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Roadmap of Molecular, Compositional, and Functional Markers during Embryonic Tendon Development

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Abstract

Tendon is a specialized connective tissue that connects muscle to bone, thereby enabling musculoskeletal movement. Tendon injury leads to formation of tissue with aberrant functional properties. Current approaches to treat tendon injuries, including surgical repair and tissue engineering, have not achieved normal tendon. A roadmap of markers could help with identifying when mis-steps occur during aberrant tendon formation and providing instructions for normal tendon formation. We propose this roadmap should be based on the embryo – the perfect model of tissue formation. Our prior studies have shown that adult mesenchymal stem cells mimic tendon progenitor cell behavior when treated with tendon developmental cues. Although transcription factors and extracellular matrix molecules are commonly used to assess tendon development, we have shown that these markers do not reliably reflect functional property elaboration. Thus, evaluating tendon formation on the basis of a combination of these molecular, compositional, and functional markers is important. In this review, we highlight various tendon markers with focus on their temporal profiles and roles in tendon development to outline a roadmap that may be useful for informing tendon healing and tissue engineering strategies.

Keywords

tendon; embryonic; crosslinks; mechanical properties; temporal; tendon markers; lysyl oxidase

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Declaration of Interests

The authors report no conflicts of interest.

I. Introduction:

Tendon is a specialized connective tissue that transmits forces from muscles to bone. Despite its critical role in facilitating skeletal movements, injured tendon heals with abnormal properties and high re-injury rate, even after surgical and physical therapy interventions [1]. An alternative treatment is to replace the injured tendon with new tissue engineered from stem cells.

For many years, differentiation of mesenchymal stem cells (MSCs) toward the tendon lineage (tenogenesis) in tissue engineering had been routinely evaluated on the basis of collagen types I and III expression, increases in mechanical strength and modulus, and alignment of cells and matrix. Notably, none of these markers were tendon-specific, and thus it was difficult to assess whether stem cells were behaving tenogenically. To overcome this obstacle, we pioneered the approach to evaluate tenogenic differentiation of stem cells by characterizing the expression of embryonic tendon markers [2]. In particular, this study first demonstrated human adult MSCs can upregulate and maintain expression of scleraxis and other tendon-specific markers when subjected to cyclic mechanical loading [2], a critical cue for embryonic tendon development [3]. Based on this seminal study, tendon tissue engineering and wound healing studies now routinely characterize expression of scleraxis and other embryonic tendon markers to evaluate stem cell differentiation and new tissue formation.

In spite of this significant advancement, tendon wound healing and tissue engineering have yet to achieve normal tendon. In both healing and tissue engineering, it is unknown where the mis-steps occur during tissue formation. Thus, these approaches would benefit from a roadmap to inform as well as guide stem cell differentiation, extracellular matrix (ECM) deposition and organization, and functional property elaboration during new tendon formation. To that end, we have looked to the embryo – the perfect model of tendon development.

In recent studies, we have established methods to harvest and culture embryonic tendon progenitor cells (TPCs) from distinct stages of tendon development [4, 5]. We have shown that these embryonic TPCs are responsive to developmental cues *in vitro*, and that these TPC responses are unique to each embryonic stage at which they are harvested [4]. We have also discovered that adult MSCs will mimic embryonic TPCs when subjected to developmental biochemical and mechanical cues *in vitro* [5]. Based on these and our other studies, tenogenically differentiating stem cells should behave differently at each phase of differentiation as they progress through new tendon formation [4, 5, 6, 7]. These novel findings suggest embryonic tendon development can be used to evaluate and guide adult stem cell-based tendon regeneration strategies.

The field's knowledge of embryonic tendon development is multi-dimensional. The identification of molecules that label tendon progenitors has enabled elucidation of signaling pathways involved in tendon lineage specification. Important earlier studies focused on collagen fibrillogenesis in embryonic tendon have provided insights into tendon ECM assembly and maturation. Based on these works, molecular and compositional markers,

including transcription factors and ECM molecules, have been used to assess tendon formation. However, as reviewed later, these markers do not consistently and reliably reflect tendon functional (mechanical) properties. In more recent years, our laboratory has pioneered methods to characterize the mechanical properties of tendons starting at the earliest stages of development when the tissue is too small and delicate for traditional tensile testing methods [8, 9]. By characterizing structure-property relationships of embryonic tendons throughout development, we have established a stage-specific profile of mechanical properties and also lysyl oxidase (LOX)-mediated crosslinks as functional markers to temporally evaluate tendon development.

