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Influence of Decorin on the Mechanical, Compositional, and Structural Properties of the Mouse Patellar Tendon

The interactions of small leucine-rich proteoglycans (SLRPs) with collagen fibrils, their association with water, and their role in fibrillogenesis suggests that SLRPs may play an important role in tendon mechanics. Some studies have assessed the role of SLRPs in the mechanical response of the tendon, but the relationships between sophisticated mechanics, assembly of collagen, and SLRPs have not been well characterized. Decorin content was varied in a dose dependent manner using decorin null, decorin heterozygote, and wild type mice. Quantitative measures of mechanical (tension and compression), compositional, and structural changes of the mouse patellar tendon were evaluated. Viscoelastic, tensile dynamic modulus was increased in the decorin heterozygous tendons compared to wild type. These tendons also had a significant decrease in total collagen and no structural changes compared to wild type. Decorin null tendons did not have any mechanical changes; however, a significant decrease in the average fibril diameter was found. No differences were seen between genotypes in elastic or compressive properties, and all tendons demonstrated viscoelastic mechanical dependence on strain rate and frequency. These results suggest that decorin, a member of the SLRP family, plays a role in tendon viscoelasticity that cannot be completely explained by its role in collagen fibrillogenesis. In addition, reductions in decorin do not cause large changes in indentation compressive properties, suggesting that other factors contribute to these properties. Understanding these relationships may ultimately help guide development of tissue engineered constructs or treatment modalities. [DOI: 10.1115/1.4006200]

Keywords: proteoglycans, structure-function, decorin knockout, tendon mechanics

Introduction

Tendon is a dynamic tissue with specialized mechanical properties and a complex composition and structure. It is defined by collagen fibrils organized in a hierarchical manner. It is composed of collagens, proteoglycans, glycoproteins, water, and cells with the extracellular matrix (ECM) components providing structural integrity to the tissue. The composition and structure of tendon are important in the proper mechanical function of the tissue, and change in their makeup results in altered mechanics both in functional adaptation and in response to injury.

The incidence of tendon injuries in the active and aging population is increasing [1] and these injuries are of concern because they heal slowly and rarely regain normal function. Surgery, rehabilitation, medication, and tendon grafts have all been employed to improve healing with limited success [2–5]. Problems such as adhesion formation, re-ruptures, and decreased function are still common, most likely due to the complexity in recreating the natural tissue architecture and resulting mechanical behavior [5,6]. However, understanding of how individual tendon constituents

and their structural arrangement affect mechanical properties in mature, uninjured tendon is limited and only becomes more complex in the response to injury. A fundamental, quantitative understanding of these relationships is needed to aid in the development of targeted pharmaceuticals or tissue engineered constructs aimed at recreating normal tissue architecture.

Tendon ECM can be described as a biocomposite material consisting of collagen fibrils surrounded by an extrafibrillar matrix with interactions between the fibrillar and extrafibrillar matrix. Many studies have focused on the role of the fibrils in determining the tensile properties of tendon; however, fibrils alone cannot completely explain the viscoelastic and nonlinear response of tendon [7–9]. Small leucine-rich proteoglycans within the ECM interact with collagen fibrils and other matrix molecules, as well as associate with water. These interactions have implicated them as having major roles in fibrillogenesis and in tendon mechanics. Further, variations in the SLRP content and type are observed based on the type of loading environment the tendon sees in vivo. The SLRP family of proteoglycans (PGs) is structurally similar, but genetically distinct and includes decorin, biglycan, fibromodulin, and lumican. The most common SLRP in tendon is decorin, which accounts for ~80% of the SLRPs in the tensile region of tendon [10].

Decorin regulates collagen fibrillogenesis. Mice null in decorin have previously been shown to have dysfunctional regulation of fibril assembly resulting in larger and more irregular fibril diameters than those in wild type mice where the magnitude of these changes is tissue and age specific [11]. Other studies have shown

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varying results on the role of SLRPs in tendon mechanics, again based on the specific tendon and age studied [11–13]. For instance, in 8–10 week old mice, decorin null mice had increased tensile modulus in the patellar tendon but not in the tail tendon fascicle or flexor digitorum longus (FDL) [12]. However, a clear definition of whether the mechanical changes were a result of alterations in the collagen hierarchy or the inherent effect of the reduction of SLRPs has not been elucidated. Additionally, these studies only evaluated tensile static properties and percent relaxation. Due to the structural location of the SLRPs between collagen fibrils and their association with water/tissue hydration, the SLRPs may play a more complex role in tendon viscoelasticity, which has not been fully evaluated.

The objective of this study was to quantitatively evaluate the structural, compositional as well as tensile and compressive mechanical changes related to alterations in decorin content. Decorin content is varied in a dose dependent manner using decorin null (*Dcn*^{−/−}), decorin heterozygote (*Dcn*^{+/-}), and wild type (WT) mouse patellar tendons. We hypothesized that in the decorin null mice compared to wild type, tensile elastic and viscoelastic properties would be increased and compressive properties decreased. In the decorin heterozygotes, tensile viscoelastic properties also would be increased and compressive properties decreased but to a lesser extent than the null mice. In both null and heterozygote mice, fibril diameters would be increased with a larger spread. Finally, we hypothesized that no changes would be seen in total collagen content.

