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Review Article

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Development of tendon structure and function: Regulation of collagen fibrillogenesis

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Abstract

In the tendon, the development of mature mechanical properties is dependent on the assembly of a tendon-specific extracellular matrix. This matrix is synthesized by the tendon fibroblasts and composed of collagen fibrils organized as fibers, as well as fibril-associated collagenous and non-collagenous proteins. All of these components are integrated, during development and growth, to form a functional tissue. During tendon development, collagen fibrillogenesis and matrix assembly progress through multiple steps where each step is regulated independently, culminating in a structurally and functionally mature tissue. Collagen fibrillogenesis occurs in a series of extracellular compartments where fibril intermediates are assembled and mature fibrils grow through a process of post-depositional fusion of the intermediates. Linear and lateral fibril growth occurs after the immature fibril intermediates are incorporated into fibers. The processes are regulated by interactions of extracellular macromolecules with the fibrils. Interactions with quantitatively minor fibrillar collagens, fibril-associated collagens and proteoglycans influence different steps in fibrillogenesis and the extracellular microdomains provide a mechanism for the tendon fibroblasts to regulate these extracellular interactions.

Keywords: Tendon, Development, Collagen Fibril Formation, Matrix Assembly, Fibrillogenesis

Introduction

The tendon is a uniaxial connective tissue which connects muscle to bone and serves to transmit force. Tendons are composed of highly aligned collagen fibrils organized as fibers. Fibers together with the tendon fibroblasts are organized into fascicles, and fascicles are bound together by connective tissue sheaths to form a tendon[1-4]. The development and maintenance of this hierarchy is a multistep process[5,6]. Development of the mechanical integrity and function of the tendon is dependent on the regulated progression through these steps during development.

In tendon, collagen fibrillogenesis results in the assembly of mature collagen fibrils with a tissue-specific structure and function[5,6]. During tendon development, there are at least three distinct steps in fibrillogenesis (Figure 1). In the first step, collagen molecules assemble extracellularly in close association with the fibroblast surface to form immature fibril intermediates. This step can be influenced at a number of points including: during packaging for secretion, vectorial secretion into the extracellular space, procollagen processing, heterotypic collagen interactions and other molecular interactions. All these factors could influence the nucleation and growth of the fibril intermediate. In the second step, linear fibril growth, the pre-formed fibril intermediates assemble end-to-end to form longer fibrils consistent with mature, mechanically functional fibrils. This linear growth from intermediates suggests a regional change in the ends of the fibril intermediate at specific developmental stages. In the third step, lateral fibril growth, the fibrils associate laterally to generate large diameter fibrils. This lateral growth step, coupled with linear growth, generates the long, large diameter fibrils characteris-

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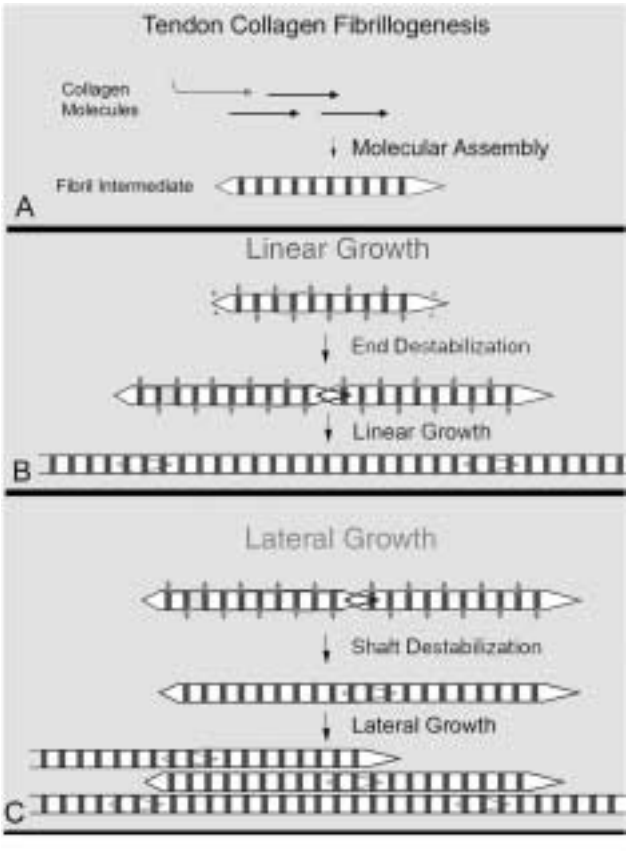

Figure 1. Tendon fibrillogenesis. (A) Molecular assembly of type I and III and/or V collagens generates the fibril intermediate. We hypothesize that heterotypic interactions are a mechanism regulating this assembly step. (B) In the linear growth step, the intermediates in (A) grow by end-to-end growth to generate longer fibrils. Alterations in molecular interactions, at the α and β ends of the intermediate, initiate and control this growth. (C) In the lateral growth step, there is a lateral association and growth of the developing fibrils. Alterations in interactions mediated by fibril-associated molecules (ovals) along the main fibril shaft regulate this step.

tic of the mature tendon. Tissue-specific fibrillogenesis would involve the independent regulation of each step.

Tendon fibrils are heteropolymeric structures assembled from two or more different fibril-forming collagens interacting with fibril-associated collagens as well as fibril-associated proteoglycans. These combinations have been described and are likely to be involved in the regulation of fibrillogenesis and matrix assembly. The tendon is composed of type I collagen with quantitatively minor amounts of types III and V collagen. The D-periodic fibrils are predominantly type I collagen with varying amounts of collagen type V and III[3]. Collagen types XII and XIV have different expression patterns during tendon development. These collagens are found on the surface of striated collagen fibrils. Because of their fibril-associated nature, these macromolecules may be involved both in the regulation of fibril formation and in modifying the interaction(s) among fibrils required for the assembly of tissue-specific extracellular matrices[7,8]. In addition, members of the leucine-rich repeat family of proteoglycans/glycoproteins are associated with tendon fibrils[1,9]. Interactions between collagen fibrils and members of this family of proteoglycans/glycoproteins have been implicated in the regulation of fibrillogenesis[9,11-13]. Interactions between the fibrils and their surrounding molecules also play a significant role in tendon mechanics.

Although the exact mechanisms by which mechanical stimuli are translated into deformations of the tendon have yet to be fully elucidated, a model is emerging as the result of recent research into this phenomenon. At very small strains (up to approximately 2%), stretching of the fibril-forming collagen molecules appears primarily responsible. Beyond this, increases in the D-period are a result of molecular and fibrillar slippage[14]. Although the exact point at which molecular deformation becomes small compared to molecular and fibrillar slippage is strain rate- and tissue-dependent, it is clear that the structure of the various matrix components and the interactions between them are an important aspect of tendon function. Thus, it follows that the development of the mature mechanical properties is dependent on the assembly of a tendon-specific extracellular matrix. This assembly process must progress through multiple steps producing the final mature structure where tendon fibroblasts and collagen fibrils organized as fibers are integrated into a functional tissue. Each step in this assembly process must be regulated, thereby directing the tendon-specific, stepwise assembly of a structurally and functionally mature tissue.

Extracellular compartments

In tendon, fibrillogenesis occurs in a series of extracellular compartments defined by the fibroblasts[2,4,5,15,16]. These compartments extend the cell's domain into the extracellular space. The first level of compartmentalization consists of a series of narrow channels containing single or sometimes, small groups of fibrils. The narrow channels originate deep within the cytoplasm often in a perinuclear position associated with the Golgi region and distally are open to the extracellular space (Figure 2). This first extracellular compartment is formed as the secretory vacuoles, containing collagen, fuse with the plasma membrane in tandem. Within this first compartment, initial assembly into fibrils occurs. The initial initiation of fibrillogenesis is mediated through macromolecular interactions dependent on the composition of the compartment. In addition, receptor-mediated interactions, with membrane receptors, such as integrins, would be expected to have an influence on the initial events. These channels add their contents to a second fibroblast delimited compartment where fibers form. The second level of extracellular compartmentalization consists of fibrils grouped as fibers in close association with the cell surface. These compartments are defined by a single fibroblast or sometimes by adjacent fibroblasts. As the tendon becomes more mature, a third level of compartmentalization is present. These compartments are defined by the apposition of two to three adjacent fibroblasts. The very large fibers characteristic of the tendon that form in this compart-

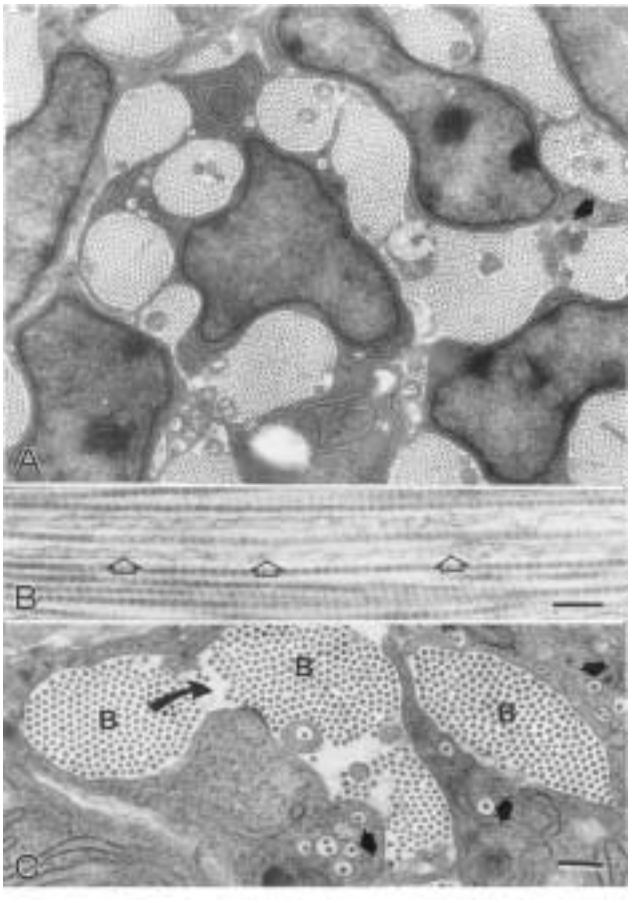

Figure 2. Compartmentalization of the extracellular matrix during tendon development. Extracellular compartmentalization of the different levels of matrix assembly is seen in the developing chicken tendon. (**A,C**) Sections cut perpendicular to the tendon axis of a 14-day chicken embryo show the fibril-forming channels (arrowheads) and the fiber-forming spaces (labeled B). The macroaggregate-forming spaces form as the cytoplasmic processes that define the fiber-forming compartments retract (curved arrow) allowing fibers (fibril bundles) to coalesce into macroaggregates. Bar 1:µm. (**B**) A section cut parallel to the axis of 14-day chicken embryo tendon illustrates a narrow extracytoplasmic channel containing a collagen fibril (intermediate). The channel has periodic indentations (open arrows) indicative of its formation by fusion of elongate secretory vacuoles. Bar 500nm. (Reproduced from Birk and Linsenmayer[6]).

