Inducible Rosa, but not αSMA or Scx, Cre driven excision achieves substantial *Col12a1* knockdown in tendon healing

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INTRODUCTION: Tendon healing follows a typical wound healing process involving transient and heterogeneous cell populations. Collagen XII, a fibril-associated collagen, regulates tendon cell and matrix organization [1], and *Col12a1* expression increases post-injury. Tamoxifen-inducible Cre mouse models permit spatial and temporal knockdown, and these models are advantageous for investigating the specific role of collagen XII in *de novo* tissue formation following injury. However, these models may also target other intrinsic or extrinsic cell populations that do not contribute to the healing response, such as vascular cells and cells in the adjacent tissue, and the efficiency of different spatial Cre drivers for collagen XII knockdown are unknown. Therefore, the objective of this study was to evaluate the efficiency of *Col12a1* knockdown in the healing tissue versus native tendon using three tamoxifen-inducible Cre mouse models (1) Rosa-CreER^{T2} model to ubiquitously target cells contributing to the healing response; 2) αSMA-CreER^{T2} model to target peritenon-derived progenitor cells that infiltrate into the injury; and 3) Scx-CreER^{T2} to target tendon-derived cells) and two tamoxifen dosage protocols (short- and long-dose). We hypothesized that 1) the Rosa-CreER^{T2} model would result in the greatest knockdown independent of region, 2) the αSMA-CreER^{T2} model would exhibit increased knockdown in the healing tissue compared to the native tissue, and 3) the Scx-CreER^{T2} model would show greater knockdown in the healing tissue with the long-dose tamoxifen protocol.

METHODS: Male Rosa-CreER^{T2};*Col12a1*$^{flox/flox}$ (RosaKO), αSMA-CreER^{T2};*Col12a1*$^{flox/flox}$ (αSMAKO), and Scx-CreER^{T2};*Col12a1*$^{flox/flox}$ (ScxKO) mice with their respective Cre- littermate controls were used (n=8/group, IACUC approved). At 90 days old, mice underwent bilateral patellar tendon injury surgery as described [2], and Cre excision of the conditional alleles was induced via four IP injections of tamoxifen (100mg/kg body weight). The short-dose group received tamoxifen at days -1, 0, 1, and 2 days post-injury, and the long-dose group received tamoxifen at -3, 0, 3, and 6 days post-injury, where day 0 is the day of surgery. Mice were sacrificed two weeks later, and left knees were fixed for three hours in 4% paraformaldehyde prior to cryo-embedding. Injured patellar tendons were sectioned axially at a thickness of 40μm, and sections were microdissected using a 25G needle to ensure isolation of the healing tissue and the adjacent, native tendon struts. The tissue was digested, RNA was isolated as described [3], and qPCR was performed for *Col12a1* expression. ΔCt values were normalized to the housekeeper gene, *Abl1*. Statistics: Two-way ANOVAs for genotype and tamoxifen dosage protocol were conducted within each region, and paired t-tests were conducted to compare *Col12a1* expression in the native and healing regions. Significance was set at $p<0.05$.

RESULTS: Supporting our hypothesis, the RosaKO model demonstrated the highest knockdown compared to control regardless of region and tamoxifen dosage protocol with substantial decreases in *Col12a1* expression (~117-2,200-fold decrease, **Figure 1A**). The short-dose protocol in RosaKO mice also resulted in greater knockdown compared to the long-dose protocol in both the native (~22-fold decrease) and healing (~5.5-fold decrease) regions. Contrary to our hypothesis, *Col12a1* expression surprisingly increased in the healing region of αSMAKO mice with the short dose protocol (~2.5-fold, **Figure 1B**), and no differences between ScxKO and control mice were observed in any region or tamoxifen dosage protocol (**Figure 1C**). However, expression was increased in the long dose group in the healing region of ScxKO compared to the short dose group. Finally, *Col12a1* expression was elevated across several groups in the healing region compared to its respective native region.

DISCUSSION: During patellar tendon healing, infiltrating peritenon-derived αSMA+ cells are the primary contributors to the healing response prior to differentiating into Scx+ cells by two weeks post-injury [4]. Our previous data showed that *Col12a1* expression is increased one-week post-injury and returns to uninjured levels by six-weeks post-injury. Therefore, we expected that targeting *Col12a1* knockdown to αSMA+ cells during early healing would result in knockdown within the healing, *de novo* tissue. However, no knockdown was demonstrated in the αSMA model, even when tamoxifen was administered through 6 days post-injury, and this suggests that this Cre driver may not be sufficient in this model. Similarly, while no knockdown in the healing region of the ScxKO model may be attributed to the timing of Scx expression during tendon healing, there was no significant knockdown in the native region either, and this model also may not be effective in *Col12a1* knockdown. Additionally, both short and long-dose protocols were tested to consider the metabolism rate of tamoxifen in maximizing knockdown to the healing tissue, and in the RosaKO model, greater *Col12a1* knockdown with the short dose protocol indicates that administering tamoxifen on consecutive days before and after injury is more efficient. Finally, as expected, we observed that *Col12a1* expression was generally greater in the healing tissue than the native region, suggesting that collagen XII plays a critical role in tendon healing. Future studies will investigate this role by inducing knockdown of *Col12a1* at the time of tendon injury in a Rosa-CreER^{T2} model.

SIGNIFICANCE: Compared to αSMA-CreER^{T2} and Scx-CreER^{T2}, the Rosa-CreER^{T2} model achieves substantial *Col12a1* knockdown when induced at time of tendon injury. Our results highlight the importance of carefully considering an appropriate tamoxifen-inducible Cre model when targeting specific genes.

REFERENCES: [1] Izu et al, Matrix Biol 2021, [2] Lin et al, J Biomech 2006, [3] Leiphart et al, ORS 2022, [4] Dyment et al, PLoS One 2014.

ACKNOWLEDGEMENTS: This study was funded by NIH/NIAMS R01AR078790 and the Penn Center for Musculoskeletal Disorders (P30AR069619).

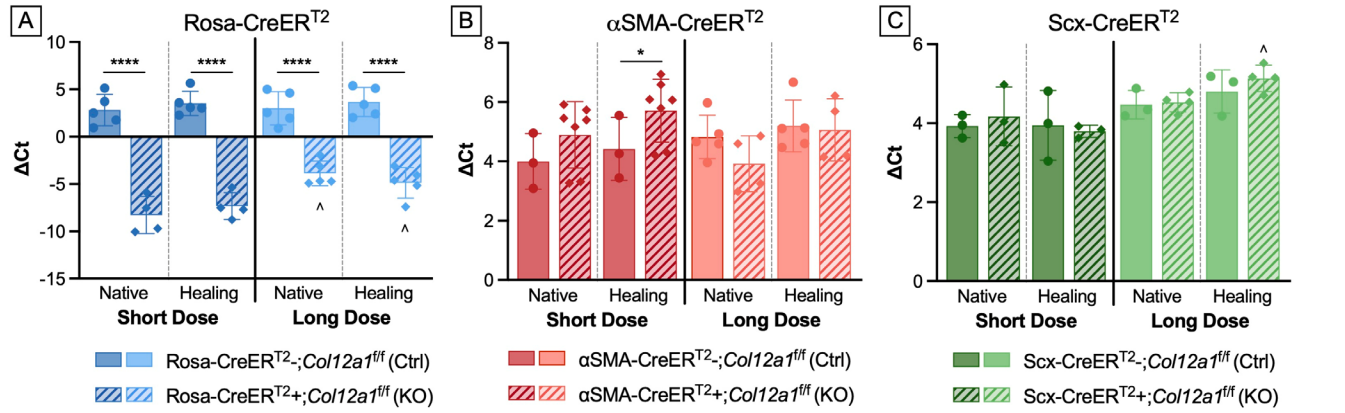

Figure 1: (A) The Rosa model achieved dramatically reduced expression of *Col12a1* expression in both the native and healing regions in mice administered the short and long dose tamoxifen protocols. The short dose groups also demonstrated greater knockdown compared to their respective long dose group. (B) In the αSMA model, *Col12a1* expression was surprisingly increased in the short dose, healing region of KO mice, while no other differences were observed. (C) In the Scx model, *Col12a1* expression was higher in the long dose, healing region of KO mice compared to its respective short dose group. (*p<0.05, ****p<0.0001, ^p<0.05 compared to short dose protocol)

Collagen III Deficiency Following Injury in Female Murine Tendons Alters Mechanical and Structural Tendon Properties

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INTRODUCTION: Tendon is composed of a highly aligned type I collagen (Col1)-rich matrix, which allows it to withstand large loads. Following injury, matrix disruption results in a shift to a type III collagen (Col3)-rich matrix and susceptibility to excessive scarring and loss of structure and mechanical function. While previous work has shown that a Col3-deficient early cutaneous healing niche failed to support healthy cellular infiltration and initial collagen deposition in granulation tissue[1], how the abundance of disorganized Col3 in the fibrotic scar affects the functional properties of the tendon is unknown. Therefore, the objective of this study was to define the role of Col3 in both tendon homeostasis and in response to injury through examination of fibril diameter and resultant changes in tendon mechanics. We hypothesized that a reduction in Col3 would lead to an increase in fibril diameters, due to the regulatory role that Col3 plays in fibrillogenesis, resulting in a stiffer matrix with improved mechanical properties.

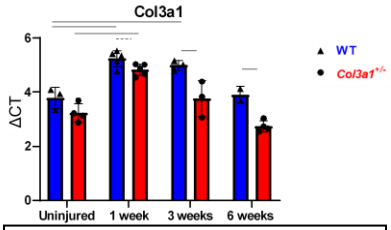


Figure 1: Expression of *Col3a1* was decreased 1, 3, and 6-weeks following injury in ***Col3a1***$^{+/-}$ tendons compared to WT.