Methods

Animals. This study was approved by the University of Pennsylvania and the University of South Florida IACUCs. Three different genotypes in a C57Bl/6 background were used and included female wild type (WT, *n* = 34), decorin heterozygotes (*Dcn*^{+/-}, *n* = 34), and decorin null (*Dcn*^{−/−}, *n* = 31) mice [14]. Decorin null and heterozygote mice had a targeted disruption of the decorin gene leading to an absence or single allele expression of this SLRP and have been described previously [14]. WT mice were obtained from Jackson Laboratories (Bar Harbor, ME). *Dcn*^{−/−} and *Dcn*^{+/-} mice were bred in house using approved protocols. Mice were sacrificed at 5 months of age to ensure skeletal maturity but before the effects of aging had begun [15]. Whenever possible, mechanical, structural, and compositional assays were performed in the same mouse. Patellar tendons were randomly dissected from either the left or right limb and assigned to biomechanical, structural, or compositional analysis. For all compositional and structural assays, tendons were cleanly dissected following sacrifice and bisected longitudinally. Each half sample was randomly assigned to a compositional or structural assay.

Tensile Mechanics. To determine the tensile biomechanical properties of the patellar tendons, tendons were removed and carefully dissected under a fine dissection microscope leaving the patella-tendon-tibia complex intact (WT *n* = 14; *Dcn*^{+/-} *n* = 14; *Dcn*^{−/−} *n* = 14). The cross sectional area was measured in the tendon midsubstance using a custom built device consisting of LVDTs, a CCD laser, and translation stages [16]. The tibia was potted in polymethylmethacrylate (PMMA), and a metal staple was placed over the tibial plateau to prevent failure at the growth plate. The tendon was coated uniformly with small speckles of Verhoeff stain using a fine bristled brush to create a textured appearance in the midsubstance for local optical strain analysis. The potted tibia was gripped and the patella held in a custom fixture, which ensured gripping of the patella bone without pinching the tendon. Specimens were submerged in a 37 °C PBS bath and tensile tested (model 5848, Instron; Norwood, MA) using a 10 N load cell. Samples were strained to 1%, preconditioned between 0.5–1.5% strain for 10 cycles at 0.25 Hz and allowed to relax at zero strain for 300 s before beginning viscoelastic testing. A stress

Fig. 1 Viscoelastic mechanical testing protocol

relaxation to 4% strain at 5%/s with a 10 min hold for relaxation was performed. Immediately following, the tendon was subjected to 10 sinusoidal strain cycles (frequency sweep) at 0.01, 0.1, 1, 5, and 10 Hz with an amplitude of 0.125% (Fig. 1) [17]. The stress relaxation and frequency sweep were repeated at 6 and 8%. Following the frequency sweep at 8% strain, specimens were returned to the preload displacement and held for 5 min before a ramp to failure at 0.1%/s was applied. Using the applied speckled coating, local tissue strain in the tendon midsubstance was measured optically with a custom MATLAB program.

A bilinear fit was applied to the ramp to failure test to obtain the elastic properties toe and linear stiffness, toe and linear modulus, and the transitional displacement or strain defined by the break-point in the bilinear fit. Peak and equilibrium load were determined from the stress relaxation tests at each strain level and percent relaxation was subsequently calculated from these values. For the dynamic response to the sinusoidal input, the last five load-displacement cycles at each frequency/strain combination were analyzed by transforming the data into the frequency domain using a fast Fourier transform. The magnitude and phase of the prevailing frequency for both the displacement and load was determined at each frequency and strain level using a custom written MATLAB program. The same process was applied to the stress-strain data to determine material properties. Dynamic stiffness, Eq. (1), was calculated as the ratio of the magnitude of the load over the displacement, dynamic modulus, Eq. (2), as the magnitude of the stress over the strain, and phase angle, Eq. (3), was defined as the difference between the corresponding phase shifts as follows where A_σ is the amplitude of the stress curve, A_ϵ is the amplitude of the strain curve, and ϕ_σ and ϕ_ϵ are the corresponding phase shifts.

