Molecular Regulation of Tendon Cell Fate During Development

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ABSTRACT: Although there have been several advances identifying novel mediators of tendon induction, differentiation, and patterning, much of the basic landscape of tendon biology from developmental stages onward remain almost completely undefined. During the New Frontiers in Tendon Research meeting, a group of developmental biologists with expertise across musculoskeletal disciplines identified key challenges for the tendon development field. The tools generated and the molecular regulators identified in developmental research have enhanced mechanistic studies in tendon injury and repair, both by defining benchmarks for success, as well as informing regenerative strategies. To address the needs of the orthopedic research community, this review will therefore focus on three key areas in tendon development that may have critical implications for the fields of tendon repair/regeneration and tendon tissue engineering, including functional markers of tendon cell identity, signaling regulators of tendon induction and differentiation, and in vitro culture models for tendon cell differentiation. Our goal is to provide a useful list of the currently known molecular players and their function in tendon differentiation within the context of development. © 2015 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 33:800–812, 2015.

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The musculoskeletal system is composed of several distinct tissues, including skeleton, muscle, and tendon. While the critical events and regulators of skeletal and muscle development are well established, relatively little is known about tendon development, despite the importance of this tissue to overall musculoskeletal function. In the vertebrate embryo, the tendons of the head, trunk and limbs are induced from mesodermal cells that then go on to form the connections between muscle and skeleton.[1] Although the cell and tissue interactions that give rise to tendons within each of these body regions possess their own unique features, there are general aspects of tendon induction and differentiation that are universal across all regions. In recent years, there have been several advances identifying novel mediators of tendon induction, differentiation, and patterning, but crucial gaps in knowledge remain. During the New Frontiers in Tendon Research meeting, a group of developmental biologists with expertise across musculoskeletal disciplines identified key challenges for the tendon development field, including the small number of labs engaged in tendon development research and the paucity of experimental tools and models. As a result, much of the basic landscape of tendon biology from developmental stages onward (including signaling, transcriptional, and epigenetic regulators) remain almost completely undefined. Overcoming these challenges and answering these questions will require a concerted community effort to coordinate resource development and research between labs and across related musculoskeletal disciplines, as well as fostering new researchers into the field. Toward that end, NIH-sponsored funding mechanisms can play a critical role in enabling such efforts.

The primary interest in tendon development research stems from the clinically important problem of degeneration and injury, which affects more than 110 million people per year.[2] The tools generated and the molecular regulators identified in developmental research have enhanced mechanistic studies in tendon injury and repair, both by defining benchmarks for success, as well as informing regenerative strategies. To address the needs of the orthopedic research community, this review will therefore focus on three key areas in tendon development that may have critical implications for the fields of tendon repair/regeneration and tendon tissue engineering: (1) Functional markers of tendon cell differentiation identity, (2) signaling regulators of tendon induction and differentiation, and (3) in vitro culture models for tendon cell differentiation. Our goal is to provide a useful "cheat sheet" of the current known molecular players and their function in tendon differentiation within the context of development. This review will present a general overview of tendon development, but is not meant to provide a comprehensive conceptual guide to patterning events, region specificity (limb vs trunk vs head) or tissue-dependent interactions (for reviews that address these important questions, see the reviews by Schweitzer et al. and Edom-Vovard et al.).[1,3] Similarly, although the past few years have seen tremendous progress in understanding enthesis formation, this review will touch on enthesis and myotendinous junction development only as it relates to the tendon cell fate (for more information, see the recent excellent review by Zelzer et al.).[4] The molecular events that regulate tendon induction and formation in mouse and chick will be the primary focus for this review, due to the large body of work using these model systems and the greater similarity of their tendon structures to that of humans compared

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to invertebrates and lower order vertebrates. The exciting potential of alternative animal models in identifying new molecular regulators of tenogenesis will be addressed, however, in the final section of this review.

FUNCTIONAL MARKERS OF TENDON CELL IDENTITY

Transcriptional Markers

Scleraxis (Scx). Historically, the slow pace of tendon development research relative to that of the other musculoskeletal tissues can be attributed to the absence of a robust tendon-specific marker. The discovery that *Scx*, a basic helix–loop–helix transcription factor, is expressed by all tendon and ligament progenitors facilitated detection of early events in tendon development.[5] In the mouse limb, *Scx* is first detected in the early limb bud, at E10.5 in the subectodermal limb bud mesenchyme.[5] *Scx* expression is initially induced as ventral and dorsal patches, which are eventually organized into the flexor and extensor tendon groups. Overt organization of *Scx*-expressing cells is not observed until E12.5, when the tendon progenitors align between muscles and cartilage, followed by condensation into aggregated structures at E13.5. While there may be continued induction of tendon progenitors in the distal limb past E12.5, the stages of tendon development in the mouse limb can be very broadly generalized as the stages of induction (E10.5 and E11.5), organization (E12.5), differentiation (E13.5), and growth (E14.5 onward). Interestingly, although *Scx* expression from the early stages of tendon development indicated that it may be a master regulator of the tendon cell fate, deletion of the *Scx* gene did not result in universal tendon loss. When the transgenic tendon reporter *ScxGFP*[6] was incorporated into the background of the $Scx^{-/-}$ mutant, reporter expression was detected in tendon progenitors in mutant embryos, demonstrating that *Scx* is not essential for regulating its own expression and the loss of *Scx* did not result in a detectable tendon phenotype until E13.5.[7] Indeed, $Scx^{-/-}$ mutants are viable and the ability to generate a tendon structure that functionally attaches muscle to bone is not lost in the absence of *Scx*. However, the tendon phenotype in *Scx* mutants is quite severe and mutants display limited mobility due to loss or disruption of all of the long tendons in the body (Fig. 1A–D), highlighting the importance of *Scx* to normal tendon development. In the $Scx^{-/-}$ mutant, embryonic expression of several tendon markers is either greatly reduced or missing entirely by E16.5,[7] and it has also been shown that *Scx* can directly drive expression of two later markers of tendon differentiation, *Col1a1* and *Tenomodulin*.[8–10] Similarly, TEM analysis showed that the amount of collagen matrix within surviving tendon structures is also reduced, although collagen fibrils can be observed in the mutant tendons. Taken together, *Scx* remains the earliest detectable tendon marker to date, is expressed by all tendon cells and regulates critical aspects of tendon differentiation during development. However, although a number of studies have now demonstrated that *Scx* can enhance tenogenic differentiation of stem cells in vitro,[11] tendon induction in vivo does not depend on *Scx* and it is not the primary nor the only driver of tenogenesis in vertebrates.