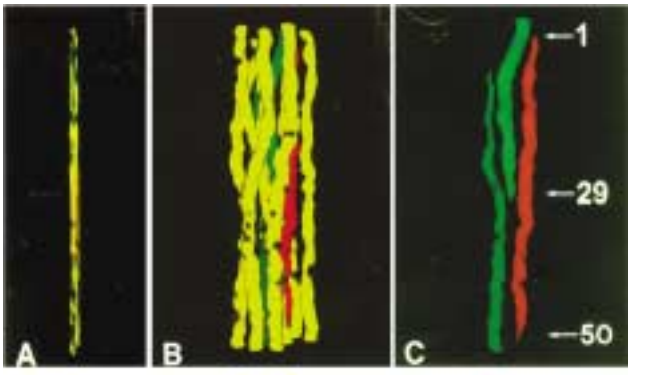

Figure 3. Collagen fibril intermediates: three-dimensional computer reconstruction of fibril intermediates. Fibril profiles were reconstructed from 55 consecutive 150 nm thick sections. For presentation, not all of the fibrils seen in the micrographs are present in this reconstruction, therefore this series of fibrils appears somewhat more open than is the case in the actual fiber. The beginning and end of a fibril intermediate (#1, red) is shown in relationship to other fibrils. Also shown is the termination of one neighboring fibril (#6, cyan) and the beginning of another adjacent fibril (#9, green) within this fiber. In (A) the relationship of the ten different fibrils is shown to scale. In (B) the reconstruction from (A) is presented with the Z scale being 10% of actual, all 10 fibrils are present. In the reconstructions presented in B-C this compressed Z scale and same orientation is maintained. In (C) only the 3 fibrils with ends in this data set are shown. (Modified from Birk et al.[17]).

ment by coalescence of fibers are partially surrounded by interdigitating processes. A model for the extracellular compartmentalization of collagenous matrix assembly during tendon development has been proposed[4]. Elongate secretory vacuoles containing procollagen in a laterally aggregated configuration fuse in a tandem fashion, similar to compound exocytosis in other tissues. This forms the long, narrow channels where the initial steps in fibril formation occur. The channels deposit the immature fibrils into a second convoluted compartment where they are integrated into developing fibers. These fiber-forming compartments laterally aggregate, as the tissue matures, with the intervening cytoplasm being retracted. Within this last compartment, fibrils mature within fibers and fibers coalesce to form large fibers characteristic of the mature tendon. This hierarchical compartmentalization affords the fibroblast with exquisite control over the stepwise process of fibril formation and matrix assembly.

Structural steps in the growth of mature collagen fibrils

During development short, immature fibril intermediates are assembled and deposited into the matrix[17,4]. As development progresses, fibril intermediates are replaced by long fibrils characteristic of the mature tissue[18,19]. Our data and that from other laboratories indicate that mature fibrils are the result of fibril growth from pre-formed intermediates[2,15,18-20]. The first appreciation that fibrils were assembled as immature fibrils of discrete length came from detailed studies of the fibril-cell relationship[2,4,17,18]. The three-dimensional course of a fibril within a narrow channel was shown to extend from the perinuclear Golgi region to an opening at the cell surface. The proximal end of the fibril terminates deep within the channel while the distal ends of fibrils terminated close to or at the site of fusion with the plasma membrane defining a fiber-forming compartment or they were observed as part of the fiber for one or more micrometers before terminating. This finding of both ends of a fibril

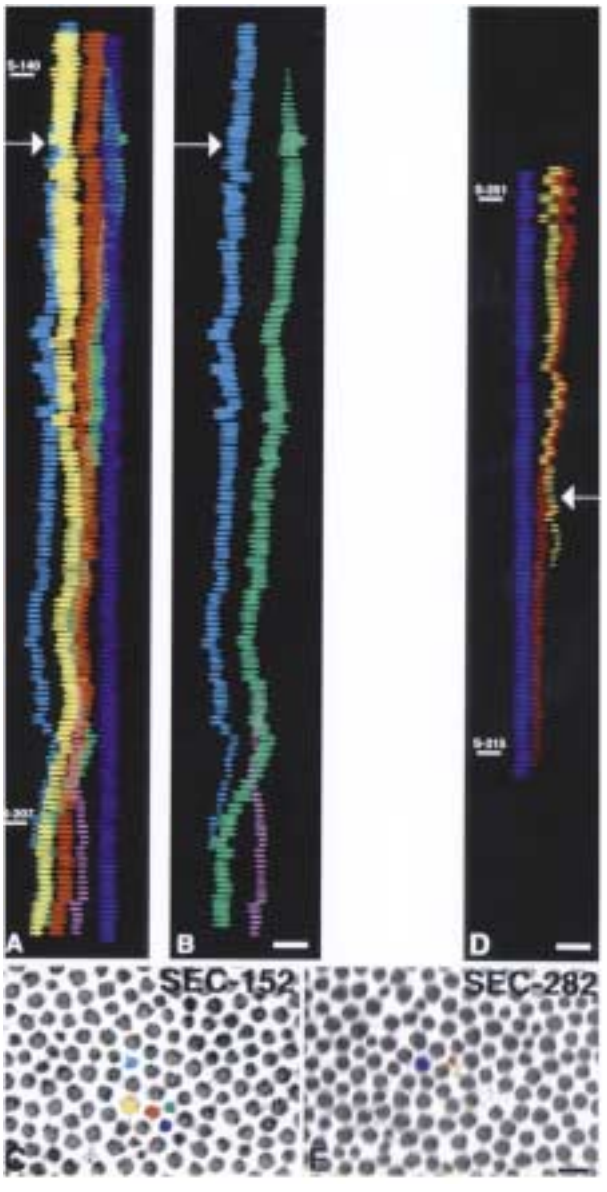

Figure 4. Three-dimensional reconstruction of 18-day tendon fibrils. This figure presents computer generated reconstructions of representative fibrils. The colors are used consistently throughout this figure to indicate a single fibril. The blue fibril is present at full diameter throughout these reconstructions (A,D). In (A) portions of 5 fibrils are presented to show their complex relationships, while in (B) only the 3 that end are shown. The green fibril terminates as a β end while the purple and cyan fibrils terminate as α ends with opposite orientations. In (D), the blue, yellow and red fibrils are continued from (A). The yellow and red fibrils both terminate as α ends. In (C) an electron micrograph from the level of the arrows in (A,B) is presented. The green fibril is a β end and is 8 sections from the end. In (E) an electron micrograph from the level of the arrow in (D) is presented. The yellow and red fibrils are both α ends and are 8 and 29 sections, respectively, from the termination. Compare the differences in diameter between α and β ends the same distance from the fibril ends (yellow vs. green in (E) and (C), respectively). The section numbers are indicated in (A and D). For presentation, not all the fibrils seen in this group are present in this reconstruction and the Z-axis was compressed. Bars 100nm, x,y axes. (Reproduced from Birk et al.[19]).

demonstrated that a fibril intermediate is initially assembled by the fibroblast.

In immature, rapidly growing tendons, the fibril intermediates are D-periodic structures with tapered ends. They are incorporated into fibers and maintained as discrete units, ~10-30μm in length (Figure 3). They are not straight, rigid structures, rather they are semi-flexible undulating through the tissue. This semi-rigid property allows the considerable change in the position of these structures along the length of the tissue with respect to nearest neighbors. In addition, the thin tapered ends are relatively flexible and not as well oriented as the major portion of the fibril intermediate[4,17]. In the later stages of tendon development the fibrils were considerably longer than those seen earlier in development (Figure 4)[17]. As tendon development proceeds a linear and lateral fusion of fibril intermediates to form mature, continuous fibrils was observed. In the more mature tendon the ends of fibrils were readily identified both within fibril-forming channels as well as within fibers[19]. Measurements of fibril intermediates within channels indicated that a length similar to that in earlier stages of development was being assembled. The presence of fibril ends within the fibers indicates that at this relatively mature stage of development the fibrils are not all continuous, i.e., from tendon origin to insertion. The ends were identified based on their significantly smaller diameters and confirmed by their disappearance in the serial data set. These data demonstrated that fibrils from the more mature tendon were significantly longer than in the immature tendon. The data supports a post-depositional maturation of fibril intermediates yielding longer and larger diameter fibrils involving a linear and lateral fusion of fibril intermediates to form mature, more continuous fibrils.

The process of fibril growth from pre-formed intermediates has been further characterized using fibrils isolated from different stages in tendon development. Immature fibrils (intermediates) were isolated by swelling the tissue in physiological buffers with protease inhibitors followed by shear. The non-disrupted tissue was removed and the isolated fibrils in the suspension were analyzed[5,18,21-24]. The structure of the initial fibril intermediate was characterized and was consistent with the *in situ* data. The fibril intermediates were short, i.e., generally less than 30~μm. There was an asymmetry with respect to the ends, i.e., long (α) and short (β) tapered ends. In addition, there was a population that was centrosymmetric with carboxyl to amino polarity at both ends. Similar structures have been isolated from echinoderms[25,26]. All of these studies indicate that a fibril intermediate is formed during tendon development and that mature fibrils are assembled from these intermediates. When fibrils were isolated from immature tendons, discrete fibril intermediates were obtained. With development the intermediates incrementally increased in mean length, due to the addition of a population of longer fibril intermediates. During the period when the limb became active, the fibril intermediates increased significantly in length. However, abruptly at 17 days of chicken metatarsal tendon development practically no intact fibrils were isolated. Only three intact fibril intermediates were analyzed in this study and these had lengths of –89, 117 and 170μm. A number of long intermedi-

ates with broken ends also were observed that had lengths in this range. The data indicate that most of the fibrils were now considerably longer than the newly formed intermediates seen in the immature tendons[18,21]. These observations were entirely consistent with the *in situ* observations. Morphological analysis of intermediates *in situ* and in tissue extracts during early stages of tendon development demonstrated linear growth through lateral associations of adjacent tapered ends. This type of interaction would yield longer fibril intermediates with a minimum increase in fibril diameter. The abrupt increase in length and diameter observed in later stages of tendon development is associated with extensive lateral associations of intermediates (Figure 5). These lateral associations (fusions) involved long stretches of the fibril (intermediate) length and were not restricted to the tapered ends. This type of interaction yields longer and larger diameter fibrils[15,16,18].