$$|k^*| = \frac{A_{\text{load}}}{A_{\text{displacement}}} \quad (1)$$

$$|E^*| = \frac{A_\sigma}{A_\epsilon} \quad (2)$$

$$\phi = \phi_\sigma - \phi_\epsilon \quad (3)$$

Compressive Mechanics. For compressive testing, tendons were removed and carefully dissected under a fine dissection microscope leaving the patella-tendon-tibia complex intact (WT *n* = 13; *Dcn*^{+/-} *n* = 12; *Dcn*^{−/−} *n* = 14). Specimens were attached to a testing stage using cyanoacrylate and flooded with PBS. This attachment creates a fixed boundary condition for subsequent data analysis [18]. A 26SW gauge steel wire was used to create a custom made, flat ended, cylindrical indenter with a diameter, *a*, of 0.018 in. (0.4572 mm). The indenter was oriented perpendicular to the tissue surface for all tests. The indenter was lowered in two locations on the cyanoacrylate, and the displacements averaged to determine the location of the test stage. Indentation testing was performed (model 5848, Instron; Norwood, MA) with a 5 N load cell. A tare load of −0.002 N was applied, and the thickness of the tendon was defined from that point to the previously measured testing stage. Six incremental indents of 4% strain at 0.3%/s were applied with 1200 s of relaxation between each step. Pilot studies confirmed that at this loading rate, the indenter did not pierce the tissue. A Poisson's ratio of 0.5, ν_{loading} , was assumed for the loading portion of the curves [19], and the Hayes model [18] with correction for finite deformation [20] was

used to calculate shear modulus, Poisson's ratio of the solid matrix, and peak and equilibrium modulus. Data from the third indentation corresponding to 12% strain was used for analysis. Shear modulus, Eq. (4), solid matrix Poisson's ratio, Eq. (5), and the instantaneous and equilibrium moduli, Eqs. (6) and (7), were calculated using (P/w) , the slope of the loading or equilibrium curves respectively [21]. κ is a correction factor dependent on a and h , the indenter diameter and tissue thickness, as well as the Poisson's ratio [20].

$$\mu = \frac{(P/w)_{\text{loading}}(1 - \nu_{\text{loading}})}{4a\kappa} \quad (4)$$

$$\nu_s = 1 - \frac{4a\kappa\mu}{(P/w)_{\text{equil}}} \quad (5)$$

$$E_{0+} = 2\mu(1 + \nu_{\text{loading}}) \quad (6)$$

$$E_{\infty} = 2\mu(1 + \nu_s) \quad (7)$$

Total Collagen Content. Total collagen was assessed using a hydroxyproline assay. Tendons (WT $n=20$; $Dcn^{+/-}$ $n=14$; $Dcn^{-/-}$ $n=18$) were dried at 65 °C for 24 h. Dry weights were obtained followed by tissue digestion in 5 mg/mL proteinase K solution at 65 °C for 18 h. For acid hydrolysis, samples were sealed in glass vials with 6 N HCL and heated to 110 °C for 16 h. Upon completion of hydrolysis, samples were neutralized by evaporating off HCL in a lyophilizer with NaOH and then resuspended in water. o-Hydroxy-proline (OHP), a measure of collagen content, was determined colorimetrically by reaction of the digest with p-benzaminoaldehyde and chloramine-T. OHP content was converted to total collagen content using a 1:14 ratio of OHP to collagen [22] and normalized by dry weight.

Gene Expression. Expression levels of biglycan, decorin, lumican, and fibromodulin were quantified using a real-time quantitative polymerase chain reaction assay (qPCR) (WT $n=18$; $Dcn^{+/-}$ $n=16$; $Dcn^{-/-}$ $n=15$). Immediately following dissection and bisection, tendon samples were flash frozen in liquid nitrogen and stored at -80 °C until analysis. Individual samples were mechanically homogenized with a mortar and pestle in RNase-free conditions. RNA was extracted using the TRIZOL isolation system (Invitrogen; Carlsbad, CA) and RNeasy Micro Kit (Qiagen Inc.; Valencia, CA) as described by the manufacturers. cDNA was produced by reverse transcription-polymerase chain reaction (RT-PCR) and amplified using the WT-Ovation RNA Amplification System as described by the manufacturer (Nugen; San Carlos, CA). For qPCR analysis, 5 ng of cDNA was amplified in a 25 μ L reaction volume in an ABI Prism 7300 Sequence Detection System (Perkin-Elmer; Applied Biosystems, Warrington, UK) with a SYBR Green PCR Master Mix (Applied Biosystems). Mouse specific primers for β -actin (Act- β : F- AGATGACCCAGATCATGTTTGAGA; R- CACAGCCTG-GATGGCTACGT), decorin (Dcn: F- GCTGCGGAAATCC-GACTTC; R- TTGCCGCCCAGTTCTATGAC), biglycan (Bgn: F- CCTTCCGCTGCGTTACTGA; R- GCAACCACTGCCTC-TACTTCTTATAA), fibromodulin (Fmod: F- GAAGGGTTGT-TACGCAAATGG; R- AGATCACCCCTAGTCTGGGTTA) and lumican (Lum: F- TCCACTTCCAAAGTCCCTGCAAGA; R- AAGCCGAGACAGCATCTCTTTGA) were used. Each individual patellar tendon sample was analyzed in triplicate. Gene specific efficiencies were calculated [23] for each qPCR plate and the relative quantity of mRNA for each gene of interest was computed using the comparative efficiency^(-ACT) method (ABI Sequence Detection System User Bulletin #2) relative to Act- β .