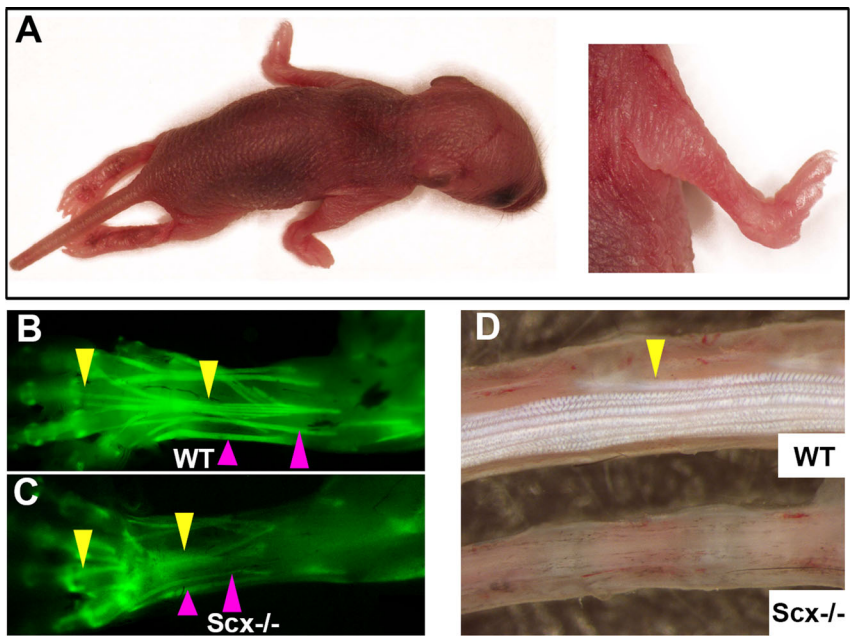

Figure 1. Long tendons are severely disrupted in $Scx^{-/-}$mice. (A) Forelimbs of newborn $Scx^{-/-}$ pups are locked in dorsal flexure due to missing flexor tendons. (B,C) *ScxGFP* expression showed reduced extensor tendons in the limbs of E18.5 $Scx^{-/-}$ embryos. (D) Tail tendons are completely lost in $Scx^{-/-}$ mutants (adapted from Murchison et al.[7]).

Mohawk (Mkx). For several years, *Scx* was the only identified transcription factor with a functional role in tendon differentiation. This changed with the generation of the $Mkx^{-/-}$ mutant which displayed a marked tendon phenotype.[12–14] The *Mkx* gene encodes an atypical homeodomain transcription factor, and is dynamically expressed in many tissues during embryogenesis.[15,16] In mouse limb and tail tendons, *Mkx* expression is not detectable until the stage of tendon differentiation at E13.5.[13] While *Mkx* is strongly expressed in embryonic tendons (Fig. 2A and B), $Mkx^{-/-}$ tendons appear largely normal during all embryonic stages, and homozygous *Mkx* mutants are both viable and fertile with no apparent tendon patterning defects.[13] The tendon phenotype in *Mkx* mutants is not observed until postnatal stages when tendon growth in mutant mice begins to diverge significantly from that of WT littermates (Fig. 2C and D). Postnatal tendon growth is characterized by dramatic collagen matrix accumulation and maturation. In wild type pups, tendon collagen matrix transforms soon after birth from a homogenous field of small diameter fibrils to a heterogeneous assembly of collagen fibrils with a stereotypic distribution of fibril diameter from large to small fibrils. In contrast, the collagen fibrils of $Mkx^{-/-}$ mutants remain much smaller and relatively uniform in size, suggesting a failure in the transition from embryonic to postnatal modes of collagen fibril growth (Fig. 2E and F).[12–14] Although *Scx* has also been shown to regulate collagen, the matrix phenotype in $Scx^{-/-}$ mice is already detectable at embryonic stages and the main feature of this phenotype is reduced fibril number (postnatal maturation of matrix has not been evaluated in *Scx* mutants since the tendon phenotype is already quite severe at birth). Expression data in both null mutants demonstrates that regulation of *Scx* and *Mkx* is mutually independent,[13] indicating non-redundant roles for these transcription factors in matrix formation and organization. A few recent studies found that like *Scx*, *Mkx* can also enhance the tenogenic potential of mesenchymal stem cells,[17,18] and others have identified *Mkx* as a transcriptional repressor.[19,20] Since the mechanical properties of tendons are directly correlated with the size, modification, and crosslinks of the collagen fibrils, *Mkx* thus regulates a critical transition in tendon differentiation and maturation that may prove central for the ability to engineer robust and functional tendons in vitro.

