Type XIV Collagen Regulates Fibrillogenesis

PREMATURE COLLAGEN FIBRIL GROWTH AND TISSUE DYSFUNCTION IN NULL MICE*

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Type XIV collagen is a fibril-associated collagen with an interrupted triple helix. This collagen interacts with the fibril surface and has been implicated as a regulator of fibrillogenesis; however, a specific role has not been elucidated. Functional roles for type XIV collagen were defined utilizing a new type XIV collagen-deficient mouse line. This line was produced using a conventional targeted knock-out approach. Col14a1(−/−) mice were devoid of type XIV collagen, whereas heterozygous mice had reduced synthesis. Both mutant Col14a1 genotypes were viable with a grossly normal phenotype; however, mature skin exhibited altered mechanical properties. Prior to evaluating tendon fibrillogenesis in type XIV collagen-deficient mice, the developmental expression patterns were analyzed in wild-type flexor digitorum longus (FDL) tendons. Analyses of mRNA and protein expression indicated tissue-specific temporal expression that was associated with the early stages in fibrillogenesis. Ultrastructural analyses of wild-type and null tendons demonstrated premature fibril growth and larger fibril diameters in tendons from null mice at postnatal day 4 (P4). However, fibril structure in mature tendons was normal. Biomechanical studies established a direct structure/function relationship with reduced strength in P7-null tendons. However, the biomechanical properties in P60 tendons were comparable in null and wild-type mice. Our results indicate a regulatory function for type XIV collagen in early stages of collagen fibrillogenesis with tissue differences.

Connective tissues, such as tendons, ligaments, dermis, cornea, cartilage, and bone, have tissue-specific structure and function. The collagen fibril is the major structural element in these tissues and is assembled from fibril-forming collagens, specifically types I, II, III, V, and XI (1–3). The development of extracellular matrices is dependent on the organization of fibrils into higher ordered structures, e.g. fibers and lamellae. The assembly of collagen fibrils is a multi-step process that is highly regulated. Specific developmental periods are associated with distinct stages in collagen fibril assembly and growth (4, 5). Early development is a pregrowth phase that is characterized by short, small-diameter fibrils intermediates. Sequentially, an end to end linear growth occurs followed by lateral growth of the preformed intermediates. Each step is independently regulated by specific fibril-associated macromolecules. These molecules associate with fibrils and modify fibrillar properties. There are two major groups of fibril-associated molecules. The first group is the small leucine-rich repeat proteoglycans and glycoproteins, which includes decorin, biglycan lumican, and fibromodulin. Previous studies found that these proteoglycans associate with collagen fibrils and regulate lateral growth during tendon development (6). A deficiency of these macromolecules in vivo resulted in unregulated lateral growth and therefore structurally abnormal tendon fibrillogenesis (7–9). The second group of fibril-associated molecules is composed of the fibril-associated collagen with interrupted triple helices (FACIT). Members of this group include type IX, XII, XIV, XVI, XIX, XX, XXI, XXII, and XXVI collagen (3). The focus of the current work, type XIV collagen, is present in developing tendon (10, 11) and is localized to the surface of tendon fibrils (12, 13).

Type XIV collagen contains two collagen triple-helical domains (COL1 and COL2) and three non-collagenous domains (NC1, NC2, and NC3) (14, 15). The triple-helical domains interact with and adhere to fibrillar collagen, the COL1 domain, in combination with the NC1 domain, interacts with type I collagen (15). The NC3 domain is a large N-terminal globular domain that extends away from the fibril. The NC3 subdomain has structural homology to von Willebrand factor A domains and fibronectin type III repeats that confer NC3 with its physical properties (3, 15, 16).

Studies of type XIV collagen in chicken (13, 17), bovine (18, 19), and human (20, 21) tissues showed that type XIV collagen is prevalent in skin, tendon, cornea, and articular cartilage. In addition, type XIV collagen is often found specifically in areas of high mechanical stress (11, 21, 22). Localization to these areas indicates that collagen XIV may affect the mechanical properties of a tissue. Evidence in support of this is that type XIV...
collagen plays a role in corneal stromal compaction (23) and promotes collagen gel contraction in vitro (24). In addition, in vivo studies of chicken tendon during embryogenesis and early post-hatching stages showed that type XIV collagen expression is high during development, but decreases during tissue maturation (13). These studies suggest that type XIV collagen is important during early development, but the specific roles remain to be elucidated.

The purpose of this study was to determine the functional role(s) of type XIV collagen in the development of tissue-specific structural and functional properties. A Col14a1-null mouse model was created and characterized to meet these ends. To elucidate the role(s) of type XIV collagen in regulation of collagen fibrillogenesis, ultrastructural analyses of fiber and fibril structure, fibril diameter distribution and evaluation of mechanical properties were conducted at multiple stages of tendon development, growth, and maturation. We hypothesized that type XIV collagen has a significant role in the regulation of fibrillogenesis and therefore the mechanics of developing soft tissues.

