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Sexual Dimorphism in the Effect of GDF6 Deficiency on Murine Tendon

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Abstract

Three members of the growth/differentiation factor (GDF) subfamily of bone morphogenetic proteins, GDFs 5, 6, and 7, have the potential to augment tendon and ligament repair. To gain further insight into the in vivo role of these molecules, previous studies characterized intact and healing tendons in mice with functional null mutations in GDFs 5 and 7. The primary goal of the present study was to perform a detailed characterization of the intact tendon phenotype in 4 and 16 week-old, male and female, GDF6 -/- mice and their +/+ littermates. The results demonstrate that GDF6 deficiency was associated with an altered tendon phenotype that persisted into adulthood. Among males, GDF6 -/- tail tendon fascicles had significantly less collagen and glycosaminoglycan content, and these compositional differences were associated with compromised material properties. The effect of GDF6 deficiency on tendon was sexually dimorphic, however, for among female GDF6 -/- mice, neither differences in tendon composition nor in material properties were detected. The tendon phenotype that was observed in males appeared to be stronger in the tail site than in the Achilles tendon site, where some compositional differences were present, but no material property differences were detected. These data support existing in vitro studies, which suggest a potential role for BMP13 (the human homologue to GDF6) in tendon matrix modeling and/or remodeling.

Keywords

Tendon; Mechanics; GDF6; BMP13; Mouse

INTRODUCTION

The bone morphogenetic proteins (BMPs) are well known for their multifunctional roles in the musculoskeletal system. Members of one BMP subfamily in particular, the growth/differentiation factors (GDFs) 5, 6, and 7, appear to play a role in tendon and ligament biology[1-6], although the precise nature of this role remains to be determined. When delivered in different forms in different animal and injury models, GDFs 5, 6, and 7 showed enhanced tendon/ligament repair[7-15], thus offering the potential for clinical use in humans. To gain further insight into the in vivo roles of these molecules, investigators sought to characterize intact and healing tendons in naturally occurring or genetically engineered mice deficient in individual members of this growth factor family[16-21].

To date, the best characterized of these knockout animals is the GDF5 deficient *brachypodism* mouse[19]. Eight week-old male mice deficient in GDF5 have compromised Achilles tendon matrix composition and mechanical behavior, and display a delay in healing

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when recovering from a full tenotomy[17, 18]. Tail tendon fascicles from these animals exhibit abnormal ultrastructural morphology of collagen fibrils, which is accompanied by altered time-dependent stress-relaxation behavior of the fascicles[16].

Adult, 16 week-old, GDF7 deficient mice of both sexes display a more subtle tendon phenotype, with a modest (but significant) reduction in Achilles tendon proteoglycan content and a slight shift toward smaller collagen fibrils, but these differences do not appear large enough to manifest themselves in any differences in material properties[20]. Tail tendons do not exhibit notable abnormalities in composition or material properties[21]. This relatively minimal phenotype may be partly due to slight over-expression of GDF5 in the tails of GDF7 deficient tendons, but further investigation is required[20, 21].

A GDF6 deficient knockout mouse line has been developed[22], but poses distinct challenges in that the post-natal mortality rate of GDF6 -/- animals is exceptionally high (knockouts are produced in Mendelian ratios prenatally, but represent only 2-5% of the offspring at four weeks of age, and, in our own colony of this line, most do not survive into adulthood). Despite these challenges, a limited characterization of tail tendon composition and mechanics from a small cohort (n = 8) of four week-old male GDF6 -/- animals from this strain showed a 33% reduction in tail tendon collagen (Hyp/DNA) and a 45-50% reduction in material properties[23]. Sub-fascicle collagen fiber size was qualitatively smaller in histological sections from -/- tail tendons. A preliminary assessment of Achilles tendon composition showed a 45% reduction in collagen (Hyp/DNA). GAG/DNA was also lower in -/- animals in both the tail and Achilles sites (-15%), but these differences were not significant. Three limitations of this study include the lack of sufficient female mice for analysis, insufficient tissue for material property characterization in Achilles tendons, and the inclusion of only very young (4 week-old) animals.

Despite the limitations of existing studies, GDF6 deficient mice clearly display a strong tendon phenotype in four week- old males. But the high mortality rates make it infeasible to perform a comprehensive assessment of tendon structure/function phenotype, and it be impossible to examine tendon healing with so few available animals. By out-crossing the existing line onto a more mixed genetic background, we have improved colony vigor and increased longevity, thus enabling a more comprehensive baseline characterization of the effect of GDF6 deficiency in tendon. Consequently, we sought to answer the following questions. Does GDF6 deficiency result in a significant tendon phenotype that persists into adulthood? Does increasing the mixed nature of the background strain affect the magnitude of the gene effect? Further, is the effect modulated by sex, age, and/or anatomical location?