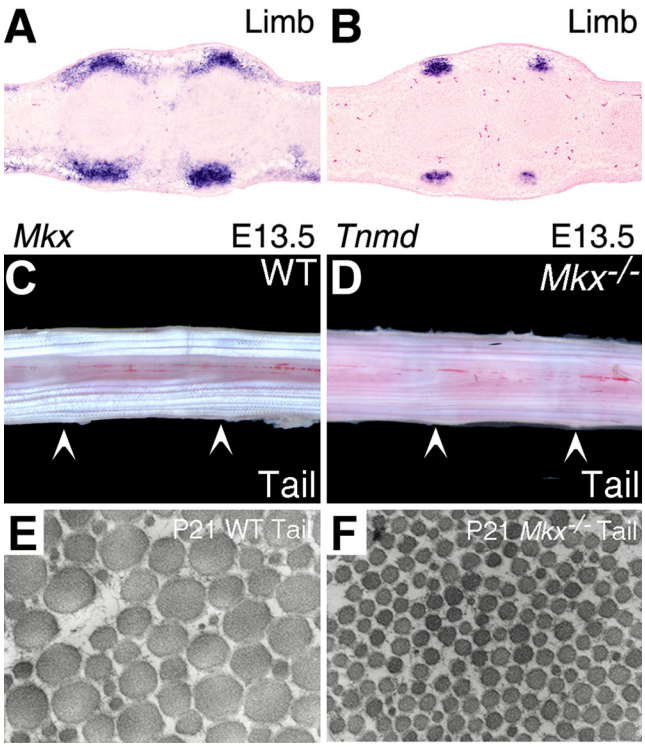

Figure 2. *Mohawk* regulates postnatal tendon matrix maturation. (A, B) Limb section ISH for *Mkx* and *Tnmd* shows strong expression of both genes in tendon progenitors at E13.5. (C, D) $Mkx^{-/-}$ mutant tail tendons are dramatically reduced in size at postnatal stage P14. (E, F) Analysis of collagen matrix morphology by TEM showed much smaller collagen fibrils in $Mkx^{-/-}$ tendons at P21 (adapted from Liu et al.[13]).

Early Growth Response (Egr). At its most basic, a "tendon" is the tissue connecting muscle to skeleton, which enables the transfer of forces and movement. Within this definition, tendons are found in a wide array of living organisms including invertebrates such as the fruit fly *Drosophila melanogaster*. *Drosophila* tendons differ from vertebrate tendons in embryonic origin (ectodermal vs mesodermal), appearance (flat sheet of cells vs linear organization), and mechanism of function (microtubules vs collagen matrix), but share a few common developmental features.[1] The key transcription factor in *Drosophila* tendon specification and differentiation is *stripe*, an Egr-like transcription factor.[21–23] Vertebrate homologues of *stripe* include four members from the Egr family of zinc finger transcription factors, Egr1 through Egr4. While *Egr3* and *Egr4* are not expressed in tendons, a recent study reported *Egr1* and *Egr2* expression in both chick and mouse tendons during embryonic development.[24] In mouse limb tendons, *Egr1* is first detected at E12.5, while *Egr2* expression is not observed until E14.5. By E16.5, *Egr1* expression is restricted to the myotendinous junctions and the epitenon while *Egr2* continues to be broadly expressed throughout the tendon until E18.5. Interestingly, ectopic expression of *Egr1* or *Egr2* led to induction of *Scx* and other tendon genes.[24] The potential to drive *Scx* expression and the analogy with *Drosophila* suggested that *Egr1* and *Egr2* may be the key regulators of the tendon cell fate, but loss of function experiments did not result in dramatic disruption of tendon induction or differentiation.[24] The overt tendon phenotype reported for single $Egr1^{-/-}$ or $Egr2^{-/-}$ mutants is a relatively modest reduction in embryonic collagen fibril number, and even in double *Egr1*/*Egr2* null mutants, strong *Scx* and collagen expression was still observed by in situ hybridization,

suggesting the Egr transcription factors are also not the primary drivers of embryonic tendon differentiation. Like *Mkx* and *Scx*, however, expression of Egr in mesenchymal stem cells was sufficient to direct them toward tenogenic differentiation and improve their effectiveness in a tendon injury model. Analysis of adult *Egr1*$^{-/-}$ tendons showed generally normal gross morphology,[25] although tendon size is reduced and mechanical properties are lower. Since *Egr2* mutants are perinatally lethal,[26] it may be that the two molecules are functionally redundant during postnatal stages and the elimination of both would result in a more dramatic matrix phenotype. Overall, the available data thus far indicates that the Egr transcription factors do not play a major role in vertebrate tendon specification or patterning during development, but may regulate aspects of matrix formation, independent of *Mkx*.

Sine Oculis-Related Homeobox (Six). The vertebrate Six genes are homeobox transcription factors that were originally cloned based on homology with the sine oculis gene in *Drosophila*. Six members of this family have been identified in mammals and of these, Six1 and Six2 were initially reported as tendon genes.[27] It was subsequently shown however, that *Six1* expression clearly defines the muscle progenitors of the limb and its expression pattern is distinct from that of *Scx*; therefore, *Six1* (and its binding partner *Eya2*) is not a tendon transcription factor.[28] Indeed, loss of *Six1* results in a clear myogenic phenotype.[29,30] Until recently, the activity of *Six2* and its role in tendon formation was almost completely ignored. In the original description of *Six2* expression, *Six2* mRNA was detected by E11.5 in the mouse limb, during the stage of tendon induction.[27] From E12.5 to E14.5, the progenitors comprising the extensor and flexor tendons of the digits continue to express *Six2* robustly. A recent study in the chick limb showed a similar pattern of expression in the developing chick autopod.[31] Like *Scx*, *Six2* in the distal limb is initially expressed as dorsal and ventral patches in the subectodermal mesenchyme. Comparison of *Scx* and *Six2* expression domains confirmed overlapping expression of these transcription factors, indicating that *Six2* is indeed expressed by tendon progenitors.[31] While *Six2* is also detected in the zeugopod, its expression domain there includes both myogenic and non-myogenic precursors. To date, the functional role of *Six2* in tendon is unknown; while it may be useful as an early marker of tendon induction, its expression in a subset of myogenic progenitors suggests it is not necessarily a marker of tendon-specific cell identity. During development, tissue patterning and positional specification are frequently driven by transcription factors that orchestrate the gross morphology and interactions between multiple tissue groups. Given the distinct developmental origin of muscle and tendon, *Six2* expression in these tissues during development suggests that *Six2* may fall into this latter category.