METHODS: Female wild-type (WT) Balb/cJ and heterozygous ***Col3a1***$^{+/-}$ mice at 90 days of age (n=80) were used (IACUC approved). Injured mice underwent bilateral patellar tendon injury surgery[2] and were sacrificed 1, 3 or 6-weeks post-injury. Uninjured age, sex and strain-matched mice were also examined. *Gene Expression:* RNA extraction and cDNA reverse transcription was completed using isolated patellar tendons. A Fluidigm 96.96 Dynamic Array with pre-amplified cDNA utilized Taqman assays to examine expression levels of 96 target genes (n=5/group, *Col3a1* expression shown). *Mechanics:* Patella-patellar tendon-tibia complexes were mechanically tested (n=10-12/group)[3] with the following protocol[4,5]: 1) preconditioning, 2) stress relaxation at strain levels of 3% and 6%, 3) a sinusoidal frequency sweep (10 cycles at 0.1, 1, 5, and 10 Hz) at each strain level, 4) return to gauge length, and 5) ramp to failure. *Statistics.* Two-way ANOVAs with post-hoc Bonferroni tests were used to assess genotype, injury and the interaction on gene expression and mechanical properties. Significance was set at $p \leq 0.05$ (solid lines) and trends at $p \leq 0.1$ (dotted lines).

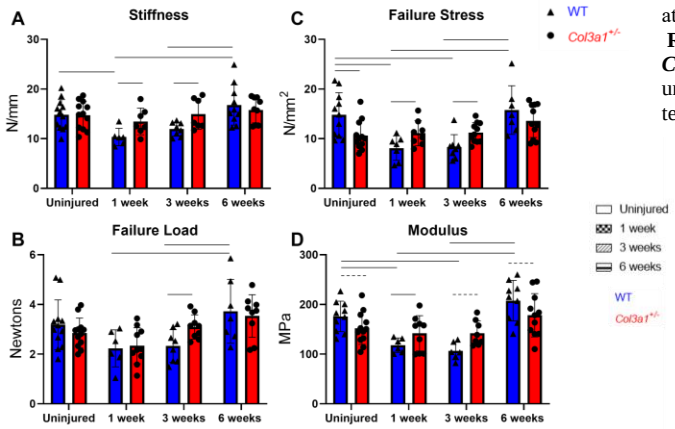


Figure 2: (A-D) Material and structural mechanical properties were increased 1- and 3w post-injury in ***Col3a1***$^{+/-}$ tendons compared to WT. WT tendon mechanical properties were decreased following injury, with no differences following injury seen in ***Col3a1***$^{+/-}$ tendons. E) Dynamic modulus at 6% strain, across all frequencies, decreased 1- and 3w post-injury in WT tendons.

RESULTS: Expression of *Col3a1* was decreased at all time points following injury in ***Col3a1***$^{+/-}$ tendons when compared to WT (Fig. 1). *Col3a1* was upregulated compared to uninjured levels 1- and 3w post-injury in WT tendons, but only 1w post-injury in ***Col3a1***$^{+/-}$ tendons (Fig. 1). Stiffness, failure stress and modulus were increased at both 1- and 3w post-injury (modulus trend at 3w), and failure load was increased 3w post-injury in ***Col3a1***$^{+/-}$ compared to WT tendons (Fig. 2A-D). Failure stress (Fig. 2C) was decreased in uninjured ***Col3a1***$^{+/-}$ compared to WT, and ***Col3a1***$^{+/-}$ tendons showed a trending decrease in both uninjured and 6w post-injury modulus (Fig. 2D). Post-injury, stiffness (1w) and failure stress and modulus (1,3w) decreased in WT tendons, but returned to uninjured levels by 6w post-injury, increasing from the 1- and 3w levels (Fig. 2A,C,D). Additionally, failure load increased from 1- and 3w levels to 6w post-injury (Fig. 2C). No effect of injury was observed in ***Col3a1***$^{+/-}$ tendons in quasistatic mechanical properties. Dynamic modulus was decreased 1- and 3w post-injury in WT tendons at 6% strain and all frequencies (5 and 10 HZ data not shown), with 6w post-injury values higher than both 1- and 3w values (Fig. 2E). A trending increase was seen between 3- and 6w timepoints in ***Col3a1***$^{+/-}$ tendons, with no other effect of injury seen. No changes were seen in dynamic modulus at 3% strain or stress relaxation (data not shown). Fibril diameters had an increased population of larger fibrils in both uninjured and 3w post-injury ***Col3a1***$^{+/-}$ tendons when compared to WT distributions (Fig. 3). Interestingly, ***Col3a1***$^{+/-}$ tendons 6w post-injury had a larger population of smaller diameter fibrils when compared to WT.

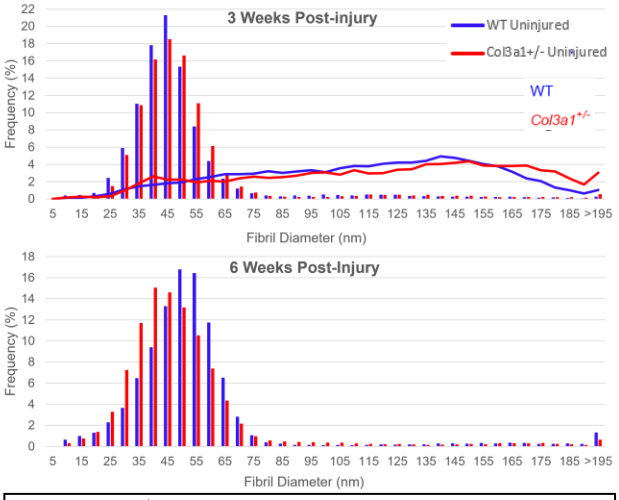
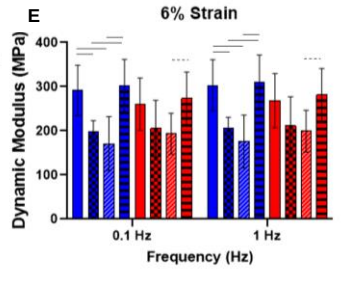


Figure 3: ***Col3a1***$^{+/-}$ tendons had an increased population of larger fibrils when uninjured and 3w post-injury. WT tendons had a greater population of larger fibrils 6w post-injury.

DISCUSSION: While the composition of healthy tendon is primarily Col1, Col3 is a main component of the open weave provisional matrix following injury[6]. In skin, a reduction in Col3 decreased the amount of granulation tissue post-injury, potentially due to reduced wound area resulting from a lack of matrix deposited and increased activation and contraction of myofibroblasts in ***Col3a1***$^{+/-}$ tendons[1]. The current study evaluated how alterations in the injured matrix composition affected tendon mechanical and structural properties. Col3 gene expression was decreased in ***Col3a1***$^{+/-}$ tendons at all time points post-injury, indicating dampened elevation in Col3 expression post-injury. Col3 expression in ***Col3a1***$^{+/-}$ tendons returned to uninjured levels by 3w post-injury, while prolonged elevated expression was seen in WT, showing that altered Col3 gene expression post-injury in ***Col3a1***$^{+/-}$ tendons resulted in changes in tendon properties. Previous studies have shown decreased mechanical properties with decreased numbers of larger diameter fibrils[7]. This supports our data that an increased population of larger fibrils 3w post-injury in ***Col3a1***$^{+/-}$ tendons likely influenced increases in mechanical properties when compared to WT. Genotypic differences in mechanics are no longer seen by 6w post-injury, potentially due to a distribution shift of WT fibrils to a larger population, while the ***Col3a1***$^{+/-}$ tendon fibril diameter distribution remains similar to 3w. This indicates that WT fibrils continue fibrillogenesis later post-injury, while ***Col3a1***$^{+/-}$ fibrils lateralize earlier in the healing process due to dysregulation of fibrillogenesis from Col3 deficiency. The relationship between stiffness, fibril diameter and cross-linking could be examined further as a potential mechanism for our findings. Work in meniscus has indicated that Col3 abundance affects fibril cross-link types and may influence mechanical strength of matrix[8]. Decreased changes in viscoelastic properties in ***Col3a1***$^{+/-}$ compared to WT tendons may be a result of increased activation of myofibroblasts and hypersecretion of Col1, combined with increased cross-linking. Future directions will include examining Col3 deficient tendons histologically as we expect the altered matrix structure and properties to contribute to myofibroblast activation and influence inflammatory cell activities and fate, as well as cross-linking analysis.



SIGNIFICANCE: The abundance of Col3 in matrix affects the structural and functional properties of the healing tendon, implicating Col3 as an important regulatory molecule of healing and a future potential therapeutic target.

REFERENCES: [1] Volk et al. *Cells Tissues Organs*. 2011. [2] Beason et al. *J. Biomech*, 2012. [3] Kjaer et al. *J. Anat.* 2006. [4] Dunkman et al. *Matrix Biol.* 2013. [5] Miller KS, et al. *J Biomech Eng*. 2012. [6] Nicols et al. *Transl Res.* 2019. [7] Sun et al. *Matrix Biol.* 2020. [8] Wang et al. *Matrix Biol.* 2019.

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Knockdown of biglycan reveals an important role in maintenance of structural and cellular properties during tendon aging

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INTRODUCTION: In tendon extracellular matrix, biglycan (Bgn), a small leucine-rich proteoglycan (SLRP), is known to regulate tendon fibrillogenesis and play a regulatory role during development and after injury [1,2]. Previous work utilized an inducible biglycan knockdown model in uninjured adult mice and observed reduced tendon viscoelastic and dynamic mechanics and altered structural parameters after 30 days, suggesting a regulatory role for biglycan during tendon homeostasis [3]. Further, knockdown of biglycan in aged tendons impaired mechanical properties by reducing maximum load, stress relaxation, and collagen fiber realignment during loading [4]. However, the mechanisms governing these effects of biglycan knockdown are unknown. Therefore, the aim of this study was to explore the underlying mechanisms that may explain the impact of biglycan on the mechanical properties of aged tendon. We hypothesized that changes in tendon structure could help explain the reduced mechanical properties after biglycan knockdown.

