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Effect of Age and Proteoglycan Deficiency on Collagen Fiber Re-Alignment and Mechanical Properties in Mouse Supraspinatus Tendon

Collagen fiber realignment is one mechanism by which tendon responds to load. Re-alignment is altered when the structure of tendon is altered, such as in the natural process of aging or with alterations of matrix proteins, such as proteoglycan expression. While changes in re-alignment and mechanical properties have been investigated recently during development, they have not been studied in (1) aged tendons, or (2) in the absence of key proteoglycans. Collagen fiber re-alignment and the corresponding mechanical properties are quantified throughout tensile mechanical testing in both the insertion site and the midsubstance of mouse supraspinatus tendons in wild type (WT), decorin-null ($Dcn^{-/-}$), and biglycan-null ($Bgn^{-/-}$) mice at three different ages (90 days, 300 days, and 570 days). Percent relaxation was significantly decreased with age in the WT and $Dcn^{-/-}$ tendons, but not in the $Bgn^{-/-}$ tendons. Changes with age were found in the linear modulus at the insertion site where the 300 day group was greater than the 90 day and 570 day group in the $Bgn^{-/-}$ tendons and the 90 day group was smaller than the 300 day and 570 day groups in the $Dcn^{-/-}$ tendons. However, no changes in modulus were found across age in WT tendons were found. The midsubstance fibers of the WT and $Bgn^{-/-}$ tendons were initially less aligned with increasing age. The re-alignment was significantly altered with age in the WT tendons, with older groups responding to load later in the mechanical test. This was also seen in the $Dcn^{-/-}$ midsubstance and the $Bgn^{-/-}$ insertion, but not in the other locations. Although some studies have found changes in the WT mechanical properties with age, this study did not support those findings. However, it did show fiber re-alignment changes at both locations with age, suggesting a breakdown of tendon's ability to respond to load in later ages. In the proteoglycan-null tendons however, there were changes in the mechanical properties, accompanied only by location-dependent re-alignment changes, suggesting a site-specific role for these molecules in loading. Finally, changes in the mechanical properties did not occur in concert with changes in re-alignment, suggesting that typical mechanical property measurements alone are insufficient to describe how structural alterations affect tendon's response to load. [DOI: 10.1115/1.4023234]

Keywords: collagen fiber re-alignment, age, proteoglycan, decorin, biglycan, polarized light, supraspinatus tendon

1 Introduction

Tendon has a hierarchical structure consisting mainly of collagen I organized into fibrils surrounded by an extracellular matrix of water, proteoglycans (PGs), and other proteins. The interplay within and between hierarchical structures plays a key role in modulating the response of tendon to mechanical load. While one might hypothesize that particular structural changes should lead to specific changes in the mechanical behavior of tendon, published data has been inconclusive. Specifically, the effects of: (1) age, and (2) proteoglycan removal on tendon biomechanics have been highly debated due to a number of conflicting studies. The goal of this study was to rigorously study these two effects by measuring

changes in the mechanical properties, coupled with tendon's response to load via re-alignment of collagen fibers.

Age-related changes in tendon biomechanics have been broadly studied, but no conclusion can be drawn from the current work. In rat tail tendons, there has been evidence to suggest that the ultimate load, modulus, and strength decrease with age [1], however, there has also been evidence to the contrary that the stiffness and strength of the tendon increases with age [2,3]. In rat patellar tendon, the mechanical properties correlated with age but there was no change over time in one study [4], and still another study showed a weaker tendon along with decreased fibril diameter with age [5]. Since the prevalence of injuries involving tendon ruptures increases with age [6], further investigation into the effects of age on tendon mechanical properties is necessary. An assessment of the re-alignment at later ages in life could provide clarification of these contradictory findings.