SRY (Sex Determining Region Y)-Box 9 (Sox9). *Sox9* belongs to the SOX family of transcription factors, members of which share a high-mobility group DNA binding domain. It is well-established that *Sox9* is one of the primary drivers of chondrogenesis and skeletal development, and loss of *Sox9* results in a total absence of cartilage.[32] While *Sox9* is indisputably a cartilage marker, lineage tracing previously revealed that the tendon cells close to the skeletal insertion sites originate from an early population of *Sox9*-expressing cells.[33] Two recent studies now shed further light on the intriguing developmental origin of these cells. These studies showed that the secondary skeletal features, including the bony eminences and the entheses, are formed from a novel population of progenitors that express both *Sox9* and *Scx*.[34,35] While some of these cells are then allocated to form the skeletal eminences (and thereby selectively down-regulate *Scx*), the remaining progenitors down-regulate *Sox9* to form tendon. Thus, by E13.5 during the stage of tendon differentiation, the expression domains of *Sox9* and *Scx* are mutually exclusive (confirming their divergent fates), although the cells at the tendon-bone insertion share a common cellular origin. Importantly, *Sox9* does not seem to have a functional role in tendon differentiation since deletion of *Sox9* in *Scx*-expressing cells leads to disruption of the skeletal eminences only, but not tendon.[34,35]

Other Markers

Prior to the discovery of *Scx*, identification of tendon frequently relied on the use of tendon matrix and non-transcriptional molecules, and several of these remain useful in distinguishing tendon-specific differentiation and maturation. These include various fibrillar and non-fibrillar collagens, small leucine-rich proteoglycans, and glycoproteins. With the exception of Type I Collagen (which is a critical matrix protein involved in the structural properties of a large number of tissues), most of these matrix molecules play relatively minor roles in tendon development; phenotypes overall do not result in tendon disruptions, are detectable only in later postnatal stages, and restricted to tissue level structural and functional properties rather than tendon cell fate decisions. For this reason, we have chosen to focus discussion on only two of these markers, however a more comprehensive catalog of known matrix-related molecules for tendon can also be found in Table 1. For a more detailed review of these structural molecules, please see the reviews by Connizzo et al., Kalamajski et al., and Zhang et al.[36–38]

Tenomodulin (Tnmd). Of the molecules that have been used to identify tendon differentiation, Tnmd is one of the few that is highly specific for tendons and

Table 1. Summary of Tendon Matrix-Related Markers

Matrix Marker	Tendon Relevance During Embryonic or Postnatal Development
Collagen type I[37,111]	Major fibrillar collagen (70–80% dry weight)
Collagen type II[112]	Compressive regions—indicator of fibrocartilage
Collagen type III[37]	Fibril assembly
Collagen type V[113,114]	Fibril assembly (nucleation)
	Null mutants are embryonic lethal at E10.5
	Heterozygous mutants display reduced tendon diameter, higher elasticity
	Compound Col5a1 and Col11a1 mutants result in severe fibril disruption
Collagen type XI[115]	Fibril assembly (nucleation)
	Compound Col5a1 and Col11a1 mutants result in severe fibril disruption
Collagen type XII[37]	FACIT collagen
	Fibril assembly, regulation of fibril diameter
Collagen type XIV[37]	FACIT collagen
	Fibril assembly, regulation of fibril diameter
Decorin[38,116]	Small leucine-rich proteoglycan (class I)
	Regulate lateral growth
	Null mutants display fibrils with larger diameters, abnormal lateral fusion
Biglycan[117]	Small leucine-rich proteoglycan (class I)
Fibromodulin[117,118]	Small leucine-rich proteoglycan (class II)
	Regulate lateral growth
	Null mutants display thinner average fibrils
Lumican[38]	Small leucine-rich proteoglycan (class II)
Tenascin C[119–121]	Glycoprotein
	Early matrix marker used to identify tendon progenitors prior to the discovery of Scx
	Also labels muscle connective tissue, perichondrium, and nerves
	Null mutants are not phenotypic

ligaments. Tnmd is a type II transmembrane glycoprotein,[39] and its expression is first observed in the mouse limb at E13.5. Loss of Tnmd however does not result in any embryonic phenotype; in *Tnmd*$^{-/-}$ mutants, tenocyte cell number, as well as tendon patterning and overall morphology appears normal at birth.[40] A modest decrease in cell proliferation is detectable in *Tnmd*-deficient mice immediately after birth, however, by P7 these differences are no longer observed. Tendon matrix is likewise largely unaffected, although collagen fibrils are slightly larger in diameter in *Tnmd*$^{-/-}$ mice at 6 months of age.[40] In early studies, Tnmd, like its cartilage homolog chondromodulin, was shown to possess anti-angiogenic properties in vitro;[41,42] however, vascularity in tendon and other tissues is also not disrupted in null mutants, indicating no essential role for Tnmd in angiogenic regulation in vivo. Therefore, despite the specificity of Tnmd in tendon, *Tnmd*$^{-/-}$ mutants do not display an appreciable tendon phenotype. As a marker for tendon cell fate, however, Tnmd remains highly relevant given that it is the most specific marker available for tendons and ligaments at this time. Although it is detected in a few other tissues (including the eye), it is completely absent from other musculoskeletal tissues such as muscle, bone or cartilage.[39]

Thrombospondin 4 (Thbs4). Thrombospondin is a glycoprotein that was first shown in *Drosophila* to have a vital role in forming the tendon–muscle attachment. *Drosophila* Thbs is secreted by tendon cells and the protein interacts with integrins produced by muscle cells.[43,44] *Thbs* null mutants die immediately after birth due to muscle detachment from the exoskeleton. In vertebrates, the Thrombospondin family is composed of five members. Thbs4, along with related family members Thbs3 and Thbs5 (also known as COMP), forms pentamers composed of five identical subunits. It was recently shown that Thbs4 is expressed in both zebrafish and mouse tendon;[45,46] while zebrafish *Thbs4* is highly specific to tendon, mouse *Thbs4* is also expressed within the muscle connective tissue though not the muscle fibers themselves. Null mutants in both model systems are viable and there are no apparent defects in gross tendon/muscle appearance or mobility. However, upon challenge, the muscle–tendon junction is profoundly disturbed, indicating impaired assembly of the muscle–tendon junction[45,46]. Taken together, Thbs4 appears to be an important molecule of myotendinous ECM assembly, but may not play a distinctive role in tendon differentiation. However, since Thbs5 is also expressed in tendon, the absence of a tendon-specific phenotype in *Thbs4*$^{-/-}$ mutants may be due to redundancy. Regardless, given the strong expression of *Thbs4* in tenocytes, we believe it is a useful marker for tendon, though far less specific than Tnmd.