Fiber assembly in tendon morphogenesis

The assembly, deposition and organization of collagen fibers and the composite fibrils were studied during morphogenesis of the chicken embryo tendon[27]. The 14-day chicken embryo is a stage when tendon micro- and macroarchitecture are being established and rapid changes in mechanical properties occur. Tendon ultimate tensile strength was shown to increase from 2.05 MPa at 14 days post-fertilization to 58.05 at 2 days post-hatching. Strain to failure was 13% at 14 days post-fertilization, 22% at 16 days and 29% thereafter[28]. The fibroblast-fiber relationship in the 14-day embryo tendon is complex. Collagen fibers branch to form a fiber network within and among fascicles. Fibers were followed in serial high voltage electron micrographs of thick sections cut perpendicular to the tendon axis and the relationship of fibers to one another and to the fibroblasts within a fascicle were studied using computer assisted three-dimensional reconstructions. Branching of fibers within a tendon fascicle was observed. Fibers were followed as they separated from one large bundle/fiber to join a different one. The fibrils within these branching fibers are discontinuous at 14 days of development. The fibrils terminated at different levels and the fibers rotated ~180° over a 10-12μm distance relative to internal markers. This may contribute to the stabilization of a structure composed of discrete fibril intermediates. The mechanical properties of a developing tendon composed of discrete fibril intermediates requires that these discrete units be stabilized in some manner. The organization of fibril intermediates into discontinuous fibers where the components could interact with the interfibrillar matrix and with adjacent fibrils through this interfibrillar matrix would contribute to the stabilization of this structure. The gradual rotation of the fibers would serve to stabilize the immature fibers through the physical twining of the composite components. In addition, the extensive branching of the fibers observed at 14 days of development and their intimate association with the cellular elements would provide a higher order of structure stabilization. At points of contact with

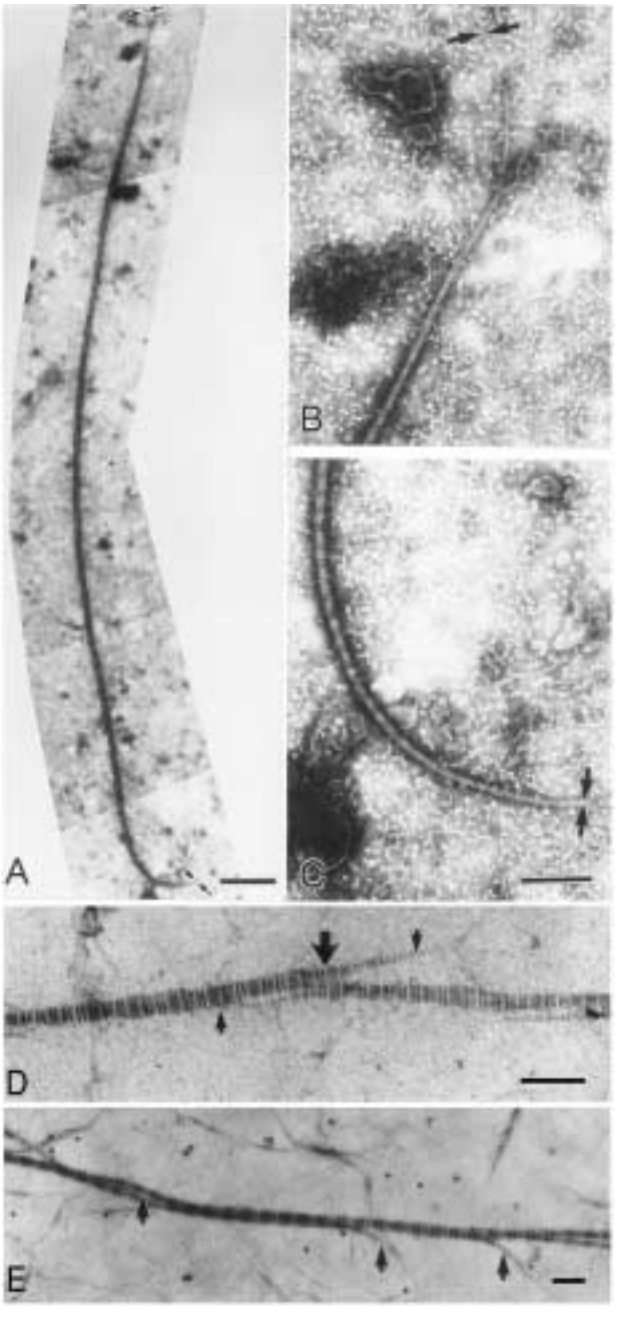

Figure 5. Collagen fibrils in tendon development. Fibril intermediates were extracted from 14-day chicken embryo tendons. The suspension was negatively stained and observed by transmission electron microscopy. (**A**) Intact fibrils of discrete lengths (intermediates) were observed. Bar, 1μm. (**B,C**) The ends of extracted fibril intermediates were asymmetric as described *in situ*, with α, long (**B**) and β, short (**C**) tapers. Bar, 250nm. Collagen fibrils grow by linear and lateral associations of fibril intermediates (**D,E**). Transmission electron microscopy of fibrils from cryosectioned tendons illustrating linear growth of fibril intermediates (**D**). The association involves the tapered ends of the intermediates. This mechanism produces fibrils of increasing length without significantly altering fibril diameter. Lateral fibril growth is illustrated in (**E**). The extensive lateral association/fusion of fibril intermediates would produce fibrils of increasing length and larger diameter. Bars 100nm. (Modified from Birk and Linsenmayer[6]).

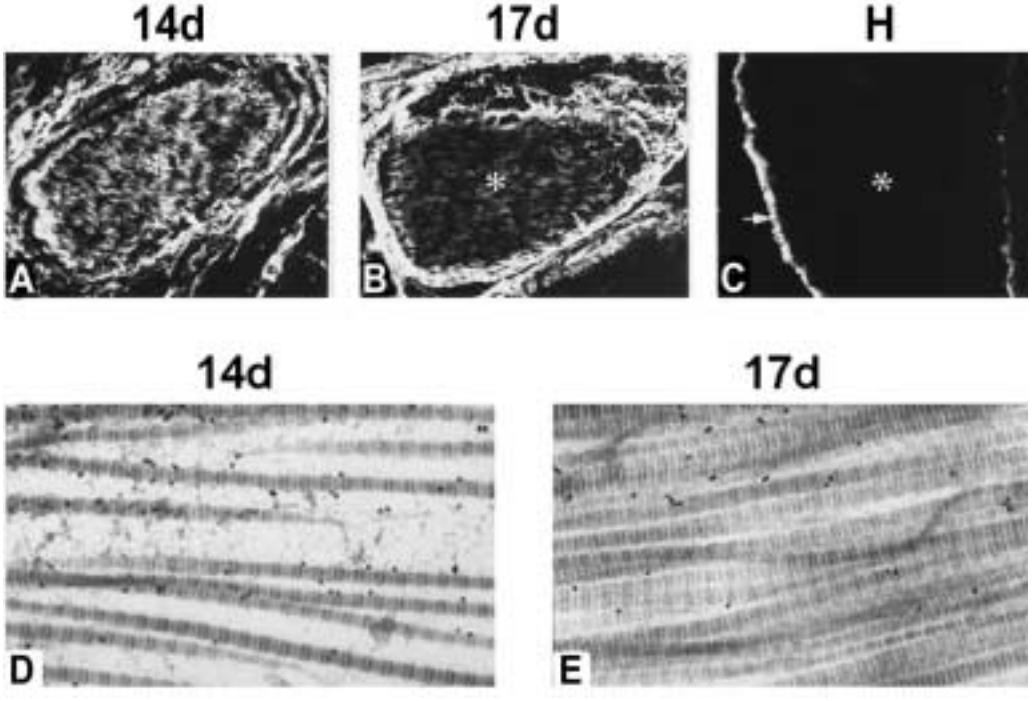

Figure 6. Type III collagen expression in developing chicken tendon. (**A-C**) Immunofluorescent localization of collagen type III at 14 and 17 days of development and hatching. Type III collagen reactivity is high at 14 days. At 17 days to hatching, reactivity becomes sequestered in the sheath. (**D,E**) Immunoelectron microscopic localization of type III collagen at 14 and 17 days of development. The type III reactivity is fibril-associated on the tendon fibrils. However, there is a significant decrease in reactivity from 14 to 17 days. (Modified from Birk and Mayne[3]).

cells, fibrils would be subjected to cytoskeletal forces that could aid in their alignment. Increased fibrillar alignment would lead to increased interactions between fibrils and thus improved tensile strength[14].

Regulation of tendon fibrillogenesis

The regulation of each of the steps described above is important in the development of a structurally and therefore functionally mature tendon. The independent regulation of each step via macromolecular interactions would provide structural diversity and controlled growth during development. Below, regulation at different steps in tendon fibrillogenesis is presented.