The role of small leucine rich proteoglycans and glycosaminoglycans (GAGs) in tendon's response to load has also been highly debated over the last decade. It has been speculated that PGs may

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mechanically connect adjacent collagen fibrils via core protein interactions or GAG interactions based on the structure of the GAG-PG-collagen complex and some modeling of tendon mechanical behavior [7,8]. While some *in vitro* work has shown that the addition of decorin, a major PG in tendon, improves the mechanical properties of self-assembled collagen fibrils [9], studies with enzymatic removal of GAGs in tendon and ligament have not provided conclusive evidence to support this mechanism [10–12]. Recently, the contribution of PGs to tendon mechanical properties has been closely studied, due to the ability to create transgenic mouse strains lacking the major proteoglycans in tendon, decorin, and biglycan [13]. Since PGs are known to play a role in the regulation of fibril structure during development [14,15], one might hypothesize that they may also affect the structure of tendon and, thus, the mechanical properties, throughout the aging process. Collagen fiber re-alignment has never been studied in these transgenic animals and could provide evidence for a role for proteoglycans in tendon's response to load.

Collagen fiber re-alignment is one mechanism by which tendon responds to load, where fibers re-orient themselves towards the direction of loading throughout the course of the mechanical testing protocol. This process is different from both fiber uncrimping, where the wavy appearance of collagen disappears with the application of load, and fiber sliding, where fibers slide past one another during loading. The re-orientation or re-alignment of collagen fibers is hindered at early ages when the interconnectivity of collagen fibrils is at a minimum. As the fibrils laterally and linearly fuse and the collagen molecules become more cross-linked, the tendons exhibit a quicker response to a smaller amount of load, suggesting maximal interfibrillar communication [16,17]. This suggests that fiber re-alignment could be an important measure of the structural alterations causing mechanical property changes. Fiber re-alignment has recently been studied throughout development in the mouse supraspinatus tendon [16]. The mouse supraspinatus model has several advantages, such as the ability to study transgenic animals, the ability to send polarized light through the tissue easily due to its small size and thickness, and the clinical relevance of the supraspinatus tendon.

Therefore, the objective of this study is clarify the effects of; (1) age, and (2) proteoglycan removal on tendon's response to load by measuring both the tensile mechanical properties and collagen fiber re-alignment of the mouse supraspinatus tendon throughout age using control (*WT*), decorin-null (*Dcn*^{-/-}) and biglycan-null (*Bgn*^{-/-}) mice. We hypothesize that: (1) tendons will get stiffer with age but will re-align later during the mechanical test as aging progresses, and (2) the mechanical and re-alignment changes will be altered in the proteoglycan-deficient mice.

2 Methods

2.1 Sample Preparation. This study was approved by the University of Pennsylvania IACUC. One hundred and four mice of C57/BL6 background in three different genotypes (wild type (*WT*), decorin-null (*Dcn*^{-/-}), biglycan-null (*Bgn*^{-/-}) and three different ages (90 days, 300 days, and 570 days) were euthanized and the supraspinatus tendons (SSTs) were removed for mechanical testing. All soft tissue was removed from around the tendon, leaving the supraspinatus muscle-tendon unit attached to the humerus. The supraspinatus muscle was then removed. Verhoeff stain lines were placed on the tendons denoting the insertion site and tendon midsubstance for regions of optical strain and alignment analysis (see Fig. 1(a)). The first stain line was placed at the tendon-to-bone insertion site on the humerus, visually determined, and the remaining stain lines were placed at 1, 2, and 3 mm from the insertion site. The tendon cross-sectional area was measured using a custom built device consisting of linear variable differential transformers (LVDTs), a CCD laser, and translation stages [18]. The top of the humeral head was carefully cut with a scalpel blade to prevent the blocking of polarized light from passing through the

tendon insertion site. The humerus was then embedded in a 1 in. diameter acrylic tube piece with the use of polymethylmethacrylate (PMMA). A second coating of PMMA was applied to prevent failure at the growth plate. The acrylic pot was inserted into a custom testing fixture. The proximal end of the tendon was glued between two pieces of sandpaper with an initial gauge length of 2.5 mm and placed in custom grips for tensile testing.