Taken together, a temporal profile of molecular, compositional, and functional markers of embryonic tendon development will offer a novel roadmap with which to evaluate and guide tendon healing and tissue engineering approaches. This review discusses three distinct but overlapping phases of embryonic tendon development: early, mid, and late development (Figure 1). Throughout the review, we cite studies performed with different animal models and refer to species-specific embryonic stages for each phase of development. It is important to keep in mind that these developmental phases may vary in embryonic stage among species (e.g. human, mouse, chick, bovine, etc.) as well as anatomical sites (e.g. axial, calcaneal, digital, etc.). For different species discussed in this review paper, human and bovine gestation terms are 280 and 283 days, respectively. Mouse embryos develop in 21 embryonic days (E). Chick embryos also develop in 21 days (to Hamburger-Hamilton stage (HH [10]) 46). While our focus in this review is on prenatal stages, it is important to recognize that further tissue development continues during early postnatal stages.

II. Transcription Factors:

There are a number of transcription factors that are involved in embryonic tendon development, including scleraxis, mohawk, and early growth response 1. These are the earliest detectable transcription factors in embryonic tendon. Their discoveries have provided markers with which to identify tendon progenitors in the embryo, though their functions have not yet been fully elucidated. As described earlier, these important discoveries inspired our group to first introduce the use of developmental markers, such as scleraxis, to evaluate the onset of stem cell tenogenesis in tissue engineering strategies [2].

Scleraxis:

Scleraxis is a basic helix-loop-helix transcription factor and the earliest known tendon marker [11]. During early development, scleraxis gene expression is first detected between embryonic days (E) 10 and 11 in mouse limb tendons and at HH21 in chick limb tendons [11]. In axial tendons, the first detection of scleraxis is reported in mouse at E10 and in chick at HH18 [12]. By E12.5 in mouse and HH27 in chick, scleraxis-expressing cells condense to form a complex pattern between developing muscle and cartilage tissue [11]. In mid development, scleraxis expression pattern starts to resemble a more distinct tendon-like shape by E13.5 in mouse and HH29 in chick. By E14.5 in mouse and HH35 in chick, embryonic tendons can be clearly distinguished by scleraxis expression [11]. Continuing into late development, scleraxis expression is reported in all tendon cells in both mouse and

chick models throughout the subsequent embryonic and postnatal days [4, 11, 13, 14]. The spatial expression pattern of scleraxis varies throughout development [11, 12]. In contrast, we characterized E13 through postnatal day (P) 7 mouse tendon cells from both axial and limb tendons and found that scleraxis gene expression levels are constant between stages [4]. While scleraxis can be used to identify tendon progenitors from the earliest embryonic stages, constant expression levels between stages makes it difficult to evaluate the extent of differentiation.

Knockout studies reveal an important role of scleraxis in the development of some tendons, though not all are universally affected [15]. Specifically, long tendons such as force-transmitting and intermuscular tendons are either entirely missing or severely disrupted. On the other hand, short-range anchoring tendons (e.g. tendons that anchor the intercostal muscles to the ribs) remain normal [15]. In the absence of scleraxis, long tendons of embryonic mouse limb fail to form at E13.5 [15]. When flexor digitorum profundum (FDP) tendons in mutant mice are examined during late development, ECM organization including matrix deposition and the network of cytoplasmic extensions is also found to be disrupted [15]. In addition, other tenogenic markers such as collagen types I and XIV and tenomodulin have been shown to be regulated by scleraxis [15, 16, 17]. These findings collectively suggest that even though scleraxis is not a master regulator of tendon development, it plays an important role in tenogenic differentiation and matrix organization in the development of a subset of tendons.

Mohawk:

Mohawk is a member of the three-amino acid loop extension superclass of atypical homeobox genes [18]. During early-to-mid development, mohawk gene expression is first detected in mouse limb and tail tendons at E12.5 [18, 19]. Overlapping spatial expression patterns of mohawk and scleraxis have been reported in mouse limb and tail tendons from E12.5 to E13.5 [18]. During late development, mohawk expression is present but decreases significantly in mouse limb and tail tendons by E16.5 [19]. In postnatal mice, mohawk expression localizes with high intensity to the outer sheath of limb and tail tendons [19].

E16.5 mouse limb and tail tendons of mohawk knockout mice express lower levels of tendon markers such as collagen type I and tenomodulin [19]. Gene expressions of other tendon components such as decorin and fibromodulin also decrease in tail and limb tendons of mutant mice at birth [19]. In postnatal tendons, structural properties including thickness, ultimate tensile strength, and fibril diameters are significantly lower in the absence of mohawk [19, 20, 21]. Interestingly, gene expression patterns of mohawk in scleraxis null mice and of scleraxis in mohawk null mice appear normal, suggesting neither transcription factor regulates the other during embryonic tendon development [19]. Furthermore, the fact that tendons still form in mohawk null mice suggests mohawk is not required for tendon formation. Based on these studies, mohawk appears to take part in regulating collagen fibril growth during tendon development.

Early Growth Response 1:

Early growth response 1 is a member of the early growth response family of zinc finger transcription factors [22, 23]. Early growth response 1 gene expression is first detected in the scleraxis-positive domain of developing chick wing tendons between HH30 and HH31 and in mouse forelimb tendons at E12.5 [23]. Early growth response 1 expression continues into late development through at least E18.5, the latest stage examined [23]. In embryonic chick limb, early growth response 1 is expressed in tendons through at least HH37 [23].