METHODS

Eight groups of animals were studied, encompassing 3 independent variables: age (4 and 16 weeks), sex, and genotype (GDF6 -/- and +/+). Out-crossing resulted in a mixed 129SV/J × C57Bl6/J × CBA background. Four week-old animals were chosen to represent the youngest age at which mechanical testing of Achilles and tail tendons could be performed prior to sexual maturity. Sixteen week-old animals were chosen to capture a time point sufficiently after sexual maturity, yet before senescence. Animals were euthanized via CO_2 inhalation in accordance with Institutional Animal Care and Use Committee guidelines and immediately weighed. To characterize tendon phenotype, 2 sites were examined: the Achilles tendon and the tail tendon fascicles. 10 animals per group were used for mechanical testing, 10 animals per group were used for compositional analyses at both sites, and 4 to 8 animals per group were used to examine GDF5 and GDF7 gene expression for the possibility of compensation by family members.

Mechanical Testing

Left and right Achilles tendons were tested in tension to failure using a materials testing system (Model 5542 Instron Corp., Canton, MA) at a strain rate of 100%/sec, as previously described[20]. Prior to the test, tendon diameter was measured at the midsubstance of the gage length using an optical LED micrometer (LS-7501, Keyence Corp., Woodcliff Lake, NJ), and cross-sectional area calculated assuming a circular cross section. Strain was measured as grip-to-grip-displacement normalized to initial gage length. Tendons were preloaded to 0.1N, but no preconditioning was performed. The following dependent parameters were calculated: structural strength (load to failure); structural stiffness; energy to failure; ultimate strength; elastic modulus; failure strain; and strain energy density. Values from the left and right sides were averaged to provide representative values for each animal. For material property determination of tail tendon fascicles, 5 individual fascicles were randomly chosen by alternating between the four tendon bundles of the tail and tested to failure in tension at a strain rate of 50%/sec using methodology described elsewhere[21]. A 20 mm gage length was used for all 4 week-old fascicles; a 30 mm gage length was used for fascicles from 16 week-old mice. The same dependent parameters as for the Achilles tendon were calculated. Values from each of the 5 fascicles per tail were averaged to provide a representative value for each animal.

Compositional Analysis

In 10 mice per group, bulk biochemical composition was measured on Achilles tendon and tail tendon fascicle bundles. Tissue was harvested and placed in 500μl of papain digest per tendon or tendon bundle (125μg/ml papain in 1X PBE, pH 6.5) for 18 hrs at 60°C. DNA content was quantified using the Hoechst 33258 dye method[24], and fluorescence intensity detected using a FLX 800 Multi-Detection Microplate Reader (Bio-Tek Instruments Inc., Winooski, VT) using calf thymus DNA as a standard. Glycosaminoglycan (GAG) content was determined using a dimethylmethylene blue assay with dermatan sulphate as a standard[25]. To assess total collagen content, Hydroxyproline was determined using a dimethylaminobenzaldehyde assay following acid hydrolysis in 6N hydrochloric acid at 110° for 24 hrs, with purified hydroxy-L-proline as a standard[26]. Each sample was analyzed in duplicate for all assays and average values used for analysis. GAG and hydroxyproline values were normalized to DNA content to account for differences in sample size.

Gene Expression

GDF5 and GDF7 gene expression analysis was performed using the Quantitative Real Time Polymerase Chain Reaction protocols.[20, 21]. Achilles tendons (left and right) and tail tendons (all 4 bundles) from 4-8 animals per group were harvested within 10 minutes of death and placed in 500μl of RNA*later* (Qiagen 76154, Qiagen Corp., Valencia, CA). Tendons were incubated overnight at 4°C and then stored at -80°C. RNA was isolated and purified using the RNeasy Fibrous Tissue Mini Kit (Qiagen 74704; Qiagen Corp.). For each sample, duplicate analyses of QRT-PCR for GDF5, GDF7 and 18S rRNA (endogenous control) were performed using an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). RT-PCR reactions were set up using Quantitect RT-PCR Master Mix (Qiagen 204443, Qiagen Corp.), forward and reverse primers, and fluorogenic probes. TaqMan Gene Expression Assays were ordered from ABI (Applied Biosystems) with the following catalog numbers (GDF5 inventoried #Mm00433564_m1; GDF7 inventoried #Mm00807130_m1). After initiating the amplification reaction[21] and determining the threshold cycle value (C_t) for each sample, the relative amount of mRNA for each gene of interest was computed using the comparative $2^{-\Delta\Delta Ct}$ method[27], with the first Δ being the C_t value of each gene relative to internal 18S rRNA and the second Δ relative to wild type within each age, site, and sex group.

