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Review

Growth and mechanobiology of the tendon-bone enthesis

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ABSTRACT

Tendons are cable-like connective tissues that transfer both active and passive forces generated by skeletal muscle to bone. In the mature skeleton, the tendon-bone enthesis is an interfacial zone of transitional tissue located between two mechanically dissimilar tissues: compliant, fibrous tendon to rigid, dense mineralized bone. In this review, we focus on emerging areas in enthesis development related to its structure, function, and mechanobiology, as well as highlight established and emerging signaling pathways and physiological processes that influence the formation and adaptation of this important transitional tissue.

1. Introduction

During development, the absence or constraint of movement can lead to musculoskeletal disorders such as fracture-prone brittle bones, malformed and dysplastic joints, respiratory and neurological impairments, and life-long mobility problems [1]. Our ability to move relies on the transmission of muscle forces to the skeleton in order to articulate joints and maintain stability during standing, walking, and sitting. Tendons are cable-like connective tissues that attach muscle to bone and are essential for the transmission of both active and passive muscle loads [2,3]. In the mature skeleton, the tendon-bone enthesis is an interfacial zone of transitional tissue located between compliant, fibrous tendon to rigid, dense mineralized bone [4–7]. This transitional tissue provides a mechanism of stress and strain reduction at the interface between two mechanically dissimilar tissues [5,8,9]. The transmission of muscle loads from tendon to bone is essential for both enthesis development as well as healing. Complete removal of loading during enthesis healing, such as following rotator cuff injury, leads to impaired mechanical integrity following surgical repair [10] and further exacerbates poor healing outcomes following chronic injury [10–14].

Paleoanthropological studies have often inferred the mechanically-dependent adaptation of fibrocartilaginous entheses to describe occupational histories of skeletons [15]. This is often evaluated from skeletal remains by examining bony structural features such as ridges, tubercles, and tuberosities (i.e., protuberances on the periosteal surface of bone). However, a direct link between bone shape and loading histories especially in paleoanthropological archives is complicated and difficult to clearly define [16,17], and the ability of the mature enthesis to adapt to mechanical loads remains contested [18–20]. Nonetheless, the enthesis is at risk of overuse injuries and pathology, clinically referred to as enthesopathies, and such injuries can affect adolescent and adult patients alike. In adolescents, sports-related injuries such as Sever and Osgood-Schlatter diseases are common clinical pathologies affecting the apophysis of bone at sites of tendon-bone entheses in children between the ages of 8–15 years [21]. In this review, we focus on emerging areas in enthesis development related to its structure, function, and mechanobiology, as well as highlight known and emerging signaling pathways that contribute to the formation of this important transitional tissue.

2. Structure, function, and development of the enthesis

Tendon-bone entheses are positioned on the periosteal surface of bone and are typically found at sites of “superstructure” ridges, known as tuberosities and tubercles. These superstructures give bones their three-dimensional shape [22–24]. The microscopic structure of the enthesis varies depending on its anatomical location and mechanical demands and is characterized as either fibrous (i.e., periosteal, bony) or fibrocartilaginous [6]. Fibrous entheses are generally found at insertion sites of stabilizing tendons, whereas fibrocartilaginous entheses are typically found at insertions of tendons that contribute to joint movement. Fibrous enthesis attach directly to bone and typically form Sharpey’s fibers, which are perforating fibers that embed into bone’s periosteal surface [25]. Fibrocartilaginous entheses consist of four distinct histological zones, including aligned tendon, unmineralized

Abbreviations: Col, Collagen; BMP, Bone morphogenetic protein; FGF, Fibroblast growth factor; FGFR, Fibroblast growth factor receptor; Gli, GLI-Kruppel family member; Hh, Hedgehog; IFT, Intraflagellar transport protein; PTHrP, Parathyroid hormone related protein; Scx, Scleraxis; Sox9, SRY-Box Transcription factor 9; Spd, Splotched-delayed, muscle-less; TGFβ, Transforming growth factor-beta.

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fibrocartilage, mineralized fibrocartilage, and subchondral bone. A smooth and uniform basophilic tidemark distinguishes the transition between the two fibrocartilaginous zones, and this tidemark is disrupted and irregular in enthesopathy. The fibrocartilage enthesis matures during postnatal growth in response to mechanical loads from skeletal muscle and consists of cells that express both tenogenic and chondrogenic factors [24,26–29] (Fig. 1).