METHODS: Female $Bgn^{+/+}$ control (WT) and bitransgenic conditional $Bgn^{floxflox}$ mice with a tamoxifen (TM) inducible Cre (I-$Bgn^{-/-}$), (B6.129-Gt(ROSA)26Sortm1(cre/ERT2)Tyj/J, Jackson Labs) were utilized (n=16/group) (IACUC approved). Cre excision of the conditional alleles was induced in aged mice (485 days) via three consecutive daily intraperitoneal injections of tamoxifen (112mg/kg). WT mice received the same injections of tamoxifen as control and mice from the two groups were euthanized 30 days after tamoxifen treatment, at 515 days old. For histology, 7 µm longitudinal sections of the patellar tendon were obtained and stained with hematoxylin and eosin (H&E). Two midsubstance sections and one insertion section (tibial insertion) were imaged per sample, and cell nuclear shape and cellularity (cells/mm^2) were quantified using ImageJ (n=4/group). For transmission electron microscopy (TEM), patellar tendons were fixed, embedded in epon, and sectioned (85 nm sections). Sections obtained through the midsubstance were stained with 2% aqueous uranyl acetate followed by 1% phosphotungstic acid, then examined at 80kV using a JEOL 1400 TEM. Collagen fibril diameters were measured (n=4/group) and fibril diameter distributions were compared between the two genotypes with a Kolmogorov-Smirnov test. Finally, samples were collected for gene analysis. After sacrifice, patellar tendons were immediately frozen in liquid nitrogen. After crushing, total RNA was extracted using the Direct-zol RNA Microprep kit (Zymo, R2062), and cDNA was synthesized using the High Capacity cDNA RT kit (Thermo). Pre-amplified cDNA was used to perform a real-time PCR with TaqMan™ Fast Advanced Master Mix. Resultant cycle threshold (Ct) values were normalized to the invariant control Abl1 and expressed as $2^{\Delta Ct}$ (n=4/group). T-tests were used to compare cellularity between the two genotypes and a Mann-Whitney test was used to compare nuclear aspect ratios and gene expression. Significance was set at $p<0.05$.

RESULTS: As expected, biglycan expression was reduced (Fig 1A) in I-$Bgn^{-/-}$ tendons. There were no compensatory increases in decorin expression (Fig 1B) or in class II SLRPs fibromodulin, lumican and keratocan (Fig 1C,D,E). Type I collagen expression was also not altered in the I-$Bgn^{-/-}$ tendons (Fig 1F). Histological analysis did not reveal any gross differences between the two genotypes (e.g., no insertion calcification, no differences in tendon thickness). No changes in cellularity were seen after biglycan knockdown within the midsubstance or the tibial insertion area. However, a significant difference in nuclear aspect ratio was observed within the tibial insertion area, with a lower ratio in the I-$Bgn^{-/-}$ tendons indicating a more spindle-like nuclear shape (Fig 2). No qualitative changes to fibril shape in TEM images were apparent after biglycan knockdown in aged tendons. However, the fibril diameter distributions were different between the two genotypes with a higher percentage of small diameter fibrils (25-45nm) and a lower percentage of medium size fibrils (150-165nm) in I-$Bgn^{-/-}$ tendons (Fig 3).

DISCUSSION: The mouse model demonstrated effective knockdown of the target gene without any compensation from other SLRPs. This allowed analysis of the role of biglycan in tendon aging without the confounding effects of altered developmental processes. Biglycan knockdown induced changes in the fibril diameter distribution. These tendon structure changes are likely due to the role of biglycan in collagen fibrillogenesis and could help explain the inferior mechanical properties of aged I-$Bgn^{-/-}$ tendons shown previously [4]. Similar to the effect observed in tendons of mature mice (120 days old) [3], biglycan knockdown induced changes in the nuclear aspect ratio in the insertion region. This could be related to the role of biglycan in organizing the tendon stem/progenitor cells (TSPC) niche. Indeed, depletion of biglycan and fibromodulin has been shown to affect the differentiation of TSPCs by modulating bone morphogenetic protein signaling and impaired tendon formation *in vivo* [5]. Future work will explore the impact of biglycan knockdown on tendon cell functions in the context of aging.

SIGNIFICANCE: As in mature tendons [3], this study showed significant effects of biglycan knockdown on structural and cellular characteristics of aged tendons (485 days old) only 30 days after knockdown. While among SLRPs, decorin is considered as the primary regulator of tendon homeostasis at maturity, these data suggest that biglycan has a major role in tendon homeostasis in the context of aging.

REFERENCES: [1] Ansorge et al., Ann Biomed Eng, 2011. [2] Dunkman et al., Ann Biomed Eng, 2014. [3] Beach et al. J Orthop Res, 2022. [4] Beach et al., ORS, 2020. [5] Bi et al., Nat Med. 2007.

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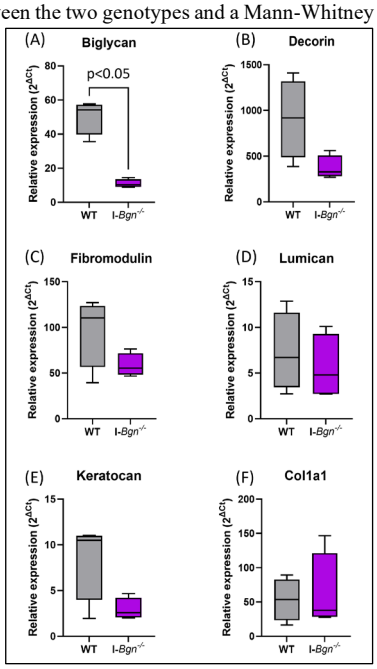

FIGURE 1. Gene expression data shows knockdown of biglycan, as expected, with no compensatory effects of class I and II SLRPs or type I collagen.

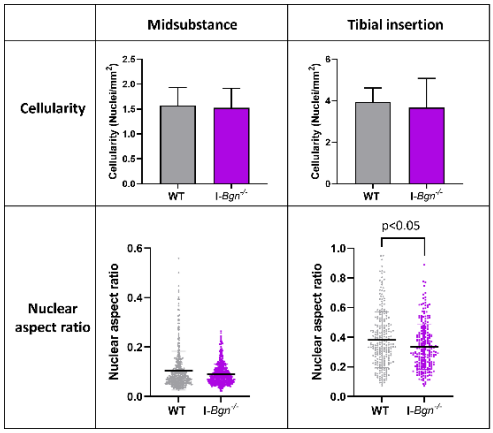

FIGURE 2. Analysis of cellularity and cell shape. There were no differences in cellularity between genotypes, while cell shape was different between genotypes with a lower nuclear aspect ratio in I-Bgn-/- tendons in the insertion area.

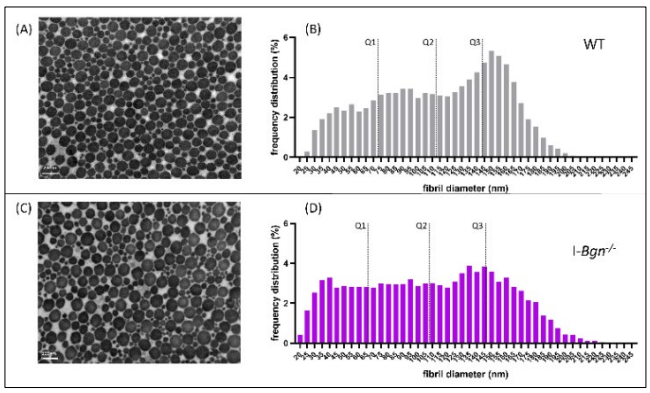

FIGURE 3. Representative TEM images (A) and (C) and fibril diameter distributions in percentages within the midsubstance (B) and (D). I-Bgn-/- tendons have more smaller and fewer medium sized fibrils compared to WT.

Scleraxis Targeted Deletion of Collagen XI Impairs Tendon Mechanical Function During Postnatal Development

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INTRODUCTION: Mutations in the *Col11a1* gene are implicated in Type II Stickler Syndrome and result in joint problems including hypermobility and early arthritis [1]. Beyond its role in Stickler Syndrome, collagen XI is highly expressed during tendon postnatal development and interacts with collagen I and II during heterotypic fibril formation [2]. We previously showed that during patellar tendon postnatal development, tendon-targeted (ScxCre) collagen XI deficiency disrupts tendon structure, resulting in tenocyte nuclear disorganization, larger diameter collagen fibrils, and increased tendon length [3]. However, whether these structural findings result in alterations in tendon mechanical function is unknown. Therefore, the objective of this study was to define the role of collagen XI in the acquisition of tendon mechanical function during postnatal development using tendon-targeted collagen XI knockout mice. We hypothesized that tendon-targeted collagen XI knockout would result in inferior tendon mechanical properties.

METHODS: Tendon-targeted ScxCre;*Col11a1*$^{flox/flox}$ (KO), ScxCre;*Col11a1*$^{flox/wt}$ (HET), and Cre- littermate control (CTRL) mice were used (IACUC approved). Patellar tendons were harvested at postnatal days (P) 10, 20, and 30 for mechanical testing (n=12/group). The tibia-patellar tendon-patella complex was dissected, tendon cross-sectional area was measured, and the tibia was potted in polymethylmethacrylate. The patella was gripped with sandpaper (P10 and P20) or clamped directly (P30) using custom fixtures, and tendons were subjected to a protocol consisting of preconditioning, stress relaxations at 3, 4, and 5% strain each followed by a dynamic frequency sweep (0.1-10 Hz), and a ramp to failure at 0.1% strain/s. For each age, genotypes were compared using a one-way ANOVA with Tukey post-hoc tests. Significance was set at $p \leq 0.05$.