Chromatin immunoprecipitation (ChIP) assays have shown that early growth response 1 can bind to the regulatory domain of the promoter of collagen type I [22, 23]. Early growth response 1 mRNA levels increase by 15-fold between E11.5 and E14.5 in mouse limb tendons. During the same period, collagen type I mRNA levels undergo a 27-fold upregulation [23]. These results suggest early growth response 1 may play a role in collagen type I deposition and fibrillogenesis in tendon. Knockout of early growth response 1 results in lower mRNA levels of scleraxis, tenomodulin, and different collagen types [22]. The mutant mice have smaller tendons with inferior mechanical properties [22]. Taken together, early growth response 1 may be involved in regulating tendon ECM formation and the expression of other tenogenic markers.

III. Transforming Growth Factors (TGF)- β s:

TGF- β 2 and TGF- β 3 are growth factors that are implicated as important signaling molecules, and also as markers, in tendon development [4, 5, 24, 25, 26]. During early development, low levels of TGF- β 3 have been detected by *in situ* hybridization in the syndetome at E10.5 [24]. In embryonic mouse limb tendons, the partially overlapping expression patterns of TGF- β 2 and TGF- β 3 with scleraxis have been reported at E12.5 [24]. We have shown that during mid-to-late development, the expression patterns of TGF- β 1, - β 2, and - β 3, as well as TGF- β receptors I and II are detectable by immunohistochemistry (IHC) staining in chick calcaneal tendons from HH39 to HH42 [26]. TGF- β 2 is present in the tertiary bundles at all examined stages with the broadest and most intense staining at HH40. TGF- β 3 is also present but becomes nearly undetectable at HH42. On the other hand, TGF- β 1 was not detected in the tertiary bundles at any stage examined in the study. TGF- β receptor I is present in the tertiary bundles at all stages but TGF- β receptor II was only detected at HH39 and HH40 [26]. Corroborating these findings, studies in embryonic chick metatarsal tendons from HH40 to HH45 [27] and in embryonic mouse limb tendons at E15.5 and E16.5 [25] have also reported the presence of TGF- β 2 at all examined stages.

We have shown that TGF- β 2 has the ability to upregulate scleraxis expression in cultured TPCs harvested from mouse embryo tendons from E13 through at least E17 [4, 5]. Disruption of TGF- β signaling in mouse model via knockout of TGF- β 2 and/or TGF- β 3, or deactivation of TGF- β receptor II, results in missing tendons throughout the body after E12 [24]. Scleraxis gene expression appears normal at E11.5 in TGF- β 2-knockout, TGF- β 3-knockout, and TGF- β receptor II-targeted knockout mouse tendon, but a dramatic loss of scleraxis gene expression is observed between E11.5 and E12.5 [24]. Based on these studies, it is suggested that TGF- β signaling is important to maintain commitment of TPCs to tendon cell fate during embryonic development.

IV. ECM Molecules:

The dynamic and complex process of ECM deposition and organization in embryonic tendon is highly active during mid development and continues throughout late development. This process overlaps with the continuous expression of the early transcription factors (discussed in Section II). Embryonic tendon includes collagen types I, III, V, VI, XI, XII, and XIV. Non-collagenous components include glycoproteins (e.g. tenomodulin and tenascin-C) and proteoglycans (e.g. the small leucine-rich proteoglycan family). The expression patterns and roles of these embryonic tendon ECM components are reviewed below.

Collagens:

Tendon is a highly collagenous tissue consisting of a hierarchical organization of parallel collagen fibrils [28, 29, 30, 31]. Collagen fibrils are first assembled extracellularly from collagen monomers. Fibrils grow in size and assemble to form fibers, and fibers, in turn, pack together to form fascicles that group together to comprise the tendon [29, 31]. This process continues postnatally until tendon maturation [32]. The process of collagen fibril growth and assembly during embryonic tendon formation is tightly regulated. There are two types of collagens: fibrillar and non-fibrillar. Fibrillar collagens, including collagen types I, III, V, and XI, are the primary components of tendon ECM and are directly involved in collagen fibril growth and assembly. Non-fibrillar collagen types, such as collagen types XII and XIV, have been implicated in regulating different aspects of collagen fibrillogenesis. Collagen deposition increases rapidly during mid development and continues through late development (Figure 2). The expression patterns of different collagen types have been shown to be distinct and dynamic during embryonic tendon development.

Collagen Type I: Collagen type I is the most abundant collagen type in tendon during embryonic development and in the adult. Studies of embryonic chick limb tendons during mid development have identified collagen type I as early as HH27 in the mesenchyme lamina [33, 34] and as early as HH29 in the autopodium [35]. Collagen type I continues to increase throughout development to become the primary constituent of tendon dry weight in the adult.