Statistical Analysis

All dependent variables were analyzed using a 3-factor ANOVA with genotype (GDF6 +/+ and -/-), sex, and age (4 and 16 weeks) as the independent variables (Statview 5.0, SAS Institute Inc., Cary, NC). If the interaction of genotype and sex was significant, males and females were subsequently analyzed separately using a 2-factor ANOVA within each sex cohort, with genotype and age as the two independent variables. If, however, the interaction of genotype and sex was not significant, males and females were pooled to examine the role of genotype and age on the dependent variable in question. For QRT-PCR data, statistical analysis was performed on $\Delta\Delta Ct$[28] values only if a ≥ two-fold difference in expression level was seen. A value of $p \leq 0.05$ was used for significance.

RESULTS

Colony Statistics

The out-crossing of the original GDF6 line[22] onto a mixed 129SV/J × C57Bl6/J × CBA background slightly increased the ratio of -/- animals that were produced (up from 4% of males and 2% of females in the original line to 9% and 7%). More significantly, mortality rates decreased, with about 90% of all -/- animals surviving in good health. Prior to out-crossing, we were unable to keep GDF6 -/- animals alive beyond about 5 weeks.

Morphometric Parameters

Sex was a significant modulator of the genotype effect on body mass ($p < 0.0001$ for sex × gene), thus males and females were subsequently analyzed separately. In both, GDF6 deficiency was associated with a lower body mass, although this effect was more pronounced in males (-30 to 35% vs. -20%, Figs. 1 A & B). For both sexes, genotype was a significant modulator of the age-related increases in body mass, with a greater percentage difference in body mass between genotypes present at 4 weeks.

Because sex was not a significant modulator of genotype for the remaining morphometric parameters (Achilles tendon cross-sectional area and length and tail tendon fascicle cross-sectional area), males and females were pooled to examine the genotype and age effects on these parameters. For all three, GDF6 deficiency was associated with a significantly smaller size, with greater differences observed between genotypes at 4 weeks than at 16 weeks (Figs. 1 C-E).

Tail Tendon Fascicle Composition and Mechanics

Because the interaction of sex and genotype was significant for both compositional parameters in the tail ($p = 0.008$ for GAG/DNA and $p = 0.023$ for Hyp/DNA), each sex was analyzed separately to determine the effect of genotype and age on composition. GDF6 -/- tail tendon fascicles from males had significantly less GAG/DNA (-26% & -23% at 4 & 16 weeks) and Hyp/DNA (-22% & -13% at 4 & 16 weeks; Fig. 2 A). Although the percentage difference between -/- and +/+ tails decreased with age, the interaction of age and genotype was not significant for either parameter. Among females, genotype did not have a significant effect on tail tendon composition (Fig. 2 B).

Male GDF6 -/- tail tendon fascicles had significantly lower values of all material properties, other than those associated with yielding (Table 1; Fig. 3A). Genotype did not have a significant effect on any material property in females (Table 2; Fig. 3 B).

Achilles Tendon Composition and Mechanics

The effect of genotype on composition was similar to that seen in the tail tendon (Fig. 4). Sex was a significant modulator of genotype for both GAG/DNA and Hyp/DNA, thus each sex was analyzed separately ($p = 0.024$ for genotype x sex for GAG/DNA, and $p = 0.0006$ for Hyp/DNA). In males, GDF6 -/- Achilles tendons had less Hyp/DNA (30-34%) and GAG/DNA (10-20%) than wild type tendons, although the differences in GAG were not significant ($p = 0.090$; Fig. 4 A). Among females, genotype did not have a significant effect on Achilles tendon composition (Fig. 4 B).

In contrast to the tail tendon, the interaction of sex and genotype was not significant for any structural or material property in Achilles tendons, indicating that sex did not modulate the genotype effect on these parameters. When males and females of the same age and genotype were pooled, GDF6 deficiency resulted in significantly lower values of all three structural parameters (ultimate load, stiffness, and energy absorbed to failure, Table 3). These structural differences were due to the size differences between -/- and +/+ tendons (Figs. 1 C & D), as genotype did not significantly affect any material property in the Achilles tendon (Table 3).

Gene Expression

No two-fold-or-greater differences in GDF5 or GDF7 expression were seen between GDF6 deficient and wild type tendons at either age or tendon site (Fig. 5) in either sex, suggesting that overcompensation by these two related BMP family members was not a factor.

