Glycosaminoglycan content decreases with age in the mouse Achilles tendon but does not alter mechanical properties

Jonathon L. Blank, Jeremy D. Eekhoff, Louis J. Soslowsky
McKay Orthopaedic Research Laboratory, University of Pennsylvania, Philadelphia, PA
jonathon.blank@pennmedicine.upenn.edu

DISCLOSURES: None.

INTRODUCTION: Injuries are common in repetitive and high load-bearing tendons such as the Achilles tendon,[1] and injuries in middle-aged and older adults are prevalent due to increased lifespans and activity.[2] Glycosaminoglycans (GAGs) are long, negatively charged polysaccharides found in musculoskeletal tissues such as cartilage and meniscus and decrease in number with age,[3,4] corresponding to altered mechanics.[5,6] Decreasing GAG content with age has also been observed in human rotator cuff.[7] While GAGs have been shown to modulate viscoelasticity in mature patellar tendon[8] and tail tendon fascicles,[9] the relationship between GAG composition and aging Achilles tendon mechanics remains unknown. Therefore, the objective of this study was to investigate the role of GAGs in dynamic tensile loading in mouse Achilles tendons. We hypothesized (1) that GAG content would decrease with age and (2) that GAG-depleted tendons would exhibit a decrease in tensile viscoelasticity, but that this effect would wane with increasing age.

METHODS: Animals: Achilles tendons from male postnatal (P) day 150, 300, and 570 C57BL/6 Charles River mice were used (IACUC approved). These age ranges correspond to roughly 25, 45, and 65 years of human age. Biochemistry: GAGs were cleaved from their proteoglycan core proteins using chondroitinase ABC (cABC). Tendons from each age group were incubated in a tris buffer (pH=8) supplemented with protease inhibitors (control buffer) or a solution of control buffer with the addition of 0.5U/mL cABC (treatment buffer) for 18 hours under gentle agitation at 37° C. Tendons were then dissected from the calcaneus and digested in a Proteinase K solution overnight at 60°C prior to quantifying GAG content using the 1,9-dimethylmethylene (DMMB) blue assay and CS as standard (n=6/group). Absorbance was read at 525 and 595 nm.[10] Mechanics and Collagen Fiber Realignment: Following digestion, cross-sectional areas (CSAs) were measured using a custom laser device. Verhoeff stain lines were applied to denote the tendon insertion (0-1 mm) and midsubstance (1-3 mm) for regional, optical strain analysis. Tendons were mounted on a loading device (4 mm gauge length) with the calcaneus fixed and the proximal tendon clamped in a sandpaper grip in a 37° C 1X PBS bath. Our protocol consisted of 10 preconditioning cycles followed by stress relaxations at 1.5% and 3% strain. Following a 10-minute hold, tendons were subjected to frequency sweeps at 0.1, 1, 5, and 10 Hz, followed by a 5-minute rest period and a ramp-to-failure at 0.1%/s (n=8-10/group). Elastic (stiffness, regional modulus) and viscoelastic properties (dynamic modulus, phase shift, and percent relaxation) were measured from the test. Fiber realignment during the ramp-to-failure in the insertion and midsubstance regions were measured using reflectance mode quantified polarized light imaging.[11] Statistics: Comparisons across age and digestion groups were conducted using two-way ANOVAs followed by Bonferroni post-hoc tests. Significance was set at $p \leq 0.05$.

RESULTS: GAG content in the Achilles tendon decreased throughout aging ($p = 0.001$) and was reduced by 50% ($p < 0.001$) following cABC treatment (**Fig. 1a**). Tendon cross-sectional area increased with age ($p = 0.01$) but was unaffected by cABC treatment (**Fig. 1b**). There were no changes in elastic mechanical properties of the Achilles tendon following cABC treatment (**Fig. 1c-e**). We did not detect any change in viscoelastic mechanical properties following cABC treatment in any age group (results for 3% strain and 1 Hz shown, **Fig. 1f-h**). cABC treatment had no effect on fiber realignment independent of strain in P150 mice in the insertion and midsubstance regions (**Fig. 1j-k**). Fiber realignment patterns were similar in P300 and P570 mice with the exception of a higher variance in angle of polarization (AoP) in the P300 insertion group at 50% of the max grip strain ($p = 0.03$, data not shown).

DISCUSSION: GAG content in the Achilles tendon decreases similarly across age as in cartilage and meniscus,[3,4] yet the role of GAGs in aging tendon mechanics in this study is minimal. We did not detect an effect of GAG depletion on elastic mechanical properties, as reported in prior studies.[12,13] Contrary to our hypothesis, GAG digestion did not alter the viscoelastic mechanical properties of the Achilles tendon in any age group, including stress relaxation, which has been reported in tendon fascicles.[9] Prior studies have shown that GAGs promote collagen fiber realignment in cartilage under tension.[14] However, a higher-than 50% digestion yield may be necessary to uncover any changes in fiber realignment and resulting changes in mechanical properties following cABC treatment in tendon, where there are fewer GAGs than in cartilage and meniscus. Future work will extend our measurements of fibril kinetics using atomic force microscopy and investigate the mechanical role GAGs serve in tendon overuse and injury.

SIGNIFICANCE: GAGs are a minor constituent in tendon and are found in abundance in tendinopathy and following injury. Understanding the role of GAGs in aging tendon structure-function is critical for developing therapies to restore tendon function.

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ACKNOWLEDGEMENTS: Penn Center for Musculoskeletal Disorders (NIH/NIAMS P30AR069619).

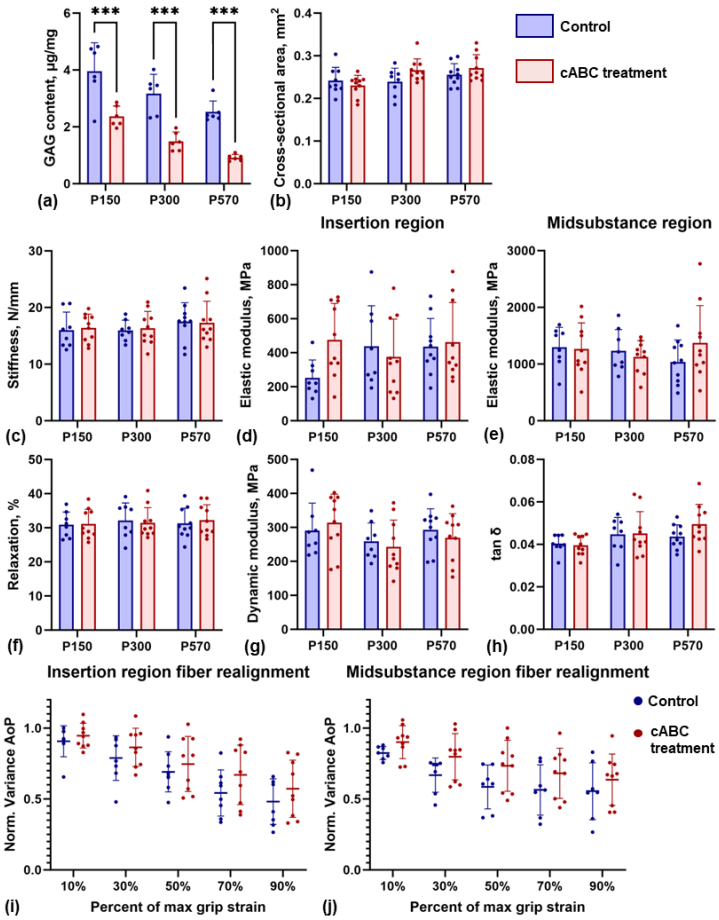

Figure 1: (a) cABC treatment reduced GAGs in the Achilles tendon, which decreased similarly across age. **(b)** CSA was unaffected by cABC treatment. **(c-e)** Elastic properties were unaffected by cABC treatment in all age groups. **(f-h)** Viscoelastic properties were unaffected by cABC treatment (properties at 3% strain and 1 Hz shown) in all age groups. **(i-j)** cABC treatment had no effect on fiber realignment independent of strain (P150 results shown) determined by variance in angle of polarization (AoP) (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Scleraxis-Targeted Collagen XII Knockout Alters Mouse Achilles Tendon Structure, Function and Gene Expression during Postnatal Development