2.2 Mechanical Testing. The samples were placed in a room temperature phosphate buffered saline bath and loaded in a tensile testing system (Instron, Norwood, MA) integrated with a polarized light setup (Fig. 1(b)), consisting of a linear backlight (Dolan-Jenner, Boxborough, MA), 90 deg-offset rotating polarizer sheets (Edmund Optics, Barrington, NJ) on either side of the test sample, and a digital camera (Basler, Exton, PA) [19]. Prior to testing, the encoder embedded in the stepper motor (Lin Engineering, Santa Clara, CA) that rotates the polarizer sheets was initialized by resetting the encoder value with the polarizer sheets set at a position corresponding to 0 deg of angular rotation. To determine the biomechanical properties, tensile testing along the long axis of the tendon was performed with a 10 N load cell for the following protocol, shown in Fig. 1(c): preload to 0.02 N (section A), followed by preconditioning (10 cycles between 0.02 N and 0.04 N, section B), a 300 s hold (section C), followed by stress-relaxation to 5% strain at a rate of 5%/s for 600 s (section D), a return to zero-displacement and a 60 s hold (section E), and finally ramp to failure at 0.1%/s (section F). The loads at which these tendons were preconditioned were chosen based on pilot studies with intact, injured, and repaired rat supraspinatus tendons. These loads were selected in order to ensure that the tendons would not be damaged during preconditioning and so that comparisons could be made across different groups and different studies. Images were obtained every 5 s for optical strain analysis. Additionally, sets of 13 images were acquired at various points during the testing protocol (Fig. 1(c), nos.1-6) and every 20 s during the ramp to failure as the polarizers rotated through a 125 deg range for measurement of the fiber alignment during loading, as previously described. [16].

2.3 Data Analysis. A custom Matlab program (Matlab, Natick, MA) was used to optically track strain lines, as previously described [20]. The stress was calculated as force divided by initial area. A bilinear curve fit was also applied to the stress-strain data to quantify the transition stress, transition strain, and the moduli in the toe- and linear-regions from the optical stress-strain data. The modulus in the toe region was defined as the slope of the stress-strain curve from the start of the ramp-to-failure to the transition point. Similarly, the modulus of the linear region was defined as the slope following the transition point to a point just before failure.

The fiber alignment was calculated from the image sets as described [16,19]. Briefly, images of the tendon surface were divided into rectangular areas. Pixel intensities were summed by the area per image and plotted against the angle of polarizer rotation. A sine wave was fit to the intensity-angle data to determine the angle corresponding to minimum pixel intensity, which represents the average direction of the area's collagen fiber alignment. The circular variance (VAR), a measure of the distribution of collagen fiber alignment, was calculated for fiber distributions before preconditioning (Fig. 1(c), no. 1), after preconditioning (Fig. 1(c), no. 2), following the initial displacement of the stress relaxation (Fig. 1(c), no. 3), at the return to zero (Fig. 1(c), no. 4), and at the transition strain (Fig. 1(c), no. 5; the intersection of the toe- and linear-regions, determined using a structural fiber recruitment model at 50% fiber recruitment [21]), and at the linear-region strain (Fig. 1(c), no. 6, at 75% fiber recruitment) (see Fig. 1(c)). This previously published structural model is based on the uncrimping of closely packed collagen fibers, where a fiber is recruited only when it is uncrimped. In this model, 75% fiber recruitment is indicative of the middle of the linear region of the

Fig. 1 (a) Image of tendon showing stain lines for regional analysis, (b) testing setup of Instron integrated with polarized light system, and (c) mechanical testing protocol. Images were taken for alignment analysis at: (1) before preconditioning, (2) after preconditioning, (3) after the initial displacement of the stress relaxation (SR), (4) after a return to zero displacement, (5) at the transition strain, and (6) at a point in the linear-region. (Not to scale)

ramp to failure, which is an appropriate point to obtain alignment data representative of the entire region.

The initial alignment of the tissue was described by the circular variance of the specimen before preconditioning (Fig. 1(c), no. 1). Alignment, defined in this context, means how large the spread of the collagen fibers is, where a higher VAR value would indicate a less-aligned tendon, with more fibers spread over many angles. Alternatively, re-alignment of the fibers was also evaluated in this study, defined as the change in the VAR from one point in the test to the next point. Since circular variance is a measure of the spread of the fiber angles, a decrease in the VAR would indicate a smaller spread of angles, or that re-alignment of the fibers towards a single direction has occurred. All of the VAR values are positive since they are a real measure of the spread of the distribution, however, changes in the VAR could be positive or negative since re-alignment is defined as the change of the VAR from one point in the test to the next (see supplementary material (Figs. S1–S3) [22]). Both positive (becoming more aligned) and negative (becoming less aligned) re-alignment will be referred to as significant re-alignment for the purposes of this study. Re-alignment during preconditioning was evaluated by comparing the VAR values from before preconditioning (BP) and after preconditioning (AP) for each age and genotype. Similar methods were used to determine fiber re-alignment during the stress relaxation (SR), after a return to zero displacement following the SR, and in the toe- and linear-regions of the stress-strain curve.