**EXPERIMENTAL PROCEDURES**

*Creation of Col14a1 Knock-out Mice*—Our group determined the entire mouse Col14a1 mRNA sequence and the genomic structure (15). This sequence was used to identify the mouse Col14a1 gene by searching the mouse genome sequence in GenBank™. Our targeting strategy was to introduce a nonsense mutation by inserting a stop codon into exon 4 of the Col14a1 gene, producing a truncated type XIV collagen protein (Fig. 1a). The 5'-targeting arm, containing exon 3, intron 3, and part of exon 4, was amplified by PCR using the forward primer: 5'-AA TCTAGA CAT GGA AAG CAC CAA GAG GG-3' and the reverse primer: 5'-AA TCTAGA TT TTAGTT CTG CTC TGG CAG AAG GC-3'. The stop codon (TAA) was introduced into exon 4 with a specifically designed reverse primer (bold sequence in above reverse primer) and the amplified 3-kb PCR template. PCR-amplified targeting arms were subcloned into a NEO-TK (neomycin resistance gene sequence and HSV thymidine kinase gene) containing vector to finish the targeting construct as previously described (25). Targeting vector was linearized by NotI digestion and electroporated into J1 (129S4/SvJae) mouse embryonic stem (ES)³ cells using standard techniques (26) at the Gene Targeting Facility of Massachusetts General Hospital (Charlestown, MA). ES cells were double-selected with G418 and gancyclovir, and Southern blots were performed to screen for positive ES cell clones using 5' and 3' gene-specific probes. Selected ES cell clones were microinjected into C57BL/6 mouse blastocysts and transferred to pseudo-pregnant B6CBA mice. Chimeras were bred to C57BL/6 mice to get germline transmission. Mouse genotypes were determined with PCR using allele-specific primers. Col14a1 heterozygous mice were inbred to derive Col14a1(−/−) homozygous mice.

*Southern Blot Genotyping*—Genomic DNA from ES cells or mouse tails was extracted using a phenol-chloroform method or high pure PCR template preparation kit (Roche Applied Science). Restriction enzymes XbaI, SpeI, and EcoRV were used to digest DNA samples individually and hybridizations were carried out separately using a 5'-probe, neomycin-resistant gene sequence (Neo) probe, and 3'-probe. All probes were [α-32P]dCTP-labeled using Prime-It random primer labeling kit (Stratagene, Cedar Creek, TX).

*PCR Genotyping*—Col14a1 genotyping was accomplished using genomic DNA isolated from mouse tail biopsies as PCR template. Allele-specific primers were as follows: Primer E4F (P1: 5'-CCA ACC AGA TGA ATC TCC AG-3') and primer I4R (P2: 5'-CCT AAT AGT CCT TAA GAG GC-3') were chosen from exon 4 and intron 4, respectively, and were used to amplify the wild-type allele producing a 410-bp PCR product. Primer neoR1 (P3: 5'-TAG GGG AGG AGG AGT AGT GG-3') was chosen from the neomycin resistance gene sequence and with primer I4R (P2) detected the mutant allele, producing a 540-bp PCR product. Positive ES cell and wild-type C57BL/6 mouse genomic DNA samples were used as heterozygous and wild-type controls.

*Analyses of mRNA Expression*—Total RNA was isolated from pooled FDL tendons. At postnatal day 4 (P4) and P10, 40 FDLs from 20 mice were used for each age. At P30 and P90, 20 FDLs from 10 mice were used at each age. Two independent cDNA preparations at each developing age were obtained by reverse transcription of total RNA (5 μg) with random primers (High-Capacity cDNA Archive kit, Applied Biosystems, Foster City, CA).

Semi-quantitative RT-PCR analyses were done as previously described (13, 27, 28). Primer sequences for XIV collagen are:

- forward primer: 5'-ACT GGT TTT CAC GGG TGT TC-3', reverse primer: 5'-TAA GTC GAG GAG AGG CAA GC-3', producing a 208-bp fragment. Classic 18S internal standard (Ambion) was used as reference gene producing a 498-bp band. The primer pair to competimer ratio yielding comparable product intensities for 18S and XIV was 2:10. From PCR linear range determination, the optimal PCR cycle was fixed at 24. PCR was: 94 °C 2 min followed by 24 cycles of 94 °C 15 s, 58 °C 25 s, 72 °C 1 min, and final extension at 72 °C for 10 min. PCR was done twice for each age.

Real-time RT-PCR was performed using the LightCycler (Invitrogen) System with the SYBR Green PCR Master Mix (Applied Biosystems) (29). Classic 11 18S internal standard (Ambion) was used as an endogenous control to standardize the amount of sample RNA. A series of 10X dilutions of cDNA mixture from each developmental time point was used to generate relative standard curves for 18S for comparison with Col14a1 samples. PCR amplification was done with cDNA derived from 25 ng of RNA input for each sample used as template with primer concentrations of 0.3 μM for type XIV collagen and 0.1 μM for 18S; the optimal 18S primer pair/competimer ratio was 1:4. The PCR cycle parameters were: 95 °C 2
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Min x 1 cycle, (95 °C 5 s, 58 °C 15 s, 72 °C 20 s) x 40 cycles. The optimal MgCl2 concentration used for the PCR was 2 mM. Real-time RT-PCR analysis of leucine-rich proteoglycans was performed using a similar approach. A StepOnePlus (Applied Biosystems) system was used with actin as an endogenous control.

Antibody Production—Antibodies against mouse type XIV collagen were produced as described previously (30). Briefly, cDNA coding for the fibronectin type III domains 5–8 of the mouse collagen XIV was amplified by PCR using the forward primer 5’-AAT GCT AGC GAA GTT CCC GCC CAG CAA TAC-3’ and the reverse primer 5’-TTG CTC GAG TTA TGA CTG TGT TTT CTT CAT GAT -3’. The cDNA was cloned into a modified pPET vector (EMD Biosciences) carrying a His6 tag with a thrombin cleavage site. The protein was produced in Escherichia coli cells (Bl21; EMD Biosciences), purified by immobilized metal ion affinity chromatography and subsequently used to immunize a rabbit and two guinea pigs from which the antisera were purified by affinity chromatography. The specificity of the antibodies was tested by immunoblotting of tissue extracts combined with collagenase digestion as described in Koch et al. (31).