DISCUSSION

This study confirms that GDF6 deficiency is associated with an altered tendon phenotype that persists into adulthood. Among males, GDF6 -/- tail tendon fascicles had significantly less collagen and GAG content, and these compositional differences were associated with compromised material properties. Among female GDF6 -/- mice, however, no differences were detected in composition or material properties. The tendon phenotype in males appeared stronger in the tail than in the Achilles tendon, where some compositional differences were present, but no material property differences were found. This work supports a possible role for GDF6 in tendon biology and, by extension, its use in therapeutic strategies for tendon and ligament repair.

The effect of GDF6 deficiency on murine tendon appears sex-specific. Such sexual dimorphism is common: in the liver, for example, a large number of genes are sex-specific, with STAT5a playing a key role in regulating sex-specific gene expression in the female liver and STAT5b in the male liver[29]. The beta(2)-adrenergic receptor on male leukocytes appears to contribute to sexual dimorphism in murine leukocyte migration[30]. In the ear, mice lacking the GABA(A) receptor subunit beta2 display a clear sexual dimorphism in cochlear phenotype[31]. In the musculoskeletal tissues, female muscle-derived stem cells have higher muscle regeneration efficiency than male cells[32]. While the basis for the sexual dimorphism the GDF6 effect on tendon remains to be determined, increasing evidence suggests that sex plays an important role in the health of most musculoskeletal tissues[33]. Recent human evidence suggests that female tendon fibroblast collagen synthesis rates may be lower than those of males[34], and that the tendon's ability to adapt to mechanical loading may be mitigated in females[34, 35]. These studies emphasize the importance of examining sex-based differences in the mechanisms underlying tendon injury and healing to optimize strategies for prevention and repair in both sexes.

Another notable finding of the present study is the more pronounced effect of GDF6 deficiency on tail tendons versus Achilles tendons. No data have been published comparing

the gene expression levels of individual GDF family members between tendons from different anatomical locations, but previous studies in GDF5 and GDF7 deficient mice demonstrated more pronounced phenotypes in the Achilles tendon versus the tail, whereas our results suggest a more prominent effect of GDF6 deficiency on the tail. These results are not surprising, as members of the larger family of BMPs exhibit overlapping functions and sites of expression[36]. Speculation regarding the functional roles of individual GDF family members based on knockout mouse models should be made with caution, however, for the high degree of amino acid homology (80-86%) seen in the C-terminal active domain of these proteins likely makes it possible for one member to compensate for another[36]. Based on our gene expression results, it does not appear that GDFs 5 or 7 are overcompensating in the absence of GDF6 in male or female tendons at either age or tendon location. Nonetheless, other factors may be partially responsible for the observed results.

The results examining whether GDF6 deficiency modulated normal age-dependent changes in tendon were inconclusive; genotype did not modulate the growth-associated changes in tendon composition ($p > 0.05$ for genotype x age, Figs. 2 & 4), but did significantly modulate age-dependent changes in some male tail tendon properties (e.g. yield strain, failure strain, post-yield strain, and strain energy density, Table 1). The age related changes in most morphometric parameters (body mass, Achilles tendon length, and tail tendon fascicle cross-sectional area) were significantly modulated by genotype, with larger differences between GDF6 -/- and +/+ animals manifest at the 4-weeks (Fig. 1). Thus, for those parameters that were significantly influenced by genotype, the presence or absence of GDF6 had a somewhat greater effect at 4 weeks than at 16 weeks, but not universally for all parameters.

A limitation of this study was the need to introduce greater heterogeneity into the background strain to increase the longevity of the GDF6 -/- mice. By comparing the effect of GDF6 deficiency on tendon properties in 4 week-old males with recently published results for the original 129SV/J × C57Bl/6J background[23], the same trends are seen, but the effect of the mutation is dampened by the outcross. For example, on the less mixed background, collagen content in 4 week-old male GDF6 -/- tail tendons was about 1/3 that of wild type mice and tail tendon fascicle material properties were 45-50% lower, while in the present study, differences in collagen content were about 25% and material property differences were about 10-25% lower in knockout animals. Larger sample sizes may be needed to detect significant differences in future studies aimed at assessing the effect of GDF6 deficiency on tendon healing.