Michael S. DiStefano, Srish S. Chenna, Louis J. Soslowsky
McKay Orthopaedic Research Laboratory, University of Pennsylvania, Philadelphia, PA
micdis@seas.upenn.edu

Disclosures: None

INTRODUCTION: Collagen XII is a Fibril-Associated Collagen with Interrupted Triple Helices (FACIT) primarily expressed during tendon growth and development [1]. Mutations in the *Col12a1* gene result in myopathic Ehlers-Danlos syndrome (mEDS), a connective tissue disorder in which patients exhibit weakness at birth, absence of deep tendon reflexes, and distal joint hypermobility and contracture [2]. We demonstrated that mature tendon-targeted knockout Achilles tendons (ATs) exhibited reduced elastic and viscoelastic mechanical properties and collagen fiber realignment relative to wild type control tendons [3]. Despite these differences in mature tendons, the roles of collagen XII on tendon structure, function, and gene expression throughout postnatal AT development remain unknown. Therefore, the objective of this study was to elucidate the roles of collagen XII on AT structure, mechanical function, and gene expression in developing postnatal mice. We hypothesized that tendon-targeted knockout of collagen XII would disrupt tendon homeostasis, leading to altered AT mechanics, morphology, and gene expression, with more notable differences at later postnatal developmental timepoints when ECM establishment and maturation are occurring.

METHODS: Animals: Male and female postnatal day (P) 10, 30 and 60 tendon-targeted collagen XII knockout (ScxCre;*Col12a1*$^{f/f}$; KO) and wild type Cre- littermate control mice (WT) were used (IACUC approved). Tendon Mechanics (n=10/group): ATs were measured with a laser displacement sensor to quantify cross-sectional area (CSA) and subjected to our mechanical testing protocol [3]. Tendon Morphology (n=5/group): Whole ankle and knee joints were fixed, decalcified, paraffin embedded, sectioned in the sagittal plane, and stained with toluidine blue to assess morphology and measure tendon length. Gene Expression (n=9-10/group): AT RNA was isolated and converted to cDNA, pre-amplified and loaded into Standard BioTools 96.96 Dynamic Array. Target genes included those of collagens, non-collagenous matrix, remodeling, cell-ECM, and cell-cell markers. Statistics: At each age, WT and KO data were compared using two-tailed t-tests with significance at $p \leq 0.05$ and trends at $p \leq 0.1$.

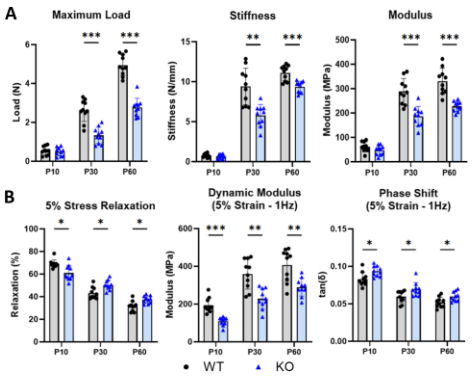

Figure 1. (A) KO tendons demonstrated reduced elastic mechanical properties at P30 and P60 and altered (B) viscoelastic mechanical properties across all ages. Data as mean ± standard deviation (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

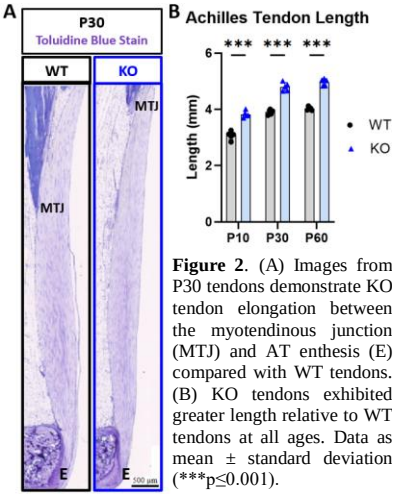

Figure 2. (A) Images from P30 tendons demonstrate KO tendon elongation between the myotendinous junction (MTJ) and AT enthesis (E) compared with WT tendons. (B) KO tendons exhibited greater length relative to WT tendons at all ages. Data as mean ± standard deviation (***$p \leq 0.001$).

RESULTS: Tendon Mechanics: CSA was not different across genotypes (data not shown). Elastic mechanical properties, maximum load, stiffness, and modulus (**Fig. 1A**) were reduced in KO tendons at P30 and P60. Percent relaxation was decreased in KO tendons at P10 and increased at P30 and P60 across all strain levels (5% strain shown in **Fig. 1B**). At all ages, dynamic modulus was decreased in KO tendons, while phase shift was increased in KO tendons across strain levels and frequencies (5% strain at 1Hz shown in **Fig. 1B**). Tendon Morphology: KO tendons exhibited elongation between the myotendinous junction (MTJ) and enthesis (E) relative to WT tendons at all ages (P30 shown **Fig. 2A**). Further, ATs in KO mice were longer than WT at all ages (**Fig. 2B**). Tendon Gene Expression: As expected, *Col12a1* expression was reduced in KO tendons (**Fig. 3A**). Principal Component Analysis (PCA) revealed clustering by genotype at P30 and P60 (P60 shown in **Fig. 3B**). Additionally, the volcano and deltaCt (dCt) plots at P60 highlight the magnitude of gene expression differences, with *Col12a1*, *Gja1* (Connexin43 gene), *Gdf5*, and *Sparc* downregulated and *Postn*, *Thbs4*, and *Mstn* upregulated in KO tendons relative to WT tendons (**Figs. 3C-D**).

DISCUSSION: This study highlights the regulatory roles of collagen XII in AT structure, mechanical properties, and gene expression. Consistent with our hypothesis, KO tendons demonstrated more notable alterations in elastic and viscoelastic mechanics, tendon morphology, and gene expression at later postnatal timepoints. Sustained increased length and decreased mechanics at later ages could be related to impaired development of the calcaneal enthesis of the AT, where the absence of an anchoring point disrupts mechanical cues critical for proper tendon development [4]. We observed decreased expression of *Gja1* and *Gdf5* in KO tendons at P60. Prior studies showed that *Gja1* and *Gdf5* are critical for regulating tendon mechanics, enthesis formation, and response to loading [4,5]. Additionally, work in osteoblasts demonstrated that absence of collagen XII resulted in decreased *Gja1* expression [6], further supporting our *Gja1* findings from P60 KO tendons. These results suggest that the absence of *Col12a1* may have a direct effect on normal AT and enthesis development. We also observed reduced *Sparc* expression in KO tendons at P60. Prior studies demonstrated load-induced regulation of AT maturation, homeostasis, and enthesis development by *Sparc* during postnatal development [7,8]. We previously showed that KO mice have reduced grip strength, indicating muscle weakness [1], which may be further supported by upregulated *Mstn*, a negative regulator of muscle mass [9], expression in the P60 KO tendons of this study. Given these results, future studies will identify the mechanisms involved with altered mechanical loading and absence of collagen XII on development of AT structure and function, particularly at its enthesis.