Recent work has defined a general mechanism of isometric scaling (i.e., proportional growth of superstructure size relative to bone size) that minimizes cumulative superstructure drift along the length of bones [22]. Superstructures form modularly from a distinct pool of cells that express both *Scleraxis* (*Scx*) and *Sox-9*, and these superstructures are reliable phenotypic readouts of enthesis development and muscle loading during embryonic and postnatal growth in the vertebrate limb (Fig. 2) [23,27]. The global patterning of superstructures is regulated by numerous factors, including the GLI-Krüppel family member 3 (*Gli3*), transforming growth factor Tgf*b*, bone morphogenetic protein (BMP4), and pre-B cell leukemia transcription factor (e.g., *Pbx1*) [23,24]. Superstructure progenitors differentiate into either chondrocytes (on the cartilage side) or fibroblasts (on the tendon side) [24,27,30], and this bi-fated cell mixture is regulated by Krüppel-like (KLF) transcription factors [26]. Depending on the type of enthesis (i.e., migratory or stationary), these progenitor populations are either replaced by or differentiate into *Gli1*+ cells that eventually become the enthesis [30,31]. In the limb, the unique molecular signature of the enthesis depends on its anatomical positioning (i.e., at the epiphysis, periosteal surface, or attached to superstructures [6,24,32,33]). The *Sox9*+/*Scx*+ cells of the enthesis, sandwiched between chondrocytes and tendon fibroblasts, have recently been identified as "bi-fated," as these cells express a mixed transcriptome of both chondrogenic and tenogenic genes (Fig. 1) [26]. This shared transcriptome between two otherwise distinct cell types may suggest that enthesis progenitors share regulatory elements with both chondrocytes and tendon fibroblasts, and these shared regulatory elements (e.g., KLF) act as enhancers to drive expression in enthesis cells as well as in adjacent cartilage (e.g., *Col11a1* associated elements) and tendon resident cells (e.g., *Col1a1* associated elements) [26].

The mechanoadaptive nature of the developing limb has been studied for decades using muscular dysgenesis models in mice. During limb development, tendon forms as an extension of the cartilage template and is later loaded by striated muscle. The formation of tendon is predominantly muscle-independent, with few exceptions [34]. However, the segregation, elongation, and maintenance of tendon typically depends on applied loads upon the migration of muscle into the limb bud from the dermomyotome [23,34–37]. Loss of muscle loading during embryonic growth impacts the growth but not the initiation of bone ridge formation, suggesting that the primordial superstructure emerges prior to the attachment and contraction of muscle (Fig. 2) [27]. These superstructures are important for skeletal function because they are three-dimensional bony structures that provide mechanical leverage to muscle for efficient movement of articular joints and also provide tendon with a stable anchorage site to bone [23]. The emergence of superstructures, where entheses attach, occurs prior to muscle migration into the limb bud and is dependent on expression of the basic helix-loop-helix transcription factor, *Scleraxis* and *Sox9* (Fig. 2) [38]. The maintenance of superstructures during embryonic growth relies on skeletal muscle contraction (Fig. 2) [23]. This has also been demonstrated during postnatal growth, and the structure and mineralization of the tendon-bone enthesis depends on muscle loading for interface maturity [28,29,39]. The mineralization patterns and multi-scale structure of the enthesis have been well described in recent experimental and computational research [4,5,8,40,41]. In the absence of postnatal muscle loading, fewer *Gli1*+ cells populate the tendon-bone interface [30] and the enthesis is less mineralized and mechanically weaker [28,39]. Conversely, in some models of muscle hypertrophy (e.g., *myostatin*$^{-/-}$ mice), tuberosities are enlarged in the postnatal skeleton [42]. Although technically challenging, new approaches to spatially and temporally control muscle contraction in vivo, such as use of light-activated muscle contraction using optogenetic stimulation, offer promise for use in mechanistic studies to improve our understanding of the mechano-adaptive response of the enthesis during postnatal growth [43–45]. Because each enthesis is uniquely loaded depending on its anatomical location and mechanical demands, there exists a broad