Immunolocalization Analyses—Skin samples were dissected from Col14a1(/−/−) and Col14a1(+/+) mice at P12 and P60. FDL tendon samples were dissected from wild-type mice at P4, P10, P30, and P60 as described above. Tissues were embedded in OCT medium, quick frozen in an ethanol/dry ice bath and stored at −80 °C. Sections (6 μm) were cut using a HM 505E cryostat. Direct immunofluorescence staining was performed as previously described (13). Rabbit anti-mouse type XIV collagen antibody (1 μg/ml) and rabbit anti-mouse type I collagen (1:1000, MD Bioscience) were used. The secondary antibody was an Alexa Fluor 568-conjugated goat anti rabbit IgG (Molecular Probes, Eugene, OR) used at 1:400. Vectashield mounting solution with DAPI (Vector Laboratories, Inc., Burlingame, CA) was used as a nuclear marker. Negative control samples were incubated identically, except the primary antibody was excluded. Images were captured using a Nikon microphot-SA fluorescent microscope (Nikon, Japan) with an Optronics digital camera (Goleta, CA). Identical conditions and set integration times were used to facilitate comparisons between samples.

Immunoblot Analyses—Skin samples (100 mg of wet weight) were collected from Col14a1(/−/−), Col14a1(+/+) and wild-type mice. FDL tendon (20 mg of wet weight) samples from wild-type mice at P4, P10, P30, and P90. At P4 and P10, 20 mice were dissected for each age group. At P30 and P90, 10 mice were dissected for each age group. For analysis of collagens, tendons were rinsed in phosphate-buffered saline, and homogenized in 5-fold excess of extraction buffer (50 mM Tris pH 8.0, 1 M NaCl, 10 mM EDTA, 10 mM N-ethylmaleimide, and proteinase inhibitor mixture, Roche). FACIT collagens were extracted at 4 °C overnight with stirring followed by centrifugation at 14,000 rpm using Eppendorf centrifuge 5415C (Eppendorf) for 20 min at 4 °C. For proteoglycan analysis, tendons were extracted in 4 M guanidine–HCl, 50 mM sodium acetate, pH 5.8 with proteinase inhibitor as previously described (28). Total protein content in the sample was determined using a BCA Protein Assay kit (Pierce). Constant total protein for each sample, 2–20 μg was resolved by SDS-PAGE and transferred to nitrocellulose membrane (Amersham Biosciences). Immunoblotting was done as previously described (32). Anti-type XIV collagen antibody was used at 1 μg/ml. Anti-decorin (LF113), anti-biglycan (LF159), and anti-fibromodulin (LF149) antibodies were used at 1:1000, 1:200, and 1:200, respectively. The antibodies were kindly provided by Dr. L. Fisher, NIH-NICDR (33). Anti-lumican antibodies were used at 1 μg/ml as previously described (27). Actin in each sample was probed with anti-actin antibody (Chemicon) as a loading control. Goat anti-rabbit IgG-peroxidase (Sigma) was used as the secondary antibody at 1:3000 with ECL as the detection system (Amer-}

Transmission Electron Microscopy—Tendon samples from Col14a1(−/−) and wild-type mice between P1 and P60 were analyzed by transmission electron microscopy. Briefly, flexor digitorum longus (FDL) tendons were dissected and fixed in 4% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M sodium cacodylate, pH 7.4, with 8.0 mM CaCl2, post-fixed with 1% osmium tetroxide and en bloc-stained with uranyl acetate/50% ethanol (34). After dehydration in an ethanol series, followed by propylene oxide, the tissue samples were infiltrated and embedded in a mixture of EMbed 812, nacryl methyl anhydride, dodecyl succinic anhydride, and DMP-30 (Electron Microscopy Sciences, Hatfield, PA). Thin sections (90 nm) were cut using a Reichert UCT ultramicrotome and post-stained with 2% aqueous uranyl acetate and 1% phosphotungstic acid, pH 3.2. Cross sections from the mid-plantar regions of FDL tendons were examined at 80 kV using a Tecnai 12 transmission electron microscope equipped with a Gatan Ultrascan US1000 2 K digital camera.

Fibril Diameter Distribution—for each genotype, 4–5 tendons from 4 different animals at P4 and 3 tendons from 3 different animals at P60 were analyzed. Fibril diameters were measured with a RM Biometrics-Bioquant Image Analysis System (Nashville, TN) using randomly chosen and masked digital images analyzed at a final magnification of ×118,790. For measurements at P4, a constant area of 0.47 μm2 per image was utilized that contained cross-sectional profiles free of cell processes. The count per image was 104–110 fibrils measured, with 24 images measured for wild-type (total fibril count of 2643) and 30 images for the null genotype (total fibril count of 3118). For measurements at P60, a constant area of 1.46 μm2 per image was utilized which contained cross-sectional profiles free of cell processes. The count per image was 62–84 fibrils measured, with 15 images measured for wild-type (total fibril count of 1266) and 15 images for the Col14a1(−/−) genotype (total fibril count of 926).