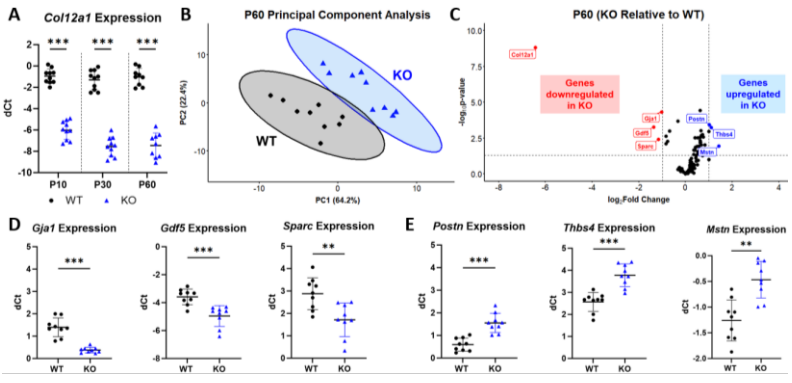

Figure 3. (A) *Col12a1* expression is reduced in KO mice at all ages. (B) PCA demonstrated clustering based on genotype at P60. (C) Volcano plot shows differential gene expression at P60, with (D) downregulated and (E) upregulated genes expressed in KO tendons relative to WT tendons. Data as mean ± standard deviation (**$p \leq 0.01$, ***$p \leq 0.001$).

SIGNIFICANCE/CLINICAL RELEVANCE: This study elucidates the critical role of collagen XII in regulating AT structure, function and gene expression during postnatal development which will inform the pathogenesis of mEDS in tendon and potentially inform future treatments.

ACKNOWLEDGEMENTS: Study supported by NIH/NIAMS F31AR083255, NIH/NIAMS R01AR078790 and NIH/NIAMS P30AR069619.

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The Temporal Role of Collagen XII in Mouse Tendon Healing

Jacob C. Nixon[1], Stephanie N. Weiss[1], Nathaniel A. Dyment[1], Louis J. Soslowsky[1]
[1]McKay Orthopaedic Research Laboratory, University of Pennsylvania, Philadelphia, PA
jacobnix@seas.upenn.edu

Disclosures: Jacob C. Nixon (N), Stephanie N. Weiss (N), Nathaniel A. Dyment (N), Louis J. Soslowsky (N)

INTRODUCTION: Collagen XII is a non-fibrillar collagen involved in cell and matrix interactions [1]. Mutations of the *Col12a1* gene are clinically associated with myopathic Ehlers-Danlos syndrome (mEDS), a congenital disorder characterized by symptoms such as distal joint hypermobility, an absence of deep tendon reflexes, and abnormal wound healing [2] – implicating a role for collagen XII in the response to tendon injury. Our previous work has shown that the patellar tendons (PTs) of tendon-targeted *Col12a1* knockout mice have increased matrix disorganization and altered cell morphology during early postnatal growth and reduced modulus and fiber re-alignment near maturity [3], indicating that collagen XII plays a critical role in cell and extracellular matrix (ECM) organization and, thus, the proper structure and function of tendons. However, the role of collagen XII in re-establishing cell and ECM organization during tendon healing remains unknown. Therefore, the objective of this study was to determine the temporal roles of collagen XII in restoring the structure, function, and composition of the healing murine tendon. We hypothesized that collagen XII knockdown would result in disrupted structure and composition and in concomitantly reduced improvements in tendon mechanical properties during healing and that knockdown at time of injury would impair the tendon healing response more than knockdown 1-week post-injury.

METHODS: Male and female global inducible collagen XII knockdown mice (*Rosa-CreER^{T2};Col12a1$^{flox/flox}$*; KO) and *CreER^{T2}*- littermate control mice (Ctrl) were used (IACUC approved). At 90 days old, mice underwent bilateral patellar tendon injury surgery as described [4], and *Cre* excision of the conditional alleles was induced via 4 consecutive daily IP injections of tamoxifen beginning at 1 day pre-injury (TM0) or 6 days post-injury (TM7). TM0 mice were sacrificed at 1-, 2-, or 4-week(s) post-injury; TM7 mice were sacrificed at 2- or 4-weeks post-injury. An additional group of uninjured control mice received tamoxifen at either of the previously mentioned time points and were sacrificed at 110 days old. Mechanical testing: PTs (n=10-12/group) were prepared and evaluated mechanically using a viscoelastic testing protocol, as described [4]. Briefly, tendons were pre-conditioned; subjected to two stress relaxations at 1% and 4% nominal strain, each followed by a dynamic frequency sweep at 0.1, 1, 5, and 10 Hz; and then loaded in a ramp to failure at 0.1%/s nominal strain rate. Transmission electron microscopy (TEM): PTs (n=4-5/group) were prepared and imaged as described [4]. The injured tissue was uniformly sampled to measure fibril diameter distribution. Gene expression: RNA was extracted from whole PTs (n=6/group), converted to cDNA, pre-amplified, and submitted to a Fluidigm Dynamic Array IFC against a panel of 93 genes (plus housekeepers *Abl1* and *Rps17*). Statistical treatment: Outliers were removed before performing analyses. For mechanical parameters, two-factor ANOVAs by genotype and healing time point with post-hoc Tukey's HSD for each induction time point were conducted. For TEM distributions, Kolmogorov-Smirnov tests by genotype for each healing time point and induction time point were conducted. For gene expression, t-tests by genotype for each healing time point and induction time point were conducted. Significance was set at $p < 0.05$.

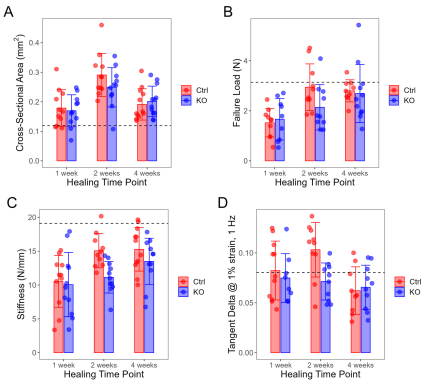

Figure 1. Knockdown of collagen XII had no effect on tendon (A) cross-sectional area, (B) failure load, (C) stiffness, or (D) tangent delta at 1% strain, 1 Hz at any healing or induction time point. Data represented as mean ± standard deviation. The average of the uninjured group is plotted as a dashed line.

RESULTS: Mechanical testing: No differences were detected between genotypes for any mechanical properties at any healing or induction time point (**Fig 1**). TEM: Knockdown resulted in an initial minor shift toward smaller fibril diameters at 2-weeks post-injury (**Fig 2A**) and a later minor shift toward larger fibril diameters at 4-weeks post-injury (**Fig 2B**). This finding was present for both induction time points. Gene expression: Tamoxifen injection resulted in an expected downregulation of *Col12a1* expression for all healing time points and induction time points. For TM0, *Acan* was downregulated due to knockdown at 2-weeks post-injury (**Fig 3A**). For TM7, *Mmp9*, *Fbn2*, *Spp1*, and *Mki67* were downregulated and *Ctgf* and *Serpine1* were upregulated due to knockdown at 2-weeks post-injury (**Fig 3B**); *Has1* was downregulated and *Col2a1* was upregulated due to knockdown at 4 weeks post-injury (data not shown).

