healing of early postnatal fibrocartilage injury. An example mouse injured on P7 and labeled with TAM 1 week after injury A)-C) Injured shoulder, D)-E) control shoulder. MicroCT reconstructions of the injured A) and control D) shoulder complex. The yellow arrow in A) indicates the mineralized fibrocartilage defect. Scale is 1 mm. (B) Epifluorescence view of the injury site. The white arrow indicates a population of Hh active cells within the tendon matrix. The yellow arrow points to a scar forming to heal the mineralized defect that contains a few Hh positive cells. Additional Hh positive cells line the fibrocartilage defect. The corresponding toluidine blue stained section is shown in C) with a black arrow instead of white indicating cells with a rounder morphology characteristic of chondrocytes within
the tendon. An epifluorescence view of the control shoulder is shown in E) and the corresponding toluidine blue stained section is shown in F). Scale bar = 100 μm.

Next, we investigated Hh activity during the fibrocartilage healing process in adult animals. Figure 6.7 shows examples of mice injured on P42 that were injected with TAM to label Hh active cells 3 days after injury (Figures 6.7A-B) and 7 days after injury (Figures 6.7E-F). The control limb corresponding to Figure 6.7A and 6.B is shown in Figures 6.7C and D. By comparison to the control level of Hh-responsive cells shown in Figure 6.7C, it appears that fibrocartilage damage initially reduces the number of Hh-responsive cells in the fibrocartilage adjacent to the injury (cyan arrows). The defect site itself appears to be filled with large, potentially immune-related, cells that are not conclusively Hh-responsive (yellow arrows, Figure 6.7A).

In samples labeled with TAM 7 days after injury (Figure 6.7E-H), Hh positive cells were observed within the fibrocartilage scar. The yellow arrows in Figures 6.7E’ and F’ indicate an injured region of fibrocartilage that contains fibroblast-like Hh positive cells. Figures 6.7E’’ and F’’ illustrates a region within the tendon that contains Hh-positive cells around the border of a fibroblast filled scar. This supports previous observations that have noted chondrogenic differentiation within tendon midsubstance scars (Rooney, Grant et al. 1992; Lui, Cheuk et al. 2012; Rui, Lui et al. 2012).
Figure 6.7: Hh activity during healing of a fibrocartilage defect in P42 animals. Panels A)-D) show a sample injured on P42 and the labeled with TAM on P45 to identify Hh-responsive cells (green). The yellow arrows in A) and B) point to the scar forming in the fibrocartilage. Less Hh-activity was observed in the remaining fibrocartilage post injury (cyan arrows) relative to the control enthesis shown in C). The corresponding toluidine blue stained images to A) and C) are
shown in B) and D) respectively. Panels E)-H) show a sample injured on P42 and labeled with TAM on P49. The boxed regions in E) and F) are shown in the corresponding insets E’), F’), E”’) and F”’). E’’) and F’’) show that the scar near the bone side of the enthesis (yellow arrow) contains Hh responsive cells (green). A region of the scar near the tendon side of the insertion is shown in E”’) and F”’) and Hh active cells (green) were observed around the edges of the scar (indicated by the yellow arrow). Scale is 100 μm.

### 6.4 Discussion

This chapter describes the development of an enthesis injury model that can be applied to neonatal mice. This model enables injury studies in genetically modified murine models, which should help to elucidate the biological mechanisms involved in the healing process. One limitation of this model is that it is not analogous to clinical injuries in the rotator cuff. The needle size can be tailored to damage approximately the middle third of the tendon attachment in neonatal through mature shoulders, analogous to the harvest of the patellar tendon for an ACL reconstruction.