Regulation of fibril intermediate assembly by heterotypic type I/III interactions

Heterotypic type I/III collagen fibrils are present early in tendon development[3]. The heterotypic interaction between two different fibrillar collagen types is important in the regulation of initial fibril assembly. Type III collagen is present in numerous developing type I collagen-containing connective tissues and is first seen in tendon at approximately the time it differentiates[29]. We determined the expression patterns of type III collagen in the developing tendon in relation to steps in fibrillogenesis. Days 14, 17 of development and hatching were chosen as representative of the period of intermediate assembly, the beginning of linear growth and as representative of a period of rapid lateral growth, respectively. Our hypothesis predicts a differential expression pattern, with type III collagen expression highest during periods of molecular assembly of intermediates. Strong immunochemical reactivity for type III collagen was present throughout the tendon at 14 days of development. At 17 days of tendon development, there was a significant decrease in the type III collagen reactivity in the tendon proper, but the sheaths were still strongly positive. At hatching, the tendon proper was virtually negative, however, the outer sheath remained positive for type III collagen (Figure 6). In contrast, the reactivity for type I collagen was constant throughout this period, as expected. The immunoelectron microscopy data also indicated that there was less type III collagen present in the fibrils of 17-day than 14-day chicken tendons and that the type III collagen content continued to decrease to hatching. The amount found in the endotendinium was relatively constant from 14-17 days, but then decreased as the fibril diameters increased in this region. At hatching, type III was confined to the outer sheath that con-

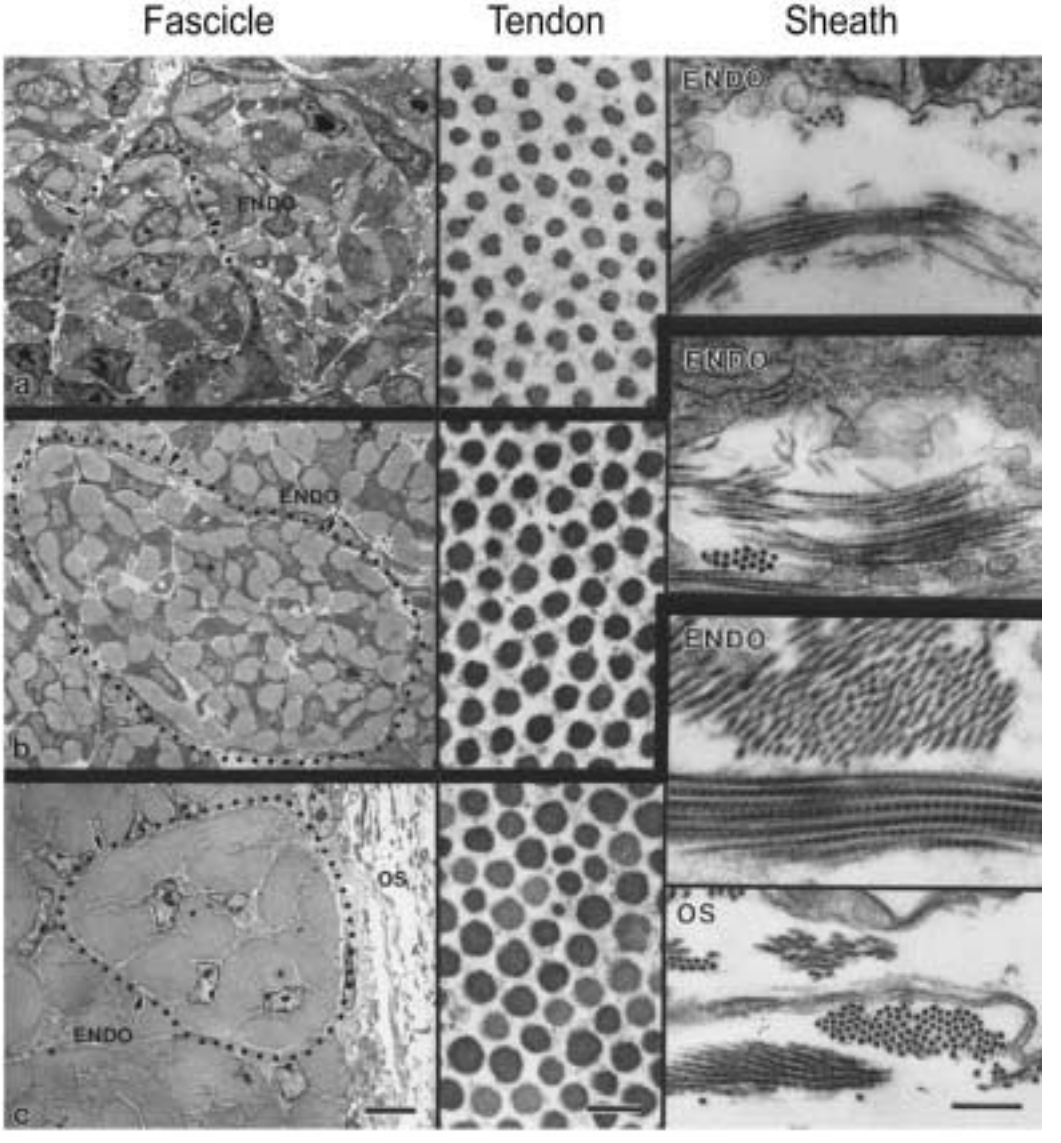

Figure 7. Fibril structure during chicken tendon development. Transmission electron micrographs of different stages of chicken tendon development. In the tendon proper, fibril diameters increase from 14 days of development to hatching. This is associated with a loss of reactivity for type III collagen (Figure 6). The fibrils in the sheaths also increase in diameter as type III collagen reactivity is lost. Bars 5μm (fascicle), 100nm (tendon), 500nm (sheath). (**A**) 14 days, (**B**) 17 days (**C**) hatching. (Reproduced from Birk and Mayne[3]).

tained numerous small diameter fibrils. The expression of type III collagen showed a complete overlap with regions of the developing tendon containing small diameter fibrils (Figure 7). Biochemical analyses of type III collagen indicate a reduction in amount with development. The decreased reactivity observed here is clearly partially related to the overall decrease in type III collagen in the developing tendon. However, the complete loss of reactivity may be due to epitope masking by the addition of type I collagen to the immature fibrils. Turnover may also be involved. However, accessible type III collagen helical epitopes are clearly associated with small diameter, immature fibrils. As expected, the type V collagen was not labeled in the untreated tendon, but there is a small, constant amount in tissues where fibril structure was disrupted.

Presumably, the regulatory properties of type III collagen are similar to those described for type V collagen[30-32]. The amino terminal domain of type V collagen is not completely processed while the amino propeptide of type III collagen is cleaved slowly[33,34]. In either case, the presence of a non-collagenous amino-terminal domain on the surface of newly forming fibrils could alter the properties of heterotypic fibrils and influence the molecular assembly of collagen into fibril intermediates. The differential processing of propeptides also may regulate other interactions involved in the stabilization of the developing matrix. For example, the gap

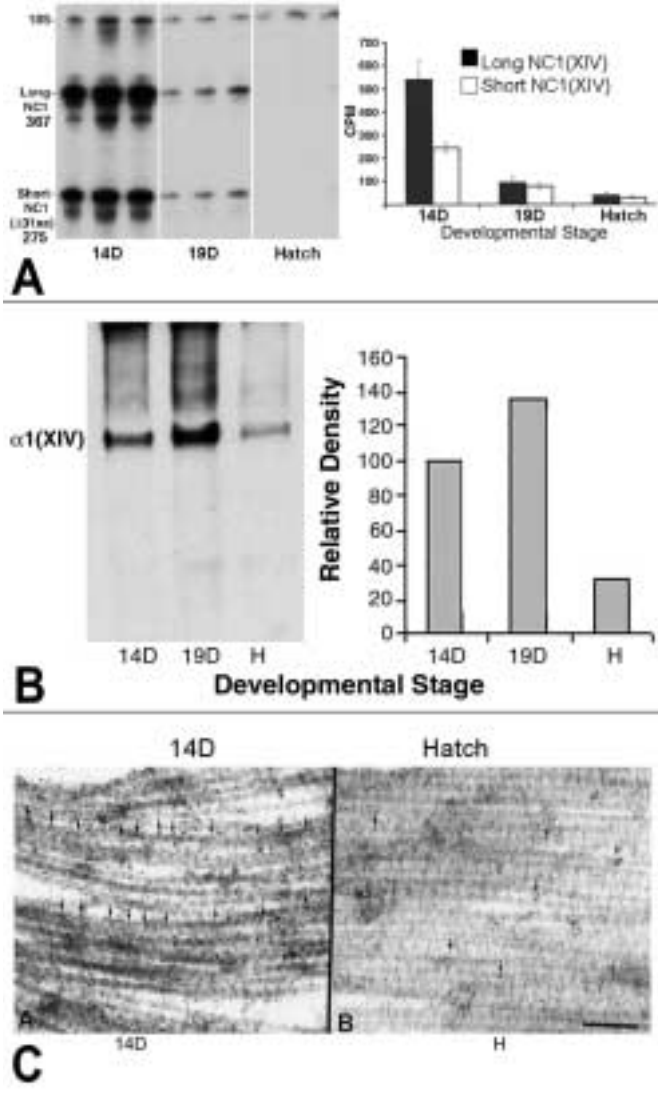

Figure 8. Type XIV collagen expression during tendon development. (**A**) Semi-quantitative RT-PCR of the collagen XIV splice variants at different stages of tendon development. This autoradiogram shows PCR product bands for both long and short NC1 splice variants and co-amplified 18S product. Three developmental stages were analyzed, 14 days, 19 days and hatching. Each PCR contained ^{32}P dCTP in addition to the cold nucleotide triphosphate mix. To amplify the NC1 variants, a pair of primers spanning the α1(XIV) NC1 splice site was used, yielding a 367 bp product for the long variant, and a 275 bp product for the short variant. In addition, each PCR contains the 18S RNA primer pair in combination with the competimer pair, in a ratio of 1:9. (**B**) Western blot analyses of type XIV collagen levels in developing tendons. At embryonic day 14 (14D) and 19 (19D) the α1(XIV) chain is more abundant than in hatchling tendon (H). The graph presents the normalized data from at least 4 Western blots for each developmental stage. This shows a 4-6 fold decrease in type XIV collagen content expression from 14/19 day to hatching. The α1(XIV) chain migrated at approximately 200 KDa. (**C**) These immunoelectron micrographs of tendons illustrate a decrease in immunoreactivity for type XIV collagen from embryonic day 14 to hatching. Antibodies against type XIV collagen localize to the striated fibril (arrows). Bar 300nm. (Modified from Young et al.[8]).

region of the fibril may inhibit interactions with fibril-associated collagens or proteoglycans. In addition, we can speculate that the presence of the propeptide may alter cross-linking. Collagen molecules within fibrils undergo covalent intra- and intermolecular cross-linking mediated by lysyl oxidase that increases with development[35,36]. In contrast to the molecular stability observed in fully cross-linked mature fibrils, the liquid crystal structure of the fibrils present in early development allows for the molecular rearrangements that are necessary for fibril growth[37].