Collagen Type III: Collagen type III is a fibrillar collagen that has been detected in embryonic tendon. Collagen type III becomes faintly detectable by IHC in developing long autopodial tendon blastemas of chick embryos between HH30 and HH31 during mid development [35]. Later in development, collagen type III can be detected by immunofluorescence throughout tendon fascicles of embryonic chick metatarsal tendons by HH40 [36]. Collagen type III expression markedly diminishes in the fascicles and becomes localized to the outer sheath by HH43 [36]. At hatching, collagen type III is completely absent from tendon fascicles and is found exclusively in the outer sheath [36]. A similar shift in collagen type III localization is observed in embryonic chick calcaneal tendons from HH39 to HH42 [26]. The effects of collagen type III knockout in tendon have not been reported, however it may play a role in regulating collagen fibrillogenesis. From HH40 to hatching, collagen type III presence diminishes in embryonic chick tendon fascicles as

collagen fibril diameters increase [26, 36]. At hatching, small diameter fibrils and collagen type III intensity become concentrated in the outer sheaths [26, 36]. Taken together, these findings suggest collagen type III regulates collagen fibril growth and assembly during embryonic tendon development.

Collagen Type V: Expression of collagen type V in embryonic tendon has been investigated primarily during late development [36, 37]. In embryonic chick metatarsal tendons, collagen type V protein is present at low levels from HH40 to hatching [36]. In embryonic mouse flexor digitorum longus (FDL) tendons, collagen type V gene expression has been reported to decrease after E18 [37]. Homozygous knockout of collagen type V leads to embryo lethality at E10 [38]. Targeted knockout of collagen type V results in tendon with altered biomechanical properties and collagen fibrils with irregular structure and organization [39]. Based on these findings, collagen type V is implicated in regulation of collagen fibrillogenesis in tendon.

Collagen Type VI: Collagen type VI is first detected in tendon during mid development. Collagen type VI is first detected in chick embryo tendon blastemas at HH30 [40]. It has also been shown to be present in chick long autopodial tendons from HH30 to HH37 [35]. During late development, collagen type VI mRNA levels increase while protein levels appear constant in embryonic chick metatarsal tendons from HH40 to HH45 [27, 41]. Collagen type VI has been detected at E18 in embryonic mouse hindlimb tendons [42]. Knockout of collagen type VI results in an increase in smaller diameter fibrils in postnatal mouse FDL tendons [43]. These FDL tendons also exhibit abnormal ECM organization and altered cross-sectional area, stiffness, and maximum load [43]. These findings suggest collagen type VI plays a role in regulating collagen fibril growth and assembly in tendon [41, 43].

Collagen Type XI: Collagen type XI expression in embryonic tendon has been studied primarily in late development [27, 37]. In embryonic chick metatarsal tendons, collagen type XI mRNA levels increase by approximately two-fold between HH40 and HH45 [27]. In embryonic mouse FDL tendons, collagen type XI has been detected at E18 and postnatally [37]. E18 collagen type XI knockout mice possess more fibrils with larger diameters and lower fibril density in FDL tendons [37], suggesting collagen type XI is involved in regulating collagen fibril growth and assembly in developing tendon.

Collagen Type XII: Studies on collagen type XII expression have focused primarily on late embryonic chick tendon development [44, 45]. Collagen type XII is present in chick metatarsal tendons from HH40 to hatching [44] and is also present in chick hindlimb tendons at HH43 [45]. Collagen type XII mRNA as well as protein localization shifts from within to outside of the fascicles from HH40 to hatching [44]. During the same period, collagen fibril diameters within and outside of fascicles increase and decrease, respectively [36]. These findings suggest that collagen type XII regulates lateral collagen fibril growth in embryonic tendon.

Collagen Type XIV: During early development, *in situ* hybridization reveals the faint expression of collagen type XIV in the sclerotome between HH28 and HH29 in the chick

embryo [45]. However, a potential role for collagen type XIV at early developmental stages has not been reported. By late development, collagen type XIV is expressed in embryonic chick metatarsal tendons from HH40 to HH45 [46]. Collagen type XIV protein levels decreased by approximately six-fold from HH45 to hatching [46]. It has also been shown that collagen type XIV protein is detected in chick hindlimb tendons at HH43 [45]. P4 and P7 collagen type XIV knockout mice possess abnormally large collagen fibrils and significantly lower maximum load, stiffness, and elastic modulus [47]. Based on these findings, collagen type XIV may play a role in regulating lateral fibril growth in developing tendon.