SIGNALING REGULATORS OF TENDON INDUCTION AND DIFFERENTIATION

Fibroblast Growth Factor (FGF)

The FGF pathway was the first signaling pathway shown to induce tendon cell identity in vivo. Members of this family have been implicated in a wide variety of biologic and developmental processes, including cell proliferation, differentiation, and growth.[47] The role of FGF in tendon induction was first established for the axial tendons in chick. During axial tendon development, *Scx*-expressing tendon progenitors are induced as a sub-compartment of the sclerotome (the somitic compartment that gives rise to the axial skeleton), called the syndetome.[48] Induction of these tendon progenitors depends on FGF signaling from the neighboring myotome (the somitic compartment that gives rise to the axial muscles).[49–51] In the absence of these signals, *Scx* expression is reduced or lost. FGF signaling is also sufficient to induce *Scx* expression; in bead experiments, ectopic FGF ligand applied to developing chick limb buds results in activation of *Scx*.[52] Several FGF ligands have been implicated in tendon induction, including FGFs 4 and 8 in chick, and FGFs 4 and 6 in mouse.[48–50,53] However, to date there is no report of *Scx* induction by FGF signaling in tissue culture or any other system except for the chick embryo, and despite the wide availability of FGF-related mutants (ligands or receptors), tendon phenotypes have thus far not been reported. This may be due to redundancy between molecules as there are currently 18 known ligands and 4 known receptors.[54] A specific role for FGF signaling in mammalian tendon induction and/or differentiation will likely require the targeted deletion of multiple family members. Uncoupling direct and indirect effects on tendon will also be challenging since FGF signaling is involved in limb growth and patterning, as well as the development of related musculoskeletal tissues, such as cartilage.[55]

Transforming Growth Factor Beta (TGFβ) Superfamily

Over the last several years, members of the TGFβ superfamily have emerged as important signaling regulators of tendon development, both as inducers and repressors.[56] The TGFβ superfamily consists of two subfamilies, members of which are organized based on their downstream receptor-Smad mediators. The TGFβ subfamily signals via the Smad 2/3 pathway while the Bone Morphogenic Protein (BMP) subfamily signals via Smads 1/5/8.[57] For all members of the superfamily, Smad-mediated signaling is initiated by ligand binding to types I and II receptors, leading to receptor Smad phosphorylation. All receptor Smads must then dimerize with Smad4, which facilitates translocation of the complex into the nucleus to activate transcription. While members of the TGFβ subfamily have been implicated as tendon inducers, the BMP subfamily functions primarily as repressors of tendon progenitor induction, although a subset of these molecules may also drive tendon differentiation. While this review will focus on Smad mediated signaling, the TGFβ superfamily also signals via a number of non-Smad pathways, including the MAPK, Jun-Fos and NF-kB pathways.[58]

TGFb and Myostatin. The Smad2/3 subfamily of ligands includes TGFβ, Myostatin, Activin, and Nodal. While classified under the same subfamily, the receptors utilized by these members are largely distinct. The TGFβs were first investigated for their chondrogenic capacity since a number of experiments showed suggestive activity during skeletal development.[59,60] Initial studies subsequently found that chondrogenic differentiation was generally unimpaired in TGFβ mouse mutants, indicating a relatively minor role in skeletal development and chondrogenesis in vivo.[61,62] Recent data, however, showed that TGFβ signaling is required for the induction of the *Sox9+/Scx+* progenitors that form the bony eminences and skeletal-tendon insertions.[34] In the absence of *TGFβRII*, this early progenitor population is not induced. As was previously shown for FGF signaling, TGFβ is also sufficient to induce *Scx* and other tendon markers in both limb explant cultures and mouse embryonic fibroblasts (Fig. 3A and B).[63] However, while FGF induction of *Scx* was so far reported only in early chick embryos, induction by TGFβ signaling has now been demonstrated in a wide range of cell types.[17,63–65] The TGFβ ligands signal via a single type II receptor; therefore deletion of the receptor is sufficient to delete all TGFβ signaling. Strikingly, elimination of TGFβ signaling, either through targeted deletion of the type II receptor (*TGFBRII*) or double deletion of the ligands *TGFβ2* and *TGFβ3*, results in a complete loss of all tendons (Fig. 3C and D).[63] While early tendon progenitor induction is not disrupted in TGFβ mutants (for example, *Scx* expression at E10.5 and E11.5 in mutant limb buds appears normal), loss of *Scx* is evident by E12.5. Interestingly, a recent analysis of the tendon progenitor transcriptome in mouse embryos found evidence only for activation of the TGFβ signaling pathway in the early forming tendons; there was no indication that FGF signaling is active in tendon progenitors during these early stages.[64] The role of TGFβ signaling in later aspects of tendon development and differentiation has still not been addressed. Given the critical role of TGFβ signaling in early tendon development, inducible deletion of TGFβ signaling at later stages in development will be required to bypass the early tendon phenotype, in order to identify requirements for signaling in tendon maintenance, differentiation or maturation.

In addition to TGFβ, other members of the Smad2/3 subfamily have also been implicated in tendon differentiation. A few years ago, it was shown that myostatin induces tendon markers in cultured cells, and adult myostatin null tendons are both mechanically inferior and smaller in size.[66] Myostatin was first