Fibril-associated collagens in tendon development

Type XII and XIV collagens are fibril-associated or FACIT collagens found in tendon. We hypothesize that interactions between this class of collagens and developing fibrils can regulate steps in fibrillogenesis and higher order matrix assembly. The expression patterns for the two collagens indicate different functions. Type XIV collagen is a good candidate to be involved in linear fibril growth while type XII collagen may function to integrate adjacent extracellular matrices. Types XII and XIV collagen are localized to the surface of fibrils[8,38,39]. However, little is known about their effects on fibril assembly or their roles during development. FACIT collagens are an expanding family with members that include collagen types IX, XII, XIV, XVI, XIX, and XX[41,40,42]. Both types XII and XIV collagen are expressed in a wide variety of tissues including the developing avian tendon[7,43-46]. Types XII and XIV collagen are structurally similar homotrimers, composed of two collagenous domains (COL1-COL2) and three non-collagenous domains (NC1-NC3)[47]. These multi-domain collagens can interact with more than one extracellular component simultaneously, allowing integration of developing matrices. For example, type XII collagen has been shown to bind to both decorin and fibromodulin[48]. In addition, the two carboxy terminal domains, NC1 and COL1, of type XII collagen are believed to interact with the type I collagen fibrils. In contrast, the large, globular, NC3 domain on the amino terminus projects into the matrix and is hypothesized to interact with other matrix components and may bridge adjacent fibrils, which would likely stabilize the structure[47].

Type XIV collagen has a developmental expression pattern in tendon consistent with a role in linear fibril growth[8]. In the chicken metatarsal tendon, immunolocalization, Western analyses and gene expression studies all demonstrate consistent, dramatic changes between 14 days of tendon development and hatching. Immunofluorescent studies showed high reactivity for type XIV collagen at 14 to 19 days of tendon development. This was followed by a dramatic decrease in the reactivity for type XIV collagen at hatching. Immunoelectron microscopy demonstrated that type XIV collagen was fibril-associated and decreased fibril-associated reactivity for type XIV collagen was seen at hatching, indicating a removal of collagen XIV from the fibril surface (Figure 8C). Western analysis was consistent and demon-

strated that type XIV collagen content was high between days 14-19, decreasing sharply at hatching (Figure 8B). Our studies of type XIV gene expression using semi-quantitative PCR demonstrated that type XIV collagen mRNA decreased significantly from day 14 to hatching. However, here the decrease was from 14 day to 19 days (Figure 8A). Our interpretation of these data is that there is decreased expression of type XIV collagen associated with the beginning of the linear growth of tendon fibrils. However, this decreased expression results in a gradual decrease in type XIV collagen in the tendon between day 19 and hatching. There may be differences in the spatial interactions of fibril-associated components along fibril intermediates. With or without the spatial differences, a gradual turnover of fibril-associated type XIV collagen could mediate a controlled linear growth phase during tendon fibrillogenesis. The virtual absence of type XIV collagen at hatching also coincides with the beginning of rapid lateral growth and therefore a role at this point cannot be excluded.

Type XII collagen is expressed throughout tendon development in the chicken embryo[7,43,45,46]. Expression of type XII collagen mRNA is observed during tendon development with a significant reduction in expression in the adult (Figure 9). The spatial pattern of type XII collagen expression was studied using immunofluorescence microscopy (Figure 9). The earliest time point studied, embryonic day 14, represents an immature tendon. The intermediate stages, day 17-19 are periods of rapid fibrillogenesis and assembly. The hatchling tendon represents a mature, weight-bearing tendon. At day 14, the immature tendon fascicles were beginning to develop and the endotendinium was not yet distinguishable. Type XII collagen was detected throughout the developing tendon with strong reactivity. At the early intermediate time point day 17, the tendon fascicles were clearly defined. Again, type XII collagen was detected throughout the developing tendon. But the fascicles showed significant reactivity in the endotendinium and the signal in tendon proper was weaker. At day 19, there was a continued decrease in reactivity in the tendon proper with an associated enrichment in the endotendinium surrounding the fascicles. After hatching, the expression of type XII collagen was predominately in the endotendinium around the fascicles. Within the tendon fascicles, reactivity appeared to be associated with processes of the tendon fibroblasts between collagen fibers while the fibers were neg-

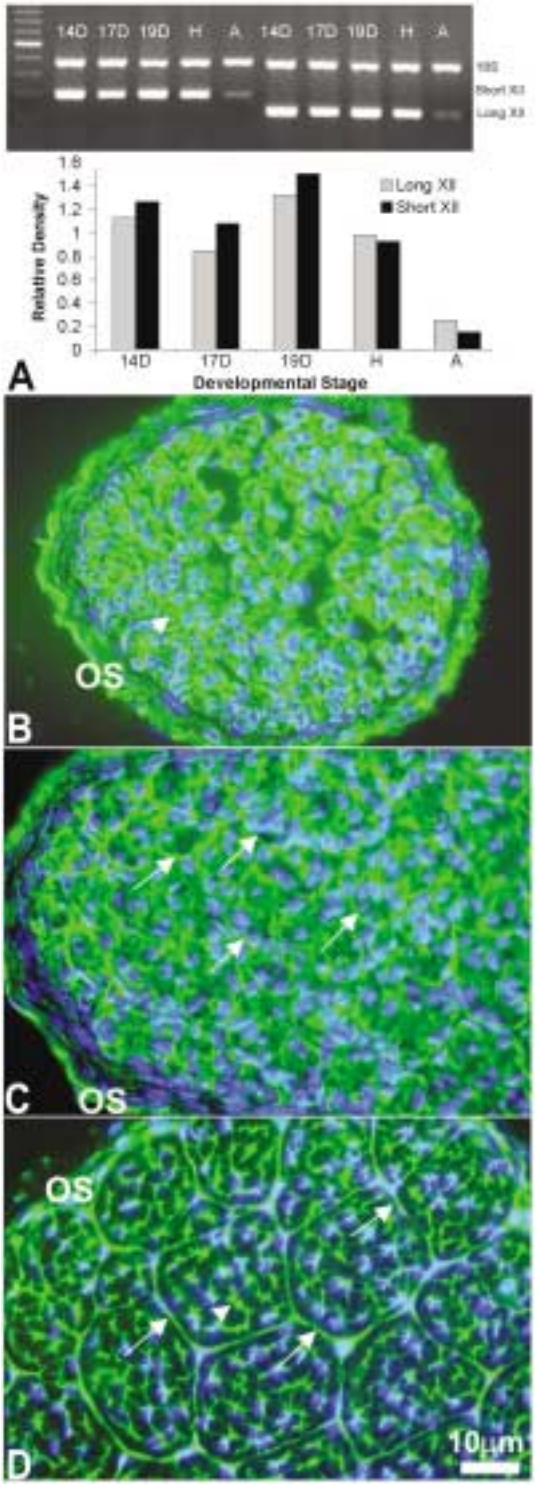

Figure 9. Type XII collagen expression in the developing tendon. Semi-quantitative RT-PCR of the type XII collagen at different stages of tendon development. (**A**) Representative ethidium bromide stained gel showing PCR product bands of both the long and short splice variant of type XII collagen and co-amplified 18S bands. Graphic analysis of the data demonstrates relatively constant expression of both splice variants throughout the fibril assembly and growth period. There is a significant decrease in both variants in the adult tendon. (**B-D**) Immunofluorescence staining of Type XII collagen on chicken tendon at developmental stages day 14, 17 and hatching. Developing chicken tendons were reacted with antibodies against type XII collagen. (**B**) Reactivity with antibodies against type XII collagen was present with strong and homogenous signal at day 14 in the tendon proper. (**C**) A localization of type XII collagen reactivity was observed beginning at day 17, in the matrix surrounding the growing fascicles **(arrows)**. (**D**) In hatchling tendons, the reactivity was preferentially localized and sequestered to the connective tissue sheaths between the fascicles (endotendinium) (arrows) and cytoplasmic processes that separate bundle-forming compartments **(arrowheads)**. OS=Outer Sheath. Bar 10µm. (Modified from Zhang et al.[7]).

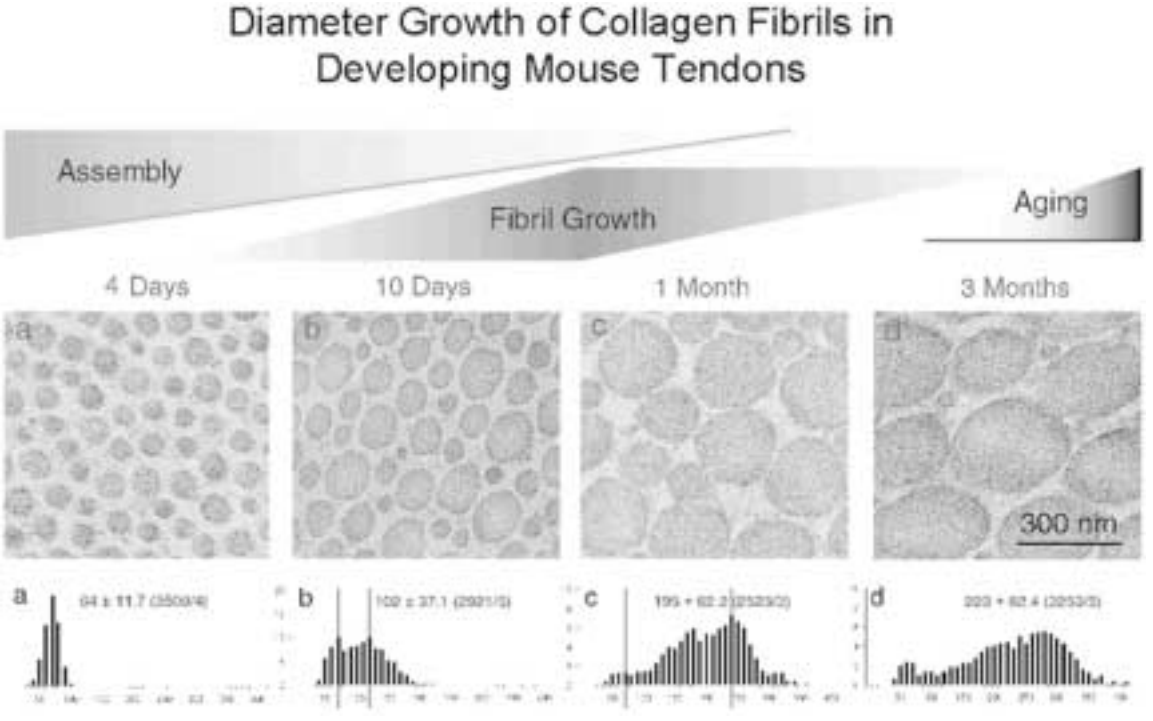

Figure 10. Collagen fibril structure during development in mouse tendons. Transmission electron micrographs of transverse sections from mouse flexor tendons and the diameter distributions at each developmental stage from normal mice (**a-d**). Bar 300nm. (Reproduced from Ezura et al.[64]).