One question that was partially examined in this study was the differential course of healing between immature and mature enthesis tissue. We hypothesized that since the enthesis fibrocartilage is still developing during the first few weeks after birth, fibrocartilage defects might heal by a more regenerative process than the classical scar-mediated healing observed in adults. Although further investigations are needed, our preliminary results suggest that early postnatal injuries heal via a scar and do not regenerate like embryonic injuries. The results of this study do suggest that mineralized defects are healed more quickly in younger animals.
We next explored the role of Hh signaling in fibrocartilage healing using the fibrocartilage punch defect model. In chapter 5, we identified a Hh-responsive cell population that is critical for mineralized fibrocartilage formation at the entheses. Here, we examined the role of this cell population in tendon healing both in early postnatal animals, before mineralized fibrocartilage is fully established, and in adult animals that have mature fibrocartilage prior to injury. Preliminary observations suggest that the Hh-responsive cell population is likely involved in the repair process of the damaged fibrocartilage tissue in the younger group of animals but once these cells have terminally differentiated into mature mineralized fibrocartilage, they do not participate in repair and remodeling of damaged fibrocartilage. This potential early regenerative capacity suggests that these cells from early postnatal animals may have some stem cell-like characteristics. Future experiments could examine the healing capacity of these cells in adult tendon-to-bone injuries.

We also performed preliminary experiments examining activation of Hh signaling during the fibrocartilage healing process. Increased Hh-signaling in tendon defects is an indicator of chondrogenesis, which leads to pathological ectopic calcifications (Rooney, Grant et al. 1992; Lui, Cheuk et al. 2012; O'Brien, Frank et al. 2012; Rui, Lui et al. 2012). In fibrocartilage defects, the level and timing of Hh activation likely requires tight regulation in order to repair the mineralized matrix defect while avoiding pathological ossifications and destruction of non-mineralized fibrocartilage. Initial observations suggest that cells recruited to the defect site one week after injury may be Hh-responsive. These cells have a fibroblast-like morphology and are probably involved in the proliferation and potentially the early remodeling stages of healing. These Hh-active cells are likely critical for repairing the mineralized fibrocartilage matrix in a similar manner to fracture healing. However, the native mineralized fibrocartilage cells down-
regulate Hh signaling likely to protect the fibrocartilage from destruction and further remodeling into bone. This probably does not occur during healing, leading to a replacement of the fibrocartilage with structurally inferior bone-like tissue. It is possible that in adult fibrocartilage defects, reduced Hh signaling might be beneficial to the later stages of healing. In support of this, decreased Hh signaling in articular cartilage defects has been demonstrated to improve cartilage healing (Zhou, Wei et al. 2013). However, the timing of reduced Hh signaling is likely critical and may correlate with the recommendations for mechanical stimuli during healing (Eliasson, Andersson et al. 2011; Killian, Cavinatto et al. 2012; Schwartz and Thomopoulos 2013). Immobilization during the initial stage of healing is beneficial, while immobilization later during healing may have detrimental effects. This would be consistent with our observations from development, which suggest that unloading throughout development results in up-regulation of Hh signaling, leading to loss of fibrocartilage. The ideal amount of Hh signaling during healing of defects sustained during early postnatal development might be more complicated. During this stage, higher levels of Hh signaling are probably required for longer durations in order to prevent developmental mineralization defects.

Some of these hypotheses might be tested in further experiments utilizing this injury model. We performed some pilot experiments using the ScxCre;Smo\textsuperscript{fl/fl} animals described in the last chapter to investigate the potential benefits of reduced Ihh signaling during healing (data not shown). However, this is likely not the best approach because these mice have significant baseline mineralization defects and this model does not allow temporal control over the level of Hh signaling. A better approach for future experiments might be to use the TAM inducible Gli1Cre to temporally control deletion of Smo or a chemical Hh inhibitor. With this approach,
Hh levels might be elevated initially to promote repair of the mineralized matrix and then reduced to protect the healing tissue from further remodeling.

6.5 Conclusion

In summary, this chapter describes pilot experiments developing a murine enthesis injury model that can be used to investigate the biological mechanisms involved in fibrocartilage healing. We showed that this model can be successfully applied to early postnatal and mature animals and is amenable to use in the numerous genetically modified murine models available. Future experiments are needed to further investigate the patterns of Hh signaling during fibrocartilage healing. Additional experiments could also use this model to manipulate Hh expression levels during healing for enhanced repair.