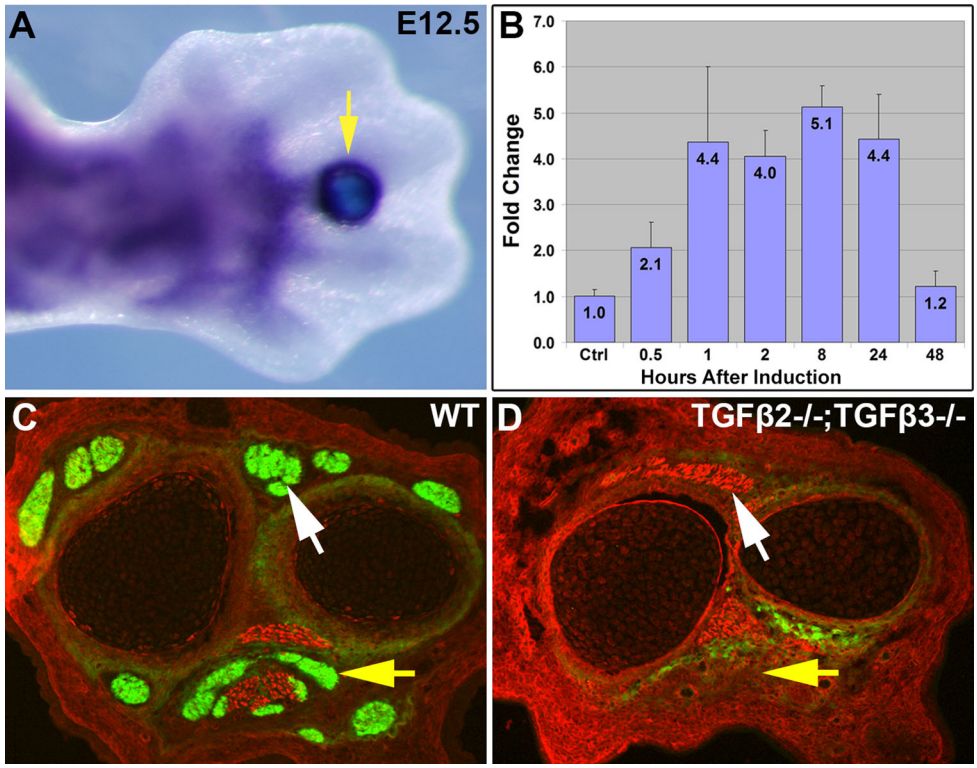

Figure 3. TGFβ signaling is required for tendon induction and development. (A) Whole mount ISH for *Scx* after incubation with Affigel beads saturated with TGFβ2 protein shows induction of *Scx*-expressing progenitors. (B) Increasing levels of *Scx* transcript (determined by qRT-PCR) in C3H10T1/2 mesenchymal cells following a pulse of TGFβ activation. (C, D) Transverse sections from E14.5 forelimbs of (C) wild type and (D) $TGF\beta2^{-/-}$; $TGF\beta3^{-/-}$ embryos showed complete loss of tendons in the absence of TGFβ signaling. Tendons were visualized using *ScxGFP* and sections were counterstained with MHC antibody to visualize muscle (adapted from Pryce et al.[63]).

established as a regulator of muscle size; in *myostatin* null mutants, muscles are considerably larger, due in part to disregulated myoblast proliferation.[67–69] Interestingly, the regulation of tendon size by myostatin appears to be inverse to that of muscle, since adult *myostatin* null tendons are both smaller and hypocellular (rather than hypercellular).[66] While TGFb signals via a single Type II receptor, there are two Type II receptors for both myostatin and activins (ACVR2A and ACVR2B). It was demonstrated that *ACVR2B* is expressed in tendons, however, tendon phenotypes have not been reported for *ACVR2A* or *ACVR2B* null mutants or for activin null mutants. Since the two receptors appear to be functionally redundant in the regulation of muscle mass, establishing the role for activin signaling in tendon may require the conditional deletion of both receptors.

Bone Morphogenic Protein (BMP) and Smad4. Although members of the Smad2/3 subfamily appear to be tendon inducers, BMP signaling was initially shown to restrict *Scx* expression during embryonic development.[5] In the chick limb, *Scx* and the BMP ligands (BMPs 2, 4, and 7) are expressed in mutually exclusive domains and ectopic application of BMPs by bead implantation results in direct repression of *Scx* expression. In addition, repression of BMP signaling during early stages via application of Noggin results in expansion of *Scx* expression. The absence of BMP signaling alone however is not sufficient to drive later events of tendon differentiation or formation, suggesting that an inducing signal is still required. A similar result was recently demonstrated in $Smad4^{-/-}$ mouse mutants, indicating that the repressive activity of BMP signaling in tendon induction may be mediated by Smad signaling.[70] In conditional *Smad4* null mutants, cartilage differentiation is disrupted and the domain of early *Scx* expression in the autopod is significantly expanded at the expense of chondrogenic domains (determined by *Sox9* expression), again highlighting the inverse relationship between cartilage and tendon as alternative cell fates. Consistent with the BMP experiments in chick, the pool of expanded *Scx*-expressing progenitors does not form organized tendons. Instead, the cells adopt a non-specific connective tissue fate, suggesting that additional instructive cues are required to drive tendon-specific differentiation. Although *Smad4* is also required for Smad-mediated TGFb signaling, the early expansion of *Scx* expressing progenitors in the *Smad4* mutant is not consistent with the early loss of tendon progenitors observed in the *TGFBRII* mutant at E12.5,[63]

suggesting that TGFβ signaling during tendon development may not be Smad-dependent. Additional studies will be required to separate the role of Smad and non-Smad signaling and the relationship between TGFβ and BMP signaling during tendon induction and differentiation.

Although BMP signaling is primarily associated with tendon repression during embryonic development, a few members of this subfamily, BMPs 12, 13, and 14 (formerly known as GDFs 7, 6, and 5, respectively), were shown to regulate some aspects of postnatal tendon matrix development.[71–75] Single mutations for these molecules result in predominantly matrix-associated phenotypes, including reduced collagen content and mechanical properties. Of these mutants, *GDF5*$^{-/-}$ mice display the most severe phenotype, although it remains unclear whether the tendon phenotype is a direct or secondary result of joint and skeletal deformities. Interestingly, ectopic implantation of BMPs 12, 13, or 14 results in formation of a tendon-like tissue in adult rats, indicating that these BMP ligands may have inductive capacity as well. The relatively mild phenotypes of single mutants suggest that there may be redundancy between these molecules; therefore compound deletions of these BMP ligands may be required to fully test their role in tendon development.