RESULTS: Tendon-targeted collagen XI knockout resulted in substantial alterations in patellar tendon mechanical properties. Cross-sectional area (**Figure 1A**) of KO tendons was greater only at P10, while KO tendons (**Figure 1B**) were significantly longer than CTRL tendons at all ages, consistent with previous histological findings. Stiffness (**Figure 1C**) and failure load (**Figure 1D**) of KO tendons were dramatically reduced compared to CTRL and HET tendons. Material properties of KO tendons showed similar trends, with marked reduction in modulus (**Figure 1E**), failure stress (**Figure 1F**), and failure strain (**Figure 1G)** at all ages compared to CTRL and HET tendons. Dynamic modulus was similarly reduced in KO tendons at all ages (data not shown).

DISCUSSION: Previous work evaluating the role of collagen XI in postnatal development found that tendon-targeted knockout disrupts tendon structure, resulting in tenocyte nuclear disorganization, larger diameter collagen fibrils, and increased tendon length [3]. Results of the present study further elucidate the importance of collagen XI in regulating tendon structure-function during early postnatal development. The mechanical differences in KO mice were particularly striking, with KO patellar tendons demonstrating substantial reductions in modulus of 45%, 78%, and 60% at P10, P20, and P30, respectively. These findings are consistent with previous mechanical findings in mature (day 60) KO tendons [4,5], but the emergence of these stark mechanical differences as early as postnatal day 10 point to a critical and previously undescribed role for collagen XI in the initial establishment of tendon hierarchical structure. In tendon, collagen XI is most highly expressed during embryonic development with minimal expression during adulthood [2], and therefore, inferior mechanical function in KO tendons may be due to alterations in fibril nucleation and assembly. Additionally, motivation for this study was primarily driven by the observation of significant patellar tendon lengthening and the presence of granulation tissue in mature tendons. Our findings suggest the absence of collagen XI leads to the deposition of substantially weakened matrix, resulting in injury and elongation following the initiation of ambulation around postnatal day 10. Preliminary gene expression findings at P10 (not shown) support a pathological response with increased expression of genes associated with non-collagenous matrix proteins and remodeling, and studies are ongoing to further elucidate the mechanisms underlying the role of collagen XI in tendon development.

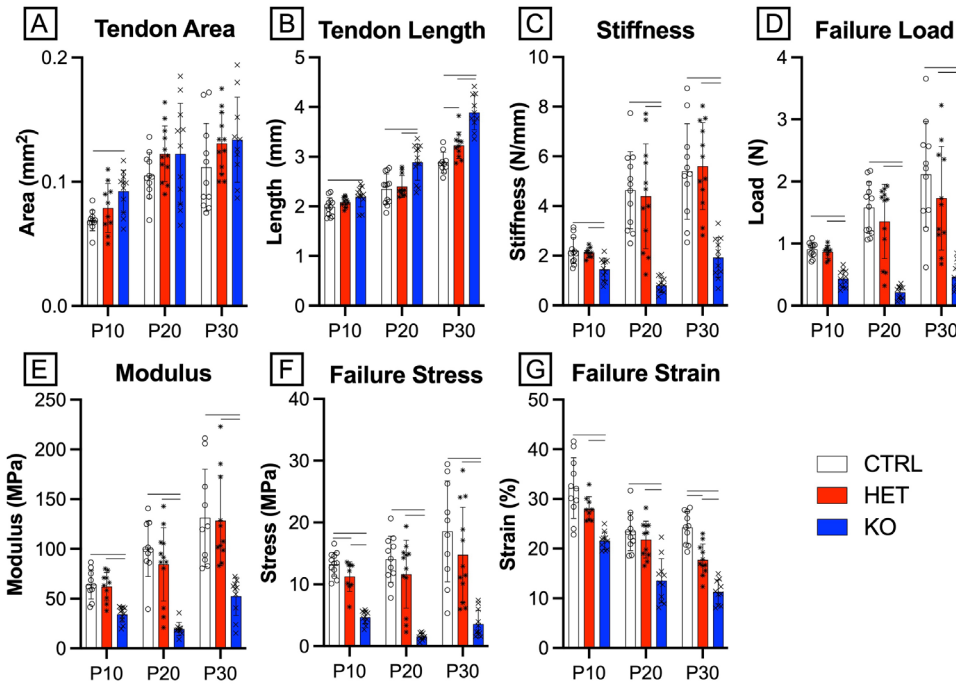

Figure 1: Patellar tendon (A) cross-sectional area was higher in KO tendons at p10, while (B) tendon length was greater at all ages. (C) Stiffness and (D) failure load were significantly reduced in KO tendons at all ages compared to CTRL and HET tendons. (E) Elastic modulus, (F) failure stress, and (G) failure strain were significantly reduced in KO mice compared to CTRL and HET tendons at all ages. (—p<0.05)

SIGNIFICANCE: Collagen XI is critical in the establishment of tendon structure and mechanical function during postnatal tendon development. These findings highlight the need to further define the regulatory role of collagen XI in tendon development and healing, which could lay the foundation for future therapeutic applications.

REFERENCES: [1] Cattalini M et al., Pediatr Rheumatol Online J, 2015. [2] Wenstrup RJ et al., J Biol Chem, 2011. [3] Stein MK. et al., ORS Annual Meeting, 2022. [4] Ye Y et al., ORS Annual Meeting, 2019. [5] Sun M et al., Matrix Biol, 2020.

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Collagen XII is a Critical Regulator of Supraspinatus Tendon Mechanics and Collagen Fiber Realignment Across Sex

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Disclosures: AF Kuntz (5, Integra Lifesciences, Orthofix, Inc., FX Shoulder; 9, Orthopaedic Research Society, American Shoulder and Elbow Surgeons, American Board of Orthopaedic Surgery), no other disclosures

INTRODUCTION: Collagen XII is a Fibril-Associated Collagen with Interrupted Triple Helices (FACIT) that regulates collagen fibril assembly and is primarily expressed throughout tendon growth and development. Mutations in the *Col12a1* gene result in myopathic Ehlers-Danlos syndrome, a connective tissue disorder in which patients exhibit weakness at birth, absence of deep tendon reflexes and distal joint hypermobility and contracture [1]. Our novel tendon-targeted collagen XII mouse model demonstrated that patellar tendons exhibited reduced elastic, viscoelastic, and dynamic collagen fiber realignment properties across sex [2]. However, the role of collagen XII on the supraspinatus tendon, which experiences a complex, region-specific (insertion and midsubstance) loading environment within the rotator cuff of the shoulder, remains unknown. Therefore, the objectives of this study are to (1) elucidate the regulatory role of collagen XII on supraspinatus tendon whole-tissue and regional mechanics and dynamic response to load in mature mice using tendon-targeted (Scleraxis-Cre) collagen XII deficient and knockout mice and (2) understand whether the role of collagen XII on supraspinatus tendon biomechanical function and dynamic response to load is dependent on sex. We hypothesized that tendon-targeted deficiency and knockout of collagen XII would result in decreased supraspinatus tendon whole-tissue and regional elastic mechanics, whole-tissue viscoelasticity, and regional collagen fiber realignment across sex.

METHODS: Supraspinatus tendons from male and female, day 60 tendon-targeted collagen XII heterozygous (HET) mice (ScxCre;*Col12a1*$^{f/wt}$, n=8-9/group), knockout (KO) mice (ScxCre;*Col12a1*$^{f/f}$, n=6-9/group) and wild-type (WT) control mice (Cre- littermates, n=7-9/group) (IACUC) were subjected to our established mechanical testing protocol and collagen fiber realignment method [3]. Tendons underwent stress relaxation testing at 3, 5, and 7% strain each with subsequent dynamic frequency sweeps at 0.1, 1, 5, and 10 Hz, followed by a quasistatic ramp-to-failure. Throughout the ramp-to-failure, dynamic collagen fiber realignment was quantified using cross-polarization imaging, and regional fiber alignment data was interpolated with a polynomial fit as a function of strain from the load-displacement data. Images were also used to optically measure strain to calculate regional moduli (insertion and midsubstance). For each sex, comparisons between genotypes were conducted using one-way ANOVAs followed by Bonferroni post-hoc tests. Significance was set at $p \leq 0.05$ and trends at $p \leq 0.1$.

RESULTS: Cross-sectional area (CSA) was not different between male tendons, while female KO tendons exhibited a trending decrease in CSA relative to WT (data not shown). Consistent with our hypothesis, linear stiffness was significantly decreased in KO mice across sex and between female HET and KO mice (**Fig. 1A**). Moreover, insertion modulus was significantly reduced in HET and KO tendons across sex whereas midsubstance modulus was significantly reduced in male KO tendons and female HET and KO tendons (**Figs. 1B, C**). Further, genotypic differences were observed in viscoelastic properties across sex. Percent relaxation was significantly increased in KO tendons across sex at all strain levels (5% strain shown in **Fig. 1D**). Additionally, dynamic modulus was significantly decreased in male KO tendons and in female HET and KO tendons, while phase shift was significantly increased in KO tendons across sex across at all strain levels and frequencies (5% strain at 1Hz shown in **Figs. 1E** and **1F**, respectively). These results are supported by reductions in collagen fiber realignment in HET and KO tendons across region and sex, as demonstrated by significantly greater normalized circular variance values for insertion and midsubstance regions from 3-7% strain (**Figs. 2A-D**), encompassing the toe and linear elastic regions of these tendons.