Transmission Electron Microscopy. The structural parameters of mean fibril diameter and fibril spread were assessed using transmission electron microscopy (TEM) (WT $n=18$; $Dcn^{+/-}$ $n=17$; $Dcn^{-/-}$ $n=18$). Following sacrifice, the patellar tendon was viewed and bisected. The half assigned to TEM was left

within the joint, and the entire leg was fixed in situ in 4% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M sodium cacodylate, pH 7.4 with 8.0 mM CaCl_2 for 15 min at room temperature [24]. The tendon was removed from the joint and fixation continued at 4 °C for 100 min. The tendons were post-fixed with 1% osmium tetroxide and dehydrated in an ethanol series followed by propylene oxide. The tendons were infiltrated over a period of 3 days and embedded in a mixture of Embed 812, nadic methyl anhydride, dodecenylsuccinic anhydride and DMP-30 (Electron Microscopy Sciences; Fort Washington, PA). Thick sections (1 μ m) were cut and stained with methylene blue-azur B for examination and selection of specific regions for analysis. Thin sections were prepared using a Leica ultramicrotome and a diamond knife followed by post-staining with 2% aqueous uranyl acetate followed by 1% phosphotungstic acid pH 3.2. Cross sections of tendons were examined at 80 kV using a JEOL 1400 transmission electron microscope (JEOL Ltd.; Tokyo, Japan) equipped with an Orius widefield sidemount CCD camera (Gatan Inc.; Pleasanton, CA).

For each tendon, micrographs were taken in the midsubstance, away from any fibers that may have been cut during tissue bisection. For analyses, 5-6 images per tendon were taken at 60,000 $\times$ from nonoverlapping regions of the central portion of the tendon. All fibrils within a predetermined region of interest (ROI) on the digitized image were measured. The ROI was placed such that 80-196 fibrils/field were measured. Fibril diameters were measured using a custom MATLAB program. All fibrils in the region of interest were measured and multiple regions of interest were used if necessary to collect at least 80 fibril diameter measurements per micrograph. Fibril diameters were determined along the minor axis of the fibril profile. For each tendon, the quantitative parameters of mean fibril diameter and fibril diameter distribution (coefficient of variation) were reported.

Statistical Analysis. For the elastic parameters of moduli, stiffness, and transition points, total collagen, gene relative expression, and TEM structural parameters, a one-way ANOVA across genotype with Bonferroni post hoc tests was performed. For percent relaxation, a two-way ANOVA across genotype and repeated strain level was performed with Bonferroni post hoc tests. For viscoelastic parameters in the frequency sweep, a three-way ANOVA comparing the effects of genotype, strain level, and repeated frequencies was performed. For significant interaction terms, simple effects were analyzed using a Bonferroni post hoc analysis. Significance for all ANOVAs was set at $p \leq 0.05$ and a trend at $0.05 < p \leq 0.1$. Significance for post hoc tests using Bonferroni corrections was set at $p \leq 0.05$ corrected by the number of comparisons n and is, therefore, evaluated as $p \leq 0.05/n$.

Results

Tendons from all three genotypes were grossly normal. However, the cross-sectional area of the patellar tendon was significantly reduced in the mutant genotypes (WT $0.25 \pm 0.03 \text{ mm}^2$, $Dcn^{+/-}$ $0.19 \pm 0.04 \text{ mm}^2$, $Dcn^{-/-}$ $0.22 \pm 0.04 \text{ mm}^2$, mean $\pm$ SD). Smaller patellar tendons were found in both $Dcn^{-/-}$ and $Dcn^{+/-}$ compared to wild type ($p \leq 0.05/3$ versus WT).

Tensile Mechanics. Contrary to our hypothesis, no differences between wild type and $Dcn^{-/-}$ tendons were seen in any tensile elastic parameter (Table 1). In addition, no differences were seen in $Dcn^{+/-}$ compared to wild type tendons in any of the elastic parameters including toe region stiffness and modulus, linear region stiffness and modulus, or transition displacement and strain. In the viscoelastic property percent relaxation, a trend was noted due to the effect of genotype (Fig. 2). Percent relaxation was shown to significantly decrease with increasing strain level, and all combinations of strain levels were different from each other (4% versus 6%, 4% versus 8%, 6% versus 8%).