ative. This pattern remained stable in the adult, i.e., 2 months post-hatching. Throughout development, type XII collagen was detected in the outer sheath of the tendon. This dramatic shift in spatial expression of type XII collagen implicates it in the integration of the developing fascicles to form a mature mechanically stable tendon.

Leucine-rich repeat proteoglycans/glycoproteins in regulation of tendon fibrillogenesis

Fibromodulin is a keratan sulfate proteoglycan found in a variety of tissues including tendon[49]. Lumican is a major keratan sulfate proteoglycan of the corneal stroma, but is present in other collagenous extracellular matrices in a glycoprotein form[50]. Decorin, a chondroitin sulfate proteoglycan, is widely expressed including during tendon development[18]. These leucine-rich repeat proteins all bind to fibrillar collagens[12,51,52]. *In vitro*, it has been shown that fibromodulin and lumican compete for the same binding site[53] and that this site is separate from the decorin binding site[54,55]. In addition, fibromodulin, lumican and decorin all interact with striated fibrils[18,56-58] and can modulate collagen fibril formation and inhibit the lateral growth of fibrils *in vitro*[12,51,55]. Also, the abnormal lateral growth of isolated fibrils was prevented by this class of proteoglycans[21]. Gene-targeting studies of decorin, lumican and fibromodulin indicate that these leucine-rich repeat proteoglycans/proteins are involved in the determination of the mature collagen fibril structural phenotype *in vivo*[59-62]. The absence of lumican affects the mature fibril architecture of connective tissues such as cornea and skin. Null mutations in fibromodulin and decorin produce abnormal fibril structures in tendons and skin. In addition, the decorin and lumican models demonstrated reduced dermal tensile strength associated with the mature fibril defects, indicating important functions of these proteins in connective tissue maturation. Investigations using fibromodulin knockout mice have shown that this proteoglycan contributes significantly to tendon strength as well[63]. However, the analysis of the fibromodulin-deficient mouse is complicated by the increased deposition of lumican into the matrix, suggesting a coordinate regulation of these closely related members of the same subfamily[62].

As has been discussed above, in the avian metatarsal tendons specific developmental periods are associated with distinct steps in collagen fibril assembly and growth[6,18]. Analogous periods of development were defined for the mouse flexor tendons. Fibrils were analyzed in tendons from 2 days postnatal to 7.5 months in wild type mice. This analysis of mouse tendons identified stages representative of different steps in fibrillogenesis (Figure 10). These are defined as the initial assembly of immature fibril intermediates (4 days); the transition from fibril assembly to growth (10 days); and fibril growth to maturation (1-3 months). The 3-month tendon is characteristic of a mature tendon that has completed growth. In the early postnatal period, characterized by the

4 day tendon, the collagen fibrils all had a normal distribution of relatively small diameters (64 ± 12 nm, mean ± sd). By 10 days postnatal, the fibril diameters were heterogenous, with larger diameter fibrils present, in addition to smaller diameter fibrils characteristic of 4 days. By 1 month, there was a broad distribution of fibril diameters with large diameter fibrils being characteristic of this stage. From 1 to 3 months, the fibril diameters changed only modestly with the largest diameter fibrils being more prominent in the oldest tendons.

The temporal expression patterns of lumican and fibromodulin were analyzed during tendon development[64]. Analysis demonstrated that each proteoglycan had peak gene expression during early stages from 2 days to 14 days (Figure 11A). Lumican expression peaked earliest at around 8 days and then decreased dramatically. Fibromodulin expression peaked at 14 days and decreased slightly to 1 month postnatal followed by a dramatic decrease in the adult expression levels. The amount of lumican and fibromodulin core protein present during normal development was also analyzed with the fibromodulin core protein increased by approximately 70% from 4 day to 1 month while lumican decreased by approximately 80% during the same period (Figure 11B). The lumican and fibromodulin core proteins were present throughout the tendon matrix at 4, 10 and 30 days postnatal, however, lumican reactivity was decreased in the 1 month matrix consistent with the mRNA expression. The co-distribution of lumican and fibromodulin with collagen fibrils is consistent with the regulation of fibrillogenesis. These data suggest that lumican functions during early stages in fibrillogenesis while fibromodulin would function throughout this period with a more prominent role in regulation of the later stages.

The formation of collagen fibrils during tendon development in mice deficient in lumican, fibromodulin or both was analyzed[64]. The fibrils from lumican-, fibromodulin- and double lumican/fibromodulin-deficient mice showed abnormalities during development relative to the wild type controls. Structurally, three distinct abnormalities were observed. First was the premature presence of fibril diameter heterogeneity in the 4-day double-deficient tendon relative to the wild type controls (Figure 12). Secondly, there was an abnormally large number of small diameter fibrils present in the later stages of development, best seen at 3 months, in the fibromodulin- and double-deficient mice (Figure 13).

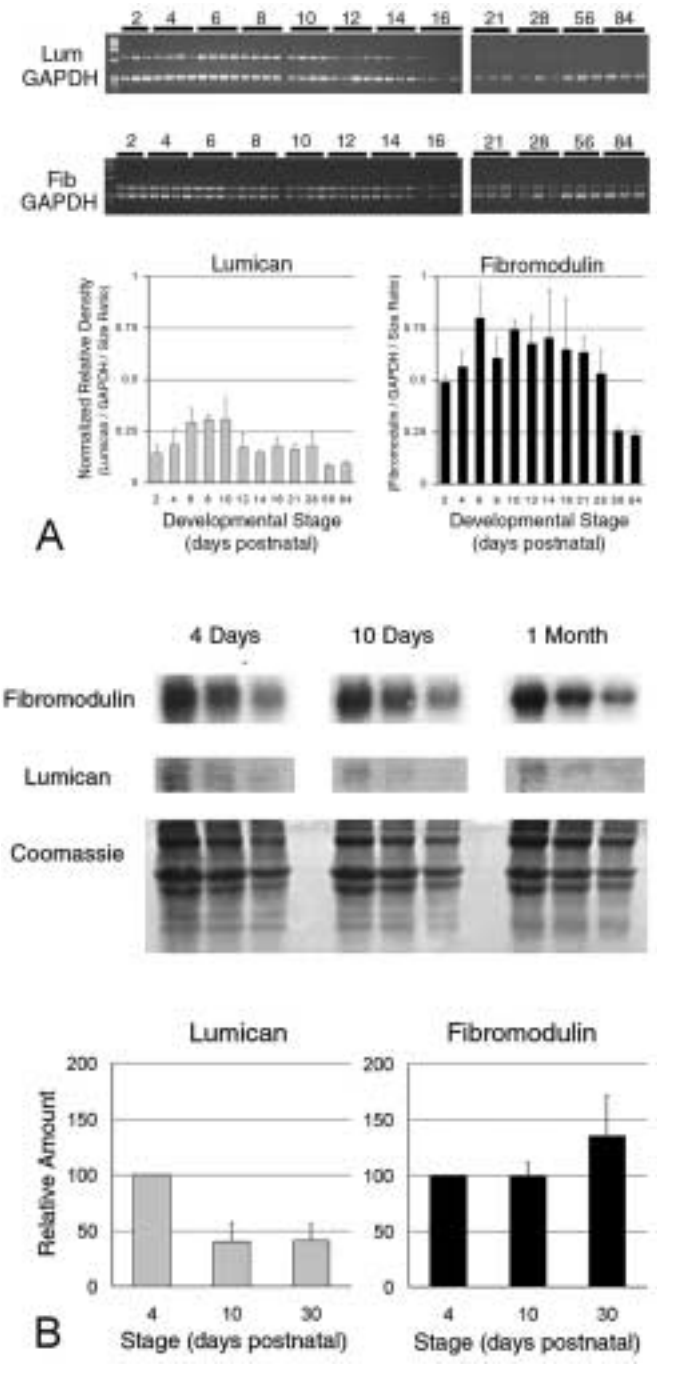

Figure 11. Expression of lumican and fibromodulin during normal mouse tendon development. (**A**) Lumican and fibromodulin mRNA expression were analyzed using semi-quantitative RT-PCR. Representative ethidium bromide stained gels showing PCR product bands for lumican, fibromodulin and co-amplified GAPDH bands. Three or four different batches of PCR products generated from independently prepared total RNA between postnatal day 4 and 16 or day 16 and 3 months were applied to a single gel. Band density was determined densitometrically and relative density was obtained by using both band density and the PCR product size ratio, lumican 656 bp, fibromodulin 418 bp and GAPDH 289 bp. The relative density was normalized using GAPDH and plotted for lumican and fibromodulin. (**B**) Lumican and fibromodulin core protein content during normal tendon development. A representative semi-quantitative Western analysis of lumican and fibromodulin core protein during development in the normal mouse tendon is presented. Tendons were extracted in 4M guanidine-HCl at 4 days, 10 days and 1 month. 20, 40, 80μg of total protein were loaded onto the gel. The core proteins were transferred, reacted with anti-lumican or anti-fibromodulin antisera followed by radiolabeled goat anti-rabbit IgG and quantitated using phosphoimaging. The relative lumican and fibromodulin content in the tendons at 4 days, 10 days and 1 month postnatal was plotted as a function of development. The values for both lumican and fibromodulin were set to 100 at 4 days. (Modified from Ezura et al.[64]).