DISCUSSION: This study investigated the temporal role of collagen XII in tendon structure, function, and composition following injury using conditional deletion both at the time of injury and at 1-week post-injury. Surprisingly, knockdown of collagen XII had no effect on the mechanical properties of injured PTs and had minimal effects on the expression of genes related to tendon healing, consistent with the effect of collagen XII knockdown on the mechanical properties and gene expression of skin granulation tissue [5]. Knockdown of collagen XII did, however, alter tendon structure during healing, resulting in an early shift to smaller fibril diameters and a later shift to larger fibril diameters. While our previous work showed a critical role of collagen XII in regulating cell arrangement rather than initial ECM assembly during tendon development [3], surprisingly, this study suggests that collagen XII may not play a similar role during tendon healing. Furthermore, our induction time points were chosen to isolate the role of collagen XII in the proliferation and arrangement of peritenon-derived stem cells during the first week post-injury. Thus, the lack of differences between induction time points indicates that collagen XII may instead play a more substantial role in ECM deposition, fibril growth dynamics, and subsequent remodeling during the later phases of healing. Further studies will assess the role of collagen XII in cell and ECM organization during tendon healing using histological methods.

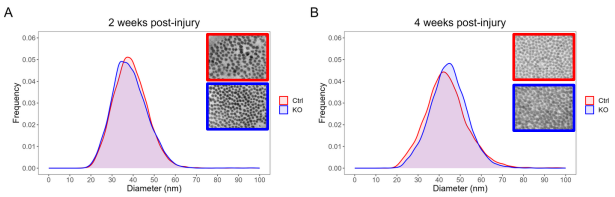

Figure 2. (A) Knockdown of collagen XII resulted in slightly decreased fibril diameters at 2-weeks post-injury. (B) Knockdown of collagen XII resulted in slightly increased fibril diameters at 4-weeks post-injury. Only TM0 data is shown. Representative images are inlaid at the top right.

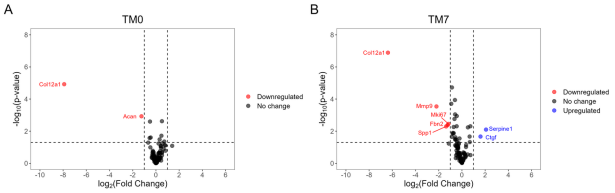

Figure 3. Knockdown of collagen XII had minimal effects on tendon gene expression at 2-weeks post-injury (A) for TM0 and (B) for TM7. Genes that were significantly ($p < 0.05$) upregulated or downregulated compared to littermate controls are labelled.

SIGNIFICANCE: Collagen XII does not regulate tendon mechanical function and composition but does alter tendon structure during tendon healing, suggesting that collagen XII may have minimal importance in re-establishing tendon structure-function at this age following injury.

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ACKNOWLEDGMENTS: The authors acknowledge the assistance of Courtney Nuss in sample preparation for TEM. This study was supported by NIH/NIAMS R01AR078790 and T32AR007132 and the Penn Center for Musculoskeletal Disorders (P30AR069619).

Reduction of Type III Collagen During Healing Worsens Mechanical Properties in Aged Mouse Tendons

Margaret K. Tamburro,[1] Jaclyn A. Carlson,[1,2] Stephanie N. Weiss,[1] Jeremy D. Eekhoff,[1] William Yen,[2] Susan W. Volk,[2] Louis J. Soslowsky[1]
[1]McKay Orthopaedic Research Laboratory, University of Pennsylvania, Philadelphia, PA
[2]School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA
Margaret.Tamburro@pennmedicine.upenn.edu

Disclosures: Margaret K. Tamburro (N), Jaclyn A. Carlson (N), Stephanie N. Weiss (N), Jeremy D. Eekhoff (N), William Yen (N), Susan W. Volk (N), Louis J. Soslowsky (N)

INTRODUCTION: The incidence of tendon injury increases with age,[1] and aged tendons heal poorly.[2] Many age-related tendon changes have been described, including dramatic reduction of expression of the gene encoding type III collagen (Col3), *Col3a1*.[3] After injury, Col3 comprises a substantial component of the provisional healing matrix and contributes to regulation of matrix and cell behavior.[4] Despite its expression in tendon being commonly associated with poor tendon healing, the age-specific role of Col3 in tendon healing remains unclear. Therefore, the objective of this study was to elucidate the mechanical impacts of reduced *Col3a1* expression during healing in aged tendons. We hypothesized that reduced *Col3a1* expression would diminish formation of scar tissue and exacerbate age-related healing deficits, yielding mechanically inferior tendons.

METHODS: Wildtype (Rosa-CreER^{T2}; *Col3a1*$^{+/+}$) and Col3 knockdown (Rosa-CreER^{T2}; *Col3a1*$^{F/F}$) male mice received tamoxifen injection at postnatal day 364 (p364) followed by patellar tendon biopsy punch injury[5] at p365 (IACUC approved). Effective Col3 knockdown was previously confirmed by qPCR.[6] Left patellar tendons were harvested at 6-weeks post-injury, and patella-patellar tendon-tibia complexes were dissected and prepared[7] for mechanical testing ($n \geq 7$/group). Patellar tendon mechanics were quantified with viscoelastic tensile testing (nominal gauge length = 3 mm) including 1) preconditioning, 2) stress relaxation at 2% and 4% strain with a subsequent sinusoidal frequency sweep (10 cycles at 0.1, 1, 5, and 10 Hz) at each strain level, and 3) quasistatic ramp to failure (0.1% strain/s). Reflectance-mode quantitative polarized light imaging at 2 Hz captured the light reflected off the tendon midsubstance in real time during the ramp to determine average degree of linear polarization (DoLP), indicative of collagen fiber alignment, and variance of the angle of polarization (AoP), indicative of collagen fiber orientation.[8] Genotype-based differences were assessed with Student's t-tests (mechanical properties) or 2-way ANOVAs (normalized average DoLP and variance of the AoP at 10, 25, 50, 75, and 100% strain; genotype, normalized strain level). Significance was set at $p \leq 0.05$.

RESULTS: Tendons with Col3 knockdown were structurally different than wildtype tendons. Knockdown tendons showed a trending increase in cross-sectional area and a significant decrease in gauge length (Fig. 1A-B). Percent relaxation at both 2% and 4% strain was not affected by Col3 knockdown (Fig. 1C, F). At 2% strain, Col3 knockdown did not affect dynamic modulus or phase shift (Fig. 1D-E). At 4% strain, knockdown reduced dynamic modulus without affecting phase shift, at all frequencies (Fig. 1G-H). Col3 knockdown did not affect stiffness, but modulus was lower in the knockdown group (Fig. 1I-J). Consistent with expected fiber uncrimping and realignment, average DoLP increased and variance of AoP decreased with increasing strain, but knockdown did not affect strain-dependent changes in either property (Fig. 2A-B).

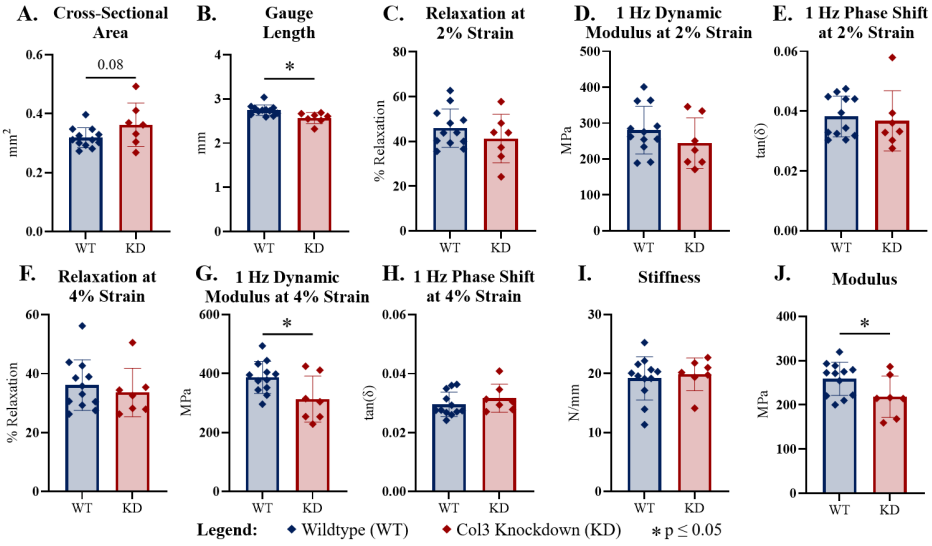

Figure 1. Col3 knockdown worsens tendon mechanics after injury. Knockdown tendons had a trend toward increased (A) cross-sectional area and had decreased (B) gauge length. At 2% strain, (C) percent relaxation, (D) dynamic modulus, and (E) phase shift were unaffected by knockdown. At 4% strain, (F) percent relaxation and (H) phase shift were unaffected while (G) dynamic modulus was reduced. Dynamic data is shown for the 1 Hz frequency (trends were consistent across frequencies). (I) Stiffness was unaffected by Col3 knockdown while (J) modulus was reduced.