Glycoproteins and Proteoglycans:

Glycoproteins and proteoglycans are important non-collagenous components of tendon ECM. Two members of the glycoprotein family, tenascin-C and tenomodulin, have been detected as early as the deposition of collagen type I during mid-development, and both continue to be expressed through late development. Knockout of tenomodulin significantly impairs tendon cell proliferation and alters tendon structural properties, whereas knockout studies of tenascin-C have not shown any detectable tendon defects. Small leucine-rich proteoglycans (SLRPs), including decorin, biglycan, and fibromodulin, have been implicated in the assembly and growth of collagen fibrils of embryonic tendon during later developmental stages.

Tenascin-C: During early-to-mid development, IHC staining of embryonic chick hindlimbs demonstrated that tenascin-C is present as early as HH24 and HH25 in the dorsal and ventral tendon primordia, respectively [48]. Other studies have also reported the presence of tenascin-C via IHC staining in mesenchyme lamina of embryonic chick limb buds at HH27 and in long autopodial tendons at HH29 [33, 35]. Tenascin-C is detected in mesenchyme lamina of chick embryo limb buds through at least HH34 [33]. Another study showed tenascin-C is present in embryonic chick long autopodial tendons through at least HH37 [35]. A role for tenascin-C in embryonic tendon development has not been identified. Knockout of tenascin-C in mouse does not result in any abnormal tendon phenotype [49, 50]. However, the specificity of tenascin-C to tendon over adjacent tissues such as bone or skeletal muscle renders tenascin-C a useful marker to identify developing tendon [51].

Tenomodulin: Tenomodulin is a type II transmembrane glycoprotein that is highly specific to tendon and ligament [17, 19, 52, 53]. In the chick embryo, using whole mount *in situ* hybridization, tenomodulin is first detected in somites at HH23 [17], and then overlaps spatially with scleraxis in embryonic chick axial tendons by HH25 [17]. Tenomodulin gene expression has also been reported in mouse limb tendons at E11.5, subsequent to the detection of scleraxis between E10 and E11 [11, 53]. During mid development, tenomodulin gene expression has been reported in embryonic chick axial tendons up to HH35, the latest stage examined [17]. In the mouse embryo, tenomodulin gene expression has also been reported in limb and tail tendons from E11.5 to E14.5 [19, 53]. Continuing into late development, tenomodulin gene expression has been found to localize to mature elongated tenocytes rather than immature oval tenocytes in embryonic chick limb tendons at HH41

[17]. In embryonic mouse limb tendons, a robust gene expression pattern of tenomodulin has also been reported at E17.5 [52].

Knockout of tenomodulin impairs cell proliferation and increases collagen fibril thickness in postnatal tendons [54]. Tenomodulin gene expression can be regulated independently by two transcription factors that are involved in early tendon development, scleraxis [15, 17] and mohawk [19]. Knockout of scleraxis in the mouse model abolishes any tenomodulin gene expression at E16.5 [15]. Overexpression of scleraxis induces upregulation of tenomodulin in embryonic chick tenocytes [17]. Knockout of mohawk in the mouse model leads to significant reduction of tenomodulin gene expression in hindlimb and tail tendons at E16.5 and birth [19]. Taken together, these findings suggest tenomodulin may play an important role during late tenogenic differentiation.

Decorin: Studies on decorin expression in embryonic tendon have focused primarily on late development [26, 55]. Decorin protein is present in embryonic chick metatarsal tendons at all stages from HH38 to HH45, with a significant decrease in both mRNA and protein levels from HH40 to HH45 [55]. Decorin is also detected in the tertiary bundles of embryonic chick calcaneal tendons from HH39 to HH42 [26]. From HH40 to HH42, decorin protein expression localizes to the periphery of the bundles and to the endotenon [26].

Mouse knockout studies suggest a role for decorin in regulating ECM organization [56, 57, 58, 59]. Postnatal decorin null mice have tendons with abnormal distribution of collagen fibril diameters, irregularly shaped (non-cylindrical) collagen fiber cross-sectional areas, and altered compositional, mechanical, and structural properties [57, 58]. Decorin has been shown to localize in the outer sheath during late development [26, 55]. During the same period, collagen fibril growth rate increases significantly [36]. These findings suggest decorin takes part in controlling collagen fibril growth during embryonic and postnatal tendon development [55].

Biglycan: Biglycan is a member of class I SLRPs that has been studied primarily during late embryonic tendon development [42, 60, 61]. Biglycan mRNA and protein are detected in mouse embryo hindlimbs from E14 to E18, with gene expression levels peaking between E16 and E18 [42]. In bovine embryo deep flexor tendons, biglycan can be detected as early as embryonic day 120 and remains present through gestation [61].

Tendon abnormalities observed in biglycan null mice suggest its role in regulating collagen fibrillogenesis [59, 62, 63]. Knockout of biglycan results in decreased collagen fibril diameter and irregular fibril organization in adult mouse tendons [59, 62, 63]. Double knockouts of biglycan and decorin or fibromodulin result in more severe tendon abnormalities [62, 63]. These findings emphasize the important role of biglycan in tendon development.