2.4 Statistical Analysis. One-way ANOVAs were run, comparing across age within a genotype, followed by *post hoc* tests when significant. Mechanical parameters were evaluated using parametric statistics and the data is presented as mean $\pm$ standard deviation (SD). Statistical comparisons were made for genotype and tendon age within a location, i.e., no statistical comparisons between the insertion and the midsubstance were made. Comparisons were made between genotypes at each age and between ages within a genotype. Alternatively, Shapiro-Wilk tests indicated non-normally distributed data for the VAR values and, as a result, nonparametric statistical tests were used for evaluating the fiber re-alignment. The VAR data was analyzed as paired comparisons and is presented as the median $\pm$ interquartile range. Changes in fiber alignment (the Friedman test) were compared for tendon location (midsubstance versus insertion) and for the mechanical test region (preconditioning, stress relaxation, return to zero, toe- and linear-region) for each genotype and age. Bonferroni corrections were used for multiple comparisons in order to remain conservative in our data interpretation (significance $*p < 0.025$).

3 Results

3.1 Age-Related Changes in WT Tendons. The whole tendon mechanical properties measured in this study were the cross-sectional area, failure stress, and stress relaxation. While no

Fig. 2 Mechanical changes are present across age and across genotype in the (a) cross-sectional area, (b) percent relaxation, (c) transition strain at the insertion site, and (d) transition stress at the midsubstance

changes with age were found with age in the cross-sectional area (see Fig. 2(a)), there were age-related changes in both the failure stress and stress relaxation. The percent relaxation was significantly decreased at 300 days and 570 days when compared to 90days (see Fig. 2(b)). In addition, the 300 day WT tendons failed at a higher stress than both the 570 day and 90 day tendons (see the supplementary material (Fig. S4) [22]).

There were age-related local changes in both the transition strain and transition stress for the WT tendons, but no changes in moduli. The insertion site transition strain was significantly increased at 570 days when compared to 300 days (see Fig. 2(c)), while the transition stress at this site was unaffected by age. The transition strain of the midsubstance of the tendon was also unaffected, but the transition stress of the midsubstance was altered, with an increase in stress at 300 days when compared to 90 day tendons (see Fig. 2(d)). Unlike the transition strain and stress, there were no age-related changes in the WT tendons for either the toe-region modulus or linear-region modulus at the insertion site or the midsubstance (see Fig. 3).

While the material property changes with age were limited in the WT tendons, there were changes with age in both the alignment and re-alignment of collagen fibers. There was an increased circular variance in the midsubstance of the tendon at both 300 days and 570 days when compared to 90 days (see Fig. 4), which indicates an increase in the spread of the fiber angles corresponding to a decreased alignment of the fibers with age. The re-alignment of collagen fibers was significantly altered with age (see Fig. 5; also, see supplementary material (Fig. S1) [22]). The WT tendons at 90 days significantly re-aligned during preconditioning, the return to zero, and the linear region at the insertion site and during all five regions of the test at the midsubstance. At 300 days, both the insertion site and the midsubstance of the tendons re-aligned during the return to zero, toe-, and linear-regions of the mechanical test. The same response occurred in the insertion site at 570 days; the tendons significantly re-aligned re-aligned during the return to zero, toe-, and

Fig. 3 (a) Significant changes with age were found in the linear modulus at the insertion site with the Bgn^{-/-} and Dcn^{-/-} tendons. (b) No changes in the midsubstance linear modulus were found.

Fig. 4 The initial circular variance of collagen fibers in the midsubstance of the tendon increased with age in the *WT* and *Bgn*^{-/-} tendons, denoting a less aligned tendon with age (larger distribution of fiber angles)

linear-regions. However, at the midsubstance of the 570 day tendons, no significant re-alignment event occurred.