DISCUSSION: This study investigated the mechanical impacts of reduced Col3 during tendon healing in aged male mice. As hypothesized, reduction of Col3 during tendon healing led to reduced mechanical properties. Interestingly, Col3 knockdown resulted in a shorter and possibly larger healed tendon. Because Col3 is the primary structural component of the provisional healing matrix, this finding is surprising and may indicate compensatory production of non-Col3 matrix in Col3 knockdown tendons. Material properties of knockdown tendons were inferior to wildtype. The possible increase in cross-sectional area in knockdown tendons may have compensated for poor material properties, yielding a comparable tendon stiffness. Because we only observed an effect of Col3 reduction on dynamic modulus at 4% strain, Col3 may be an important contributor to dynamic tendon mechanics at higher strains. Based on the similarities in collagen realignment between wildtype and knockdown groups, Col3 may not be an influential contributor to dynamic collagen realignment in healing tendon. In summary, these results highlight an important role for Col3 in promoting mechanical integrity after injury in aged tendons. Results are consistent with studies in other tissues that demonstrate pathologic changes with age in male mice with reduced Col3.[9] To fully understand contributions of Col3 to aged tendon healing, future studies will evaluate tendon structure and gene expression, include female mice, and investigate earlier healing time points.

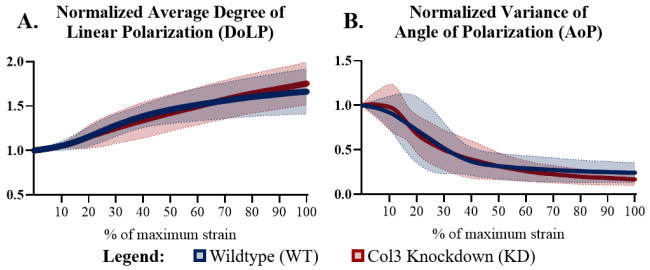

Figure 2. Col3 knockdown does not influence dynamic collagen realignment. (A) Average DoLP and (B) variance of AoP are unaffected by genotype across the strain profile. Data are presented normalized to the initial value as a percent of maximum strain. Initial values for average DoLP and variance of AoP were not different between groups.

SIGNIFICANCE: As therapeutic Col3 modulation through pharmacologic, biomaterial, and mRNA-based approaches becomes more feasible, understanding contributions of Col3 to tendon healing, particularly in aged tendons where injury is prevalent and devastating, is imperative for improving treatment strategies.

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Reduction of Type III Collagen During Healing has Minimal Impact on Young Adult Mouse Tendon Healing

Margaret K. Tamburro,[1] Jaclyn A. Carlson,[1,2] Emma E. Kroll, [1] Miranda K. Doro,[1] Stephanie N. Weiss,[1] Jeremy D. Eekhoff,[1] William Yen,[2] Louis J. Soslowsky[1], Susan W. Volk,[2]

[1]McKay Orthopaedic Research Laboratory, University of Pennsylvania, Philadelphia, PA

[2]School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA

Margaret.Tamburro@pennmedicine.upenn.edu

Disclosures: Margaret K. Tamburro (N), Jaclyn A. Carlson (N), Emma E. Kroll (N), Miranda K. Doro (N), Stephanie N. Weiss (N), Jeremy D. Eekhoff (N), William Yen (N), Louis J. Soslowsky (N), Susan W. Volk (N)

INTRODUCTION: Injured tendons heal poorly with fibrovascular scar, leading to functional deficits and high reinjury risk. As in skin, increased type III collagen (Col3) is a hallmark of the healing tendon matrix.[1] In healing skin, Col3 regulates cell activities and fate in the early healing niche and is a suppressor of scar formation.[2] However, the role of Col3 in tendon healing remains unclear. Therefore, the objective of this study was to elucidate the regulatory role of Col3 throughout tendon healing in young adult mice by reducing *Col3a1* expression at the time of tendon injury. We hypothesized that Col3 knockdown at time of injury would limit cell recruitment and differentiation, leading to poor structural and functional healing outcomes.

METHODS: At postnatal day 89 (p89), wildtype (WT, Rosa-CreER^{T2}; *Col3a1*$^{+/+}$) and Col3 knockdown (KD, Rosa-CreER^{T2}; *Col3a1*$^{F/F}$) mice (mixed sex) received a tamoxifen injection (200 mg/kg). Injured groups underwent bilateral patellar tendon biopsy punch injury[3] at p90 (IACUC approved). Injured patellar tendons were harvested at 1-, 3-, and 6-weeks post-injury (wpi), and uninjured tendons were harvested at the middle (3-wpi) timepoint. Tendons were randomized to composition assessment with gene expression and immunohistochemistry (IHC), structure assessment with second harmonic generation (SHG) imaging and transmission electron microscopy (TEM), and function assessment with tensile testing. Gene expression (n ≥ 8/group): RNA was processed, and expression of 96 genes was quantified with a Fluidigm 96.96 Dynamic Array.[4] Tamoxifen-treated knockdown samples used for gene expression analysis were confirmed to have >50% reduction in *Col3a1* expression relative to uninjured wildtype tendon. IHC (n ≥ 4/group): Tendons were fixed, cryopreserved,[5] and sectioned along the longitudinal axis of the tendon (8 µm thickness). Sections were blocked, permeabilized, and stained for Col3 (Goat Anti-Type III Collagen-UNLB, SouthernBiotech) and nuclei (SYTOX^{TM} Green, Thermo). Col3 fluorescence for regions of interest containing healing matrix was quantified with Fiji.[6] SHG (n ≥ 5/group): Tendons were optically cleared[7] and imaged (SP8 Multiphoton Microscope, Leica) with SHG. To quantify matrix alignment, regions of interest containing healing matrix were analyzed with OrientationJ.[8] TEM (n ≥ 4/group): Tendons were fixed, prepared, sectioned, stained, and imaged (60,000x).[9] Fibril diameter distributions were quantified. Tensile Testing (n ≥ 16/group): Tendons were prepared[10] and tested with a viscoelastic protocol: 1) preconditioning, 2) stress relaxation at 2% and 4% strain with a subsequent sinusoidal frequency sweep (10 cycles at 0.1, 1, 5, and 10 Hz) at each strain level, and 3) quasistatic ramp to failure (0.1% strain/s) with image capture (2 Hz). After outliers were removed (ROUT method, Q = 1%), data were compared with 2-way ANOVAs (genotype, healing timepoint). Fibril diameter distributions were compared with Kolmogorov-Smirnov tests. Significance was set at $p \leq 0.05$.