Fibromodulin: Fibromodulin is a member of class II SLRPs [60]. Expression of fibromodulin in embryonic tendon has been investigated mostly during late development [27, 64]. Studies using northern blot analysis have reported fibromodulin in embryonic chick metatarsal tendons from HH40 to HH45. The mRNA levels of fibromodulin are shown to

increase up to eight-fold between these stages [27, 64]. Fibromodulin expression in tendons continues to be detected after birth [65].

Knockout of fibromodulin results in thinner and irregularly shaped collagen fibrils, fewer fiber bundles, and decreased stiffness of tendons in postnatal mutant mice compared to WT mice [65, 66]. Interestingly, the expression level of lumican, another member of class II SLRPs that has been shown to compete for a same binding site on collagen type I fibrils with fibromodulin [67], increases sharply in the tendons of adult fibromodulin null mice [66]. Taken together, these findings suggest the coordination between these two SLRPs might regulate collagen fibrillogenesis in developing tendon [66, 68].

V. Morphological Markers:

During mid-to-late development, cells secrete and organize an abundance of ECM molecules that impart tendon with unique morphological and functional properties. This process is highly dynamic, with statistically significant changes in quantitatively measurable properties from one day to the next. Our lab has recently characterized such changes in organization of the actin cytoskeleton as well as collagen fibers of the developing embryonic tendon [8, 69]. Based on such studies, actin and collagen patterns may be useful as novel morphological markers to assess normal tendon formation.

Tendon Cells:

We previously characterized cellular density of embryonic chick calcaneal tendon tissue from HH28 to HH43 [8, 26, 69]. Biochemical assay quantitatively showed DNA-to-dry mass ratio decreases considerably from HH28 to HH43, with the largest decreases from HH28 to HH30 and from HH38 to HH43 (Figure 3A) [8]. It is interesting that DNA-to-dry mass ratio remains relatively constant between HH35 and HH38. We observed similar trends in cell content using DAPI staining of HH34 to HH37 calcaneal tendons (Figure 3B) [69] and haematoxylin and eosin (H&E) staining of HH39 to HH43 calcaneal tendons [8, 26]. We also showed cell nuclei appear to conform the parallel alignment and crimping of collagen fibers from HH34 to HH37 (Figure 3B) [69]. In the same study, phalloidin staining revealed that actin filaments organize parallel to each other, also in a similar fashion to collagen fibers and along the axis of tension (Figure 3B). Furthermore, the actin filaments appear to form a contiguous network that spans adjacent TPCs (Figure 3B) [69]. Intriguingly, image analysis showed that amplitude and period of the crimp pattern exhibited by actin filaments is identical to that of collagen fibers during the stages that were examined (HH35 to HH37) (Figure 3C) [69]. Based on these data, cell density and actin filament organization change dynamically during embryonic development and thus may be used as novel morphological markers to temporally evaluate tendon formation and development.

We have shown that disruption of actin cytoskeleton in HH36 chick calcaneal tendons leads to lower tendon elastic modulus [69]. In another study, disassembly of actin filaments resulted in fewer collagen fibrils and abnormal collagen fibrillogenesis in HH39 chick metatarsal tendons and E15.5 mouse tail tendons, respectively [70]. Based on these results, actin filaments may play a critical role in collagen fibril growth and fibrillogenesis during embryonic tendon development.

Collagen Fibers:

Collagen deposition increases rapidly during mid-to-late embryonic tendon development. We used trichrome staining and SHG imaging to characterize chick embryo calcaneal tendon development from HH28 to HH43. Fibrillar collagen is detectable by HH35, increases rapidly in density and alignment from HH35 to HH43, and by HH43 begins to resemble an adult tendon (Figure 2A) [8]. Quantitative measurements by biochemical assays and liquid chromatography–mass spectrometry corroborate these qualitative findings with nearly exponential increases in hydroxyproline content from HH28 to HH43 (Figure 2B) [8, 71].

In addition to changes in collagen content, we have measured developmental stage-specific changes in collagen crimping during mid-to-late chick embryo calcaneal tendon development [8, 69]. In embryonic chick calcaneal tendons, collagen crimping has been observed between HH34 and HH43 (Figures 2A & 3B) [8, 69]. The amplitude and period of the collagen crimp pattern are further quantitatively characterized by image analysis from HH35 to HH37 (Figure 3C) [69]. Corroborating our findings, other studies have also reported collagen crimp pattern in embryonic chick metatarsal tendons from HH39 to HH42 [72, 73, 74]. Since fibrillogenesis continues throughout the embryonic development, it is likely that the development of collagen crimp pattern persists beyond the developmental stages reported thus far. Collectively, these findings demonstrate collagen crimp pattern exists during embryonic tendon development and can be a useful morphological marker to temporally assess tendon formation and development.