Wnt

The Wnt signaling pathway is a highly conserved pathway that governs a wide range of biological processes, including cell differentiation, migration, and polarity. During limb development, the surface ectoderm is the source of multiple Wnt ligands, including Wnt3 and Wnt6.[76–78] It was previously demonstrated that Wnt signaling suppresses chondrogenesis, and promotes a proliferative undifferentiated state when combined with FGF signaling.[79] Interestingly, prolonged exposure to Wnt3a ligand in tissue culture induces a connective tissue fate at the expense of chondrogenesis, although *Scx* is not one of the markers induced. A few studies however, suggest a more direct role for Wnt signaling in tendon progenitor induction. It was recently shown that *Scx* induction in the autopod was disrupted when secretion of Wnt ligands from limb bud ectoderm cells was blocked by ectodermal targeting of the *Wntless* gene.[80] Moreover, in another study it was shown that Wnt signaling induces *Six2*-expressing progenitors in the autopod and loss of Wnt signaling (via the application of a Wnt antagonist) results in loss of *Six2* expression.[31] Collectively, the available data suggests that ectodermal Wnt signaling during limb development may play a role in establishing the domains of connective tissue and cartilage formation, however, the specificity to tendon is still unclear. It may be that Wnt promotes differentiation toward alternative connective tissue fates such as muscle connective tissue or perichondrium.

IN VITRO CULTURE MODELS FOR TENDON CELL DIFFERENTIATION

Organ Explant Culture

Organ explant cultures are frequently used for in vitro studies of tissue development and biology, since they provide unique possibilities for manipulation and imaging while the structural, cellular and environmental cues for the tissue are largely preserved intact.[81] The application for tendon studies, however, has been limited by a few major challenges of these systems for tendon research. Successful organ culture (limbs, trunks or tails) is restricted to relatively early stages in tendon development, during the stages of induction (E10.5–E12.5 for the mouse forelimb) since diffusional constraints apply once the limb increases in size. Culture durations are also limited to ~18–24 h after harvest as the organs generally fail to further develop in culture, and native gene expression and signaling begins to break down resulting in rapid loss of endogenous transcripts. This culture system was therefore mostly useful for testing questions related to the regulation of tendon induction within the context of the native embryonic environment (Fig. 3A).[63,82] One recent study, however, showed that the addition of Kartogenin, a small pharmaceutical molecule first identified for its chondrogenic effects, can enhance the development of limbs in culture, and tendon development in these limbs extended beyond induction to aggregation and organization of distinct tendon structures.[83] The use of Kartogenin may therefore open exciting new possibilities for the investigation of tendon formation by incorporating time lapse microscopy and various experimental manipulations (such as bead implantation or gene expression through electroporation or viral delivery). Despite this advance, organ explant culture is still limited by the difficulty in controlling the mechanical environment of tendons; active muscle forces are also completely abolished once the organ is harvested. Taken together, organ explant culture is a powerful method for investigating early tendon induction with high in vivo relevance and context; however, later events in tendon differentiation, matrix regulation, and mechanotransduction are difficult to address using this culture system. We therefore present some alternative in vitro culture models that have been used to evaluate tendon differentiation and maturation, with their attendant strengths and weaknesses.

Two Dimensional (2D) Culture Systems

Conventional cell culture relies on the maintenance and growth of cells adhered to flat, coated plastic. The main advantage of this system is experimental ease, which enables rapid screening of novel molecular mediators and provides a simple platform for testing signaling pathways. Mesenchymal cell lines, such as the C3H10T1/2 line, have been used to evaluate induction of tendon markers in response to exogenous factors (Fig. 3B), although primary cells isolated from

a variety of sources, including embryos, tendons, or bone marrow, have also been used.[18,25,63,84] While an attractive model, the in vivo relevance of cells maintained in this culture environment is questionable for many cell types, including tenocytes. Under 2D conditions, tenocytes rapidly adapt to the underlying plastic substrate and lose expression of characteristic tendon markers. Although mechanical stimulation in 2D culture (using commercially available or custom systems to apply direct tension or fluid flow) can induce or rescue some aspects of tendon cell identity,[85–87] proper tendon differentiation and the formation of a mature, tendon-specific matrix likely requires a more representative 3D environment.

Micromass

One of the classic methods of evaluating chondrogenesis in vitro is an assay known as micromass culture. In this system, limb bud mesenchymal cells are typically suspended at a high density in media, and then plated as individual droplets of very small volume ($\sim$10 μl) to encourage aggregate formation of cells.[88] This environment forces cells to adopt a more three-dimensional morphology, mimicking their native morphology in the limb bud. Under standard media conditions, cell aggregation occurs between 12–24 h of culture and Alcian Blue positive cartilage nodules begin to appear within the micromass culture by day 2.[88] While cartilage formation is spontaneous for a subpopulation of cells, not all of the limb-derived cells undergo chondrogenesis. Some of these non-chondrogenic cells are myogenic in origin, while other cells may be tenogenic or otherwise unspecified.[89] One study showed that fibroblasts derived from embryonic tendon do not spontaneously differentiate into cartilage under micromass conditions.[90] A few recent studies have applied micromass culture as a platform to investigate chondrogenic versus tenogenic differentiation in vitro.[65,91] Since Alcian Blue staining is a rapid and easy readout of cartilage-specific differentiation, analysis of tenogenic (or fibrous) differentiation is first indicated by the absence of chondrogenesis. Confirmation of tenogenesis then depends on standard assays for gene or protein expression (PCR, in situ hybridization or Western blotting). Although promising, this culture system does not replicate the native mechanical environment of tenocytes and additional studies to test the utility of this culture system for tendon is still required.

Three Dimensional (3D) Engineered Constructs

One promising method for testing tendon differentiation and mechanotransduction within a more representative three-dimensional environment utilizes design principles derived from the field of tissue engineering. During embryogenesis, developing tendons are highly cellular with minimal extracellular matrix, and are anchored under tension between the skeleton and muscle. Using the developmental state as design guidelines, there are now several available methods to engineer linear constructs of cells fixed between two anchors.[25,87,92–98] In most of these protocols, cells are seeded at high densities (either scaffold-free or embedded within a hydrogel biomaterial) and formed first as a sheet. The cell-laden sheet then self-assembles over time into a tissue structure around two stationary posts. Cell-mediated forces drive this contraction and the construct is subject to static tension once anchored. This type of engineered construct is amenable to additional mechanical stimulation and a few studies have shown that dynamic stimulation through cyclic loading or gradually increasing strain can drive better alignment of cell-secreted collagen matrix and enhance tenogenic markers.[87,99–103] In addition, while most constructs employ soft hydrogels such as collagen or fibrin as a carrier for cells, nanofiber-based scaffolds have also been explored to mimic the organization and fiber maturity of the collagenous tendon matrix.[104,105] Like most in vitro culture models, the degree to which this system recapitulates the in vivo biology is still an open question. The general methods described here for construct generation and mechanical stimulation have also been widely applied in the cardiac tissue engineering field,[106–108] indicating that these cues alone are not sufficient to drive tendon-specific differentiation, in the absence of additional molecular cues. To date, analysis of the matrix by TEM showed some features that are similar to native tendon (aligned collagen fibrils), but also significant non-fibrillar matrix.[92,97] For all methods, the collagen fibrils within constructs appear consistently immature (uniformly small diameter) and mechanical properties are low, suggesting that this model system best captures aspects of early tendon differentiation, but not maturation. Logistical challenges include the large numbers of cells required to generate each construct, which may limit the number of experimental conditions that can be tested at once, especially if primary cells are used. Therefore, while this is one of the most promising model systems available, the ideal culture conditions for driving tendon differentiation in this system have yet to be fully established. Identifying the markers specific to tendon differentiation will further these efforts.