3.2 Changes in Null Tendons With Age. The cross-sectional area, stress relaxation, and failure stress were all altered with age in the *Bgn*^{-/-} and *Dcn*^{-/-} null tendons. The tendon cross-sectional area was increased in the *Bgn*^{-/-} tendons from 300 days to 570 days and decreased across all ages in the *Dcn*^{-/-} tendons (see Fig. 2(a)). At 300 days, the *Bgn*^{-/-} tendons were smaller than the *WT* and the *Dcn*^{-/-} tendons. In addition, the 300 day *Bgn*^{-/-} tendons failed at a higher stress than the 90 day tendons (see the supplementary material (Fig. S4) [22]). The percent relaxation of the whole tendon was decreased in the *Dcn*^{-/-} tendons when comparing the 300 day and 570 day tendons to the 90 day tendons, in the same fashion as the *WT* tendons (see Fig. 2(b)).

There were no changes in the transition stress, transition strain, or toe modulus with age in the null tendons, however, there were significant changes in the linear modulus at the insertion site (see Fig. 3(a)). The linear modulus at the insertion site was increased in the *Bgn*^{-/-} tendons at 300 days, compared to 90 days and 570 days and in the *Dcn*^{-/-} tendons at 300 days and 570 days compared to 90 days. However, there were no significant changes in the linear modulus at the midsubstance of the tendons (see Fig. 3(b)).

There were changes in both the initial alignment and re-alignment with age in the null tendons. The initial alignment was decreased (the circular variance increased) at 570 days in the *Bgn*^{-/-} tendons relative to 90 days (see Fig. 4). There were no significant changes in the initial alignment in the *Dcn*^{-/-} tendons. At all three ages, the insertion site of the *Dcn*^{-/-} tendons re-aligned during the return to zero, toe-, and linear-regions of the mechanical test (see Fig. 6; also, see the supplementary material (Fig. S2) [22]). At the midsubstance, 90 day tendons re-aligned at each point of the test except the toe-region. At 300 days re-alignment only occurred during the return to zero and the linear-region and at 570 days re-alignment occurred during the stress relaxation, return to zero, and the linear-region. In the *Bgn*^{-/-} tendons, the midsubstance of all three ages re-aligned during the stress relaxation, return to zero, and linear-region of the test (see Fig. 7; also, see the supplementary material (Fig. S3) [22]). At the insertion site of the 90 day *Bgn*^{-/-} tendons, re-alignment occurred during each point of the test except the toe-region, while the 300 day tendons re-aligned during the stress relaxation, return to zero, and linear-region and the 570 day tendons re-aligned only during the return to zero and linear-region.

4 Discussion

The purpose of this study was to answer two closely-related questions: (1) what is the effect of age on the ability of tendon to respond to load, and (2) how is that effect of age altered with the

Fig. 5 Re-alignment of wild type (*WT*) tendons at later ages shows a progressively later response of tendons to the application of load in both the insertion site and the midsubstance. Data is presented as a representative sample for each group with population statistics noted. Each line connects the alignment at one point of the mechanical test to the alignment at the next point, i.e., the 'B' segment connects the 'before preconditioning (no. 1 in Fig. 1)' point to the 'after preconditioning (no. 2 in Fig. 1)' point. This segment therefore represents the change in alignment, or the re-alignment, occurring during the preconditioning segment of the mechanical test. A significant change in re-alignment is represented as a line segmented in bold with a significance star (* $p < 0.025$).

Fig. 6 Re-alignment of decorin-null (*Dcn*^{-/-}) tendons at later ages shows a progressively later response of the tendons to the application of load at the midsubstance but not at the insertion site. Data is presented as representative samples with population statistics for nonparametric data.

removal of key proteoglycans. To address these questions, we studied whole tendon and local mechanical properties, along with the alignment and re-alignment of collagen fibers, first in *WT* animals and then in *Dcn*^{-/-} and *Bgn*^{-/-} animals. Contrary to recent studies, only minimal mechanical property changes were found in

the *WT* tendons with age, with the exceptions of the percent relaxation and transition point. In the *WT* tendons, there is a decrease in the percent relaxation, which is indicative of viscoelastic behavior, at 300 days that remains at 570 days. There has been some support for the idea that the dynamic properties of tendon

Fig. 7 Re-alignment of biglycan-null (*Bgn*^{-/-}) tendons at later ages shows a progressively later response of the tendons to the application of load in the insertion but not in the midsubstance. Data is presented as representative samples with population statistics for nonparametric data.