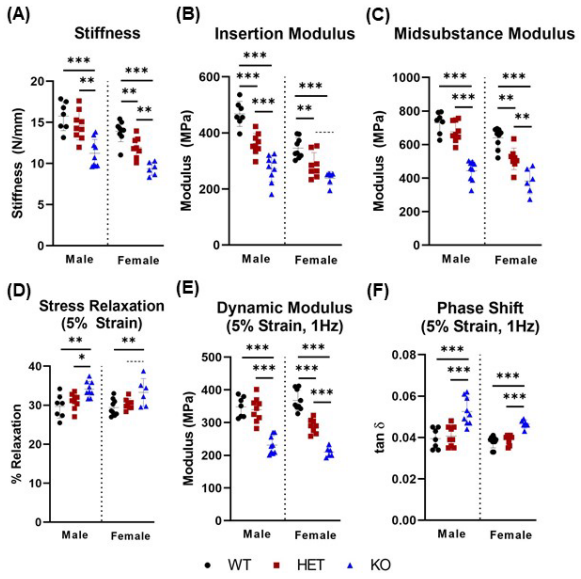

Figure 1. Differences between male and female elastic (A-C) and viscoelastic (D-F) mechanical properties of WT, HET, and KO supraspinatus tendons. Data as mean ± standard deviation (---$p \leq 0.1$ *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

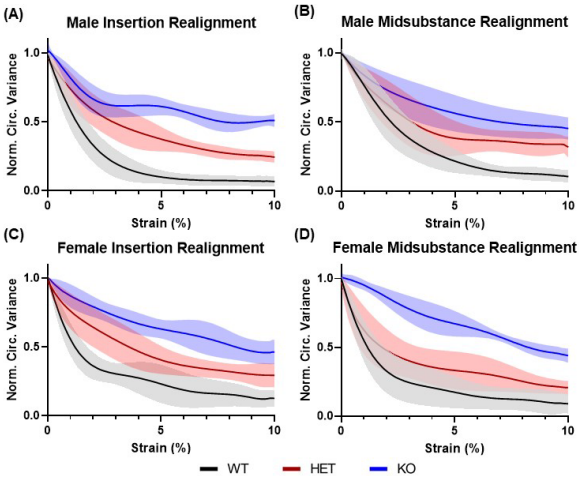

Figure 2. Collagen fiber realignment distribution differences for male (A-B) and female (C-D) WT, HET, and KO supraspinatus tendon insertion and midsubstance regions. Decreased normalized circular variance is indicative of increased collagen fiber realignment.

DISCUSSION: This study investigated the role of collagen XII on supraspinatus tendon elastic and viscoelastic mechanics and dynamic collagen fiber realignment using tendon-targeted male and female ScxCre;*Col12a1*$^{f/wt}$ and ScxCre;*Col12a1*$^{f/f}$ mice. Consistent with previous data [2], we showed that tendon-targeted collagen XII knockout resulted in striking reductions in regional and whole-tissue elastic and viscoelastic mechanical properties and regional collagen fiber realignment. Further, reductions in these properties in our collagen XII deficient HET tendons, highlight the allele-dependency of collagen XII on tendon mechanical function and dynamic collagen fiber realignment. These mechanical deficits could be due to the improper hierarchical assemblies of HET and KO tendons resulting in disorganized tendon matrices with an inferior ability to respond to load. This was evidenced by marked reductions in the HET and KO tendons' responses to realign resulting in inferior mechanical properties, especially whole-tissue stiffness, regional moduli, and dynamic modulus. Although similar differences in elastic and viscoelastic mechanical properties were present across sex in response to collagen XII deficiency and knockout, more genotypic differences were present in female mice. Genetic variations in the *Col12a1* gene have been associated with an increased risk of ACL ruptures in women [4], implicating potential sex-specific effects of collagen XII deficiency and knockout. Overall, our results demonstrate that decreased collagen XII expression detrimentally affects supraspinatus tendon mechanical properties and dynamic collagen fiber realignment across sex.

SIGNIFICANCE/CLINICAL RELEVANCE: This study elucidates the critical role of collagen XII in regulating male and female supraspinatus tendon regional and whole-tissue mechanics and dynamic structural response to load within the complex loading environment of the rotator cuff of the shoulder. Clinically, understanding the effects of collagen XII in tendon across sex can be used to develop and evaluate potential treatments modalities for myopathic Ehlers-Danlos syndrome.

ACKNOWLEDGEMENTS: We thank Ashley Fung for her assistance. This study was supported by NIH/NIAMS R01AR078790 and Penn Center for Musculoskeletal Disorders (NIH/NIAMS, P30AR069619).

REFERENCES: [1] Punetha et al. *Muscle Nerve*. 2017. [2] Fung et al. *ORS Annual Meeting*. 2021. [3] Connizzo et al. *Interface Focus*. 2016. [4] Posthumus et al. *Br J Sports Med*. 2010.

Tendon-Targeted Collagen XII Knockout Attenuates Male and Female Achilles Tendon Function Similarly

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Disclosures: None

INTRODUCTION: Collagen XII is a Fibril-Associated Collagen with Interrupted Triple Helices (FACIT) that regulates collagen fibril assembly and is primarily expressed throughout tendon growth and development [1]. Mutations in the *Col12a1* gene result in myopathic Ehlers-Danlos syndrome, a connective tissue disorder in which patients exhibit weakness at birth, absence of deep tendon reflexes and distal joint hypermobility and contracture [2]. Our novel tendon-targeted collagen XII mouse model demonstrated that flexor digitorum longus (FDL) tendons exhibited reduced elastic mechanical properties in both male and female mice [1]. However, the role of collagen XII on the elastic, viscoelastic, and dynamic response to load in the Achilles tendon, which experiences a complex and distinct mechanical loading environment, remains unknown. Therefore, the objectives of this study were to (1) elucidate the role of collagen XII on Achilles tendon mechanics and dynamic response to load using tendon-targeted (Scleraxis-Cre) collagen XII knockout mice and (2) understand whether the role of collagen XII on Achilles tendon biomechanical function and dynamic response to load is comparable between sexes. We hypothesized that tendon-targeted knockout of collagen XII would result in consistent reductions in elastic and viscoelastic function and collagen fiber realignment in both sexes.

METHODS: Achilles tendons from male and female, day 30 tendon-targeted collagen XII knockout (KO) mice (ScxCre;*Col12a1*$^{f/f}$, n=4/group) and wild-type (WT) control mice (Cre-littermates, n=4/group) (IACUC approved) were subjected to our mechanical testing protocol [3]: stress relaxation testing at 3, 5, and 7% strain each with subsequent dynamic frequency sweeps at 0.1, 1, 5, and 10 Hz, followed by a quasistatic ramp-to-failure. During the ramp-to-failure, dynamic collagen fiber realignment was quantified using cross-polarization imaging [3], and alignment data was interpolated with a polynomial fit as a function of strain from the load-displacement data. For each sex, comparisons between genotypes were conducted using two-tailed, t-tests with significance set at p≤0.05 and trends at p≤0.1.

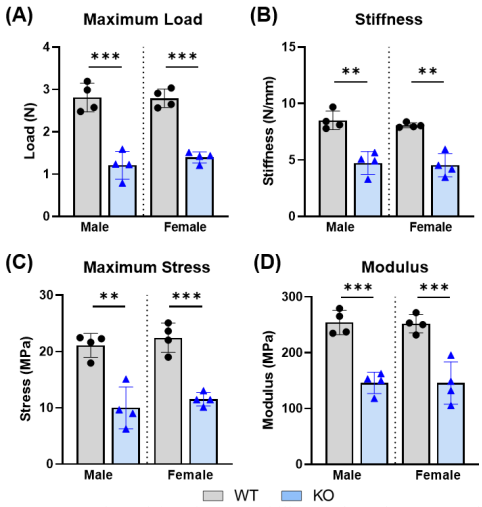

Figure 1. Male and female KO Achilles tendons demonstrated significant reductions in structural (A-B) and material (C-D) properties relative to WT controls. Data as mean ± standard deviation (*p≤0.05, **p≤0.01, ***p≤0.001).

RESULTS: Cross-sectional area (CSA) was not different across genotypes for both male and female tendons (data not shown). Consistent with our hypothesis, structural properties, maximum load and linear stiffness (**Figs. 1A, B**), and material properties, maximum stress and linear modulus (**Figs. 1C, D**), were significantly decreased in both male and female KO tendons. Further, genotypic differences were observed in viscoelastic properties in both sexes. Percent relaxation was significantly increased in KO tendons at all strain levels (7% strain shown in **Fig. 2A**). Additionally, dynamic modulus was significantly decreased in male and female KO tendons, while phase shift was significantly increased in male and female KO tendons across at all strain levels and frequencies (7% strain at 1Hz shown in **Figs. 2B** and **2C**, respectively). These results are supported by reductions in collagen fiber realignment in both male and female KO tendons, as demonstrated by significantly greater normalized circular variance values from 3-9% strain (**Figs. 3A, B**), encompassing the toe and linear elastic regions of these tendons.

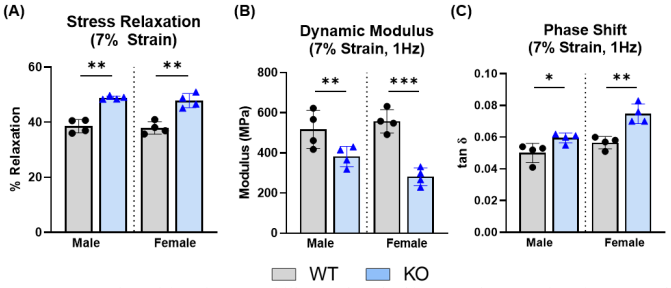

Figure 2. Male and female KO Achilles tendons had increased stress relaxation (A) and significantly decreased dynamic modulus (B) and increased phase shift (C) relative to WT controls. Data as mean ± standard deviation (*p≤0.05, **p≤0.01, ***p≤0.001).