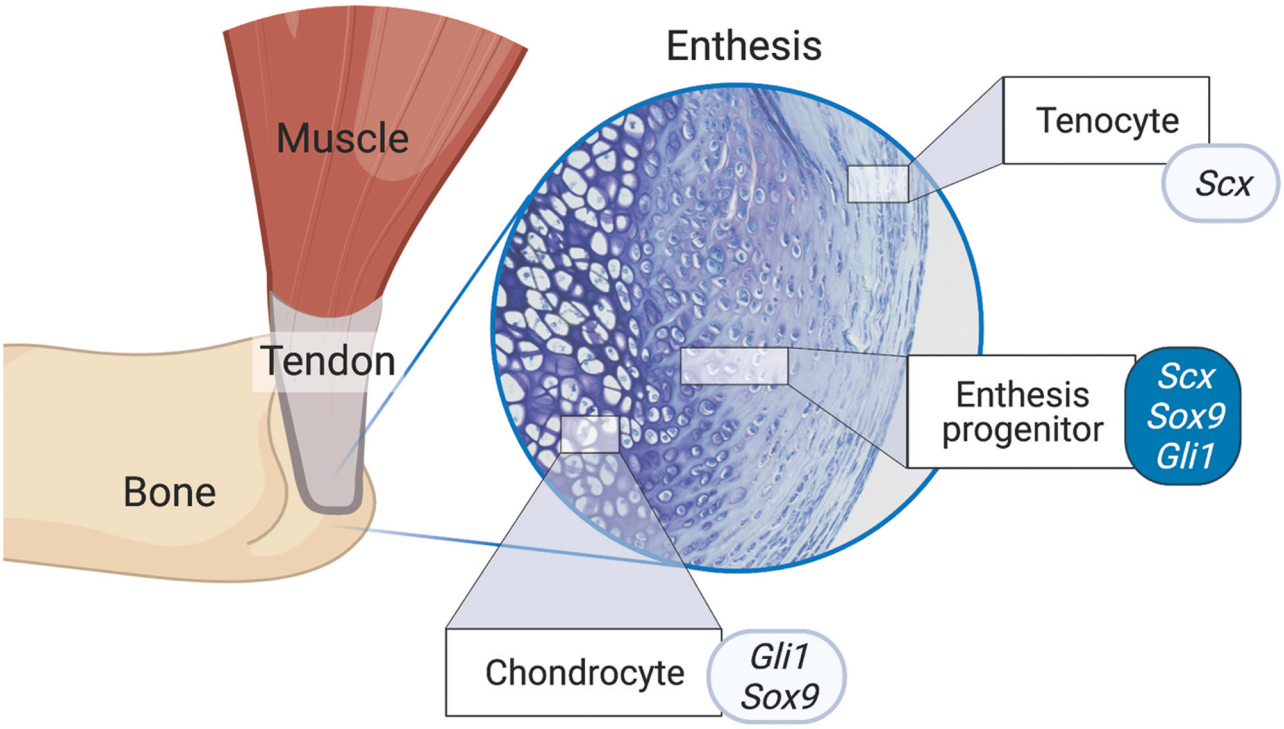

Fig. 1. The enthesis is a transitionally graded tissue positioned between bone and tendon. The primordial enthesis develops from bi-fated progenitor cells expressing chondrogenic and tenogenic factors (i.e., *Scx*, *Sox9*, and *Gli1*). Created with BioRender.com.

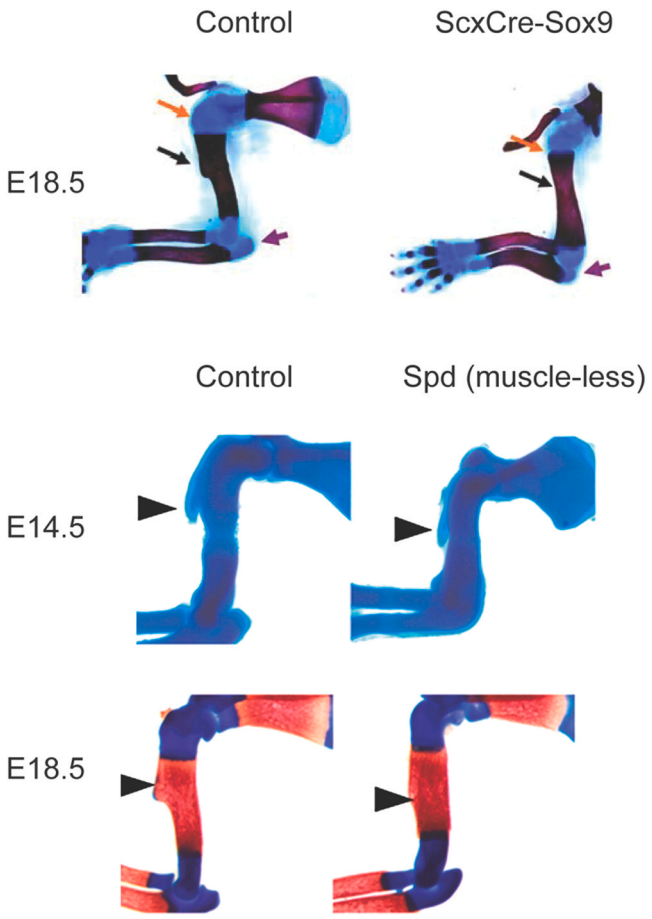

Fig. 2. The deltoid tuberosity is a superstructure on the humerus and is a reliable readout of tendon and enthesis development as well as muscle loading in the embryonic limb. In ScxCre-Sox9 mutant mouse embryos (top row), the deltoid tuberosity (black arrow) and triceps insertion (purple arrow) fail to initiate formation. In *muscleless* mouse embryos, the formation of the deltoid tuberosity is initiated at embryonic day (E) 14.5; however, without muscle contraction, the tuberosity is not maintained by E18.5. Images modified with permission from [23,27].

diversity of enthesis structure and size. For example, Felsenthal et al. recently identified divergent cell programming in migratory and stationary entheses that depends on the dynamic maintenance and replacement of *Sox9*+ and *Gli1*+ cells during postnatal growth (Fig. 3) [31]. Thus, developing a better understanding of enthesis diversity and mechanoadaptation is needed.

3. Signaling pathways regulating enthesis formation and remodeling

The development of tendon and maturation of the enthesis plays a crucial role in joint shape and alignment [7,23,29,39]. Tendon-bone entheses and long bone growth plates develop with overlapping and divergent cell behaviors. Most long bones develop via endochondral ossification, a process of mesenchymal differentiation and sequential replacement of cartilage with bone through expansion of growth plates. This process is mirrored in the tendon-bone enthesis, with establishment of chondrogenic cells (e.g., *Sox*9+) and sequential replacement or differentiation into fibrocartilage cells (e.g., *Gli1*+) [31]. Morphologically, the development of the enthesis has been likened to a "miniature" or arrested growth plate [6,8,30,47]. However, unlike growth plates in long bones which eventually fuse at skeletal maturity, the fibrocartilage of the enthesis retains the morphological features of fibrocartilage and maintain Gli1+ cells at the interface throughout postnatal growth [30].