A second limitation is the normalization of compositional measures to DNA content, rather than a more direct measure of sample volume such as wet weight. Without verifying that cell density is comparable between wild type and GDF6 deficient tendons within each age, sex, and site group, we cannot rule out that the observed differences in GAG/DNA and Hyp/DNA in males are due to higher cell density rather than lower GAG and total collagen content per tissue volume. However, given that male tail tendon material properties were significantly reduced in GDF6 deficient animals compared to wild types, consistent with lower matrix constituents, this possibility seems unlikely. Similarly, the lack of compositional differences seen in female GDF6 deficient tendons might be explained by a *reduction* in cell density in GDF6 -/- female tendons in combination with a reduction in GAG and total collagen content per tissue volume. Again, this seems unlikely, given that genotype did not have a significant effect on female tail tendon material properties, consistent with a lack of compositional difference between female GDF6 deficient and wild type tendons. Nevertheless, given the less clear reconciliation of composition and material property results in Achilles tendon, it will be important to examine the potential for cell density differences between genotypes in future work.

While GDF6 (a.k.a CDMP2 and BMP13) was first identified as a component in cartilage (hence the name Cartilage Derived Morphogenetic Protein)[37], it is present in tendon and ligament[4,6,38], particularly in the more rounded, metabolically active tenoblasts and in perivascular mesenchymal cells that can serve as progenitors for healing[6]. In vitro, BMP13 can stimulate the expression of type I collagen[6] in human fibroblasts; in isolated bovine fibroblasts, increased total proteoglycan synthesis and [^{35}S]sulphate incorporation is found[38]. Our study is consistent with these reports, in that the absence of GDF6 (the murine homologue of BMP13) was associated with decreased levels of total collagen and, to a lesser extent, sulphated GAG. In a series of investigations on the augmenting tendon healing via exogenously delivered CDMPs[5,7,8,10-12], Aspenberg's group suggests that CDMP2/BMP13/GDF6 function may depend on the local mechanical loading environment[5, 10]. If GDF6 function is indeed sensitive to mechanical loading, this could provide an alternative explanation for the more notable phenotype in GDF6 deficient tail tendons (lower in vivo loads) versus Achilles tendons (higher loads).

In conclusion, in 4 and 16 week-old, male and female, GDF6 -/- and +/+ mice, GDF6 deficiency: resulted in a significant tendon phenotype, which persisted into adulthood; had a sexually dimorphic effect on tendons, with male tendons exhibiting a gene effect, while female tendons did not; had a more notable effect on composition and material properties of tail tendons than Achilles tendons; and may have had a slightly more pronounced effect on tendons of younger versus older mice. Whether GDF6 deficiency has an adverse effect on tendon healing remains to be determined.

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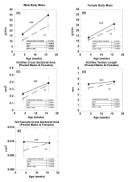

Figure 1.

The effect of GDF6 deficiency on animal body mass in male [A] and female [B] mice at 4 and 16 weeks of age. Pooled male and female Achilles tendon cross-sectional area [C], Achilles tendon length [D], and tail tendon fascicle cross-sectional area [E]. Males and females of the same age and sex were pooled only if the interaction of genotype and sex was not significant. Wild type data: solid lines; GDF6 -/- data: dashed lines. Mean ± SD.