6.6 Acknowledgements

We would like to thank Dr. Leesa Galatz for performing all of the enthesis injuries.
Chapter 7: Conclusions and Future Directions

7.1 Summary of the Dissertation

Tendon-to-bone injuries such as tears to the rotator cuff or anterior cruciate ligament (ACL) are consistently plagued by high failure rates (Friedman, Sherman et al. 1985; Robertson, Daniel et al. 1986; Harryman, Mack et al. 1991; Galatz, Ball et al. 2004). This is because current repair protocols do not lead to regeneration of the complex transitional tissue critical for modulating the stress transfer between tendon and bone (Galatz, Sandell et al. 2006; Silva, Thomopoulos et al. 2006). In order to devise better repair strategies, it is useful to study the natural developmental process of the tissue. Tendon enthesis development represents a successful biological solution to the fundamental problem of joining two materials with very different material properties. Understanding the development of this tissue may enable us to elucidate the critical factors that might be applied to improve tissue regeneration after injury.

As a first step, we investigated mineralization patterns in the developing enthesis. Previously, a mineral gradient had been identified in the rat supraspinatus enthesis (Wopenka, Kent et al. 2008) and this unique structural feature was found to have important mechanical consequences (Genin, Kent et al. 2009; Liu, Thomopoulos et al. 2012). Additionally, it was noted that this structural feature is lost during injury and repair (Wang, Su et al. 2013). We observed a narrow mineral gradient at the edge of the mineralization front that remained constant throughout postnatal development. This region was located within the epiphyseal cartilage at P7 and P10 and within the developing fibrocartilage thereafter. The structure and composition of
the organic component of the extracellular matrix dictated the nano-scale morphology of the mineral.

Next, we sought to estimate the mechanical consequences of a constant mineral gradient throughout development. Estimated muscle forces were much lower at P7 and P10 than in the mature enthesis. This suggested that the mineral gradient may not be as important for reducing peak stresses in young animals and may have a different function. By combining the above results describing the mineral gradient with additional morphological data and histomorphometry, we found that the mineral gradient resulted in elevated local stresses near mineralizing hypertrophic chondrocytes within the epiphyseal cartilage. This high local stress could provide a mechanical stimulus that triggers mineralization and advancement of the mineral front.

We followed these studies with an examination of the role of muscle forces in the development of a functionally robust enthesis. Mechanical cues are critical not only to the development of musculoskeletal tissues, but also for mature tissue homeostasis and the healing process (Nowlan, Sharpe et al. 2010; Killian, Cavinatto et al. 2012). Previous work in the Thomopoulos laboratory has demonstrated that muscle forces are required for supraspinatus enthesis development (Thomopoulos, Kim et al. 2007; Kim, Galatz et al. 2009; Das, Rich et al. 2011). In this thesis, using the same model of botulinum toxin unloading, we demonstrated that removing muscle forces throughout postnatal development resulted in a functionally inferior enthesis. These functional losses could be partly attributed to changes in the extracellular matrix collagen structure and to alterations to the nano-scale and micro-scale mineral composition.

We next sought to investigate biological factors that might be critical to enthesis development. The indian hedgehog (Ihh) signaling pathway was determined to be a promising
candidate since this molecule is a critical regulator of endochondral bone formation (Kronenberg and Chung 2001; Lai and Mitchell 2005). Additionally, this pathway has identified as mechano-responsive in a number of previous studies (Ng, Chiu et al. 2006; Shao, Wang et al. 2012). We demonstrated that this pathway is required for enthesis mineralization during postnatal development. Additionally, we identified a unique cell population in the neonatal enthesis that is responsible for populating and maintaining the mineralized fibrocartilage at the enthesis. This cell population was initially hedgehog (Hh)-responsive during fibrocartilage development, but Hh pathway activation was turned off in cells in the mature enthesis. We hypothesized that this down-regulation of Hh signaling was required for tissue homeostasis because it protects the mineralized fibrocartilage from degradation and remodeling. This idea was supported by a set of experiments in limbs that develop without muscle forces, where Hh-activity increased in the enthesis and was accompanied by an increase in the number of osteoclasts, the cells responsible for matrix resorption. Consistent with the modeling results demonstrating elevated local stress around mineralizing chondrocytes at the enthesis, these in vivo results suggest that fibrocartilage cells require mechanical stress to down-regulate Hh activity. This Hh activity may then protect the fibrocartilage from being remodeled into trabecular bone, thus maintaining the thickness of the mineralized fibrocartilage layer, and allowing for effective transfer of muscle forces from tendon to bone.