RESULTS: Col3 knockdown was effectively induced, as evidenced by gene expression and IHC (Fig. 1A-C). Col3 increases after injury and remains elevated compared to uninjured baseline throughout the 6-week healing period. Despite effective knockdown, principal component analysis (PCA) of gene expression did not show separation between WT and KD groups at any timepoint (Fig. 1D). Eleven genes were differentially expressed between WT and KD groups overall; post hoc comparisons of genotype at each timepoint showed five genes were differentially expressed at 3-wpi (Fig. 1E). Unlike skin, fibrillar collagen alignment (Fig. 2A-B) was unaffected by loss of Col3 in healing tendon, but fibril diameter distribution showed a shift toward larger fibrils in the KD group at 3- and 6-wpi (Fig. 2C). Tendon mechanics were not affected by genotype at any healing timepoint in p90 mice (selected properties shown, Fig. 3A-C).

DISCUSSION: We investigated the compositional, structural, and functional impacts of lasting reduction of Col3 at the time of tendon injury on tendon healing. Contrary to our hypothesis, reduction of Col3 during tendon healing had a nuanced impact on tendon healing in these young mice. This challenges the existing paradigm that persistent Col3 in the healing matrix directly contributes to poor tendon healing outcomes. Our results suggest Col3-independent mechanisms can contribute to poor physiologic tendon healing. Assessment of the molecular composition of the healing matrix in KD tendons will provide additional insight into possible compensatory production of other matrix molecules to form a provisional healing matrix that supports reparative cell recruitment and differentiation. We will also consider possible sex-specific effects of Col3 reduction and effects of different timing of Col3 reduction on healing outcomes.

SIGNIFICANCE: Understanding regulators of poor tendon healing is critical for design and use of improved tendon injury prevention and treatment strategies.

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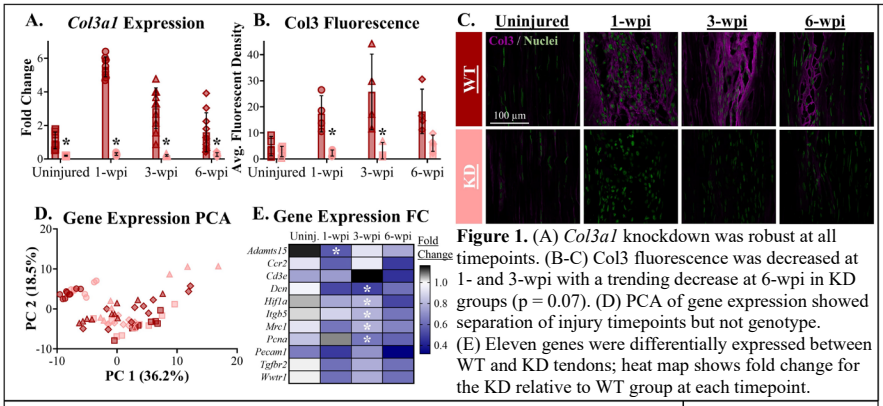

Figure 1. (A) *Col3a1* knockdown was robust at all timepoints. (B-C) Col3 fluorescence was decreased at 1- and 3-wpi with a trending decrease at 6-wpi in KD groups ($p = 0.07$). (D) PCA of gene expression showed separation of injury timepoints but not genotype. (E) Eleven genes were differentially expressed between WT and KD tendons; heat map shows fold change for the KD relative to WT group at each timepoint.

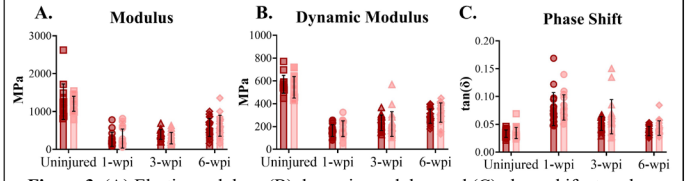

Figure 3. (A) Elastic modulus, (B) dynamic modulus, and (C) phase shift at each healing timepoint were unaffected by Col3 knockdown. Representative data are shown for 1 Hz at 4 % strain; results were similar at both strains and all frequencies.

Universal Legend:
- WT
- KD
- Uninjured
- 1-wpi
- 3-wpi
- 6-wpi
- * $p \leq 0.05$ relative to WT at same timepoint

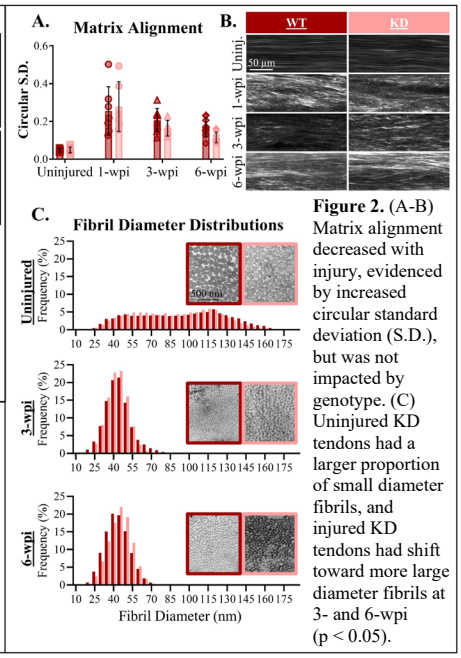

Figure 2. (A-B) Matrix alignment decreased with injury, evidenced by increased circular standard deviation (S.D.), but was not impacted by genotype. (C) Uninjured KD tendons had a larger proportion of small diameter fibrils, and injured KD tendons had shift toward more large diameter fibrils at 3- and 6-wpi ($p < 0.05$).

Tendon-Targeted Type III Collagen Reduction Impairs Development of Mouse Tendon Mechanical Properties

Margaret K. Tamburro,[1] Miranda K. Doro,[1] Stephanie N. Weiss,[1] Susan W. Volk,[2] Louis J. Soslowsky[1]
[1]McKay Orthopaedic Research Laboratory, University of Pennsylvania, Philadelphia, PA
[2]School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA
Margaret.Tamburro@pennmedicine.upenn.edu

Disclosures: Margaret K. Tamburro (N), Miranda K. Doro (N), Stephanie N. Weiss (N), Susan W. Volk (N), Louis J. Soslowsky (N)

INTRODUCTION: After injury, tendons heal poorly, failing to effectively and completely remodel the type III collagen (Col3)-rich fibrovascular scar into healthy tendon matrix. While embryonic[1] and neonatal[2] tendons are also Col3-rich, collagen fibrillogenesis and matrix assembly during development yield a highly-aligned, type I (Col1)-rich matrix with high tensile strength. Col3 is known to regulate matrix deposition and remodeling in other Col1-rich tissues including skin,[3] meniscus,[4] and bone,[5] but the role of Col3 in tendon development remains unknown. Therefore, the objective of this study was to define the role of Col3 in development of tendon matrix structure and function. We hypothesized that Col3 is a critical regulator of matrix formation such that tendons with reduced Col3 develop with compromised structure of the collagen-rich matrix and reduced tensile mechanical properties.