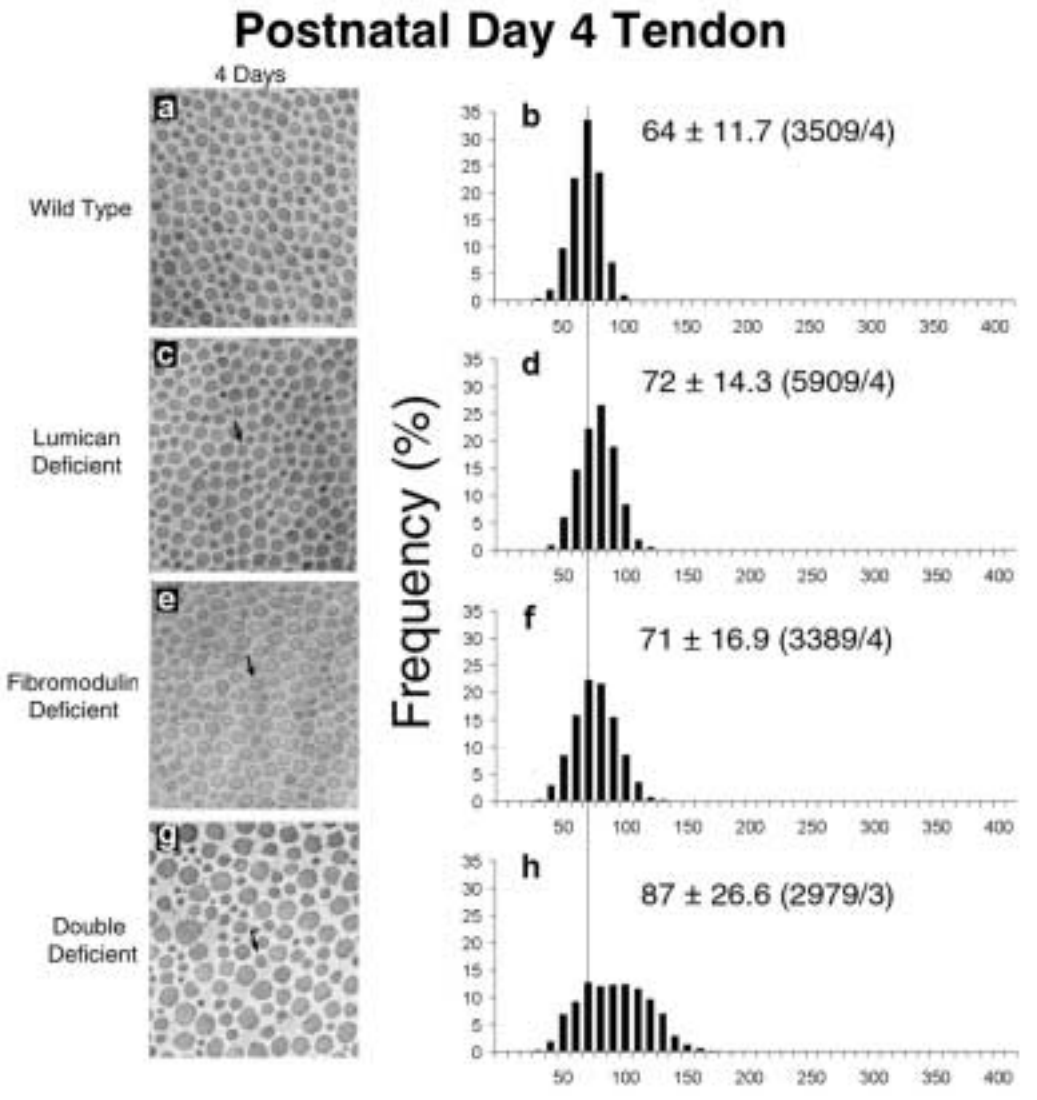

Figure 12. Collagen fibril structure at 4 days postnatal in normal wild type, lumican-, fibromodulin- and double lumican/ fibromodulin-deficient mice. Transmission electron micrographs of transverse sections from mouse flexor tendons from normal mice (**a**) and mutant mice (**c,e,g**). Arrows indicate fibrils with diameters of ~64nm, the diameter seen in normal 4 day postnatal tendons. The fibril diameter distributions are presented in (**b,d,f,h**). (Modified from Ezura et al.[64]).

Finally, all three deficient conditions had fibrils with irregular profiles, indicative of abnormal lateral association or a defect in molecular rearrangement after fusion. The 1 to 3 month fibromodulin- and double-deficient tendons had large numbers of very abnormal "cauliflower" fibrils, with the double-deficient tendons showing the most severe phenotype. In contrast, the lumican-deficient tendons contained fibrils only slightly irregular in profile at 1 to 3 months.

To characterize the diameter distributions, all the fibril diameters measured were plotted in a single histogram for each group at each developmental point. At 4 days postnatal the lumican- and fibromodulin-deficient tendons had fibril diameter distributions that were similar to that observed from wild type tendon with a mean diameter of 64 nm. However, the diameter distributions for lumican- and fibromodulin-deficient mice were shifted to larger diameters, i.e., 72 nm and 71 nm, respectively. The double-deficient tendons had a broader diameter distribution and there was a shift to even larger diameter fibrils, i.e. 87 nm (Figure 12). Significant differences between the wild type distribution and the different deficient distributions were shown using a Kolmogorov-Smirnov test. This demonstrated that the effect of the single mutations was additive during this period of fibrillogenesis and that this class of leucine-rich repeat proteins influenced the assembly of immature fibril intermediates.

The three-month postnatal lumican-deficient tendons had a fibril diameter distribution comparable to the wild type tendon. In contrast, the fibromodulin- and double-deficient

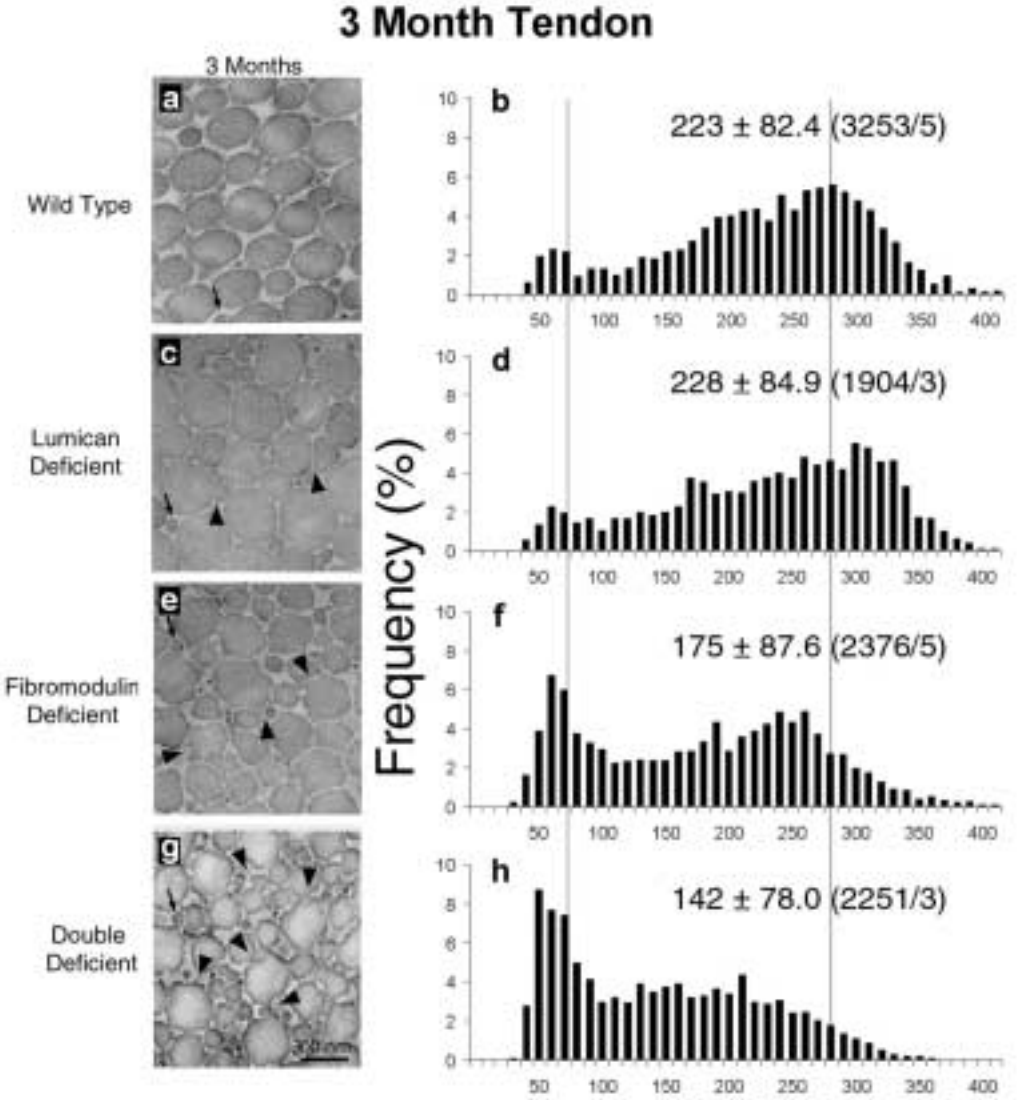

Figure 13. Mature collagen fibril structure in 3 month normal wild type, lumican-, fibromodulin- and double lumican/fibromodulin-deficient mice. Transmission electron micrographs of transverse sections from mouse flexor tendons from normal mice (**a**) and mutant mice (**c,e,g**). Arrows indicate fibrils with diameters of ~64nm, the diameter seen in normal 4 day postnatal tendons. Arrowheads indicate irregular fibril profiles. Bar 300nm. The fibril diameter distributions are presented in (**b,d,f,h**). (Modified from Ezura et al.[64]).

tendon fibrils had diameter distributions distinctly different from the normal tendon (Figure 13). At three months these mutant tendons demonstrated a significant increase in the number of small diameter fibrils, i.e., 65nm. The double-deficient and fibromodulin-deficient distributions were quite similar. Fibromodulin expression appeared to be required for the progression through fibril growth associated with the later stages of tendon development. A statistical analysis indicated that after 3 months of development the distribution of fibril diameters in the wild type tendon was significantly different from the distribution of fibril diameters in the fibromodulin- and double lumican/fibromodulin-deficient tendons. No significant difference between the wild type and lumican-deficient tendons at 3 months was found[64].