Biomechanics—for analyses of skin, samples were dissected at P60 from the back of wild type and 7 Col14a1(−/−) littermates. The hair was shaved, and two dumbbell-shaped skin specimens per mouse, oriented normal to the sagittal plane, were prepared. The right side of each sample was marked to ensure consistency of direction. Cross-sectional area of the skin was calculated from measurements of the width and thickness (35, 36). Skin samples were glued (cyanoacrylate) to sand paper 10-mm apart and stain lines were placed 5-mm apart in the
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mid-substance to track strain optically (37). Samples were clamped in custom test fixtures. Standard mechanical testing protocols were used, including preload to 0.03 N and loading to failure at 0.33 mm/s (1.67%/s) (38). Both skin samples for each mouse were tested and parameters were averaged within each animal. For all tests, samples were immersed in a 37 °C saline bath and tested with an Instron 5543 (Instron Corp., Canton, MA). Maximum stress was determined and modulus was calculated using linear regression from the near-linear region of the stress-strain curve. Student’s t-tests were performed on original tissue cross-sectional area, maximum stress, and elastic modulus across genotype (significance at p ≤ 0.05, trend at p ≤ 0.1).

For analyses of P7 FDLs 6 wild type, 13 Col14a1(−/−) and 12 Col14a1(+/−) littermates were utilized. Cross-sectional area of the P7 FDL was measured using a custom built device consisting of LVDTs, a CCD laser, and translation stages. Briefly, the specimen was translated beneath the laser while displacement data were simultaneously acquired in two directions from two LVDTs, as well as thickness data from a CCD laser displacement sensor (39). Because of their extremely small size and fragile nature, P7 mouse tendons have not been mechanically tested previously. Each end of the P7 tendon was glued (cyanoacylate) to sandpaper 4-mm apart. Stain lines were placed 2-mm apart within the mid-substance to track the strain optically (37). Tendons were placed in custom grips and a custom holding fixture was secured to the grips. Importantly, the holding fixture was designed to ensure that the tendons remained unloaded prior to testing. The tendons were preloaded to 0.005 N and held for 5 min. The load was returned to 0 N and held for 1 min. The tendons were then loaded at a constant ramp to failure at 0.004 mm/s (0.1%/s). Both the right and the left FDL were tested, and parameters were averaged within each animal.

For analyses of P60 FDLs, 8 wild-type, 12 Col14a1(−/−), and 8 Col14a1(+/−) littermates were analyzed. Cross-sectional area of the P60 tendon was calculated from measurements of the width and thickness (35, 36). P60 tendons were glued (cyanoacylate) to sandpaper 5-mm apart, and stain lines were placed 2.5-mm apart within the mid-substance to track strain optically (37). Samples were clamped in custom test fixtures, and standard mechanical testing protocols were used including preconditioning, stress-relaxation, and ramp to failure as described (40).

For all tendon tests, samples were immersed in a 37 °C phosphate-buffered saline bath and tested with an Instron 5543 (Instron Corp.). Maximum stress and modulus were calculated as previously described. One way ANOVAs were performed on cross-sectional area, maximum load, maximum stress, stiffness, and elastic modulus comparing across genotype within each age group (significance at p ≤ 0.05, trend at p ≤ 0.1).

RESULTS

Creation of a Type XIV Collagen A-null Mouse Model—To address the functional role(s) of type XIV collagen in vivo, a Col14a1 knock-out mouse line was created using a gene-targeting vector that was designed to introduce a nonsense mutation into exon 4 of the Col14a1 gene. This mutation generates a premature stop codon, thus truncating the synthesis of type XIV collagen. DNA sequence analysis determined that no other downstream start codon existed that could potentially allow type XIV collagen peptide synthesis. Only a small, non-functional peptide of 94 amino acids can be potentially translated from the initial start codon located in exon 2. This putative 94 amino acid peptide contains the signal peptide and the initial portion of the FNIII-1 domain. Collagen domains (COL1 and COL2), non-collagenous domains (NC1 and NC2), and the rest of the NC3 domain could not be translated, and the truncated type XIV collagen peptide would be unable to form a stable triple helix. The targeting strategy is presented in Fig. 1a.

Targeted ES clones were identified by Southern blotting. DNA samples were digested individually with XbaI, SpeI, or EcoRV restriction enzymes for hybridization using a 5′-probe, Neo probe, and 3′-probe as shown (Fig. 1b). A 9.9-kb DNA fragment and a 7.2-kb DNA fragment detected the wild-type and mutant alleles, respectively, with the 5′-probe. Hybridization with the 3′-probe detected a 6.3-kb wild-type allele fragment and a 7.5-kb mutant allele fragment, respectively. These results demonstrated that the mutant gene fragments had been correctly integrated into the mouse genome. The Neo probe detected a 10.4-kb DNA fragment confirming that the Neo gene also had been inserted into the genome.

Correctly targeted ES clones were microinjected into C57BL/6 blastocysts and germ-line transmission was obtained. Breeding of the F1 heterozygous (Col14a1(+/−)) mice produced offspring with the three expected genotypes (Col14a1(+/+), Col14a1(+/−), Col14a1(−/−)). Mice were genotyped using allele-specific primers (Fig. 1c). A total of 240 offspring produced by breeding of heterozygous Col14a1 mice were genotyped. The ratio of Col14a1(+/+), (+/−), and (−/−) mice was not statistically deviant from the 1:2:1 ratio expected by Mendelian inheritance of the targeted mutation (Fig. 1d). The Col14a1(+/−) and Col14a1(−/−) mice were fertile and were grossly normal in appearance.