From the frequency sweep tests, dynamic modulus was significantly affected by genotype. There was an interaction between

Table 1 Tensile elastic parameters, mean $\pm$ SD

	Toe stiffness (N/mm)	Linear stiffness (N/mm)	Stiffness transition point (mm)
WT	0.99 $\pm$ 0.30	12.26 $\pm$ 1.96	0.19 $\pm$ 0.02
<i>Dcn</i> ^{+/-}	0.95 $\pm$ 0.48	12.26 $\pm$ 2.12	0.20 $\pm$ 0.02
<i>Dcn</i> ^{-/-}	1.09 $\pm$ 0.51	12.66 $\pm$ 1.92	0.21 $\pm$ 0.03

Fig. 2 Percent relaxation was significantly decreased with increasing strain level. There was a trend toward an effect of genotype. Mean and standard deviation shown. Strain level significantly different ($p \leq 0.05/3$) from 4% (*) and 6% (#), respectively.

frequency and genotype with Bonferroni post hoc tests for simple effects showing that *Dcn*^{+/-} had a significantly higher dynamic modulus than wild types at all frequency levels but not *Dcn*^{-/-} (Fig. 3). In addition, dynamic modulus increased with increasing strain level and frequency, and there was a significant interaction between the two. Simple effects tests show that at a given frequency, all strain levels are significantly different from each other, i.e., 4% versus 6%, 4% versus 8%, 6% versus 8% (Fig. 4(a)). Also, at a given strain level, all frequencies are significantly different from each other except 0.01 Hz versus 0.1 Hz at 6% strain and 0.01 Hz versus 0.1 Hz and 5 Hz versus 10 Hz at 8% strain (Fig. 4(b)). The dependence of dynamic modulus on frequency can be difficult to visualize graphically due to sample variation (Fig. 4(b)), but due to the design of the study, repeated measures ANOVA testing allows for analysis of the within sample variation. When each sample is visualized separately, the frequency dependence is clearer (Fig. 5). Similar findings can be seen in all genotypes.

Fig. 3 At every frequency level, *Dcn*^{+/-} is significantly increased from WT. Mean and standard deviation shown pooled across strain level. At the given frequency, $p \leq 0.05/3$ * *Dcn*^{+/-}.

Fig. 4 (a) At every frequency level, dynamic modulus significantly increased with increasing strain level. (b) At a given strain level, all combinations of frequencies were significantly different from each other (paired comparisons) except 0.01 Hz versus 0.1 Hz at 6% and 8% as well as 5 Hz versus 10 Hz at 8%. Mean and standard deviation shown pooled across all genotypes. At the given frequency level, $p \leq 0.05/3$ for * 4% versus 6%, # 4% versus 8%, +6% versus 8%. At the given strain level, bar denotes $p \leq 0.05/10$, paired comparisons.

pendence is clearer (Fig. 5). Similar findings can be seen in all genotypes.

No difference was seen in dynamic stiffness as a function of decorin genotype (Table 2). However, similar to dynamic modulus, there was a significant increase in dynamic stiffness with increasing strain and frequency as well as an interaction between the two. Simple effects tests show that at all frequencies, there is a significant difference between all strain levels (4% versus 6%, 4% versus 8%, 6% versus 8%), and at a given strain level, all frequencies are significantly different from each other except 1 Hz versus 10 Hz at 4%, 0.01 Hz versus 0.1 Hz at 6%, and 0.01 Hz versus 0.1 Hz and 5 Hz versus 10 Hz at 8% strain.

Similarly, no change was seen in the phase shift across decorin genotypes (Table 3). Phase shift was significantly decreased with increases in both strain and frequency with a significant interaction between the two. At 0.1 Hz and 1 Hz, all strain levels were significantly different from each other (Fig. 6(a)). At 0.01 Hz, 5 Hz, and 10 Hz, only 4% versus 6% and 4% versus 8% were different. At a given strain level, all frequencies were significantly different from each other except 0.01 Hz versus 0.1 Hz at 4% (Fig. 6(b)). Finally, a significant interaction between frequency and genotype was seen in the measurement of phase shift denoting that the change with frequency differs based on genotype.

Compressive Mechanics. Decorin genotypes were shown to have no significant effect on any compressive property, contrary to our hypothesis (Table 4). This included compressive shear modulus, peak modulus, equilibrium modulus, and Poisson's ratio of the solid matrix.

Table 2 Dynamic stiffness, mean $\pm$ SD (N/mm)

		Freq (Hz)				
		0.01	0.1	1	5	10
4%	WT	5.03 $\pm$ 2.24	5.2 $\pm$ 2.24	5.59 $\pm$ 2.36	5.72 $\pm$ 2.42	5.61 $\pm$ 2.45
	<i>Dcn</i> ^{+/-}	6.43 $\pm$ 2.71	6.85 $\pm$ 3.15	7.32 $\pm$ 3.35	7.50 $\pm$ 3.44	7.39 $\pm$ 3.48
	<i>Dcn</i> ^{-/-}	5.58 $\pm$ 4.16	5.92 $\pm$ 4.39	6.36 $\pm$ 4.58	6.52 $\pm$ 4.69	6.42 $\pm$ 4.74
6%	WT	11.05 $\pm$ 2.72	11.18 $\pm$ 2.92	11.72 $\pm$ 3.05	12.00 $\pm$ 3.12	11.96 $\pm$ 3.16
	<i>Dcn</i> ^{+/-}	12.34 $\pm$ 3.54	12.49 $\pm$ 3.39	13.21 $\pm$ 3.66	13.56 $\pm$ 3.77	13.53 $\pm$ 3.81
	<i>Dcn</i> ^{-/-}	10.76 $\pm$ 5.23	11.27 $\pm$ 5.34	11.88 $\pm$ 5.55	12.18 $\pm$ 5.67	12.14 $\pm$ 5.72
8%	WT	16.63 $\pm$ 2.52	17.23 $\pm$ 3.61	17.97 $\pm$ 3.68	18.41 $\pm$ 3.77	18.43 $\pm$ 3.79
	<i>Dcn</i> ^{+/-}	17.45 $\pm$ 3.36	17.68 $\pm$ 3.67	18.53 $\pm$ 3.88	19.02 $\pm$ 4.02	19.04 $\pm$ 4.06
	<i>Dcn</i> ^{-/-}	16.39 $\pm$ 4.73	16.64 $\pm$ 4.63	17.37 $\pm$ 4.76	17.79 $\pm$ 4.89	17.80 $\pm$ 4.93