In the final chapter of the thesis, we developed a fibrocartilage injury model that could be applied to early postnatal animals to study the healing process in young animals relative to mature animals. Preliminary results from this study indicate a possible role of Hh signaling in healing of tendon enthesis injuries. This model will be be used in future studies with genetically
modified mice to examine the biological factors critical for fibrocartilage healing and regeneration.

7.2 Limitations and Alternative Approaches

All of the work presented in this thesis was performed using murine models. The structure of the mouse skeleton is somewhat simpler than human or other larger animals. Mouse bones, tendons and cartilage layers are much smaller than the corresponding tissues in larger animals, while the size of the cells is roughly the same. Based on this, it is unknown how comparable the enthesis structural features are between species. Further studies are required to establish how the width of the mineral gradient, for example, scales between species and what mechanical consequences result from this. Furthermore, the larger distances, for example, between zones of cells in human cartilage compared with murine cartilage, might also confound paracrine signaling patterns, such as Ihh and PTHrP, that rely on diffusion through the extracellular matrix. Additionally, the human genome is much larger and contains more redundancy than the murine genome. For these reasons, further studies are required to verify that the biological signaling patterns observed in the mouse models presented here are applicable to the human condition.

We used Raman microprobe analysis to investigate spatial variations in mineral content in freshly frozen tissue sections. The samples were air dried briefly before analysis, which led to preferential shrinkage of the non-mineralized areas of the enthesis. This was observed by the sample visibly moving during some of the experiments. This might confound the results by making the mineral gradient appear shorter. This effect was limited by using thin tissue sections

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on adhesion slides. Furthermore, all samples were prepared in the same manner, so inter-group comparisons should not be affected. An alternative approach would be to use a larger volume of liquid to submerge the sample, but this makes it difficult to determining the optimum focal distance for the measurements. This drying effect was similarly observed in the samples used for the X-ray diffraction measurements. These samples were mounted vertically with the bone side of the insertion facing down, which limited movement during shrinking. However, the drying process of these tissue sections likely affected the local strain environment of the mineral compared to native, fully hydrated tissue. As an alternative, these experiments could be performed in a custom water bath, although this might adversely affect the X-ray signal intensity.

The results presented in chapter 3 depend on a linear elastic axisymmetric model of the developing enthesis. The assumptions and simplifications used in the model were necessary because many of mechanical and geometric parameters were not readily available. The major model parameters investigated were the gross changes in the geometry of the attachment while maintaining a constant mineral gradient size, and the size and volume fraction of the cells. However, one important enthesis feature neglected in this model was the role of the local extracellular matrix structure and composition. Collagen fiber alignment and density have been shown to affect enthesis material properties (Genin, Kent et al. 2009). Throughout the developmental stages, the material properties of the “fibrocartilage” layer of the model were assumed to be identical to tendon. In fact, at the early time points P7 and P10, the extracellular matrix is cartilaginous, which has significantly different mechanical behavior than tendon due to a random fiber orientation, lower collagen content, and higher water and proteoglycan content. This could be accounted for in the model by changing the fibrocartilage material properties as a function of age. Another model limitation is that the axisymmetric model, motivated by the
action of the rotator cuff tendons on the humerus, does not account for the more complex loading environment of many entheses including the supraspinatus, which experiences a mixture of tensile and compressive loads, depending on the position of the joint. Future models might account for more complicated tendon enthesis geometries.

In Chapter 4, a botulinum toxin A (BtxA) unloading mouse model was used to examine the necessity for muscle forces during postnatal development. In several assays, differences were observed between the saline injected limbs and normal control limbs. The saline limbs originate from the animals injected with BtxA. However, control experiments with paired saline injected and normal limbs did not display significant differences. The differences observed in the saline group outcomes compared to normal controls likely result from decreased mobility of the animals due to the paralyzed limb.