Col14a1(−/−) Mice Are Null for Type XIV Collagen—Type XIV collagen was immunolocalized in skin of P12 and P60 mice. At P12, positive anti-type XIV collagen antibody reactivity was present throughout the dermis of wild-type mice. The dermis of homozygous mutant mice was not statistically deviant from the 1:2:1 ratio expected with the 3′-probe—tests were performed on original tissue cross-sectional area, maximum load, maximum stress, stiffness, and elastic modulus across genotype (significance at p ≤ 0.05, trend at p ≤ 0.1).

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en-deficient skin at P60. The maximum stress was significantly decreased ($p < 0.05$) in the skin of the Col14a1 (−/−) mice compared with that of wild-type controls. Additionally, the deficient mice showed a trend toward decreased modulus when compared with wild-type mice (Fig. 2c).

**Restricted Temporal Expression of Type XIV Collagen in Developing Mouse Tendon**—The regulatory role(s) of type XIV collagen in fibrillogenesis were analyzed in developing tendons. Transcription of Col14a1 was analyzed from birth to maturity in the FDL tendon. Expression of mRNA in tendons at P4, P10, P30, and P90 was analyzed using semi-quantitative and quantitative RT-PCR. Quantitative PCR demonstrated the highest amount of Col14a1 mRNA at P4 that then decreased sharply between P4 and P10. A very low level of expression was reached by P30 and this was maintained in the mature (P90) tendon (Fig. 3a). Semi-quantitative PCR confirmed these results (Fig. 3b). The expression of type XIV collagen was analyzed using immunoblot analyses. Protein immunoblot results were consistent with the mRNA analyses. Tendons at P4 demonstrated the highest amount of type XIV collagen with a sharp reduction by P10. Virtually no type XIV collagen was present in extracts from P30 and P90 tendons (Fig. 3c).

The temporal and spatial expression patterns of type XIV collagen in the tendon were investigated utilizing immunofluorescence microscopy (Fig. 4). These findings demonstrate homogeneous type XIV collagen expression throughout the tendon at P4 and P10. However, by P30 and maintained at P90, reactivity against type XIV collagen was undetectable. Consistent with the immunoblot and qPCR analyses, a reduction in type XIV collagen reactivity was observed between P4 and P10. At all stages studied, the expected homogeneous distribution of type I collagen was observed. The temporal and spatial expression patterns indicate a role(s) for

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**FIGURE 1. Creation of Col14a1 knock-out mice.** a, targeting strategy. Functional domains of type XIV collagen and genomic structure of mouse Col14a1 are shown in the upper part of the diagram. In the gene-targeting vector, exon 4 was split into two parts, and a stop codon was inserted into the 5′-half of E4 by PCR. Genomic sequences of exon 3 through the first part of exon 4 and the second part of exon 4 through exon 5 were chosen as 5′- and 3′-targeting arms. Both arms are about 3 kb in size. Neomycin-resistant gene was inserted between the two arms, reversely (black arrows show transcription direction). Probes (short color rectangles) and restriction enzymes used for genomic DNA digestion and Southern blotting are indicated in the diagram. Green arrows represent gene-specific primers for genotyping. Primer 1 (P1) and Primer 2 (P2), from the second part of exon 4 and intron 4, respectively, produce a PCR product from the wild-type and mutant alleles. Primer 3 (P3 from the Neo gene, produces a DNA fragment only from the mutant allele when used with P2. The translation termination codon, which is located in FNIII-1, is marked on the protein structure diagram. The anti-type XIV collagen antibody epitope also is marked in this diagram. b, genotype analysis of Col14a1 knock-out mice using Southern blotting. A 9.9-kb and a 7.2-kb DNA fragment corresponding to the wild-type and null alleles, respectively, were detected by 5′-probe hybridized with XbaI digested mouse genomic DNA. Neo probe was used for hybridization with Spl-digested DNA and a 10.4-kb DNA fragment was detected. A 6.3-kb DNA fragment indicating the wild-type allele and a 7.5-kb DNA fragment indicating the mutant allele were recognized by 3′-probe after hybridization with EcoRV-digested DNA. c, PCR genotyping. A 410-bp wild-type allele PCR product and a 540-bp mutant allele PCR product were generated by PCR genotyping, which identified the three Col14a1 genotypes. Col14a1 (+/+) ES cell and wild-type C57 mouse genomic DNA samples were used as controls. d, Mendelian inheritance analysis. A total of 240 offspring from heterozygous Col14a1 breeders were genotyped. The ratio of wild type, heterozygous, and homozygous null mice is roughly 1:2:1.
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a. Type XIV Collagen Immuno-Localization In Skin

![Image](image.png)

**FIGURE 2. Absence of type XIV collagen and alteration of biomechanical properties in Col14a1-null mouse skin.** a, immunofluorescence staining of Col14a1 knock-out mouse skin using anti-type XIV collagen antibody was completely negative in Col14a1-null mice skin. DAPI staining shows skin structure (blue nuclear). b, Western blotting of protein samples extracted from mouse skin showed complete absence of type XIV collagen in Col14a1-null mice and reduced synthesis in heterozygous mice. c, biomechanical studies showed a significant decrease in the maximum stress in the P60 skin of the col14a1(-/-) mice compared with that of wild-type controls. Additionally, the modulus showed a trend toward lower modulus when compared with wild-type mice. No significant differences were found in the cross-sectional area of the P60 skin. *, p < 0.05; #, p < 0.1.