DISCUSSION: This study investigated the role of collagen XII on Achilles tendon elastic and viscoelastic mechanics and dynamic collagen fiber realignment using tendon-targeted male and female ScxCre;*Col12a1*$^{f/f}$ mice. Consistent with previous data from collagen XII knockout FDL tendons [1], we showed that tendon-targeted collagen XII knockout resulted in striking reductions in Achilles tendon elastic and viscoelastic mechanical properties and collagen fiber realignment in both sexes. These mechanical deficits could be attributed to the improper hierarchical assemblies of KO tendons resulting in disorganized tendon matrices with an inferior ability to quasi-statically and dynamically respond to load. This was evidenced by marked reductions in the KO tendons' responses to realign resulting in inferior mechanical properties, especially maximum load, modulus, and dynamic modulus. Although similar differences in elastic and viscoelastic mechanical properties were present in both male and female tendons in response to collagen XII knockout, more genotypic differences were present in female mice. Genetic variations in the *Col12a1* gene have been associated with an increased risk of ACL ruptures in women [4], implicating potential sex-specific effects of collagen XII knockout. Our results demonstrate that decreased collagen XII expression detrimentally affects male and female Achilles tendon elastic and viscoelastic properties and dynamic collagen fiber realignment in response to load.

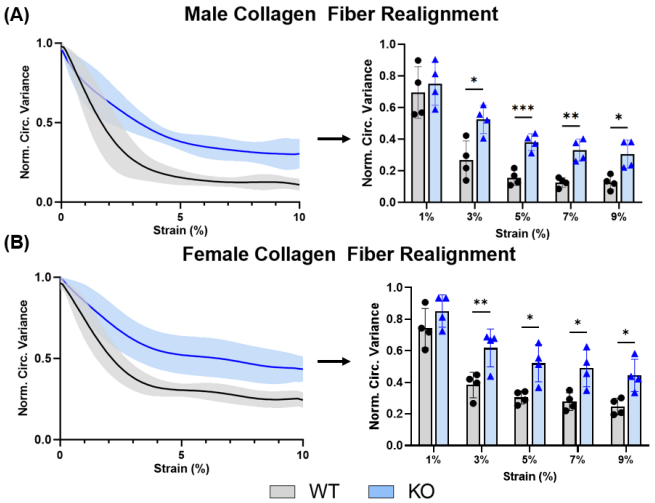

Figure 3. Male (A) and female (B) KO Achilles tendons exhibited significantly reduced collagen fiber realignment with increasing strain. Decreased normalized circular variance is indicative of increased collagen fiber realignment. Data as mean ± standard deviation (*p≤0.05, **p≤0.01, ***p≤0.001).

SIGNIFICANCE/CLINICAL RELEVANCE: This study further elucidates the role of collagen XII in regulating tendon function. Future studies will evaluate the mechanisms that contribute to these results. Understanding the effects of collagen XII in both male and female tendons can be used to develop potential treatments modalities for myopathic Ehlers-Danlos syndrome.

ACKNOWLEDGEMENTS: We thank Ashley Fung for her assistance. This study was supported by NIH/NIAMS R01AR078790 and Penn Center for Musculoskeletal Disorders (NIH/NIAMS, P30AR069619).

REFERENCES: [1] Fung et al. *Matrix Biology Plus*. 2022. [2] Punetha et al. *Muscle Nerve*. 2017. [3] Connizzo et al. *Interface Focus*. 2016. [4] Posthumus et al. *Br J Sports Med*. 2010.

Collagen XII Regulates Cell and Matrix Organization and Structure During Postnatal Tendon Development

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INTRODUCTION: Collagen XII is a fibril-associated collagen with interrupted triple helices (FACIT), and mutations in the *Col12a1* gene are associated with myopathic Ehlers-Danlos syndrome (mEDS), a connective tissue disorder resulting in symptoms such as joint hypermobility and contractures [1]. Collagen XII interacts with type I collagen to mediate fibrillogenesis and has also been shown to regulate tendon cell organization and the formation of interacting cellular processes [2]. In mature tendon-targeted collagen XII knockout (ScxCre;*Col12a1*$^{flox/flox}$) mice, we previously showed that patellar tendons demonstrated reduced viscoelastic properties and collagen fiber realignment [3], further suggesting a critical role for collagen XII in establishing matrix organization for proper mechanical function. However, whether these mechanical differences in the absence of collagen XII are due to the initial deposition of disorganized matrix or disordered cell organization early in development leading to disorganized matrix is still unknown. Therefore, the objective of this study was to evaluate the role of collagen XII in regulating cell and matrix organization and structure during postnatal tendon development, prior to the establishment of significant matrix deposition. We hypothesized that collagen XII disrupts cell organization, resulting in alterations in tendon structure and organization.

METHODS: Postnatal day 10 tendon-targeted collagen XII knockout (KO, ScxCre;*Col12a1*$^{flox/flox}$) and control (CTRL, Cre- littermates) mice were used (IACUC approved). Tendon morphology: Knees (n=4-6/group) were fixed, paraffin processed, and sectioned in the sagittal plane. Sections were stained with toluidine blue to measure tendon length and quantify cell density. Matrix & cell organization: Knees (n=5-6/group) were fixed, and patellar tendons (PTs) were dissected from the joint. PTs were blocked and permeabilized, stained with AF 647 phalloidin and Hoechst, and optically cleared using increasing fructose concentrations (20-115% wt/vol) [4]. Z-stacks were acquired (40μm thickness) using a multiphoton microscope to visualize collagen with second harmonic generation (SHG) imaging, actin, and nuclei. Collagen density (SHG intensity), matrix alignment, and nuclear shape (sphericity) were calculated. Matrix alignment was quantified using a fast Fourier algorithm to calculate circular standard deviation of the fiber direction distribution. Fibril structure: PTs (n=4/group) were fixed, processed, sectioned, stained, and imaged via transmission electron microscopy (TEM) as described [5]. Gene expression: RNA was extracted from PTs (n=4-6/group), converted to cDNA, pre-amplified, and loaded into a Fluidigm Dynamic Array. Target genes included those of collagens, non-collagenous matrix, remodeling, cell-ECM, and cell markers. Statistics: Nuclear shape and fibril diameter distributions were compared using Kolmogorov-Smirnov tests, and all other parameters were compared using two-tailed, Student's t-tests with significance set at $p \leq 0.05$.

RESULTS: Tendon Morphology: PTs in KO mice were significantly longer than CTRL (**Fig 1A-B**). Matrix & cell organization: KO tendons demonstrated higher average forward SHG signal (**Fig 1C**), indicative of greater collagen density, and increased circular standard deviation of fiber directions, signifying greater collagen matrix disorganization in KO tendons (**Fig 1D-F**). Additionally, cell density was higher in KO tendons (**Fig 1G**), and nuclei were rounder (**Fig 1H**). In contrast to CTRL tendons, where actin filaments were arranged parallel with the long-axis of the tendon, actin appeared disordered and less aligned in KO tendons, consistent with matrix disorganization (**Fig 1I-J**). Irregular cell shape was also observed in TEM tendon cross-sections (**Fig 2A**). In CTRL tendons, cell protrusions interacted with those of neighboring cells towards establishing defined fibril bundles. In KO tendons, however, cell protrusions were fragmented with abundant fibripositors (white arrows). Fibril structure: The collagen fibril diameter distribution in KO tendons was more heterogeneous with a greater percentage of larger diameter fibrils compared to CTRL (**Fig 2B**). Gene expression: As expected, *Col12a1* expression was significantly reduced (**Fig 3A**), while expression of *Fn1, Mmp2, and Serpine1* (**Fig 3B-D**) was increased in KO tendons. Expression of tendon-related genes (*Col5a2, Dcn, Bgn, Tnc, Tnmd*) and those associated with cell-cell and cell-matrix interactions (*Cdh11, Cdh2, Cd44, Itgb1*) were also increased in KO tendons (data not shown).

DISCUSSION: During tendon development, proper cell and matrix organization is essential for establishing tendon hierarchical structure and function, and our findings indicate that collagen XII is critical in this process. In the absence of collagen XII, postnatal day 10 patellar tendons have disrupted matrix and cell organization, altered cell and nuclear shape, increased fibril diameter, and increased expression of tendon and cell-matrix related genes. Though findings support previous work in mature mice [2], interestingly, our results during early postnatal growth suggest that alterations in cell organization may precede or result in disorganized matrix deposition. Preliminary findings at postnatal day 30 show similar but less striking alterations in matrix organization and fibril diameter, further supporting a more prominent cell-mediated mechanism during early development. Additionally, increased tendon length, collagen and cell density, and fibril diameter point to a hypertrophic phenotype due to collagen XII knockout. Cornea and skin studies showed that collagen XII may be necessary for storing latent TGF-β, and its absence increased TGF-β activity [6,7]. Gene expression findings in this study support a similar mechanism in tendon, where TGF-β responsive genes such as *Serpine1* are upregulated despite no changes in *Tgfb1, 2,* or *3* expression. Studies are ongoing to explore this mechanism and further elucidate the role of collagen XII in regulating initial cell organization during embryonic tendon development.

SIGNIFICANCE: Collagen XII regulates cell and matrix organization and structure during postnatal tendon development, highlighting its importance in the establishment of tendon hierarchical structure and function.