Table 3 Phase shift, mean $\pm$ SD (Degrees)

		Freq (Hz)				
		0.01	0.1	1	5	10
4%	WT	3.25 $\pm$ 1.12	3.37 $\pm$ 1.05	2.67 $\pm$ 0.65	2.39 $\pm$ 0.53	2.31 $\pm$ 0.97
	<i>Dcn</i> ^{+/-}	3.79 $\pm$ 1.31	3.64 $\pm$ 0.88	2.40 $\pm$ 0.25	2.07 $\pm$ 0.27	1.76 $\pm$ 0.44
	<i>Dcn</i> ^{-/-}	3.68 $\pm$ 1.60	3.99 $\pm$ 1.85	2.76 $\pm$ 0.89	2.42 $\pm$ 0.74	2.01 $\pm$ 0.76
6%	WT	2.39 $\pm$ 0.45	2.09 $\pm$ 0.46	1.75 $\pm$ 0.25	1.62 $\pm$ 0.34	1.35 $\pm$ 0.75
	<i>Dcn</i> ^{+/-}	2.66 $\pm$ 0.70	2.41 $\pm$ 0.18	1.98 $\pm$ 0.15	1.76 $\pm$ 0.28	1.32 $\pm$ 0.34
	<i>Dcn</i> ^{-/-}	2.91 $\pm$ 0.85	2.25 $\pm$ 0.54	1.89 $\pm$ 0.26	1.59 $\pm$ 0.22	1.13 $\pm$ 0.24
8%	WT	2.13 $\pm$ 0.61	1.84 $\pm$ 0.21	1.71 $\pm$ 0.18	1.60 $\pm$ 0.46	1.38 $\pm$ 0.83
	<i>Dcn</i> ^{+/-}	2.84 $\pm$ 0.64	2.07 $\pm$ 0.12	1.84 $\pm$ 0.11	1.58 $\pm$ 0.15	1.04 $\pm$ 0.08
	<i>Dcn</i> ^{-/-}	2.50 $\pm$ 0.64	1.90 $\pm$ 0.29	1.67 $\pm$ 0.21	1.33 $\pm$ 0.12	0.91 $\pm$ 0.18

Total Collagen Content. The total collagen content was significantly affected by change in decorin genotype (WT 837.9 $\pm$ 267.3 μ g/mg, *Dcn*^{+/-} 546.0 $\pm$ 199.4 μ g/mg, *Dcn*^{-/-} 652.8 $\pm$ 275.6 μ g/mg, mean $\pm$ SD). Specifically, there was a significant reduction in total collagen in the *Dcn*^{+/-} compared to wild type tendon. The difference between *Dcn*^{-/-} and wild type tendons was not significant.

Gene Expression. Relative expression of decorin mRNA was significantly decreased in *Dcn*^{-/-} compared to that in both wild type and *Dcn*^{+/-} tendons. A trend toward decreased expression was found in the *Dcn*^{+/-} tendons versus wild type tendons (Fig. 7(a)). A trend also was seen with the effect of decorin genotype on the

Table 4 Compressive parameters, mean $\pm$ SD

	WT	<i>Dcn</i> ^{+/-}	<i>Dcn</i> ^{-/-}
μ (kPa)	11.5 $\pm$ 7.6	9.0 $\pm$ 4.1	12.1 $\pm$ 7.6
ν	0.37 $\pm$ 0.10	0.41 $\pm$ 0.06	0.39 $\pm$ 0.05
E ₀₊ (kPa)	34.6 $\pm$ 22.9	26.8 $\pm$ 12.4	36.3 $\pm$ 22.9
E _∞ (kPa)	31.2 $\pm$ 20.4	25.2 $\pm$ 11.8	33.4 $\pm$ 20.7

relative expression of biglycan (Fig. 7(b)). No change in the relative expression of fibromodulin or lumican was seen between any of the decorin genotypes (Fig. 7(c) and 7(d)).

Fig. 5 Dynamic modulus of representative WT samples at 4% strain demonstrating frequency dependence. Similar trends were seen in all genotypes.