Type XIV collagen in regulation of the early stages in tendon fibrillogenesis.

**Absence of Type XIV Collagen Is Associated with Altered Fibril and Fiber Assembly**—To analyze fibril and fiber assembly during early tendon development; ultrastructural analyses were performed using transmission electron microscopy of Col14a1-deficient mice and wild-type littermate controls. At early stages of development between P1-P5, tendon architecture was disorganized in the Col14a1(-/-) compared with wild-type tendons (Fig. 5). This was seen as altered organization of fibrils into fibers with a disruption of the associated fibroblast compartmentalization in the null tendons. In addition, in the P4-null and wild-type tendons, the normal circular cross-sectional fibril profiles were observed in both genotypes. However, the collagen fibrils in the null tendons were larger and less uniform in size than fibrils from wild-type tendons (Fig. 6, a and b). In the mature type XIV collagen-deficient tendons, general fiber, and fibroblast organization was comparable to that observed in the wild-type tendons (Fig. 5). However, the P60 type XIV collagen-deficient tendons contained fibrils with larger cross sectional profiles compared with fibrils from wild-type controls (Fig. 6, a and b).

**Premature Fibril Growth in the Type XIV Collagen-null Tendon**—The fibril diameter distributions for both P4 and P60 tendons were analyzed (Fig. 6c). At P4 the fibril diameter distribution of wild-type and Col14a1(-/-) tendons were both unimodal, however there was a broadening of the distribution and shift toward larger diameter fibrils in the Col14a1-deficient tendons. This observation was consistent with a premature entry into the fibril growth phase in tendon development. In contrast, at P60, the fibril diameter distributions in both Col14a1(-/-) and wild-type tendons were multimodal with a comparable range of diameters. However, the fibrils in Col14a1(-/-) tendons had a prominent subpopulation of large diameter fibrils in the 150–190-nm range that was not present in the wild-type tendons. This subpopulation is consistent with the progression of fibril growth of the fibrils seen at P4 in the null tendons.

**Altered Biomechanical Properties Associated with Abnormal Fibril and Fiber Assembly in the Collagen Type XIV-null Tendon**—Biomechanical studies of Col14a1(+/+), Col14a1(+/−) and Col14a1(-/-) tendons were conducted for developing (P7) and mature (P60) tendons. Cross-sectional area as well as maximum load, maximum stress, stiffness, and modulus were measured. At both developmental stages the cross-sectional areas were comparable in all genotypes (data not shown). The developing Col14a1(-/-) tendons (P7) demonstrated a significant reduction in maximum load, stiffness and modulus when compared with wild-type tendons (Fig. 7a). In addition, FDLs from the developing Col14a1(-/-) mice showed significantly decreased maximum load and a trend toward decreased maximum stress and stiffness when compared with the heterozygote mice. FDLs from heterozygous mice had significantly decreased stiffness compared with wild-type mice. In contrast, the mature tendons demonstrated no significant differences between the wild type, Col14a1(−/−) or Col14a1(+/−) tendons (Fig. 7b).

The possibility that the observed alterations in fibrillogenesis and biomechanical properties were secondary effects due to a compensatory up-regulation of other regulatory molecules in
the Col14a1(−/−) mouse was addressed. Expression of biglycan, decorin, lumican, and fibromodulin was analyzed at P4, a period when type XIV collagen is normally expressed and at P30, a period when type XIV is not expressed. An analysis of transcript in null and wild-type mice demonstrated no significant difference (p > 0.1) in expression at either developmental stage for biglycan, decorin, lumican, or fibromodulin (Fig. 8a).

A semi-quantitative immunoblot analysis supported these results, demonstrating no changes in expression of any of the core proteins at either developmental stage (Fig. 8b). These data indicate a direct relationship between the absence of type XIV collagen and the structural and functional changes observed in the tendon.

In summary, a Col14a1-null mouse model was created and characterized. The absence of collagen type XIV in mature skin was associated with reduced mechanical attributes. To address the regulatory roles in fibrillogenesis the developing tendon was utilized. Type XIV collagen expression is restricted to the early stages in normal development. There was no up-regulation of decorin, biglycan, fibromodulin, or lumican associated with the type XIV collagen-null phenotype. Loss of type XIV collagen in the null mice was associated with altered regulation of fibril growth and consequent changes in biomechanical properties.

DISCUSSION

Development of a Targeted Col14a1(−/−) Mouse Line—The only known Col14a1-null mouse line is presented in the current work. This novel line is used to elucidate the regulatory role(s) of type XIV collagen in fibrillogenesis. The Col14a1 heterozygous and homozygous mice are viable with a standard Mendelian inheritance pattern. No gross phenotypic markers were observed. In particular, no differences in body weight or size were observed and the null mice did not develop any abnormalities with age.
FIGURE 6. Altered collagen fibril formation in Col14a1(−/−) tendons. Transmission electron micrographs of FDL tendon at postnatal day 4 (P4) from (a) Col14a1(+/+) and (b) Col14a1(−/−) mice. At P4, the fibrils of the wild-type control are more uniform in shape and smaller than the fibrils in the Col14a1(−/−) tendon, which are more irregular and larger. At P60, the mature fibrils from the wild-type tendon are of variable size with small to larger cross-sectional profiles. In the Col14a1(−/−) tendon, there are fewer smaller fibrils and more fibrils of larger diameter as compared with the wild-type control. Bar, 100 nm. c, histograms representing fibril diameter measurements of tendons at P4 and P60. In the P4 wild-type tendon, the fibril diameter distribution demonstrates a unimodal pattern with the majority of fibril diameters between 35 and 45 nm. In the P4 mutant tendon, the fibril diameter measurements also show a unimodal (50–60 nm) distribution; however, there is a greater frequency of larger diameter fibrils. At P60, the wild-type diameter measurements have a much broader multimodal distribution. The fibril diameter measurements in the mutant tendon also have a multimodal distribution. In contrast to the wild-type distribution, there is a reduced frequency of smaller diameter fibrils in the null tendons. In addition, a distinct subpopulation of larger diameter fibrils, absent in the wild-type tendons, is present in the null tendons. We suggest that this represents the fibrils that prematurely enter the fibril growth phase in the absence of type XIV collagen.
Dysfunctional Fibrillogenesis in Collagen XIV-null Mice