A major challenge of studying enthesis progenitors at the transcriptional or epigenetic level is the limited number of cells that reside within a matrix dense tissue, which makes isolation for high-quality sequencing approaches difficult. Additionally, there does not yet exist an exclusive marker for enthesis progenitors that can be exclusively used for enthesis lineage tracing and targeting with Cre-lox strains in mice (Table 1). Kult et al. recently generated a compound mouse line with transgenes for Sox9-CreER, Scx-GFP, and tdTomato in an effort to sort enthesis progenitors using fluorescence-activated cell sorting [26]. This approach, coupled with advancements in single-cell RNA sequencing, established a useful method to identify enthesis-specific promoters that do not overlap with adjacent limb tissues. Additionally, the use of high-precision microdissection approaches to isolate region-specific cells, such as laser-capture microdissection microscopy [29,30,48], have also shown promise, and improvements in RNA preservation prior to laser-capture methods could further improve RNA quality and rigorous downstream molecular analyses. Ideally, the identification of promoter(s) that exclusively target enthesis progenitors would allow for mechanistic studies using Cre-lox; however, there does not yet exist an "enthesis-specific" Cre strain that does not significantly overlap with other tissues (Table 1). For example, although used extensively for enthesis-related investigations, both *Scx*-Cre and *Sox9*-Cre lineages are also localized to tendon, cartilage, and/or perichondrium, as well as organs such as the kidney, brain, and lungs. Additionally, depending on the timing of induction, *Gli1*-CreERT2 targets a broad range of tissues with hedgehog-responsive cells including the growth plate of long bones, mesothelium, kidney, spinal cord, forebrain, and vasculature. This known and significant overlap in lineage specificity with other tissues is a challenge for enthesis-related research. Therefore, new discoveries in the identification of novel promoters that could be used for Cre-lox or CRISPR-based recombination have the potential to advance the field. For example, the development of paired recombination and inversion strategies using Cre-lox and FLP-FRT to exclusive target *Sox9/Scx* co-expressing cells and not *Sox9*- or *Scx*-only cells could strengthen enthesis-only specification and could improve robustness of targeting only entheseal progenitors instead of flanking tissues.

In spite of these technical challenges related to enthesis specificity, much of what we know and understand of enthesis development has been discovered using transgenic mouse lines for the controlled specification, expression, and deletion of specific genes. Phenotypic readouts, such as the size and shape of tuberosities, provide a first-pass assessment of enthesis-related changes. The gradient morphology, mineralization, and mechanical strength of the enthesis are also important readouts for assessing the contributions of specific genes to enthesis development in transgenic strains. Some emerging pathways in enthesis development, described below, could provide insight into mechanisms of enthesis degeneration. The use of mouse and other vertebrate models (e.g., zebrafish) provide tools to mechanistically test biological pathways of enthesis development which is also critical for identifying potential regenerative strategies following injury.

3.1. TGF-β and BMP signaling

The transforming growth factor-β (TGF-β) superfamily includes a family of proteins, such as TGF-βs (TGF-β1 and TGF-β3) and bone morphogenetic proteins (BMPs, e.g., BMP2 and BMP4). TGF-β signaling is a critical pathway for joint and tendon development [60,61,68–71]. The recruitment and maintenance of differentiated tendon cells is regulated by TGF-β and its receptors, including TGF-β2, TGF-β3, and TGFβR2 [60,69]. Additionally, deletion of either TGFβR2 in the limb bud or BMP4 in tendon in mice leads to a complete loss of the deltoid tuberosity, suggesting it also regulates enthesis and superstructure specification [23,27,69,72]. The requirement of TGFβR2 for tuberosity growth likely depends on tendon (e.g., *Scx*Cre) but not cartilage (e.g., *Col2a*Cre). Canonical TGFβ signaling involves ligand binding via receptors on the cell surface followed by translocation to the nucleus via