Transmission Electron Microscopy. Overall, collagen fibrils were observed to have uniformly circular cross sections (Fig. 8). In some fibrils, the outer contours were irregular; however, this was not noted to differ between genotypes and was minor in comparison to previous studies in the tail tendon [11]. The mean tendon fibril diameter was significantly affected by decorin genotype (WT 95.4 $\pm$ 7.1 μ m, *Dcn*^{+/-} 98.0 $\pm$ 5.3 μ m, *Dcn*^{-/-} 90.1 $\pm$ 7.7 μ m, mean $\pm$ SD). The *Dcn*^{-/-} tendons had significantly smaller fibril diameters than WT and *Dcn*^{+/-} tendons. A trend was noted due to the effect of genotype on the fibril diameter spread (coefficient of variation) (WT 0.42 $\pm$ 0.04 mm, *Dcn*^{+/-} 0.41 $\pm$ 0.04 mm, *Dcn*^{-/-} 0.43 $\pm$ 0.03 mm, mean $\pm$ SD). Qualitatively, the fibril diameter distribution histogram from *Dcn*^{-/-} tendons demonstrated an increase number of smaller fibril diameters compared to wild type and resulted in a more bimodal appearance of the distribution (Fig. 9).

Discussion

This study quantitatively evaluated the changes in mature mouse patellar tendon mechanical, compositional, and structural parameters as a function of decorin content using decorin mutant

Fig. 6 (a) At all frequencies, phase shift is significantly decreased between 4% and 6% as well as 4% and 8%. At 0.1 Hz, 1 Hz it is also decreased between 6% and 8%. (b) At a given strain level, all frequency combinations are significantly different from each other (paired comparisons) except 0.01 Hz versus 0.1 Hz at 4%. At the given frequency level, $p \leq 0.05/3$ for * 4% versus 6%, # 4% versus 8%, + 6% versus 8%. Mean and standard deviation shown pooled across all genotypes. At the given strain level, bar denotes $p \leq 0.05/10$, paired comparisons.

Fig. 7 The relative expression of decorin was significantly reduced between the WT and $Dcn^{-/-}$ groups as well as between $Dcn^{+/-}$ and $Dcn^{-/-}$. (b) There was a trend toward an effect of genotype in the biglycan relative expression ($p = 0.1$). No significant difference was found in (c) fibromodulin or (d) lumican. Mean and standard deviation shown. Bar denotes $p \leq 0.05/3$.

genotypes ($Dcn^{-/-}$, $Dcn^{+/-}$) compared to wild type ($Dcn^{+/+}$). This study is the first to evaluate these parameters concurrently in mature tendons and will lead to a better understanding of structure-function relationships in tendon. This study aims to

Fig. 8 Representative TEM images of fibril cross sections in (a) wild type, (b) $Dcn^{+/-}$, and (c) $Dcn^{-/-}$. Scale bar 200 nm.

describe relationships between mechanics and both the physical role of SLRPs in vivo and the changes that occurred during collagen fibrillogenesis.

No changes were seen in the elastic tensile properties of either $Dcn^{-/-}$ or $Dcn^{+/-}$ tendons compared to wild type. These results are in contrast to previous studies. In 8-10 week old mice, increases in linear region modulus were found in $Dcn^{-/-}$ patellar tendons but not in mouse tail tendon fascicles or the FDL [12]. However, studies in older mice have shown that changes in mechanical properties are age dependent. For instance, in the FDL, the linear region modulus between WT and $Dcn^{-/-}$ was different at 5 months of age but not 2 months of age [11]. These studies demonstrate the importance of considering age and specific tendon type when drawing conclusions across studies as well as the need to examine compositional changes in the specific model to fully characterize the changes in mechanics, especially when analyzing mutant genotypes.

Interestingly, the smaller fibril population in the null mice is not directly reflected as a change in any of the mechanical parameters. It is possible that this is a result of the overlapping functions

Fig. 9 Histograms of fibril diameters. Qualitatively (a) the shape of the *Dcn*^{+/-} distribution is similar to WT while (b) the *Dcn*^{-/-} has an increased small fibril population

of both smaller collagen fibrils and decorin, the only measured changes in the *Dcn*^{-/-} genotype. Smaller diameter collagen fibrils are thought to resist creep [25], and in this case, the increase in smaller diameter fibrils may balance the mechanical consequences of removing decorin. On the other hand, in the *Dcn*^{+/-} mice, where the fibril characteristics are similar to WT in TEM measurements and total collagen is lower than WT, a significantly stiffer dynamic response is seen (Fig. 3(d)), a combination of both viscous and elastic components. This could be a combined result of both changes in collagen content and decorin; however, if collagen were the primary player in this mechanical response, a decreased linear and/or dynamic modulus corresponding with less collagen would have been expected, which was not observed. It has been hypothesized that SLRPs are responsible for maintaining space between collagen fibrils, and a reduction in the decorin content would allow the collagen fibrils to interact more, reducing fibril sliding and resulting in a stiffer response [26], and these results support this concept. In addition, these results suggest that the ratio of collagen to SLRP may be more important than either parameter alone in determining structure-function relationships [27,28].