Restricted Temporal Expression of Type XIV Collagen in Developing Mouse Connective Tissue—Type XIV collagen is highly expressed in development in a variety of connective tissues (10, 17, 19, 32, 41). In this study, type XIV collagen was highly expressed early in mouse FDL development. Furthermore, type XIV collagen expression rapidly decreases as the tendon matures becoming virtually absent by P30. On the other hand, skin from P60 wild-type mice still contained type XIV collagen, even though it was greatly reduced from P12. These findings are consistent with previous studies that demonstrated expression of type XIV collagen is maintained longer in skin than in tendon (21). The decrease in type XIV collagen during development suggests a regulatory role in fetal and early postnatal fibrillogenesis. Previous work from our laboratory supports these findings (13, 42).

Type XIV collagen decorates the surface of fibrils and its large non-collagenous domain (NC3) projects into the interfibrillar space (12, 13). This domain has been implicated in the regulation of fibril packing (15, 16, 32) and these interactions would be expected to inhibit lateral fibril growth, therefore, decreased expression of type XIV collagen would be associated with the beginning of lateral fibril growth. The controlled replacement of type XIV collagen with other fibril-associated molecules such as type XII collagen or leucine-rich proteoglycans would allow continued regulation of the growth steps. Our current work indicates a regulatory role determining entrance into the lateral fibril growth stage in addition to fiber assembly.

Type XIV Collagen Regulates Fiber and Fibril Assembly in Tendon—During mouse FDL development, P1 represents a stage of immature fibril intermediate formation, P10 represents initial stages of lateral growth and P90 represents a mature tendon (27). This work supports the assumption that type XIV collagen functions in the formation of mature fibrils from fibril intermediates. Our finding of a premature entry into lateral fibril growth in the null tendon provides support for this role. Interestingly, null and wild-type fibrils had normal circular cross sectional profiles, but both the null developing and mature FDL fibrils had a broadening of the distribution of fibril diameters with a shift toward larger fibrils. The increase in fibril diameter seen at all stages of development, again suggests that type XIV collagen functions to regulate entry into the lateral fibril growth phase during development. Young et al. (13) suggested that type XIV collagen could stabilize immature fibrils, preventing lateral association and therefore, lateral growth in tendon. In addition, immature fibrils undergo significant linear growth during the period where type XIV collagen expression is down regulated (4, 27, 42). Therefore, whereas type XIV collagen may not play an active role during lateral growth, the current data indicate that it regulates the fibril’s entry into the lateral growth phase.

Type XIV collagen on the fibril surface also would be expected to be involved in the integration of fibrils into fibers. In this study, a disruption of developing fiber structure in the null tendon was observed. Deficiency in type XIV collagen was associated with disorganization of tendon architecture that was observed as early as P1 in the null mice. There was an altered organization of the fibrils into fibers with a disruption of the associated fibroblast compartmentalization in the null tendons. This suggests a role for type XIV collagen in mediating interfibrillar interactions involved in packing into fibers. Absence of these interactions also may be associated with altered biomechanical properties.

Altered Biomechanics in the Connective Tissue of Col14a1-null Mice—The assembly and final properties of the collagenous fibrillar matrix are not only dependent on the main constituents of the fibrils, but also fibril-associated molecules that participate in fibrillogenesis. In this study, type XIV collagen played a significant role in the biomechanics of the developing mouse FDL tendon. Maximum load, stiffness, and modulus were all significantly reduced when comparing the Col14a1(−/−) with wild-type tendons. However, by P60 the differences seen at P7 were absent. There were no differences...
seen in the cross-sectional area of any genotypes at either age. These results once again support the hypothesis that type XIV collagen plays a significant role in fibrillogenesis. It is also interesting to note that the heterozygote FDL tendons had mechanical properties intermediate to those of the wild-type and null tendons. This implies that type XIV collagen has a dose-dependent effect on the developing tendon. Type XIV collagen has previously been implicated in playing soft tissue mechanics (11, 21, 22). It was shown to aid in the compaction of collagen gels in vitro (24) and is up-regulated during the compaction stage of the corneal stroma (23). This study indicates that type XIV collagen has a significant role in establishing the mechanical properties in developing tendons.

Extracellular matrix architecture is largely responsible for connective tissue diversity. Different combinations of fibril and fibril-associated molecules provide each tissue with specific structural and functional properties. The disruption of tendon architecture observed early in development, but not in mature tendons may account for these mechanical differences.