change with age [23], however, more evidence is required to draw conclusions from this result. Although there were no changes in the insertion or midsubstance moduli for either the toe- or linear-region of the mechanical test in the *WT* tendons, there were age related changes in the transition strain at the insertion and transition stress at the midsubstance in these tendons (see Fig. 2). At the insertion site, the 570 day tendons had a significantly higher transition strain, indicating that they responded later to the application of load with a delayed transition from the toe to the linear region. At the midsubstance, the transition stress was increased at 300 days, when compared to 90 days. This suggests that a higher stress was required to transition to the linear region. Although no changes in moduli were found, molecular changes in collagen that naturally occur with age, such as increased molecular cross-linking and decreased thermal stability [24], could affect the fibril-fibril interactions (changes on a hierarchical scale closer to the molecular level) more than whole tendon mechanical properties. The changes in the local transition strain/stress and re-alignment of collagen fibers together could suggest that there is a diminished capacity of the tendon to respond to load that is not indicated in the mechanical properties alone.

Overall, changes in the initial alignment and re-alignment in the *WT* tendons showed a decreased response to load with age. The initial fiber alignment in the midsubstance of the *WT* tendons was decreased with age and there was a diminished response to the application of load via re-alignment at both locations. This result, easily seen in the *WT* midsubstance (see Fig. 5; also, see the supplementary material (Fig. S1) [22]), is most notably indicated by the progressively later time point (in terms of the region of the test) where the first significant re-alignment occurs. Tendons at 300 days and 570 days do not re-align their fibers until the return to zero region of the test, whereas the 90 day tendons re-align at the first application of load. A delayed response to load could be indicative of a breakdown of the structural organization of tendon over time and is a mirror effect of that seen during development [16]. Since these changes in re-alignment with age did not occur in concert with the changes in the mechanical properties, these results suggest that typical mechanical property measurements alone are insufficient to describe how structural alterations affect tendon's response to load. Since re-alignment is a structural measure, changes in structure on a lower hierarchical scale may not always lead to changes in whole tendon mechanical properties, which is a potential explanation behind the mixed results in the literature. Additionally, these changes are likely location-dependent (insertion site versus midsubstance) and tendon-dependent, which could also explain the disagreement of the published work in whole tendon properties [1–3,5].

When looking at the null tendons throughout age, there were no differences in the transition stress or strain, however, there were significant differences in the linear modulus (see Fig. 3). The *Bgn*^{-/-} tendons had a significantly increased linear modulus at the insertion site of the 300 day tendons when compared to the 90 day and 570 day groups. One possible explanation is that the *Bgn*^{-/-} tendons are at their strongest at 300 days, before the effects of aging take place. This trend is seen in all of the data presented here, where the *Bgn*^{-/-} tendons seem to be elevated or decreased at 300 days, compared to the other ages, suggesting that 300 days could be a midpoint for the effects seen throughout life. The *Dcn*^{-/-} tendons had an increase in the linear modulus at the insertion site of 300 day tendons and 570 days, compared to 90 day tendons. This could mean that the absence of decorin in aging tendons increases the strength or stiffness of aged tendon, perhaps to counteract the effect of delayed re-alignment seen throughout age. These effects could also be explained by changes in fibril diameter throughout age, similar to changes seen in the development of tendons in *Dcn*^{-/-} animals [25].

Notable changes in re-alignment with age were found between the *Dcn*^{-/-} and *Bgn*^{-/-} tendons and the *WT* tendons (see Figs. 6 and 7; also, see the supplementary material (Figs. S2 and S3) [22]). Both the insertion site and the midsubstance of the *WT* ten-

dons re-aligned during preconditioning, while re-alignment only occurred in one of the locations for each null animal (midsubstance for *Dcn*^{-/-}, insertion site for *Bgn*^{-/-}). This suggests a diminished capacity to respond to load at the *Dcn*^{-/-} insertion and the *Bgn*^{-/-} midsubstance, but not in the other locations. While a diminished capacity at those locations is present when comparing three genotypes at 90 days, this capacity does not degrade further after 90 days. This supports a role of decorin and biglycan in the aging process and implies that the lack of these PGs may shield the tendon from deteriorating effects. Since it is already known that the effect of loss of these proteoglycans changes from one tendon to another [26], it is not surprising that the effect is also altered from the insertion site to the midsubstance along a single tendon. However, this could also advocate for a stronger role for decorin in the insertion site, where high loads are transferred in a small area of transitional tissue. Recent studies analyzing the size of dermatan sulfate filaments found location-dependent length and orientation of filaments between collagen fibrils in horse superficial digital flexor tendon [27], providing further evidence for location-dependent functions of decorin. Similarly, these results could also advocate for a stronger role for biglycan in the midsubstance where water flow and viscoelasticity play a large function.