METHODS: Mice with constitutive, tendon-targeted *Col3a1* knockdown (Scx-Cre; *Col3a1*$^{F/F}$) were compared to Cre-negative littermate controls ($n \geq 10$/group mixed sex). Patellar tendons were harvested bilaterally at postnatal day 28 (p28). For structural evaluation, patellar tendons were assessed with second harmonic generation (SHG) imaging for collagen density and matrix alignment or transmission electron microscopy (TEM) for fibril diameter distribution. For SHG ($n \geq 4$/group), tendons were optically cleared,[6] and the tendon midsubstance was imaged (SP8 Multiphoton Microscope, Leica). Collagen density was quantified as the average of the intensity of the forward and backward channels, and matrix alignment was quantified with circular standard deviation using Orientation J.[7] For TEM ($n \geq 6$/group), prepared tendons were sectioned, stained, and imaged.[8] Fibril diameter distribution was quantified for the tendon midsubstance. For mechanical assessment ($n = 10$/group), patella-patellar tendon-tibia complexes were dissected, tendon cross-sectional area was measured,[9] stain lines were applied for optical tracking, tibias were potted, and patellas were gripped with custom fixtures. The viscoelastic tensile testing protocol included 1) preconditioning, 2) stress relaxation at 2% strain with a subsequent sinusoidal frequency sweep (10 cycles at 0.1, 1, 5, and 10 Hz), and 3) quasistatic ramp to failure (0.1% strain/s, imaging at 2 Hz). After removal of outliers (ROUT method, Q = 1%), matrix and mechanical properties were compared with two-tailed, Student's t-tests. Fibril diameter distributions were compared with a Kolmogorov-Smirnov test. Significance was set at $p \leq 0.05$.

RESULTS: Constitutive, tendon-targeted knockdown of Col3 resulted in nuanced alterations in structure of the tendon matrix. Knockdown did not affect collagen density or matrix alignment of the tendon midsubstance (Fig. 1A-C). However, Col3 knockdown altered collagen fibril diameter distribution in the tendon midsubstance whereby knockdown tendons had increased frequency of large diameter fibrils ($p < 0.05$, Fig. 1D-E). Wildtype and knockdown tendons had comparable cross-sectional areas (Fig. 2A). Knockdown tendons demonstrated decreases in several mechanical properties, including reduced stiffness (Fig. 2B). Despite having similar moduli in the tendon midsubstance (Fig. 2C), defined as the central 1 mm along the length of the tendon, knockdown tendons had reduced modulus at the tibial insertion (Fig. 2D), defined as the distal 1 mm of the tendon, compared to wildtype tendons. Knockdown tendons also had increased relaxation at 2% strain (Fig. 2E), but dynamic modulus and phase shift were unaffected by knockdown (Fig. 2F-G) at all frequencies.

DISCUSSION: We studied the impact of Col3 reduction during tendon development on structural and functional outcomes. Despite the well-established role of Col3 in regulating developmental processes[10-11] as well as matrix density and alignment[3-5] in other tissues, tendon-targeted Col3 knockdown had a modest influence on development of the tendon midsubstance. Observed changes in fibril diameter distribution are consistent with the established role of Col3 in regulating Col1 fibril diameters in skin,[12] but these changes were not mechanically consequential in the tendon midsubstance. Interestingly, Col3 reduction had a detrimental impact on development of the tendon insertion, causing reduced modulus. While type II collagen is classically associated with the enthesis, we previously demonstrated increased Col3 content near the tendon insertion,[13] and enthesis progenitor cells have enriched expression of *Col3a1*.[14] Thus, Col3 may be critical for physiologic development of the tendon insertion. We will explore this further by investigating morphological and gene expression changes in the Col3-deficient tendon insertion. We will also evaluate earlier timepoints to better understand the temporal role of Col3 in tendon development.

SIGNIFICANCE: Efforts to mimic tendon development for experimental and therapeutic purposes rely on an improved understanding of in vivo developmental processes. Understanding contributions of Col3 to tendon development can inform strategies to improve functional tendon formation after injury.

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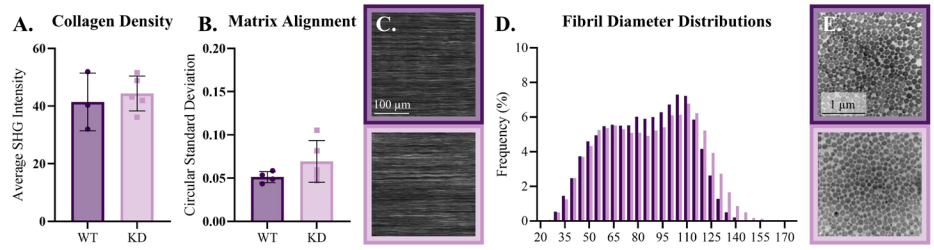

Figure 1. Col3 knockdown affected fibril diameter distribution. Knockdown did not affect (A) collagen density or (B) matrix alignment (representative images shown in C). (D) Reduction of Col3 resulted in a shift toward more large diameter (> 110 nm) fibrils (representative images shown in E).

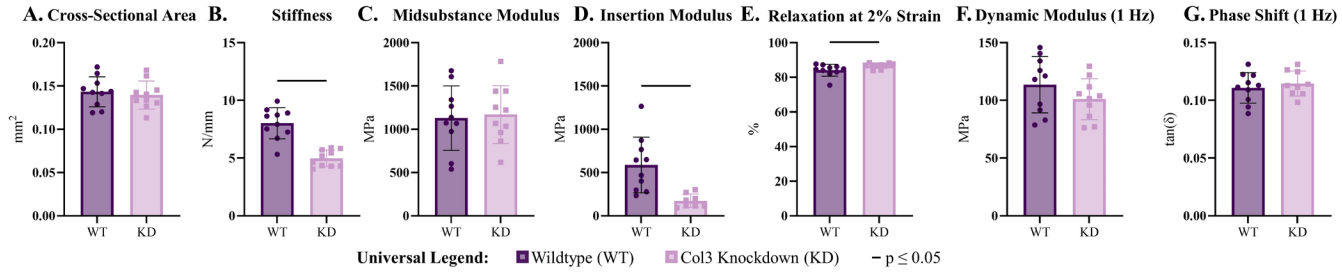

Figure 2. Col3 knockdown reduced stiffness and insertion modulus and increased relaxation. Col3 reduction did not influence (A) tendon cross-sectional area but did reduce (B) tendon stiffness. While (C) midsubstance modulus was unaffected by knockdown, (D) insertion modulus was dramatically reduced. (E) Relaxation at 2% strain was increased in knockdown tendons. (F) Dynamic modulus and (G) phase shift were unaffected by Col3 knockdown. Representative data is shown for the 1 Hz frequency; trends were consistent across frequencies.

Coordination of Collagens V and XI Guide Fibril Assembly during Mouse Patellar Tendon Postnatal Development

Nat A. Thurlow, Jeremy D. Eekhoff, Meera Ratnagiri, Courtney A. Nuss, Stephanie N. Weiss, Nathaniel A. Dyment, Louis J. Soslowsky
McKay Orthopaedic Research Laboratory, University of Pennsylvania, Philadelphia, PA
nthurlow@seas.upenn.edu

Disclosures: None

INTRODUCTION: Collagens V and XI are minor fibril-forming collagens that assemble with major collagens, such as collagens I and II, to facilitate fibrillogenesis. Interestingly, collagens V and XI have related roles in fibril assembly including fibril nucleation, organization, lateral growth, and fusion [1]. In tendons, lack of *Col5a1* expression leads to larger diameter fibrils, smaller tendon cross-sectional area, and reduced mechanical properties [2]. Lack of *Col11a1* expression leads to tendons with disrupted fibril organization, more fibrils with small diameters, and decreased elastic properties [3]. Collagens V and XI also have similar structures, can assemble together in triple helices, and have comparable gene expression levels in tendon until postnatal day 30 (p30) [1]. Together, this suggests that a potential interaction between these two collagen types during early development is critical for proper fibril growth and mechanical response. However, the structural and functional outcomes due to cooperation between collagens V and XI in tendon are unclear. The objective of this work is to assess the impact of cooperation between collagens V and XI during early development by comparing tendons with tendon-specific knockdown of *Col5a1* and *Col11a1* (VHet-XIHet and VKO-XIHet) to tendons with altered expression of *Col5a1* only (VHet and VKO). We hypothesized that tendons with compound deficiencies would have more severe dysregulation of fibril growth, altered tendon structure, and mechanical deficits compared to their counterparts lacking only *Col5a1*.