The biomechanical assays performed throughout this thesis tested the entire supraspinatus tendon-bone complex. This tissue has a complicated geometry and variations in tissue properties that might confound a detailed analysis of the mechanical behavior. The cross section varies along the length of the tendon. The load-bearing midsubstance of the tendon is completely enclosed in an outer tendon sheath, the epitenon, which has visually different mechanical properties. A stain pattern that is applied to this exterior layer may therefore not accurately represent the local deformations within the tendon substance. As this layer was too thin to reproducibly remove in mouse tendons, this is a necessary limitation to testing mouse supraspinatus tendons. As all the tendons were treated identically, these methods still provide useful information about overall tissue function. However, other methods are required to test the mechanical implications of micro-scale structural features such as the mineral gradient described in Chapter 2. A potential future experiment might test laser micro-dissected beams from the
mineral gradient region using a custom atomic force microscope modified for this purpose (Hang and Barber 2011; Hang, Lu et al. 2011; Hang, Gupta et al. 2014).

While it is well established that the BtxA unloading model causes gross morphological joint patterning defects and loss of trabecular bone, it remains unclear how BtxA affects the volume and mineral density of the mineralized fibrocartilage. This could be determined using microCT analysis. We showed that there are micro- and nano-scale mineralization differences within the mineralized fibrocartilage of unloaded limbs compared to controls. However, how these changes in mineral crystal properties translate to enthesis structure and function requires further investigation. The mineral crystals could be visualized using TEM to examine their morphology and location with respect to the organic matrix components.

Tamoxifen was used to label the Hedgehog active cells during the lineage tracing experiments. This lipophilic drug must be dissolved in oil and was injected into the scruff to limit trauma to neonatal animals. Upon dissection, in many animals, small pockets of oil were observed at dissection up to several weeks after injection. It is unknown if these oil droplets still contain TAM. This observation suggests that the timing of TAM administration may not be precise. However, the administered volume was scaled to the animal weight, so the TAM dose remaining would likely be negligible after several weeks of rapid murine growth. Additionally, lineage tracing experiments showed a different labeled cell population that overlapped very little with the population labeled later in development. This increased duration of TAM delivery conceivably also affected the cell ablation model. The timing of TAM administration might be improved in older animals by switching to an oral gavage method where the oil would be cleared by the digestive track. However, this is not a feasible alternative in early postnatal animals, which are very small.
Another shortcoming of the lineage tracing experiments is that this experiment assayed for Hh-responsive cells but did not determine the cellular source of the secreted Ihh signal itself. This may be inferred from the literature for the initial stages of postnatal development (Vortkamp, Pathi et al. 1998). In situ hybridization experiments using a radio-labeled probe for Ihh could be used to localize the source of the Ihh secreted protein in late postnatal enthesis. Laser-capture microdissection with RT-qPCR analysis could also be used to verify the incidence of Ihh in the enthesis fibrocartilage, but this technique would not localize the signal to specific cell populations within the enthesis.

One surprising drawback related to the cell lineage tracing experiments was the lack of Col2Cre expression observed in the enthesis. Two different TAM inducible Cre lines (Nakamura, Nguyen et al. 2006; Hilton, Tu et al. 2007) were investigated and neither model showed any expression in the enthesis fibrocartilage. Immunostaining for two isoforms of collagen II (data not shown) and in situ hybridization (Galatz, Rothermich et al. 2007) demonstrated that collagen II is present in the enthesis fibrocartilage throughout development, albeit at a lower level than the adjacent articular cartilage. One possibility is that the low expression level produces an intracellular concentration of Cre protein that is below the threshold required for detection with a reporter. Another explanation could be that the genetic regulation of collagen II expression in the enthesis is different than in the articular cartilage and the promoters used to drive Cre expression are lacking a regulatory element needed for expression within the fibrocartilage.

Postnatal tendon and fibrocartilage are not very metabolically active and these tissues have very low rates of turnover and remodeling. This likely confounded the proliferation experiments, as the most commonly used proliferation markers (BrdU, EdU, PCNA, and p-
HistoneH3) are optimized for use in rapidly proliferating cell populations common to developmental studies. For instance, the residence time of EdU in a mouse is only ~2 hours. Even repeated administration of EdU labeled very few cells in the postnatal enthesis. One alternative approach that might facilitate comparisons of the rate of enthesis cell proliferation throughout development might be to use implanted pellets of EdU/BrdU. These pellets provide a continuous release of drug for an extended duration.