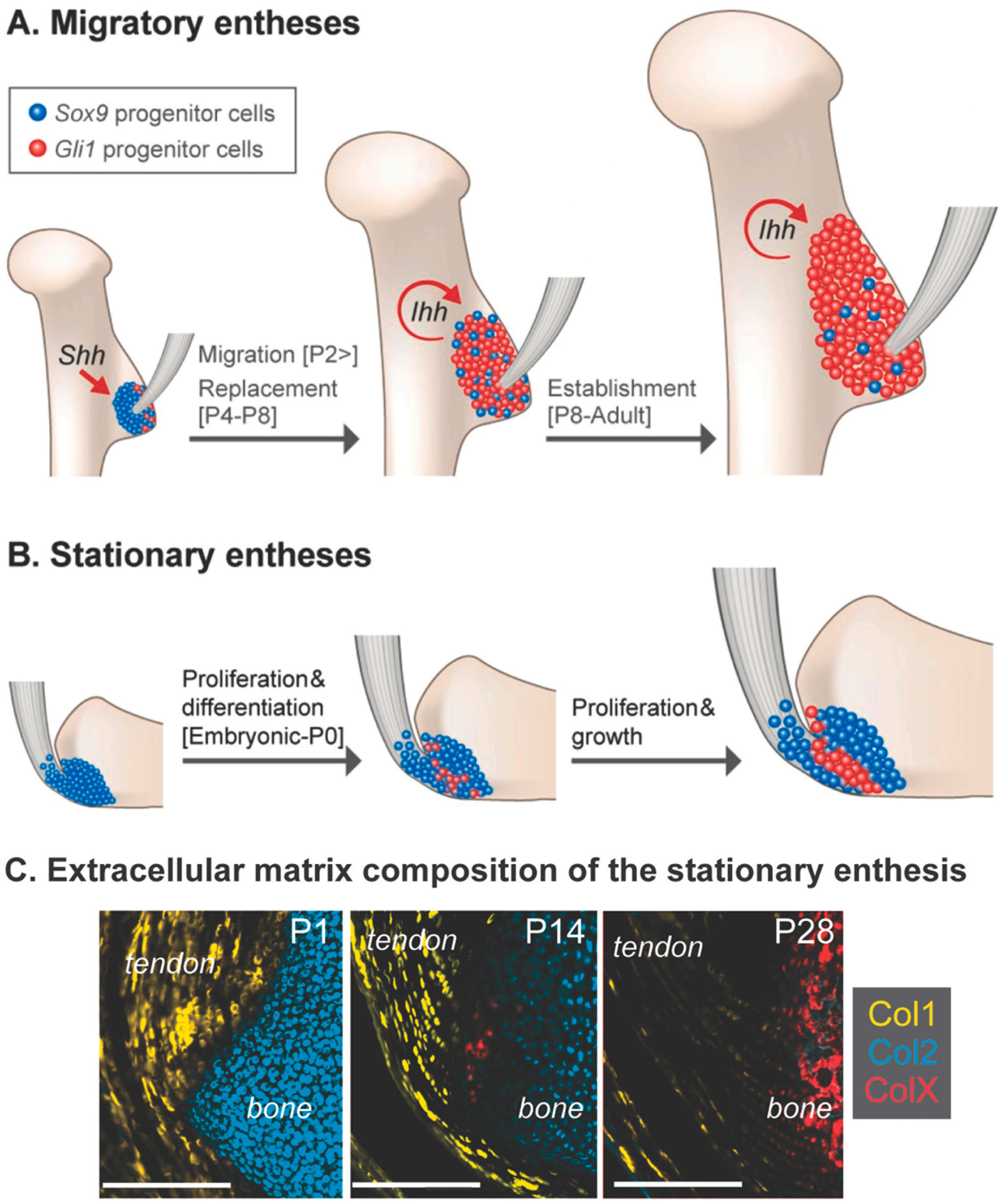

Fig. 3. The establishment of the postnatal enthesis depends on either (A) migratory (e.g., replacement) or (B) stationary (e.g., maintenance and differentiation) of the progenitor cells at the interface between tendon and bone. (C) Enthesis maturation results in dynamic remodeling of collagen-rich extracellular matrix, with predominantly collagen type I (Col1) in tendon, collagen type II (Col2) in the cartilage template, and deposition of collagen type X at the enthesis and in the secondary ossification center. Timelines shown are representative of mouse enthesis development; P = postnatal day. Modified from [31,46].

the cytoplasm. These ligands may be derived from or diffuse into the tendon and enthesis from surrounding muscle, and the secretion of TGFβ is thought to be, in part, mechanically mediated [73]. TGFβRs plays a role in musculoskeletal tissue crosstalk and has the potential to promote tendon regeneration [74,75]. Downstream, the TGFβ and BMP subfamilies canonically signal via Smads; specifically, TGFβ signals via Smad 2/3, and BMP signals via Smads 1/5/8 [76]. Recent work by Schlesinger et al. showed that loss of Smad4 leads to thinner tendons and induction of joint contracture, suggesting an essential role of BMP signaling during tendon growth [52].

Table 1
Previously reported Cre strains used for targeting the tendon-bone enthesis in mice.