METHODS: Animals: Male and female p30 mice with tendon specific (ScxCre) alterations in *Col5a1* and/or *Col11a1* expression were collected (VHet, VHet-XIHet, VKO, and VKO-XIHet; n=12/genotype) and ScxCre- littermates served as controls (Ctrl, n=12; IACUC approved). Mechanics: Patella-patellar tendon-tibia complexes were finely dissected from hindlimbs, and cross-sectional area was measured using a custom laser device. The patella was secured in neoprene with cyanoacrylate glue, and the tibia was potted in polymethyl methacrylate. Tendons were tested with a viscoelastic protocol consisting of preloading to 0.05N, preconditioning for 10 cycles, stress relaxations at 2% and 4% strain followed by a frequency sweep of 10 cycles at 0.1, 1, 5, and 10 Hz, and quasistatic ramp-to-failure at 0.1% strain/sec. VKO-XIHet patellar tendons (PTs) only underwent preconditioning and ramp-to-failure due to tissue fragility. Transmission Electron Microscopy: PTs (n=4/genotype) were isolated, fixed, embedded, sectioned, stained, and imaged as described [4]. Fibril diameters were measured in MATLAB (n=10 images/PT). Tendon Morphology: Knees were fixed, cryoembedded, sectioned at 8 µm, and stained with toluidine blue to visualize morphology. Statistics: Mechanical properties were compared using ANOVA followed by Bonferroni corrected t-tests when appropriate ($p \leq 0.05$).

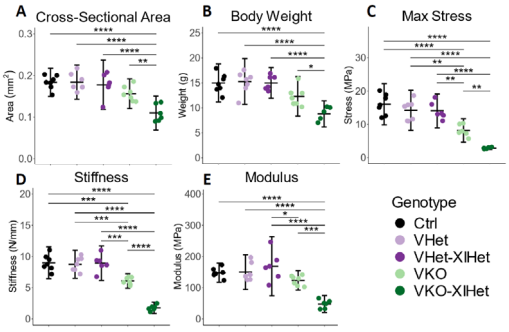

Figure 1. VKO-XIHet mice had (A) reduced tendon CSA and (B) body weight. (C) Maximum stress and (D) stiffness were lower in the VKO compared to VHet. Maximum stress, stiffness, and (E) modulus were lower in VKO-XIHet compared to VKO (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.001$).

RESULTS: Mechanical changes due to the addition of *Col11a1* knockdown were larger than *Col5a1* knockout alone: VKO-XIHets had smaller PT cross-sectional areas (CSA) (Figure 1A) and lower body weight (Figure 1B) than the other four genotypes. There were no differences detected in maximum stress, stiffness, or modulus in Ctrl, VHet, and VHet-XIHet tendons (Figure 1C-E). VKO tendons had reduced maximum stress and stiffness compared to Ctrl and VHet (Figure 1C-D). Compared to VKO tendons, VKO-XIHet had dramatically lower maximum stress, stiffness, and modulus. Viscoelastic properties were similar between genotypes (data not shown). VHet-XIHet and VKO-XIHet had populations of larger fibrils: Ctrl, VHet, and VKO fibril diameters were similar with only small deviations (~5nm) in Q1, median, and Q3 values (Figure 2A-B). Conversely, the VHet-XIHet distribution had a large population of larger fibrils with a shift of 23.4nm in median diameter compared to VHet fibrils (Figure 2C, F, G, J). Similarly, the VKO-XIHet fibril sizes shifted toward larger fibrils with a shift of 18.6nm in median diameter and an increase in 22.6nm in the interquartile range (Figure 2D, H-J). Tendon morphology was altered in VKO-XIHet PTs: VKO and VKO-XIHets had visibly smaller anterior-posterior thickness which approximated the differences in CSA measured during mechanical testing (Figure 3). VKO-XIHet PTs also appeared shorter than the other four genotypes and had abnormal morphology at the tibial insertion.

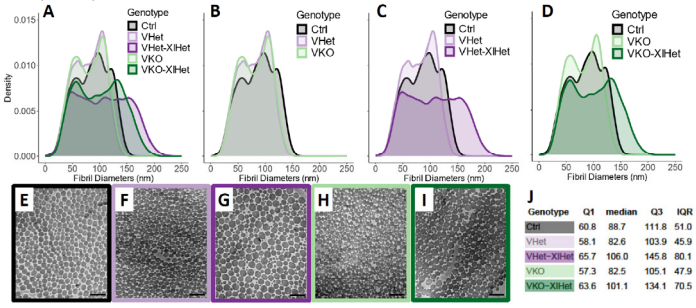

Figure 2. Fibril diameter distributions of (A) all genotypes. (B) *Col5a1* knockdown had little effect on fibril diameters, but (C, D) the addition of *Col11a1* knockdown caused larger fibril diameters. These changes are reflected in (E-I) representative images (scale bar = 400nm) and (J) a numerical summary.

DISCUSSION: We demonstrated that VKO-XIHet PTs have considerable deficits in mechanical properties and dysregulation of fibril assembly, while the effects of collagen V alone are comparatively minor. During the first 30 days of postnatal growth, tendon fibrils are organized and undergo a phase of lateral growth. Both collagen V and XI have N-terminal regulatory domains that extend to the fibril surface [3] and have been hypothesized to regulate fibril growth and assembly. Fibril diameters in p30 PTs were largely unchanged due to *Col5a1* knockout; however, the addition of *Col11a1* knockdown to *Col5a1* knockout led to dramatic fibril growth beyond 150nm suggesting that collagen V/XI interaction is a critical regulator of fibril growth. Interestingly, the increases in fibril diameters in the VHet-XIHet and VKO-XIHet tendons are coupled with unchanged and decreased overall cross-sectional areas, respectively. In previous work, tendon-targeted knockdown of a single copy of *Col11a1* (XIHet) in PTs caused no differences in maximum stress, stiffness, or modulus by p30 [5], while knockout of both copies (XIKO) resulted in significant decreases in the same properties. In this study, the mechanical property changes due to *Col5a1* knockout alone were minor, but the addition of *Col11a1* knockdown caused drastic decreases in mechanical properties. Together, these results indicate that there is a coordinated role between collagens V and XI during this phase of tendon growth, with collagen XI having a more prominent role. Given the abundance of collagen I in tendon, this finding is surprising given the canonical roles of collagen V and XI in regulating collagen I and II fibrillogenesis, respectively.

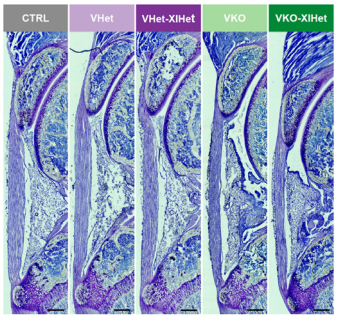

Figure 3. Tendon morphology is similar between Ctrl, VHet, and VHet-XIHet. VKO and VKO-XIHet PTs show a slight narrowing in anterior-posterior thickness. VKO-XIHet tendons also appear shorter and have abnormal bone morphology near the insertion (scale bar = 250µm).

SIGNIFICANCE: Collagen fibril organization and structure are vital to proper tendon function. Collagens V and XI have known roles in the regulation of fibrillogenesis, and their similar structure, expression levels, and roles suggest that coordination of these two collagen types occurs during tendon development. Defining these interactions is essential to understanding the mechanisms of collagen fibril formation and establishment of tendon structure.

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ACKNOWLEDGEMENTS: This study was supported by NIH/NIAMS (R01AR073231) and Penn Center for Musculoskeletal Disorders (P30AR069619).