In the viscoelastic properties, decorin genotype did not significantly affect phase shift in contrast to our hypothesis (Table 3). Phase shift is a measure of the viscoelastic damping of the material and for a linearly elastic solid, $\delta = 0$ and for a viscous fluid, $\delta = 90$ deg [29]. No change in phase shift suggests that the mechanical damping of the tissue is not altered with decreases in decorin content. However, a trend toward increased percent relaxation with a reduction in decorin in the mutant genotypes was noted, and in a previous study, *Dcn*^{-/-} tail fascicles were shown to relax significantly more than WT tail fascicles [9]. These combined results are surprising since SLRPs are hydrophilic and bind

water, and therefore both phase angle and percent relaxation would be expected to change [30]. Previous studies have shown varying results when investigating this phenomenon. It has been demonstrated that although patellar tendons relax more with more water (hypotonic versus hypertonic test baths), [31] fascicles and collagen fibrils do not show changes in energy dissipation with changes in environmental salts [32]. It is possible that the relationship between collagen, SLRPs, and water are more complex than evaluated here. For instance, as mentioned above, a decrease in PGs may cause both an increase in fibril-fibril frictional effects and allow for adhesion between collagen fibrils, thus influencing internal damping in multiple ways [26]. Additional research is needed on multiple scales (sub-fibril, fibril, fiber, fascicle, etc.) to fully understand these relationships.

Overall, the viscoelastic parameters determined in this study exhibited strain level dependence (Fig. 4(a)). Dynamic stiffness and modulus increased with increasing strain level. These results suggest that as the tendon is increasingly strained, more of the tissue is engaged, which results in a stiffer dynamic response. At this point, it is unknown how much of that response is from the fibrillar matrix versus extrafibrillar matrix although the dominating component may differ at each strain level (i.e., the extra-fibrillar matrix at low strains and the fibrillar at high strains). Also, phase shift and percent relaxation generally decreased with increasing strain (Figs. 2, and 6(a)). These results suggest that at lower strain levels, fluid moves more easily in and out of the tissue. At higher strain levels, the matrix is tensioned and the fluid cannot flow through the tissue as easily. These strain level dependencies demonstrate the nonlinear nature of the tissue, an important characteristic needed for describing tendon in mathematical modeling [8,33].

All properties measured during the frequency sweep also demonstrated a small, but significant influence of frequency (i.e., Figs. 5, and 6(b)). It should be noted that due to the constant amplitude during the frequency sweep, both frequency dependence and strain-rate dependence are incorporated in this measure. Previous studies have shown similar frequency/strain rate dependence in the bovine Achilles tendon [34] and human MCL [35].

Surprisingly, no changes were seen in any of the compressive properties of the patellar tendon in the genotypes with decreased decorin compared to wild type tendons. Instantaneous modulus is the apparent tissue modulus under fluid pressurization. It is a result of both the fibrillar and extra-fibrillar networks and is indicative of how the solid matrix is retaining the fluid [36]. Although compression tests were performed transverse to the fiber direction, due to fluid pressurization, collagen tensile properties still play a role. The equilibrium modulus in indentation, on the other hand, is after the fluid flow has ceased, and although it is less dependent on the collagen network, it is not independent. In addition, it is also affected by the confining effects of the surrounding tissue and fluid [37]. Finally, the shear modulus μ is a flow-independent property and, when compression tests are performed transverse to the fiber direction as they were in this study, is a description of the extra-fibrillar matrix.

Evaluating the sensitivity of the current compression study given an $\alpha = 0.05$ and $\beta = 0.8$, it was possible to detect a $\sim 30\%$ change from WT with this sample size. Since compensatory increases of SLRPs were not noted, results from this study suggest that at this age, decorin does not play a large enough role in the nonfibrillar matrix stiffness to overcome the effects of collagen tensile properties and the confining effects of the surrounding tissue, contrary to our hypothesis. This is not to say that decorin does not play a role in time-dependent properties. A previous study in the porcine MCL showed that intrinsic permeability, which is not correlated with either instantaneous or equilibrium moduli [36], is significantly increased after sulfated GAG digestion [38]. Future studies will continue investigating the role of SLRPs/GAGs in controlling the time-dependent fluid flow both through the tissue and parallel to the collagen fibrils.

In conclusion, this study quantitatively characterized the mechanical, compositional, and structural properties of mature

tendon at a defined age and in a specific tendon with changes in the decorin content. Through this mouse model, both inherent effects of decorin in vivo as well as changes in collagen structure were explored. This study demonstrated that changes in collagen content and structure cannot completely account for changes in tendon viscoelastic properties, and a complex relationship with decorin exists. Also, reductions in decorin do not cause large changes in compressive properties suggesting that other factors contribute to these properties.

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