Cre	Enthesis prevalence	Inducible	Overlapping expression with other tissues	References
Constitutive Cre strains				
Scx-Cre	High	No	Yes; tendon, ligament, periosteum, trabecular bone, kidney, lung, brain, endothelial cells	[23,27,30,47,49–54]
Prrx1-Cre	High, for appendicular skeleton	No	Yes; all lateral plate mesoderm-derived tissues	[7,24,29,55,56]
Wnt1-Cre	High, for neural crest cells only	No	Yes; midbrain, dorsal spinal cord	[57]
Gdf5-Cre	High, for intra-articular attachments	No	Yes; articular joint tissues including ligaments, cartilage, meniscus, tendons	[46]
Inducible Cre strains				
Gli1-CreERT	High	Yes, tamoxifen (postnatal)	Yes; broad expression in other tissues during embryogenesis, including growth plate, mesothelium, kidney, neural stem cells, alveoli, hair follicles, heart, and vascular smooth muscle cells.	[30,31]
Sox9-CreERT	High, for stationary attachments	Yes, tamoxifen (embryonic)	Yes; cartilage/tendon as well as brain, lung, heart, pancreas and kidney. Sox9+ cells labeled during embryonic development are replaced by Gli1+ cells.	[24,31,58]
Gdf5-CreER	Moderate to low	Yes, tamoxifen (embryonic; E11.5–12.5)	Yes; proximal chondrocytes, ligaments	[59]
Col2-CreERT	Low or none	Yes, tamoxifen (early postnatal; <P14)	Yes; secondary ossification center, articular cartilage	[30]
Scx-CreERT2	Low or none	Yes, tamoxifen	Does not label embryonic tendons or the postnatal enthesis	[60–62]
Cre strains (potential for enthesis targeting)				
Prrx1-CreERT2	Not reported	Yes, tamoxifen	Yes; periosteum	[63]
Fgf18-CreERT2	Not reported	Yes, tamoxifen	Yes; lung, limb bud, palate, skeleton, central nervous system, and hair follicle	[64]
Col1a1-CreER	Not reported	Yes, tamoxifen	Yes; osteoblasts, odontoblasts, some tendons	[65]
Postn-Cre	Not reported but likely	No	Yes; myofibroblasts, tendon	[66,67]

The TGFβ superfamily also signals via non-Smad pathways such as MAPK and NF-κB [77]. While the link has yet to be fully established in the tendon-bone enthesis, recent work by Abraham et al. showed that targeting of the NF-κB pathway via IKKβ can dramatically influence enthesis maturation as well as its ability to repair following injury [50]. This work and that of others supports the need for further investigations related to crosstalk between immunomodulation and tendon-bone enthesis development.

3.2. Fibroblast growth factor (FGF) signaling

FGF ligands are secreted signaling proteins that bind to and activate a family of high affinity protein tyrosine kinase receptors (FGF receptors, FGFRs) [78]. Most bones grow via endochondral ossification, a process of mesenchymal differentiation and sequential replacement of cartilage with bone through growth plates. Several key steps of endochondral ossification are dependent on activation and repression of FGFR [79] and FGF ligands such as FGF2, FGF9, and FGF18 [80–82]. FGFR activation elicits a wide breadth of cellular processes and is especially important during bone development. In the growth plate of long bones, resting zone chondrocytes express low levels of *Fgfr2*, proliferating and pre-hypertrophic cells express high levels of *Fgfr3*, and hypertrophic chondrocytes express high levels of *Fgfr1* [78]. FGFR3 signaling in growth plate chondrocytes is especially critical for regulating bone growth and activating mutations of FGFR3 result in decreased chondrocyte hypertrophy and proliferation [79,83,84]. Developing bone has distinct patterns of the FGF9 and FGF receptor expression in both intramembranous and endochondral bone formation [78]. Endochondral bone development relies on the expansion of the embryonic and postnatal growth plate, which is regulated by both FGFR1 and FGFR3, whereas intramembranous formation occurs in the absence of FGFR3 and depends on FGFR1. The patterns of FGF ligands and receptors in developing bone are well established; however, the patterns of FGF signaling in the developing enthesis have only recently been elucidated [57,85,86]. In the developing mouse patellar tendon, *Fgfr1* is highly expressed in the tendon mid-substance and tibial insertion [85]. Liang et al. showed, in mice, the mature enthesis fibrocartilage expresses *Fgfr3* and *Klotho*, but not *Fgfr1* [86], which may underly its mineralized expansion and susceptibility to enthesopathy.

As previously mentioned in this review, the tendon-bone enthesis forms as an arrested growth plate with an endochondral-like zone. However, the developing enthesis differs from the growth plate during endochondral ossification in several ways. For one, the resident progenitor pool of the enthesis remains static even into postnatal maturity, whereas the expansion of the growth plate dynamically remodels and is replaced with trabecular bone throughout longitudinal bone growth. Additionally, the developing enthesis lacks a resting zone of proliferating chondrocytes, which is a critical regulator of growth plate expansion; instead, the enthesis is constrained by tendon fibroblasts. Tendon extends via cell proliferation and differentiation in the tendon anlage and then elongates following recruitment of mesenchymal progenitors [35]. The enthesis relies on matrix synthesis from both fibroblasts (e.g., *Scx*+ cells), chondrocytes (e.g., *Sox9*+ cells), and fibrochondrocytes (e.g., *Gli1*+ cells) (Fig. 1) [23,24,27,30,31]. This cell fate is unique to the enthesis and strikingly different than the growth plate. Recent work studying enthesis development in the mouse mandible showed the cell fate of enthesis progenitors, specifically *Scx*+ cells, is regulated by FGF signaling via *Fgfr2-Fgf2* signaling [57]. Although the formation of the craniofacial bones and limb bones have divergent cell origins (cranial neural crest [87] vs. lateral plate mesoderm [88], respectively) and undergo different patterns of bone formation (intramembranous vs. endochondral, respectively), these findings suggest a potent role of FGF signaling during enthesis development.