Although changes in the mechanical properties with the removal of the PGs and glycosaminoglycans have been debated [10–13], this study shows that decorin and biglycan contribute to tendon's response to load. While there were minimal differences between genotypes at each age (supporting previous studies negating the role of PGs and GAGs in tendon mechanics), there were changes with the effect of aging on those properties and changes in the re-alignment of collagen fibers. Recent studies suggest that GAGs and PGs may play a bigger role in fiber-fiber and fiber-matrix interactions at low strain levels, which is supported in this study, where changes in fiber re-alignment were found at small strain changes, such as preconditioning and in the toe region [28]. Overall, this data suggests that mechanical property measurements alone do not fully account for the functional changes that occur with structural alterations, especially when concerning alterations at much lower hierarchical levels.

Despite the interesting results of this study, it is not without limitations. Specifically, the function of decorin and biglycan cannot be fully elucidated in conventional transgenic models, which are inherently confounded by developmental and compensatory effects. While these models do present an excellent platform for studying natural aging without these molecules, further research could utilize inducible knockout models, allowing for a focused investigation of the impact during aging. In addition, performing additional biological and histological assays could further elucidate the mechanisms governing the changes found in this study. Compositional assays, such as collagen content or proteoglycan content, and structural assays, such as fibril diameter and distribution, could explain the changes between the three genotypes, especially since these assays have never been performed on a mouse supraspinatus tendon at any age. This study focused on the fibril level and whole tendon mechanical behavior, however, there are several other possible structural features that exist, such as the assembly of structural features from the fibril to the whole tendon. The fibrils form fiber networks that can branch and rotate during development [29]. These branches could help to stabilize the tendon's structure; thus likely affecting the whole tendon mechanical properties. Furthermore, while this study measured fibril re-alignment, it did not investigate fibril uncrimping or fibril sliding during mechanical loading, which are two processes that contribute to the mechanical behavior of tendon.

The data analysis in this study also has some limitations. Due to the small size of these tendons and the inherent variability of tissue, the variability in the data is large. This is especially evident in the bilinear fit analysis, which determines the transition strain, transition stress, toe modulus, and linear modulus. In a sensitivity analysis, it was determined that the transition stress and toe modulus can change appreciably with only small perturbations in

standard deviation. Despite sufficient power based on *a priori* analysis, this could suggest that changes in these particular parameters that were not detected in this study could exist. While there is a large variance in some of the results, that variance is consistent among the parameters studied and is common in these types of studies. Importantly, even with conservative Bonferroni corrections for multiple comparisons, large and significant changes in both the mechanical properties and fiber re-alignment were able to be detected, which provides confidence in our findings.

In conclusion, this study aimed to answer two closely-associated questions: (1) what is the effect of age on tendon's response to load, and (2) how is that effect of age altered with the removal of key PGs, such as decorin and biglycan. This study addresses these goals by quantifying local collagen fiber re-alignment and mechanical properties throughout tensile mechanical testing in wild type, decorin-null, and biglycan-null mouse supraspinatus tendons at three different ages (90 days, 300 days, 570 days). Although some studies have found changes in the mechanical properties with age, this study did not support that claim in the *WT* tendons, however, did show re-alignment changes at both locations with age that suggested a breakdown of tendon's ability to respond to load with later ages. In the proteoglycan-deficient tendons, however, there were changes in the linear modulus at the insertion site and changes in the cross-sectional area over time. These changes were only accompanied by re-alignment changes in the *Dcn*^{-/-} midsubstance and the *Bgn*^{-/-} insertion site, suggesting a location-dependent role for these molecules in loading. Finally, changes in the mechanical properties did not occur in concert with changes in the re-alignment, suggesting that typical mechanical property measurements alone are insufficient to describe how structural alterations affect tendon's response to load.

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