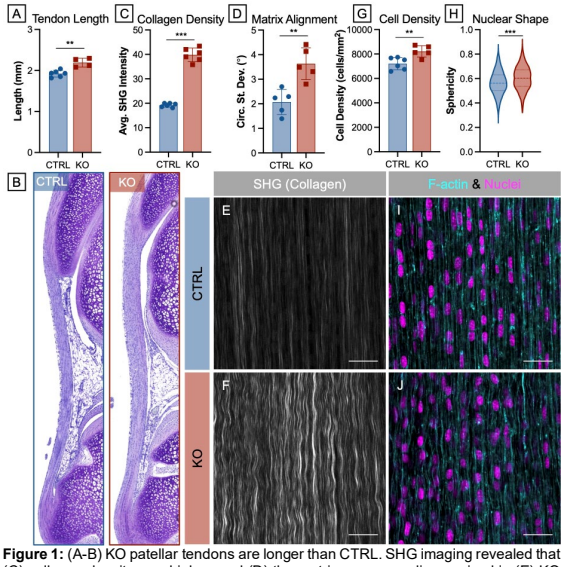

Figure 1: (A-B) KO patellar tendons are longer than CTRL. SHG imaging revealed that (C) collagen density was higher, and (D) the matrix was more disorganized in (E) KO tendons compared to (F) CTRL. (G) Cell density was higher and (H) nuclei were rounder in KO tendons. (I-J) Actin staining also revealed that cells were less aligned. (**p<0.01,***p<0.001, scale bar = 25μm)

REFERENCES: [1] Zou et al, Hum Mol Genet, 2014. [2] Izu et al, Matrix Biol, 2021. [3] Fung et al, ORS Annual Meeting 2021. [4] Ke et al, Nat Neurosci 2013. [5] Dunkman et al, Matrix Biol 2014. [6] Sun et al, Am J Pathol 2022. [7] Schönborn et al, Matrix Biol 2020.

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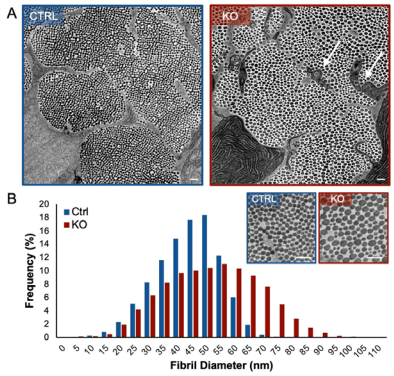

Figure 2: (A) Cell protrusions in CTRL tendons interact with neighboring cells and form fibril bundles, while those in KO tendons are fragmented and contain several fibripositors (white arrows). (B) KO tendons have a heterogenous collagen fibril diameter population with a greater percentage of larger fibrils. (scale bar = 200nm)

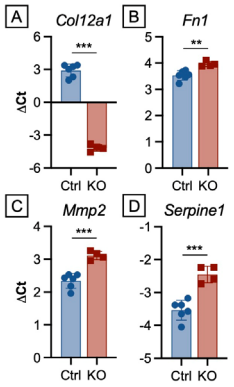

Figure 3: (A) *Col12a1* expression is significantly reduced in KO mice, while expression of (B) *Fn1*, (C) *Mmp2*, and (D) *Serpine1* is increased. (**p<0.01, ***p<0.001)

The Mechanosensor Focal Adhesion Kinase Regulates Cell Shape and Tendon Development

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INTRODUCTION: Throughout development and postnatal growth, resident tendon cells respond to mechanical cues from the nascent tendon extracellular matrix (ECM) to regulate tissue properties. Focal adhesion kinase (FAK, gene: *Ptk2*) is an intracellular protein kinase that regulates the actin cytoskeleton and cell-ECM adhesions. In tendon cells, FAK activity is required for tenogenic gene expression in response to growth factor stimulation and mechanical stretching.[1–3] In addition, pharmacological inhibition of FAK in explanted tendons significantly attenuates ECM to nuclei strain transmission.[4] Despite these known roles for FAK in tendon, the mechanism by which FAK activity regulates cell mechanotransduction as well as the role of FAK-dependent mechanotransduction in tendon development remain unknown. Therefore, the objective of this study was to evaluate the regulatory role of FAK in (1) tendon cell-ECM mechanical interactions and in (2) tendon development. We hypothesized that (1) FAK activity regulates tendon cell *in vitro* focal adhesion morphology and cell spreading behavior, and that (2) reduced FAK expression will negatively impact tendon development.

METHODS: ***In Vitro* Cell Culture**: Tail tendon cells were isolated from P30 WT male and female mice (3 mice in 2 independent experiments; n = 30 cells/treatment/mouse). Cells were cultured on fibronectin-coated coverslips and treated with a FAK inhibitor (10 μM PF-573228; FAK-I) or vehicle (DMSO) control. Immunofluorescence staining was performed to quantify cell morphology and pFAK localization 6 hours post-treatment. ***In Vivo* Mouse Model**: Tendon targeted FAK knockout (Scx-Cre;FAK$^{F/F}$; FAK-KO) mice were generated.[5] Achilles tendons (ATs), flexor digitorum longus tendons (FDLs), and patellar tendons (PTs) from P30 male and female FAK-KO and WT littermate controls were used for gene expression analysis, paraffin histology, and viscoelastic mechanical testing. Gene Expression: RNA was isolated from tendons to evaluate *Ptk2* expression using Taqman assays, with *Abl1* as a housekeeping control (n=6/genotype/sex). Paraffin Histology: Whole ankle and knee joints were fixed, decalcified, paraffin embedded, and sectioned in the sagittal plane (n=5/genotype/sex). Hoechst nuclear staining was used to quantify cell density and nuclear aspect ratio (nAR). Overall tissue morphology was evaluated via toluidine blue staining. Viscoelastic Mechanics: Tendon cross-sectional areas (CSAs) were measured (n=7-9/genotype/sex), and tendons were subjected to a viscoelastic mechanical testing protocol (preconditioning, viscoelastic stress relaxation and dynamic frequency sweep, and a quasi-static ramp to failure).

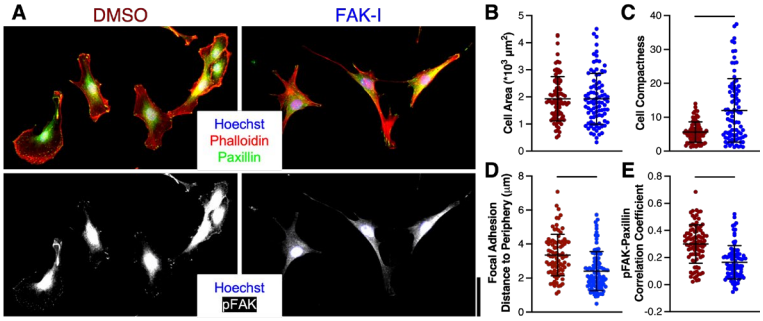

Figure 1. A) Representative images of DMSO and FAK-I treated tail tendon cells. Scale: 50 μm. B) Cell area, C) cell compactness, D) focal adhesion distance to the cell periphery, and E) pFAK-Paxillin correlation coefficient quantifications of DMSO and FAK-I treated tail tendon cells. Data was analyzed with t-tests comparing groups. Bars represent sig. diff. between groups ($p < 0.05$).

RESULTS: ***In Vitro* Cell Culture**: FAK-I treated cells developed pronounced cell protrusions compared to DMSO treated cells (Fig. 1A). While cell area was not different between groups (Fig. 1B), FAK-I treated cells had significantly higher cell compactness values relative to DMSO treated cells (Fig. 1C), which is indicative of the increased protrusion phenotype. Focal adhesions were closer to the cell periphery and colocalized less with pFAK staining in FAK-I treated cells relative to DMSO treated cells (Fig. 1D-E). ***In Vivo* Mouse Model**: Ptk2 expression was reduced in all FAK-KO tendons relative to WT tendons, thereby validating our conditional knockout mouse model (Fig. 2). FAK-KO tendons were not remarkably distinct from WT tendons histologically, and there were no differences between groups in quantified cell density or nAR (data not shown). Interestingly, FAK-KO tendons were consistently smaller compared to WT tendons (Fig. 3A), while there was no difference in animal body weight (body weight data not shown). Despite the decreased size in all FAK-KO tendons, stiffness was only decreased in PTs (Fig. 3B), and modulus was generally comparable and, in fact, superior in ATs (Fig. 3C). Failure properties of FAK-KO tendons demonstrated decreased maximum load in the AT and PT, while maximum stress was increased in the FDL (Fig. 3D-E). Viscoelastic stress relaxation was not different between groups (data not shown), though dynamic modulus was increased in all FAK-KO tendons relative to WT tendons at all frequencies evaluated (1Hz dynamic modulus data shown as representative in Fig. 3F).

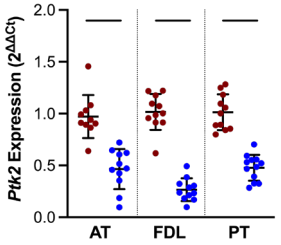

Figure 2. *Ptk2* expression for ATs, FDLs, and PTs from WT and FAK-KO tendons. Data was analyzed with t-tests comparing groups. Bars represent sig. diff. between groups ($p < 0.05$).

DISCUSSION: Consistent with our hypothesis, inhibition of FAK activity in tendon cells significantly affected focal adhesion morphology and cell spreading behavior. Taken together with our previous results demonstrating attenuated ECM to nuclear strain transmission with reduced FAK activity,[4] these findings help explain FAK's regulatory role on tenogenic gene expression.[1–3] In our *in vivo* model, all tendons from FAK-KO mice were smaller, which is consistent with our hypothesis and indicates that FAK plays an essential role in tendon development. Interestingly, structural properties were not consistently reduced, and material properties were comparable or increased in FAK-KO tendons relative to WT tendons, potentially indicating changes in matrix assembly.