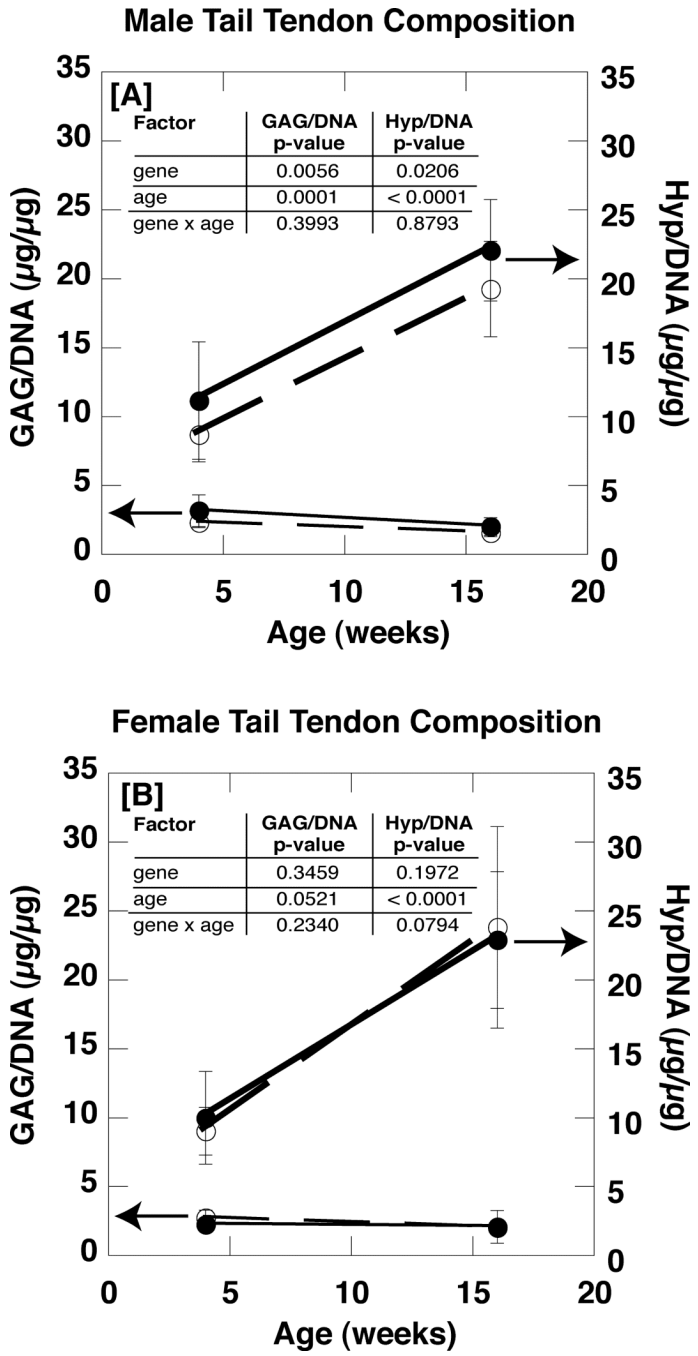

Figure 2.

The effect of GDF6 deficiency on tail tendon composition in males [A] and females [B]. Each plot displays the scale for GAG/DNA (lower lines) on the left and Hyp/DNA (upper lines) on the right. Wild type data: solid lines; GDF6 -/- data: dashed lines. Mean ± SD.

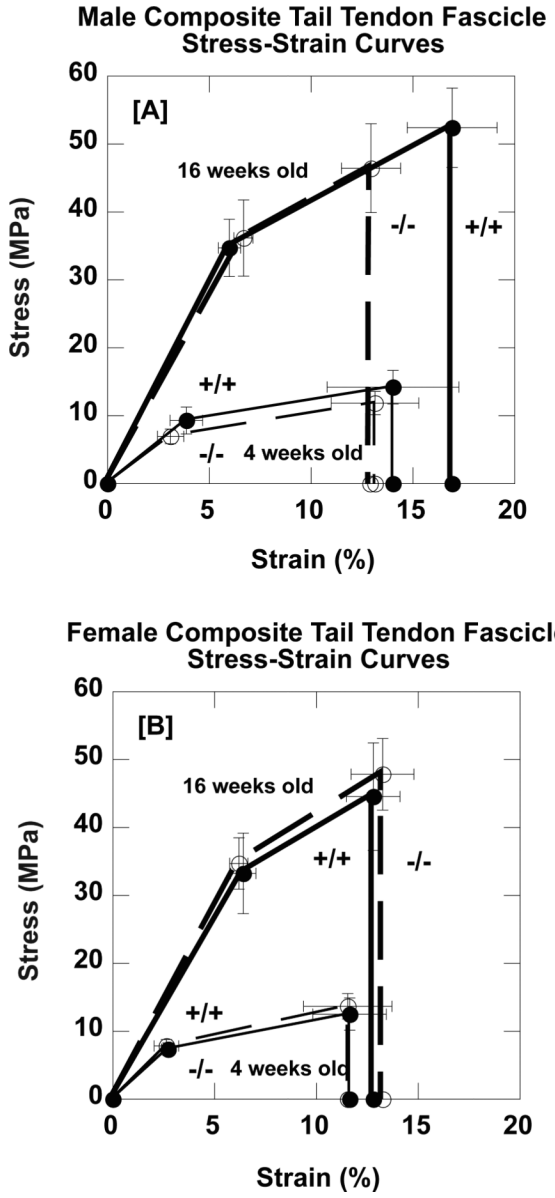

Figure 3.

Composite tail tendon fascicle curves for 4 and 16 week-old GDF6 deficient and wild type males [A] and females [B]. Curves were constructed using the mean ± SD of both stress and strain at the yield and failure points and are not meant to capture a typical failure curve. Wild type curves: solid lines; GDF6 -/- curves: dashed lines.

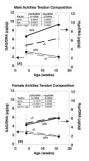

Figure 4.
The effect of GDF6 deficiency on Achilles tendon composition in males [A] and females [B]. Each plot displays the scale for GAG/DNA (lower lines) on the left and Hyp/DNA (upper lines) on the right. Wild type data: solid lines; GDF6 -/- data: dashed lines. Mean ± SD.