DISCUSSION

One of the most basic unknowns the tendon community faces is the fundamental question of tendon cell identity. The challenge in defining this cell type in molecular terms is one of the main impediments to tendon research today. The defining feature of the tendon is the collagen matrix, and what distinguishes it from other collagen-rich tissues such as skin, is the highly ordered, heterogeneous assembly of collagen fibrils. Removed from this context, the characteristics that normally define a cell type in vitro (morphology, molecular profile, matrix deposition) are surprisingly

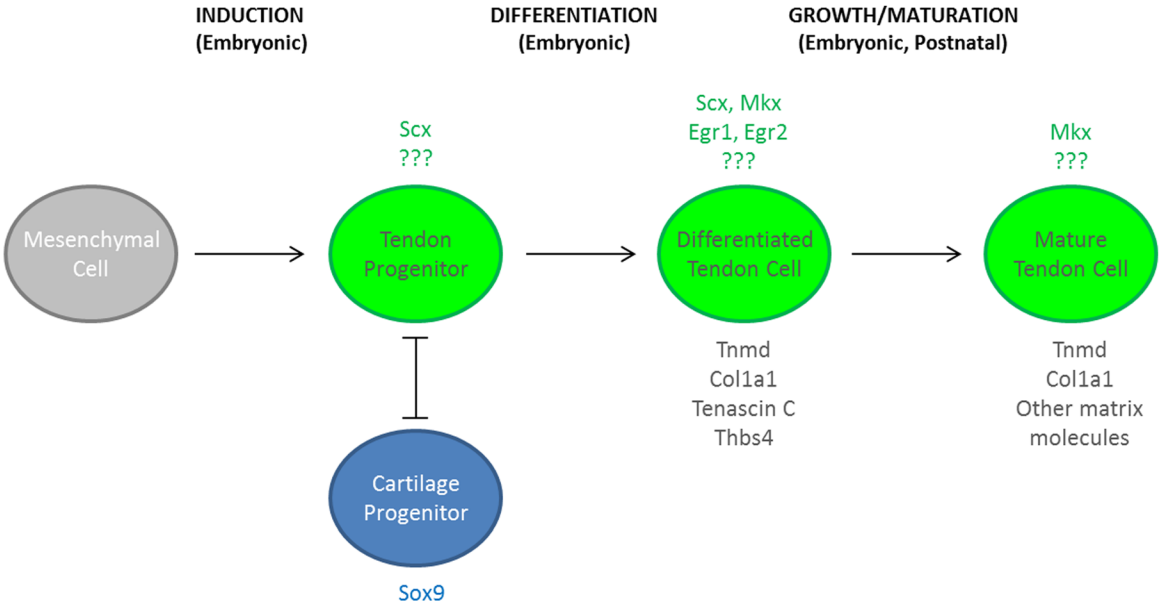

Figure 4. Benchmarks for tendon induction, differentiation, and maturation. This schematic extrapolates the developmental sequence of tendon formation and the markers expressed during these stages to define a preliminary framework for defining the tendon cell fate. Note that tendon maturation is defined by the postnatal transition from the immature matrix (homogeneous distribution of small fibrils) to the mature matrix (heterogeneous distribution of small and large fibrils).

difficult to apply to tendon cells, which lose native morphology upon extraction, and are incapable of forming a mature matrix under current culture protocols. The molecular markers that can be used to define the differentiated tenocyte remain few in number, and many of those identified have limited functional role and/or specificity. As described in this review, not all markers are necessarily specific to tendon, but collectively they serve as a starting point for discussion, and serve as benchmark criteria for evaluating the robustness of in vitro models as well as guiding tendon regeneration strategies (Fig. 4). The difficulties establishing the distinct stages of tendon differentiation and the specific molecular or structural criteria to identify a tenocyte further emphasize the need for a centralized community wide effort to generate these critical information resources.

To date, the molecular mediators of tendon development have been discovered by distinct expression in tendons and often by identifying a tendon phenotype using existing mutant models generated for other purposes. The absence of a reliable tissue culture model is one reason that new molecular regulators of tendon biology are difficult to uncover. While much of the work in tendon development have utilized mouse and chick models, a few recent studies now highlight zebrafish as a promising new model for tendon research.[45,109] The primary advantage of the zebrafish model system is the ability to perform high throughput screening assays (genetic or small molecule) in a living organism.[110] While rapid assays are also possible in *Drosophila*, zebrafish possess long tendon structures in the head that resemble mammalian tendons,[109] in contrast with *Drosophila* tendons, which do not share significant molecular or structural similiarities with vertebrate tendons and possibly share more resemblance with myotendinous junctions. The zebrafish is also a well-established model system with a wide array of available reagents and tools. Moreover, zebrafish embryos are amenable to embryological manipulations and time lapse imaging since the egg develops outside the body and embryos are transparent. While the tendon size, organization, and mechanical features may be significantly different between zebrafish and mammals, the potential for genetic screens in zebrafish to uncover conserved molecules pertinent to tendon development is thus far unrivaled.

AUTHOR CONTRIBUTIONS

This review paper was conceptualized, written, and edited by A.H., H.L., and R.S. All authors have read and approved the final manuscript.

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