Laser-capture microdissection was used to isolate the approximately 1 mm x 200 μm area of the enthesis fibrocartilage in mice. Due to the extremely small size of this region, the less than ideal tissue processing conditions, and the high laser power required to cut mineralized tissue, the RNA yields obtained from these experiments were extremely low and the RNA quality analysis indicated likely degradation. In spite of this, the tissues from both experimental and control groups were handled identically and the RNA quantity and quality was not significantly different between groups. Therefore, in spite of the poor RNA quality, it is likely that any trends observed between groups are valid. As an alternative, a larger number of sections could be pooled to improve the RNA quantity, but this would not necessarily improve the quality. Alternative tissue processing techniques could be explored including using methacarn to fix the RNA prior to processing. In general, our observations indicate that using fresh frozen tissue for laser capture is the best option for RNA. Another alternative is to macro-dissect the insertion fibrocartilage and extract the RNA directly. However, this method results in significant contamination from other tissue types that might overshadow localized gene expression trends in the fibrocartilage.

To delete Hh signaling from the developing enthesis, we used a Smoothened conditional knockout model that was targeted to the entire tendon and enthesis progenitor population
(ScxCre). This model was not inducible, which resulted in gene deletion early in embryonic development. Because of this, the required timing of Ihh signaling during fibrocartilage development remains unknown. This might be investigated using the inducible Gli1Cre\textsuperscript{ERT2} or ScxCre\textsuperscript{ERT2} mice to delete Smo.

The fibrocartilage injury model described in Chapter 6 had several shortcomings. First, the needle punch defect employed in this model is not analogous to typical rotator cuff injuries. There are alternative tendon injury models that are more clinically relevant, such as removing the middle third of the patellar tendon, which mimics the tendon harvest used for ACL reconstructions (Dyment, Liu et al. 2013); however, this technique is too challenging to use with early postnatal mice. Additionally, our model completely transects the mineralized fibrocartilage layer, introducing cells from the bone marrow into the defect site. These cells are not native to a tendon injury and may confound the healing process. This study was preliminary in nature and requires more animals to verify the results presented regarding Hh expression during injury. Furthermore, a sham group that receives the surgical procedure without the needle-punch defect should be included as an additional control.

7.3 Future Directions

One question pertaining to the mineral gradient described in Chapter 2 is to determine where the mineral accumulates in relation to the collagen fibers in the regions of low mineralization and the process by which it is deposited. We observed different patterns of mineralization in the epiphyseal cartilage compared to the fibrocartilage of the mature enthesis. This suggests that the composition of the organic matrix template to be mineralized may guide
hydroxylapatite crystallization patterns. Previous in vitro experiments have suggested that mineral might accumulate in the gap regions within the interior of collagen fibrils prior to accumulation on the exterior of the fibrils (Nudelman, Pieterse et al. 2010). Our initial observations suggest that the opposite might be true within the enthesis fibrocartilage. This is not necessarily the case in all mineralization situations; it is possible that osteoid might accumulate mineral on the interior of collagen fibrils. This makes sense in the case of tendon, a material that is biologically resistant to mineralization in vivo. It is possible that the collagen fibrils that extend into the fibrocartilage from tendon are resistant to mineralization, so at the interface, mineral preferentially accumulates on the exterior of fibrils. As the collagen composition changes into the mineralized fibrocartilage, mineral begins to accumulate within collagen fibrils as well as around them. To test this hypothesis, there are several possible approaches. One is to directly label newly deposited mineral in vivo in a manner that is detectable at the nano-scale, possibly by using an elemental substitution of barium or strontium for calcium within the mineral and using TEM-EELS to detect substitution patterns in the mineralized matrix. This approach has the limitation that altering the chemical properties of the mineral might affect the mineralization process. An alternative method might be to investigate in vitro mineralization of various de-calcified natural matrices, such as tendon, fibrocartilage, osteoid, etc. This method is also limited in that the various mineral removal techniques also affect the organic matrix. It does have the advantage of being able to more precisely control mineralization conditions than the in vivo models.