VI. Functional Markers:

Formation of a functional tendon relies heavily on mechanical property elaboration during development. While transcription factors (Section II) and ECM components (Section IV) describe tendon differentiation, composition, and structure, they do not reliably reflect tendon functional properties. Our recent studies identified LOX-mediated crosslinks as key regulators of tendon elastic modulus elaboration and showed that crosslinks are more reliable markers than ECM content and morphology [8, 71]. Based on this, functional markers such as elastic modulus and LOX-crosslink density must be evaluated to monitor whether newly forming tendon is developing normally.

Mechanical Property Elaboration:

Using force volume-atomic force microscopy (FV-AFM), we characterized the elastic moduli of chick embryo calcaneal tendons from HH28 to HH43 (Figure 4A) [8]. Between HH28 and HH43, both nanoscale (not shown) and microscale elastic moduli (Figure 4A) increase from 7 kPa to 21 kPa and from 5 kPa to 108 kPa, respectively, in a non-linear fashion. Specifically, the elastic modulus trends up from HH28 to HH30, plateaus from HH30 to HH38, and then increases significantly from HH38 to HH43 [8]. In embryonic chick extensor tendons, elastic modulus has been characterized by bulk tensile testing from HH40 to hatching [9]. Interestingly, between HH40 to HH42 and HH42 to HH43, tendon bulk elastic modulus approximately doubles between stages from 216 kPa to 540 kPa and to 1020 kPa, respectively [9]. This doubling trend is also observed in our study at the same

stages in calcaneal tendon microscale elastic modulus, which increases from 31 kPa to 56 kPa and to 94 kPa [8].

We then asked whether tendon mechanical properties may play a role in regulating embryonic tendon cell behavior during development. To investigate this, we engineered 3-dimensional hydrogel scaffolds that possess the elastic modulus of embryonic tendon at each developmental stage (Figure 5A) [75]. We then cultured HH37 embryonic TPCs within these gels to examine how tissue moduli of select embryonic stages from HH28 to HH43 influenced cell behavior (Figure 5) [75]. After 7 days in culture, gene expression levels of key tendon markers, such as scleraxis and collagen type XII, were significantly lower in embryonic cells cultured within the softer hydrogels that represented earlier embryonic stage tendons (Figures 5B and 5C). In contrast, expression levels were statistically higher in the stiffest hydrogel that represented the latest embryonic stage tendon. During embryonic development, the elastic modulus of tendon increases [8] and TPCs become increasingly committed to the tendon lineage [11, 17]. Based on this, TPCs would be expected to express higher levels of key tendon markers when cultured within stiffer hydrogels that represent tendon tissue of later embryonic stages, as we observed [75]. Taken together, our data suggest that tissue mechanical properties play a role in modulating embryonic cell behavior during tendon development.

Lysyl Oxidase (LOX)-Mediated Crosslinks:

We have shown that elastic modulus exhibits a stage-specific temporal pattern during embryonic tendon development (Figure 4A) [8]. To identify contributors to mechanical property elaboration, we characterized ECM content and organization during development. Despite a nearly exponential increase from early to late development, collagen content and organization were found to only weakly correlate with elastic modulus quantitatively and spatially, respectively [8]. Other ECM components such as glycosaminoglycans (GAGs) showed no correlation with elastic modulus [8]. These data demonstrated changes in ECM content and organization do not reliably reflect changes in mechanical properties.

We then investigated LOX-mediated crosslinks, hydroxylysyl and lysyl pyridinoline crosslinks (HP and LP, respectively), as potential regulators of elastic modulus elaboration during tendon development. Inhibition of LOX activity via β -aminopropionitrile (BAPN) treatment resulted in significant reductions in both elastic modulus and LOX-mediated crosslink density, where LOX-mediated crosslink density (pmol/mg) is defined as the amount of HP and LP crosslinks per tendon dry mass (Figures 4B and 4D) [8, 71]. Statistical analysis showed LOX-mediated crosslink density and elastic modulus are highly correlated ($r^2 = 0.80$, $P < 0.0001$) and that this correlation holds when LOX activity is perturbed and LOX-mediated crosslink density is altered (Figure 4C) [71]. Strikingly, significant reductions in elastic modulus occurred without any detectable changes in collagen content or organization. Cellularity, dry mass, GAG content, and cell functions including viability, proliferation, and metabolic activity were also unaffected by BAPN treatment [8]. These findings collectively demonstrated ECM content and organization are insufficient as markers to evaluate functional properties of developing tendon.

Similar to elastic modulus, LOX-mediated crosslinks, primarily HP and LP, also exhibit stage-specific temporal trends. Specifically, LOX-mediated crosslink per dry mass increases by six-fold from HH28 to HH35 and then plateaus from HH35 to HH40 before increasing dramatically by five-fold from HH40 to HH43 (Figure 4D) [71]. Based on our findings, LOX-mediated crosslink density is an important functional marker to monitor development of tendon mechanical properties.