3.3. Hedgehog signaling and cilia

Mineralization processes during endochondral bone formation are regulated by hedgehog (Hh) signaling [89,90]. Chondrocyte maturation in the growth plate is regulated by Indian Hh, expressed by pre-hypertrophic and hypertrophic chondrocytes, via a negative-feedback loop with parathyroid hormone-related protein

(PTHrP). Indian Hh induces PTHrP expression in chondrocytes further away from the growth plate in the periarticular region, which suppresses chondrocyte maturation. This process is recapitulated in the postnatal enthesis [30,31,46]. Phenotypic characteristics of ScxCre-*Pthrp* mutant mice have enlarged superstructures at sites of fibrous entheses [47], and unloading of entheses results in reduced expression of PTHrP [91]. Conversely, enthesis unloading leads to increased *Gli1* expression [30, 49] and ablation of *Gli1*+ cells as well as Hh signaling in enthesis progenitors results in a nearly complete loss of the mineralized fibrocartilage zone [30].

In vertebrates, Hh signaling relies on bidirectional intraflagellar transport (IFT) of proteins in cilia [92,93]. Localization of IFT88 in the postnatal enthesis has been correlated with *Gli*1+ cells and tendon unloading leads to increased expression of primary cilia genes [49]. Additionally, loss of cilia motor proteins such as *Kif3a* leads to formation of synchondroses [94], which resemble entheseal fibrocartilage. In the postnatal growth plate, primary cilia are important in Hh signaling and are required for Hh activation as well as the proteolytic processing of *Gli3* to either an activator or repressor form [95]. Specifically, in the absence of Hh, *Gli3* is proteolytically cleaved into a short form with repressor activity [96]. However, in the presence of Hh, *Gli3* cleavage is inhibited and it then acts as a transcriptional activator [97]. The overlap and divergent behavior of cilia and ciliary Hh signaling is an unexplored area in enthesis development that warrants further investigation.

4. Physiology and pathophysiology of the enthesis

4.1. Extracellular matrix (ECM)

The structure and function of the enthesis relies on the establishment and remodeling of its ECM. The primordial matrix of the enthesis is predominantly collagen and includes types I, III, VI, IX, and XI collagen [98]. As it matures, the ECM of the enthesis undergoes a dynamic remodeling for spatially segregation of regions that richly express and deposit type I, II, and X collagens (Fig. 3) [31,46,99]. ECM markers of the developing enthesis include sustained expression of *Col12* and *Tnc*, as well as *Bgn* during its postnatal development [31]. Indeed, the enthesis is rich in hyalectans (chondroitin sulfate proteoglycans, specifically aggrecan and versican) [4,6] and small leucine-rich proteoglycans (e.g., chondroadherin and biglycan, which control fibril size and interaction with collagen) [4,26,31,85,100]. These recent studies using proteomic analyses have helped better describe the ECM patterns of the enthesis, yet the dynamics and remodeling throughout growth are still unknown.

Tendon and enthesis regeneration is challenging in adult mice, however zebrafish are capable of fully regenerating tendons and this process is regulated by BMP signaling [74]. In zebrafish, muscle and cartilage connective tissues may also contain signaling cues such as BMPs that, after cell ablation in these tissues, can promote directional cell recruitment [74]. That ECM can be exploited for tissue regeneration is not new [101–103], yet what the composition of the matrix should be still remains unclear. Some processes of enthesis development have been mimicked in engineered strategies of enthesis regeneration, including the use of gradients in mineralization, alignment, and stiffness [104–106]. Parallels in matrix remodeling during enthesis development may also be elucidated using models of scarless wound healing and tissue regeneration. During wound healing in other tissues, such as muscle and bone, the provisional matrix functions to promote proliferation and matrix deposition by migrating fibroblastic cells [102,107]. This matrix-guided development has been primarily studied in organisms capable of regenerating limbs following amputation, such as in amphibians (newts, axolotl, and Xenopus), as well as in tissues that can undergo regeneration following injury, such as mouse skin [108–114]. Hyaluronic acid and sulfated proteoglycans (e.g., heparan sulfate) are two key ECM components that contribute to the "pattern following" and "pattern forming" processes, respectively, of positional biochemical properties in developing and regenerating tissues. The "pattern following" cells (in newts) rely on positional information (e.g., retinoic acid [110] and hyaluronic acid [108]) in order to migrate towards or within a transitional ECM, whereas the "pattern forming" cells are responsible for synthesizing this transitional matrix [115]. In axolotl, "pattern forming" cells deposit heparan sulfate proteoglycans in order to control growth factor signaling, including FGF and BMP signaling [109]. The discovery of enthesis-specific ECM turnover and dynamics is likely with use of innovative techniques to visualize ECM composition and proteolysis, such as non-canonical amino acid labeling [116–118] and damaged collagen hybridization [119,120], respectively.