In this thesis, we examined two models that displayed aberrant enthesis mineralization patterns, BtxA unloading and Smo ckO. As discussed above, it is unknown how changes in the organic components of the extracellular matrix might affect the mineralization process. We
showed in Chapter 4 that the organization of the collagen fiber alignment is affected by BtxA unloading. It is likely that these two models will also have other differences in organic matrix structure and composition relative to normal entheses. Differences in proteoglycan content, collagen content and type could be investigated using immunostaining or comparative proteomics analysis.

Using a lineage tracing approach, we identified a potential progenitor population of cells early in development that is necessary for fibrocartilage formation. Preliminary results using a fibrocartilage defect model hinted that these cells might have some regenerative capability during early postnatal development. To further test the regenerative capabilities of this cell population, these cells could be isolated from early postnatal animals, sorted using FACS and implanted into fibrocartilage defects in mature animals.

Furthermore, the lineage tracing experiments demonstrated that activated Hh signaling is required early in development but expression is down-regulated in mineralized areas at later developmental stages. We hypothesized that this might be required to protect the fibrocartilage from the remodeling into bone that occurs after terminal differentiation of growth plate chondrocytes. This hypothesis was supported by the result that in BtxA unloaded limbs, where there was an increase in Hh activity accompanied by increased remodeling of the endosteal surface underlying the fibrocartilage. This hypothesis might be reinforced by examining the role of markers linked to matrix remodeling, such as matrix metalloproteinase 13 and pro-angiogenic factors in normal enthesis development and in BtxA unloaded animals and Hh deficient animals.

A related question that remains to be addressed is: what is the involvement and timing of Ihh signaling during fibrocartilage healing? Our preliminary results and those of others suggest that Ihh is likely involved in the remodeling process that repairs a mineralized defect in the
fibrocartilage. It remains to be determined what the optimal timing and level of Ihh signaling might be during the healing process in order to repair mineralization defects without leading to pathological calcifications within the tendon. Experiments using the fibrocartilage defect model combined with an inducible conditional knockout model such as the Gli1Cre\textsuperscript{ERT2};Smo\textsuperscript{fl/fl} animals or Hh inhibitory drugs could be used to explore this.

One aspect of the role of Ihh signaling in enthesis development that was not explored in this work is the role of PTHrP. Ihh has been shown to act in both PTHrP dependent and PTHrP independent manners in endochondral bone development (Long and Ornitz 2013). PTHrP is highly expressed and plays a critical role in the development of many fibrous entheses (Wang, Nasiri et al. 2013; Wang, VanHouten et al. 2013). These authors have noted that fibrous entheses form via intramembranous ossification instead of endochondral ossification. Additionally, they have implied that PTHrP expression is much lower in fibrocartilagenous entheses. Based on this observation, it is unclear whether Ihh signaling acts in a PTHrP-independent manner in entheses and if so, what other signals might be involved in Ihh regulation in entheses.

7.4 Conclusions

This dissertation examines the roles of muscle loading and Hedgehog signaling in the developmental processes leading to the complex structure and function of fibrocartilaginous entheses. Previously, it was not well understood how the mineralization patterns critical to the functional behavior of the mature enthesis arose during development and what their mechanical consequences might be. Furthermore, it was unknown what biological and physical factors impact this developmental process. We investigated postnatal mineralization patterns across multiple length scales and presented hypotheses regarding the mechanical consequences of these
structural features based on a multi-scale modelling approaches. Next, we investigated changes in the multi-scale structural and compositional features of entheses that developed in the absence of muscle forces and identified the mechanical consequences of these changes. We identified the Ihh signaling pathway as a critical modulator of enthesis development and mineralization patterns and showed that this process was dependent on muscle loading. Finally, we developed a fibrocartilage injury model that was used to examine the involvement of Hh signaling in enthesis healing. Taken together, the work presented in this thesis investigated factors critical to the natural development of fibrocartilagenous entheses and demonstrated that this work has the potential to guide the development of novel therapeutics for tendon-to-bone repair.
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