The structural differences suggested alterations in the mechanical properties of the tendons deficient in fibromodulin, lumican or both. Biomechanical testing of the flexor digitorus longus tendon revealed remarkable loss of tendon stiffness, in the double nulls, causing joint hypermobility. Fibromodulin deficiency alone causes significant reduction in tendon stiffness in the $Lum^{+/+}Fmod^{-/-}$ mice, with further loss in stiffness in the $Lum^{+/-}Fmod^{-/-}$ in a lumican gene dose-dependent way (Figure 14)[63]. When compared with wild type, $Lum^{+/+}Fmod^{-/-}$, $Lum^{+/-}Fmod^{-/-}$ and $Lum^{-/-}Fmod^{-/-}$ tendons exhibit a decrease in stiffness of 25%, 45% and 61%, respectively. Tensile modulus was also reduced by 29% and 49% in $Lum^{+/-}Fmod^{-/-}$ and $Lum^{-/-}Fmod^{-/-}$, respectively, relative to wild type controls. These results establish fibromodulin as a key

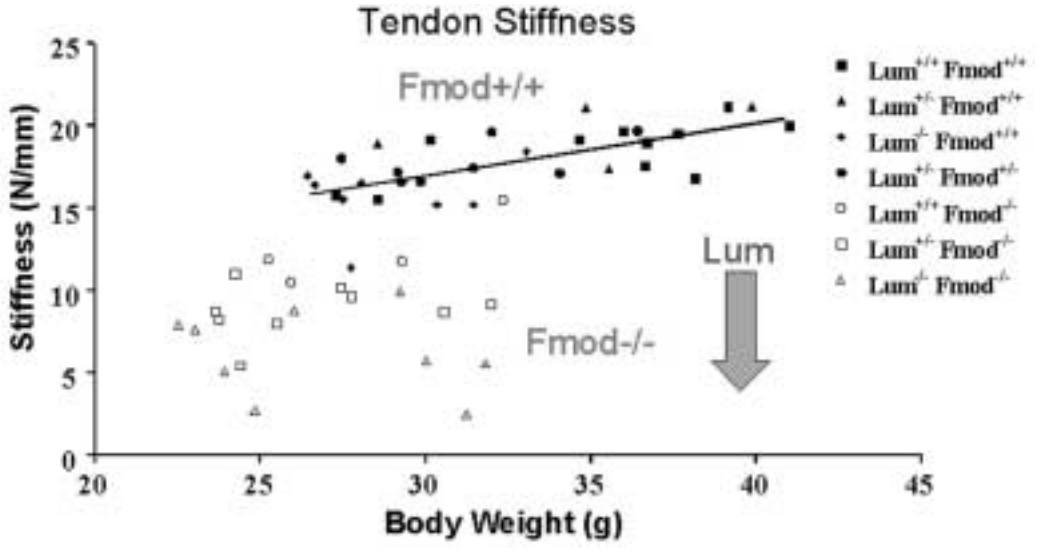

Figure 14. Tendon function is dependent on fibromodulin and modulated by lumican expression. Relationship between whole tendon stiffness and body weight. Whole tendon stiffness was determined from tension tests of FDL tendons and plotted against body weight $Lum^{+/+}Fmod^{+/+}$, $Lum^{+/-}Fmod^{+/+}$, $Lum^{-/-}Fmod^{+/+}$, and $Lum^{+/-}Fmod^{+/-}$ (shown as solid symbols) clustered along a similar linear regression indicating that tendon stiffness was similarly adapted to body weight for these four fibromodulin expressing genotypes. The three $Fmod^{-/-}$ genotypes (open symbols) clustered separately revealing that tendon stiffness was dramatically reduced relative to their body size. (Modified from Jepsen et al.[63]).

regulator of tendon strength and lumican as a modulator of this function, and confirm that loss of these proteoglycans alters the mechanical properties of the tissue. A disproportionate increase in small diameter, immature collagen fibrils and a lack of progression to mature, large diameter fibrils in the fibromodulin-deficient background may be the underlying cause of tendon weakness and suggests that fibromodulin aids fibril maturation. To determine if the functional difference between $Lum^{+/+}Fmod^{-/-}$ and $Lum^{+/-}Fmod^{-/-}$ is directly related to quantitative differences in the lumican gene product, levels of lumican protein and RNA in the different genotypes were examined[63]. The $Lum^{+/+}Fmod^{-/-}$ tendons contain about twice as much lumican protein as the $Lum^{+/-}Fmod^{-/-}$ tendons. Moreover, excess protein appears to down regulate transcription of lumican in the fibromodulin-deficient mouse tendon; the immunoblotting data indicate excess lumican protein while the real-time RT-PCR data indicate a down regulation of the lumican message. Svensson and co-workers also have noted a 4-fold increase in lumican protein and a decrease in its message in the tail tendons of $Fmod^{-/-}$ mice[62]. These findings suggest that the leucine-rich repeat proteoglycans/proteins regulate their mutual levels in tissues. The over expression of lumican in the Fmod-null background may compensate for fibromodulin-deficiency and prevent further loss in tendon strength in the $Lum^{+/+}Fmod^{-/-}$ genotype.

Decorin and biglycan are two additional, closely related members of the leucine-rich repeat proteoglycan family[9,65]. Mice deficient in one or both have alterations in fibril structure in a variety of connective tissues, including tendon. A synergistic interaction between biglycan and both decorin and fibromodulin has been reported[65-67].

Studies conducted using decorin-deficient knockout mice suggest a critical role for this proteoglycan during tendon development as well. Decorin is regularly and specifically associated with the surface of type I collagen in extracellular matrices[68] and it has been hypothesized that decorin limits collagen fibril diameter by inhibiting the lateral fusion of fibrils[18,59,69-71]. In addition to its influence on fibril diameter, decorin also induces alterations in collagen orientation. In the absence of decorin, fibrils in the periodontal ligament display a random, rather than parallel, arrangement (Figure 15)[72]. The tendons of decorin knockout mice undergo a larger and faster stress relaxation than their wild type counterparts, indicating more rapid dissipation of their viscoelastic properties[73]. It has also been proposed that decorin can act as a bridge between two adjacent collagen molecules, thereby helping to stabilize and align fibrils during fibrillogenesis[14]. Considering that the shear stiffness of proteoglycan aggregates has been estimated to be only on the order of 10^{-5} MPa[74], it is improbable that the presence of leucine-rich repeat proteoglycans/proteins in the matrix of a tendon directly affects its function. However, their influence on fibril morphology and their role in the interactions between collagen molecules and fibrils likely have a profound impact, as evidenced by the effect of fibril defects on mechanical properties.

Conclusions

During tendon development, collagen fibrillogenesis generates a tendon-specific extracellular matrix that determines the functional properties of the tissue. During development

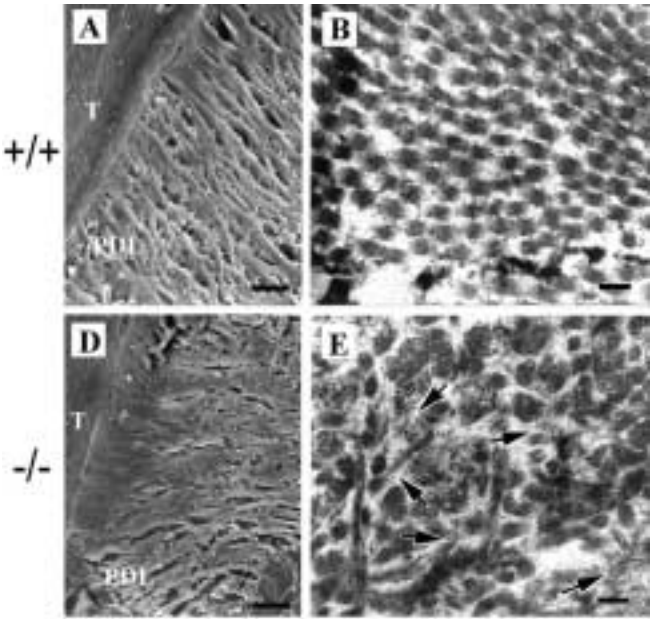

Figure 15. Decorin-deficient mice have alteration in fibril structure and organization. (**A,D**) Scanning electron micrographs of collagen fibrils in the wild type (**A**) and decorin-deficient mouse (**D**) molar periodontal ligament. In the decorin -/- mouse, collagen fibrils are larger in diameter and more randomly arranged compared with the wild type mouse. (**B,E**) Transmission electron micrographs of collagen fibrils in the wild type (**B**) and decorin-deficient mouse (**E**) periodontal ligament. In the decorin -/- mouse, collagen fibrils are more randomly arranged and irregularly shaped, and show more heterogeneity in cross-sectional size compared with the wild type mouse. In the decorin -/- mouse periodontal ligament, there are very large diameter collagen fibrils (**long arrows**) and also many small diameter collagen fibrils separated from the larger collagen fibrils (**short arrows**). PDL, Periodontal ligament; T, tooth. (**A,D**) Bar 0.2μm; (**B,E**) Bar 0.1μm. (Reproduced from Hakkinen et al.[72]).

and growth, collagen fibril intermediates are assembled and undergo linear and lateral growth within immature fibers. This allows intercalation of fibrils necessary for growth and generates mature fibrils and fibers necessary for mechanical integrity. These steps occur within a hierarchy of extracellular compartments defined by the tendon fibroblasts and are regulated by macromolecular interactions within the fibroblast controlled extracellular domains. The interactions of 2 or more fibril-forming collagens, and interactions with fibril-associated collagens and proteoglycans have all been implicated in the regulated development of tendon structure and therefore its functional properties.

Acknowledgments

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