We have also characterized expression patterns of the LOX molecule and examined correlations with mechanical properties [3]. We found that LOX gene expression, protein, and enzyme activity levels each demonstrate stage-specific increases during embryonic tendon development. In particular, proLOX and LOX activity levels exhibit temporal profiles that correlate significantly with that of embryonic tendon elastic modulus ($r^2=0.93$, $p=0.034$; $r^2=0.97$, $p=0.016$, respectively) [3]. These novel findings suggest LOX molecular and enzyme activity levels can also be characterized as markers to temporally evaluate tendon formation.

VII. Conclusions:

Tendon wound healing and tissue engineering strategies generate tissues with aberrant composition and mechanical properties compared to native tendons. We propose that this is because current approaches do not follow a sequence of events that is required for normal tendon formation. Evaluations of tissue engineering and healing commonly rely on sustained expression of tendon transcription factors and increases in extracellular matrix content, with little attention to when and how long these events should occur. For instance, studies have concluded that MSCs are tenogenically differentiating based on sustained expression of tendon transcription factors (e.g., scleraxis) and extracellular matrix molecules (e.g., collagens, proteoglycans), coupled with continual increases in construct elastic modulus or tensile strength. In contrast, heightened scleraxis and tenomodulin gene expression levels only overlap at specific stages during embryonic development [4]. Furthermore, increases in tendon elastic modulus occur primarily during earlier and later developmental stages, with no significant changes in mechanical properties during mid-development [8], whereas collagen (hydroxyproline) content increases continuously during these same stages [8, 71]. Importantly, these events need to be accompanied at later stages by increases in LOX-mediated crosslink density. As we previously showed, embryonic tendons can elaborate ECM composition and organization without changes in mechanical properties if LOX-mediated crosslinking is compromised [8]. It is possible that the commonly observed increases in ECM content without sufficient elaboration of mechanical properties in tendon tissue engineering and wound healing are due to insufficient crosslinking. Thus, effective tissue engineering or wound healing strategies should promote LOX-mediated crosslinking during key timepoints of collagen synthesis and remodeling. Taken together, we propose that wound healing and tissue engineering strategies that follow a coordinated sequence of events, as occurs during embryo development, is necessary to generate a tissue that is more compositionally and mechanically similar to native tendon.

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Figure 1.

Key events reported during embryonic tendon development. In early development, embryonic tendon is highly cellular with minimal to no extracellular matrix (ECM) deposition. In mid development, cellularity starts to decrease while ECM deposition and organization become detectable. In late development, ECM deposition and organization increase substantially compared to earlier time points while cellularity continues to decrease.

Figure 2. Collagen content and organization of chick embryo calcaneal tendons from HH28 to HH43. (A) Second harmonic generation (SHG) imaging (top row) shows collagen fibers increase in content and alignment throughout development (Scale bar, 2 μ m). Trichrome staining (bottom row) shows cells (red) decrease while collagen (blue) increases during tendon development (Scale bar, 20 μ m). (B) Collagen content, measured by hydroxyproline assay, increases in a nearly exponential manner throughout embryonic development. (Figure adapted from Marturano et al. 2013).

3A.

3C.

Figure 3.

Cell density, collagen fiber, and actin filament morphology of chick embryo calcaneal tendons from HH34 to HH37. (A) DNA content per dry mass decreased non-linearly from HH28 to HH43. (B) Actin filaments (green, phalloidin staining), and collagen fibers (red, SHG imaging) both exhibit crimp patterns during development. Cell nuclei (blue, DAPI staining) conform with the parallel alignment and crimp pattern of collagen (Scale bar, 10 μm). (C) Measurements of amplitude (top) and period (bottom) of actin filament and collagen crimp patterns revealed statistically identical morphological patterns during

development starting at HH35. (Figure 3A adapted Marturano et al. 2013; Figures 3B and 3C adapted from Schiele et al. 2015).

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A.

B.

C.

D.

Figure 4.

Chick embryonic tendon tissue elastic modulus and LOX-mediated crosslink density. (A) Tendon elastic modulus characterized via FV- AFM as a function of developmental stage increases nonlinearly during development. (B) BAPN treatment significantly reduced tendon elastic modulus at HH40 and HH43. (C) LOX-mediated crosslink density is significantly correlated to elastic modulus. (D) HP+LP-to-dry mass ratio (crosslink density) is significantly reduced by BAPN treatment from HH35 to HH43. (Figures 4A and 4B adapted from Marturano et al. 2013; Figures 4C and 4D adapted from Marturano et al. 2014).

Figure 5:

Effects of hydrogel elastic moduli on TPC gene expression. (A) Hydrogels were fabricated to present chick embryonic tendon elastic modulus of different developmental stages from HH28 to HH43. Gene expression levels of scleraxis (B) and collagen type XII (C) were significantly higher in chick embryo TPCs in stiffer gels that represented later embryonic stage tendons. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (Figure adapted from Marturano et al. 2016).