Mechanical differences were not seen in mature null tendons, the mechanics in the mature Col14a1(−/−) skin were inferior to wild-type skin. Most notably, the null skin had a reduced maximum stress and modulus. Regional and temporal differences in the presence of type XIV collagen in skin and tendon could help explain the tissue-specific differences observed in the null mouse (21). While skin and tendon are both connective tissues consisting mainly of collagen fibrils organized as fibers, they have important differences. Most notably, the skin has a decreased fibril content compared with the tendons and the collagen fibers are loosely packed, small and non-aligned. In contrast, tendons are composed of densely packed, large, and well aligned fibers. Another important feature of connective tissues is the type of stresses they see in vivo. The skin experiences multi-axial stresses whereas the tendon sees mainly tensile stresses. Each of these tissues gives a unique view into how these components work together in a functioning tissue. Therefore, while the basic fundamentals of fibrillogenesis are the same in these two tissues, it is not surprising that we see a differential effect of the absence of type XIV collagen.

It was first proposed that fibril diameter parameters may predict mechanics by Parry et al. (43) in a large, multiple species study. Several groups have since examined the relationship between mechanics and fibril parameters including: mean fibril diameter size, fibril cross-sectional area fraction and fibril distribution variation (44–46). While some of these studies have found significant correlations between fibril parameters and mechanics, they were usually accompanied by a low correlation coefficient. A low correlation coefficient indicates that there are other parameters that also play a significant role in tendon mechanics. For example, proteoglycans and fibril area fraction were both found to be significant predictors of mechanical

![FIGURE 8. Leucine-rich proteoglycan expression in the Col14a1-null mouse.](image)

### mRNA Expression

|            | P4 | P30 |
|------------|----|-----|
| Col14a1 mRNA | ![](chart1) | ![](chart2) |
| Bgn mRNA    | ![](chart3) | ![](chart4) |
| Dcn mRNA    | ![](chart5) | ![](chart6) |
| Lum mRNA    | ![](chart7) | ![](chart8) |
| Fmod mRNA   | ![](chart9) | ![](chart10) |

### Protein Expression

|            | P4 | P30 |
|------------|----|-----|
| Col XIV    | ![](chart11) | ![](chart12) |
| Bgn        | ![](chart13) | ![](chart14) |
| Dcn        | ![](chart15) | ![](chart16) |
| Lum        | ![](chart17) | ![](chart18) |
| Fmod       | ![](chart19) | ![](chart20) |

Error bars represent the pooled standard deviation.

Col14a1(+/−) mice demonstrated no significant changes in mRNA expression between wild-type and Col14a1(−/−) mice (p > 0.1). This was the case for biglycan, decorin, lumican, and fibromodulin at both developmental stages. b, semi-quantitative immunoblot analysis. Extract (10 mg) prepared from P4 and P30 FDLs was subjected to immunoblot analysis with antibodies specified to collagen type XIV, biglycan, decorin, lumican, fibromodulin, and actin. Actin reactivity was used as loading control. Relative expression levels of indicated proteoglycan showed that the core protein expression in the wild type compared with null mice was comparable for all four proteoglycans at both developmental stages.
parameters (44). Therefore, while we see differences in fibril diameter distribution in both the P4 and P60 tendons it is very likely that at P60 another parameter, such as proteoglycan or collagen content, is significantly affecting the mechanics.

Types XIV and XII collagen are closely related members of the FACIT collagen class (3). Type XII collagen is more widely expressed throughout development, maturation, and aging in most connective tissues (1, 17, 47). It is possible that type XII collagen replaces type XIV collagen, both structurally and functionally at later stages of tendon development. Therefore, compensation in the null mouse is possible; however, compensation by type XII collagen was not observed in the developing tendon (data not shown). Work on other closely related fibril-associated molecules, the leucine-rich proteoglycans, supports this suggestion. Both decorin/biglycan and fibromodulin/lumican have been shown to be important regulators of collagen structure and mechanical function (7, 8, 27, 28, 48). However due to high similarity of these proteoglycans, they may share redundant function(s). For example, in the fibromodulin-deficient mouse biglycan was increased (28) and in the decorin-deficient mouse lumican was increased (9) and in the decorin-deficient decorin or fibro-modulin. If a similar interaction between types XII and XIV collagen exists, then a compensation with up-regulation of type XII collagen would be predicted and not the reverse. Future analyses of type XII collagen-XIV collagen in the absence of type XII collagen would be required and not the reverse. Future analyses of type XII collagen-null and compound type XII/XIV collagen null mouse lines will disagree with the order of compensation in any of these proteoglycans.

The NC3 domain of the type XIV collagen molecule is thought to act similarly to proteoglycans. The published work on the leucine-rich proteoglycans demonstrated compensation only when the proteoglycan expressed throughout development, maturation and aging was deleted, i.e. decorin or fibromodulin. If a similar interaction between types XII and XIV collagen exists, then a compensation with up-regulation of type XIV collagen in the absence of type XII collagen would be predicted and not the reverse. Future analyses of type XII collagen-null and compound type XII/XIV collagen null mouse lines will provide insight into this and other possible interactions, but will require the availability of a type XII collagen-null mouse model.

Overall, our data suggest a tissue-specific role of type XIV collagen in the structure-function relationship of connective tissues. Specifically, type XIV collagen functions early in development regulating the entry of fibril intermediates into lateral fibril growth. Our finding of altered fiber architecture also indicates that type XIV collagen functions in the integration of fibrils into fibers. Differences in fibril and fiber architecture in developing tendon, but not mature tendon may account for mechanical differences. Interestingly, mechanical differences are seen in mature skin that could be due to longer temporal expression of type XIV collagen in the skin as compared with the tendon.

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