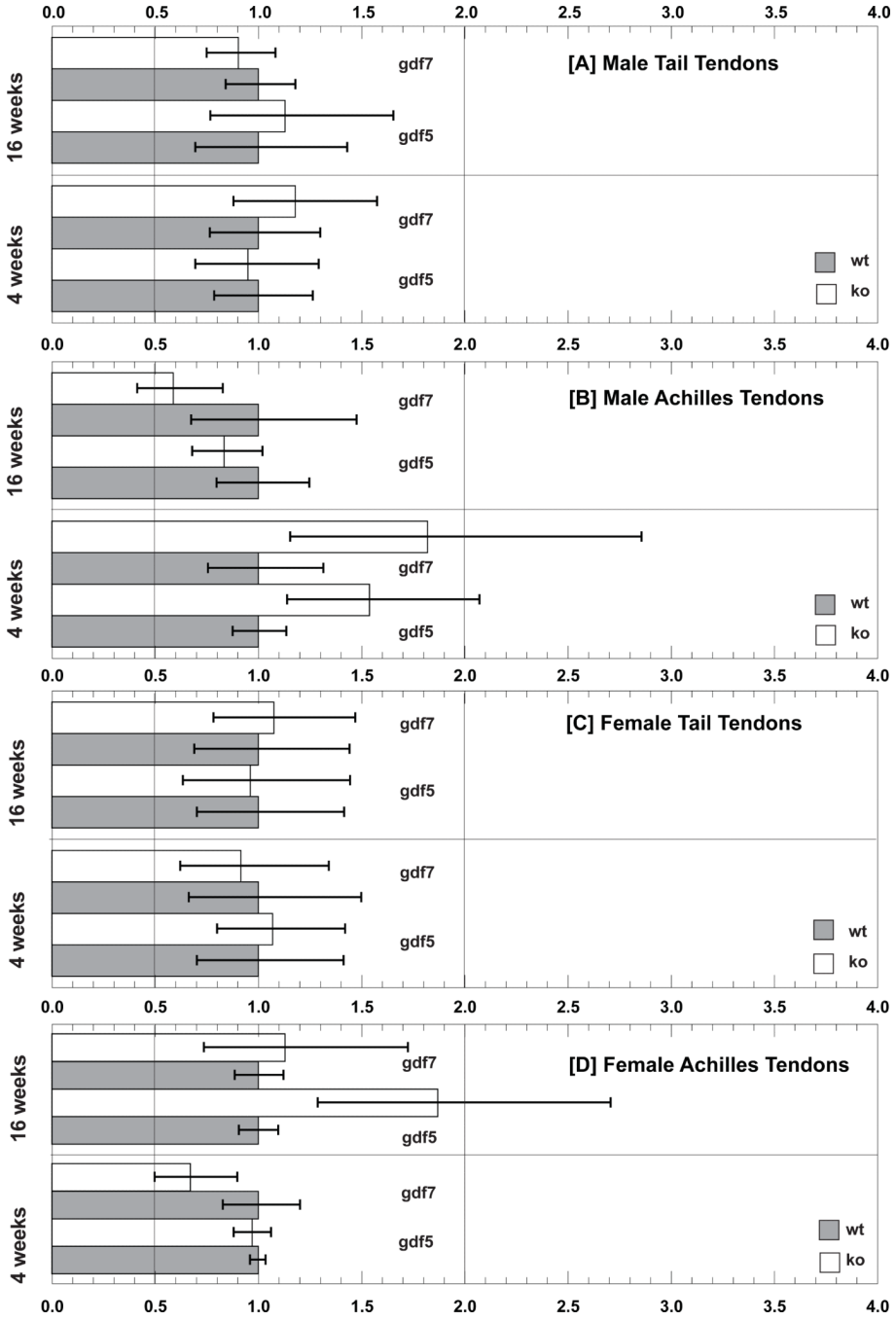

Figure 5.

Gene expression for Gdf5 and Gdf7 in GDF6 deficient (ko, shown in white) and wild type (wt, shown in grey) [A] male tail tendons; [B] male Achilles tendons; [C] female tail tendons; and [D] female Achilles tendons. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method, with the first Δ relative to internal 18s rRNA, and the second Δ relative to wild type in each age, sex, and site group. n = 4-8 per group. No two-fold or greater difference in expression was seen between GDF6 deficient and wild type tendons. Error bars calculated as described in reference [27].