SIGNIFICANCE: Due to the mechanical role and mechano-responsiveness of tendons, defining the key transductive pathways that regulate cell and tissue properties will be critical to better understand disease and to develop improved therapies. Our results indicate that FAK-dependent tendon cell mechanotransduction may drive tissue assembly during growth and development.

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REFERENCES: 1. Liu *et al.*, Stem Cells Int, 2018; 2. Xu *et al.*, J Cell Physiol, 2012; 3. Li *et al.*, J Orthop Res, 2019; 4. Leahy *et al.*, ORS Annual Meeting, 2022; 5. Beggs *et al.*, Neuron, 2003;

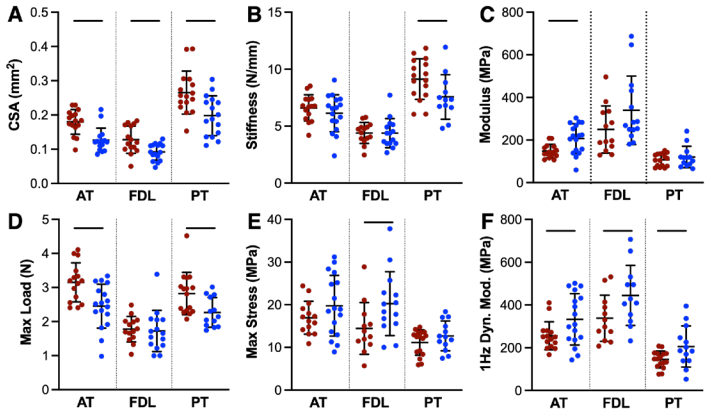

Figure 3. A) Cross-sectional area (CSA), B) stiffness, C) modulus, D) maximum load, E) maximum stress, and (F) 1Hz dynamic modulus measurements for WT and FAK-KO tendons. Data was analyzed with t-tests comparing groups. Bars represent sig. diff. between groups ($p < 0.05$).

Collagen III Haploinsufficiency Alters Fibril Size but Not Mechanical Properties in Uninjured, Young Adult Male Murine Tendons

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INTRODUCTION: Clinically, *Col3a1* mutations present as vascular Ehlers-Danlos syndrome (vEDS), a rare but life-threatening condition due to abnormalities in the matrix of the vasculature and hollow viscera. Additional patient morbidity from tendon pathology[1,2] indicates a consequential role for type III collagen (Col3) in tendon. While a role for Col3 in matrix homeostasis has been established in the vasculature,[3] cutaneous skin,[4] articular cartilage,[5] meniscus,[6] and bone,[7] the involvement of Col3 in tendon structure and function is poorly understood. Early investigations focused on understanding the role of Col3 in female murine tendon,[8] but biological sex is known to influence tendon health, Col3 levels,[9] and vEDS presentation,[10] motivating investigation of the role of Col3 in male tendon. Therefore, the objective of this study was to elucidate the role of Col3 in tendon homeostasis using a murine model of male vEDS. We hypothesized that Col3 haploinsufficiency would alter fibrillogenesis and fibril maintenance yielding decreased large diameter fibrils and mechanically inferior tendons.

METHODS: Tendons from male wild-type (WT) Balb/cJ and heterozygous *Col3a1*$^{+/-}$ mice at 90 days of age were assessed (IACUC approved). Patella-patellar tendon-tibia complexes were dissected and prepared as described[11] for mechanical testing ($n \geq 9$/group). Tendons were assessed with a viscoelastic testing protocol consisting of: 1) preconditioning, 2) stress relaxation at 3% and 6% strain with a subsequent sinusoidal frequency sweep (10 cycles at 0.1, 1, 5, and 10 Hz) at each strain level, 3) return to gauge length, and 4) ramp to failure at a strain rate of 0.1% strain/s. Images were captured during the ramp to failure for elastic modulus measurement. Patellar tendons were fixed, processed, and imaged using transmission electron microscopy as described[12] to measure collagen fibril diameters (n=3/group). T-tests were used to determine the impact of genotype on mechanical properties. A Kolmogorov-Smirnov test was used to assess the effect on collagen fibril diameter distributions. Significance was set at $p \leq 0.05$.

RESULTS: Compared to WT tendons, *Col3a1*$^{+/-}$ tendons were not significantly different in any quasistatic or viscoelastic mechanical property including stiffness (Fig 1A), elastic modulus (Fig 1B), dynamic modulus (Fig 1C), phase shift (Fig 1D), and percent relaxation (data not shown). In contrast, collagen fibril size distributions were significantly different between genotypes (Fig 2, $p < 0.0001$). WT tendons had a characteristic bimodal fibril diameter distribution (Q1: 70.0 nm, Q2: 116.5 nm, Q3: 154.2 nm). *Col3a1*$^{+/-}$ tendons had a tighter fibril diameter distribution (Q1: 80.6 nm, Q2: 125.0 nm, Q3: 154.0 nm) with a smaller proportion of small diameter fibrils (< 70 nm) and a greater proportion of intermediate and large diameter fibrils (> 110 nm).

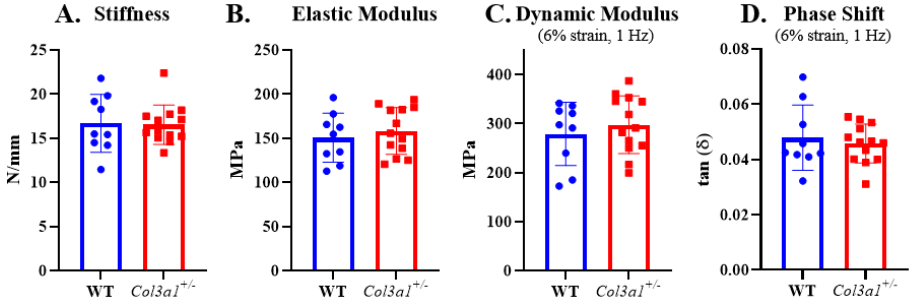

Figure 1. Collagen III haploinsufficiency does not impact male tendon mechanical properties. No differences in stiffness (A), elastic modulus (B), dynamic modulus (C), phase shift (D), or percent relaxation (data not shown) were observed between genotypes. No differences were seen in other strain or frequency levels (data not shown).

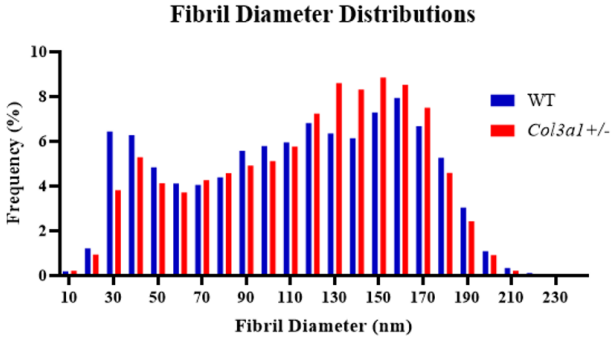

Figure 2. Collagen III haploinsufficiency alters collagen fibril size. Collagen fibril diameter distribution was characteristically bimodal in WT tendons. *Col3a1*$^{+/-}$ tendons had significantly different fibril distribution ($p < 0.0001$) with a lower proportion of smaller fibrils (< 70 nm) and a higher proportion of intermediate and large fibrils (> 110 nm).

DISCUSSION: We studied the role of Col3 in tendon homeostasis using a murine model of male vEDS. In contradiction to our hypothesis, Col3 haploinsufficiency in young adult male mice did not alter uninjured patellar tendon quasistatic or viscoelastic mechanical properties. However, in support of our hypothesis, Col3 haploinsufficiency did significantly alter the fibril diameter distribution. This alteration in matrix structure may have mechanical consequences in settings where fibrillogenesis and maintenance are altered, such as in injury and/or aging. Importantly, genotype-dependent changes to fibril diameter in male mice differ from those observed in female mice. In developing female mice, Col3 haploinsufficiency resulted in a decrease in large diameter fibrils, [8] in contrast to the increase in large diameter fibrils observed in the present study. Interestingly, this indicates a differential influence of biological sex on tendon matrix structure in the context of Col3 haploinsufficiency. Conclusions from this study should be interpreted in the context of a conventional haploinsufficiency mouse model which conflates developmental and regulatory effects. Moreover, dose-dependent effects of Col3 insufficiency cannot be investigated due to perinatal lethality of *Col3a1*$^{-/-}$ mice. Further, in recognition of the critical importance of Col3 in the provisional healing matrix, subsequent investigations will define the effects of Col3 knockdown in injury and advanced aging contexts. Future studies will leverage the power of inducible Col3 knockdown to further delineate the sex-, dose-, and age-dependence of the tendon response to Col3 knockdown in homeostatic and injury environments.

SIGNIFICANCE: In addition to direct implications for patients with *Col3a1* mutations, insights from this study reveal contributions of Col3 to tendon structure which serve as an important foundation for future investigations of sex-based differences in the regulatory role of Col3.

REFERENCES: [1] Byers et al. Am J Med Genet. 2017. Review. [2] Shirley et al. Sports Health. 2012. [3] Mizuno et al. J Biochem. 2013. [4] Volk et al. Cells Tissues Organs. 2011. [5] Wu et al. J Biol Chem. 2010. [6] Wang et al. Matrix Biol. 2020. [7] Miedel et al. J Orthop Res. 2015. [8] Carlson et al. ORS Poster. 2022. [9] Stevenson et al. Clin Interv Aging. 2007. [10] Cooper at al. Vet Pathol. 2010. [11] Miller et al. J Biomech Eng. 2012. [12] Dunkman et al. Matrix Biol. 2013.
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