4.2. Enthesopathies

The presentation of inflammation, damage, and outgrowth of the mature enthesis is a clinical problem resulting in pain and dysfunction that is challenging to treat [32,121]. In children, apophyseal injuries are associated with increased loading at the enthesis and can lead to painful disorders such as Sever disease, Osgood-Schlatter syndrome, and Little Leaguer's elbow [122]. Many of these conditions, especially for pediatric patients, are treated non-surgically, primarily with rest and stretching [123]. Arthritic conditions, such as diffuse idiopathic spinal hyperostosis (DISH) and spondylosis, are linked to mechanical loading as well as metabolic dysfunction [124,125]. Enthesopathy can be initiated by loading-induced microdamage, such as in tennis elbow and Achilles insertional tendinopathy [2,126]. Degeneration and inflammation likely contribute to unresolved enthesopathy and related joint diseases, such as osteoarthritis, tendinopathy, and rotator cuff disease [50,127–129]. Hallmark characteristics of unresolved tendinopathy and enthesopathy include neovascularization and increased innervation of the tendon and enthesis [130], yet the healthy enthesis is not well vascularized or innervated [131,132]. Rheumatological conditions, such as fibromyalgia and psoriasis, can also manifest entheseal changes and damage which are likely not purely mechanically derived. X-linked hypophospathemia (XLH) is also a risk factor for enthesopathy, which manifests in pervasive osteophyte formation in fibrocartilaginous entheses [86]. This disease, which effects mineralization of the enthesis, has been studied using Hyp mice, a model of the XLH mutation that mimics the human syndrome (including via hypophosphatemia and elevated circulating FGF-23) [86].

4.3. Future directions in enthesis research

In adult tendon, hypervascularity induces a "state-switch" that likely leads to advancement in pathology and matrix degradation [133]. Yet we do not fully understand if and how the tendon-bone enthesis, and tendon more generally, is vascularized during its development or how tendon and enthesis vascularity influences its ability to heal following injury. Recent work using in vitro models of mechanical-stress deprivation models of tendon fascicles has shown that mimicking a pathophysiological environment under normal oxygen tension culture conditions can lead to pathophysiological processes associated with increased oxidative stress (e.g., activation of hypoxia-inducible factor (HIF-1) and NADPH oxidase, which produce reactive oxygen species in response to hyperoxia) [133]. *In vitro*, low oxygen conditions (i.e., hypoxia) can promote tendon cell differentiation and maturation and limit tendon fascicle contraction [133–135]. It is likely that the standard culture conditions of normoxia are in fact mimicking a pathological condition for tendon. Hypoxia can also induce a phenotypic switch from tenocyte to fibrochondrocyte in vitro which may depend on Rho/Rac GTPase signaling [136], a well-established pathway controlling cellular mechanosensing [137]. The ability of a cell to respond to hypoxic stress can lead to depletion of ATP in cells [138] and Rac1 activation [139], further influencing its ability to adhere to substrates, migrate, and undergo gene transcription. However, if and how oxygenation and vascularization contribute to the development of the tendon-bone

enthesis remains unexplored.

The environment of the mammalian embryo is predominantly hypoxic prior to the establishment of the cardiovascular system and availability of oxygen [140]. Cells are able to survive hypoxic stress depending on the stability of HIF1α [141]. Additionally, when Hif1α is positively regulated, oxygen consumption and cell proliferation are tamped down while collagen synthesis is elevated even in spite of low oxygen availability [141–143]. Several elegant studies have demonstrated that the fetal growth plate maintains an oxygen gradient and growth plate development relies on expression of the transcription factor HIF-1α [140–142,144]. In the hypoxic growth plate, increased levels of Hif1α also lead to decreased mitochondrial respiration and oxygen consumption, ultimately promoting cell survival [145]. Like the fetal growth plate, the enthesis is an avascular tissue [131]. Thus, the existence of an oxygen gradient during enthesis development may be essential for collagen synthesis and metabolic demands and reprogramming.

New directions in the field related to the metabolic bioenergetics during tendon and enthesis development and adaptation are ripe for exploration, especially focused on glycolysis, lactate production, and oxidative phosphorylation. For example, the role of hypoxia and stability of Hif1α during tendon and enthesis development is unclear, as is the function of mitochondria during formation of these ECM-dense tissues. Additionally, the ability of enthesis progenitors to balance energy demands and regulate autophagy during its rapid and expansive growth remains unexplored.

5. Conclusion

The enthesis is an interfacial collagen-rich tissue essential for the joint motion and stability and functions as a stress-reducer between tendon and bone. Its development in the vertebrate skeleton is complex and mechano-adaptive, and recent discoveries have identified a unique pool of mixed tenogenic and chondrogenic cells that form and maintain this unique tissue. These bi-fated cells between tendon and bone have overlapping and divergent characteristics to an arrested growth plate, including TGF-β/BMP, FGF, and Hedgehog signaling. Future research will be required to more clearly understand the physiology of the enthesis, including identifying cellular patterns and ECM composition during the dynamic postnatal remodeling process and also elucidating the role of hypoxia and cellular metabolism during enthesis development and pathogenesis.

Conflict of interests

None.

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