Table 1

Tail Tendon Fascicle Material Properties - Males

	4 weeks		16 weeks		p-values		
	GDF-6 +/+ (n = 10)	**GDF-6 -/- (n = 9)**	**GDF-6 +/+ (n = 10)**	**GDF-6 -/- (n = 10)**	**gene**	**age**	**gene × age**
Ultimate Strength (MPa)	14.2 ± 2.5	11.9 ± 1.7	52.4 ± 5.8	46.4 ± 6.5	0.009	< 0.0001	0.235
Yield Strength (MPa)[1]	9.3 ± 2.0	7.0 ± 1.1	34.7 ± 4.2	36.2 ± 5.6	0.716	< 0.0001	0.120
Modulus of Elasticity (MPa)	224 ± 35	200 ± 31	600 ± 60	558 ± 62	0.047	< 0.0001	0.613
Yield Strain (%)[1]	3.88 ± 0.80	3.10 ± 0.65	5.99 ± 0.55	6.68 ± 0.46	0.842	< 0.0001	0.0008
Failure Strain (%)	14.02 ± 3.23	13.14 ± 2.15	16.93 ± 2.21	12.94 ± 1.45	0.003	0.080	0.046
Post-yield Strain (%)	10.13 ± 3.77	10.00 ± 1.89	10.95 ± 2.27	6.26 ± 1.36	0.005	0.077	0.007
Strain Energy Density (MPa)	1.4 ± 0.3	1.2 ± 0.3	6.0 ± 1.2	3.9 ± 0.8	< 0.0001	< 0.0001	0.0004

[1] $p > 0.05$ for *gene* × *sex* interaction using entire data set & for *gene* using pooled males and females, but data shown are separated by sex for consistency with remaining data in Tables 1 & 2.

Table 2

Tail Tendon Fascicle Material Properties - Females

	4 weeks		16 weeks		p-values		
	GDF-6 +/+ (n = 10)	**GDF-6 -/- (n = 9)**	**GDF-6 +/+ (n = 10)**	**GDF-6 -/- (n = 10)**	**gene**	**age**	**gene × age**
Ultimate Strength (MPa)	12.5 ± 2.4	13.7 ± 1.8	44.6 ± 7.9	47.8 ± 5.2	0.170	< 0.0001	0.511
Yield Strength (MPa)[1]	7.4 ± 0.9	7.9 ± 0.9	33.2 ± 5.9	34.7 ± 3.8	0.400	< 0.0001	0.685
Modulus of Elasticity (MPa)	211 ± 28	222 ± 37	530 ± 93	558 ± 50	0.304	< 0.0001	0.635
Yield Strain (%)[1]	2.71 ± 0.32	2.63 ± 0.61	6.41 ± 0.62	6.19 ± 0.45	0.364	< 0.0001	0.670
Failure Strain (%)	11.63 ± 1.81	11.54 ± 2.18	12.79 ± 1.32	13.25 ± 1.54	0.736	0.0139	0.621
Post-yield Strain (%)	8.95 ± 1.73	8.91 ± 1.87	6.37 ± 1.09	7.06 ± 1.26	0.515	< 0.0001	0.453
Strain Energy Density (MPa)	1.1 ± 0.2	1.2 ± 0.3	3.7 ± 1.0	4.2 ± 0.9	0.228	< 0.0001	0.310

[1] $p > 0.05$ for *gene* × *sex* interaction using entire data set & for *gene* using pooled males and females, but data shown are separated by sex for consistency with remaining data in Tables 1 & 2.

Table 3

Achilles Tendon Mechanical Testing Parameters – (Pooled Males and Females)

	4 weeks		16 weeks		p-values		
	GDF-6 +/+ (n = 16)	**GDF-6 -/- (n = 18)**	**GDF-6 +/+ (n = 20)**	**GDF-6 -/- (n = 20)**	**gene**	**age**	**gene × age**
Ultimate Load (N)	5.6 ± 1.3	3.8 ± 0.7	16.0 ± 2.8	13.2 ± 2.5	< 0.0001	< 0.0001	0.2861
Stiffness (N/mm)	8.4 ± 2.5	5.7 ± 1.7	18.8 ± 3.4	15.3 ± 1.8	< 0.0001	< 0.0001	0.5030
Energy-to-Failure (mJ)	2.8 ± 1.2	2.0 ± 0.6	8.4 ± 3.2	6.9 ± 2.3	0.0316	< 0.0001	0.4920
Ultimate Strength (MPa)	24.0 ± 4.2	24.3 ± 6.3	43.6 ± 8.4	38.1 ± 7.5	0.1143	< 0.0001	0.0798
Elastic Modulus (MPa)	113 ± 39	106 ± 37	192 ± 50	162 ± 43	0.0734	< 0.0001	0.2449
Failure Strain (%)	39 ± 15	42 ± 11	38 ± 11	40 ± 8	0.3771	0.5145	0.8982
Strain Energy Density (MPa)	4.1 ± 2.2	4.6 ± 1.9	6.3 ± 3.0	5.7 ± 1.8	0.9056	0.0026	0.3195