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

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Introduction

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

Methods

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

Results

Tendon Morphology: PTs in KO mice were significantly longer than CTRL (Figure 1A-B). Matrix & cell organization: KO tendons demonstrated higher average forward SHG signal (Figure 1C), indicative of greater collagen density, and increased circular standard deviation of fiber directions, signifying greater collagen matrix disorganization in KO tendons (Figure 1D-F). Additionally, cell density was higher in KO tendons (Figure 1G), and nuclei were rounder (Figure 1H). In contrast to CTRL tendons, where actin filaments were arranged parallel with the long-axis of the tendon, actin appeared disordered and less aligned in KO tendons, consistent with matrix disorganization (Figure 1I-J). Irregular cell shape was also observed in TEM tendon cross-sections (Figure 2A). In CTRL tendons, cell protrusions interacted with those of neighboring cells towards establishing defined fibril bundles. In KO tendons, however, cell protrusions were fragmented with abundant fibripositors (white arrows). Fibril structure:

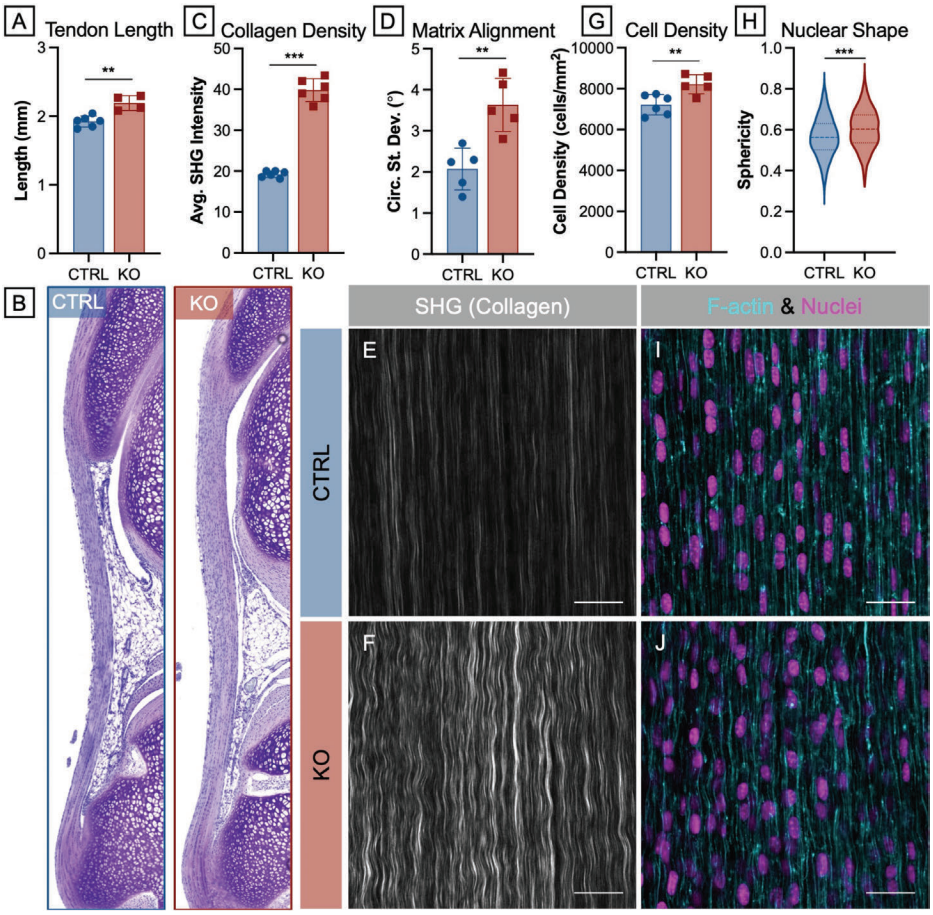

Figure 1. (**A-B**) KO patellar tendons are longer than CTRL. SHG imaging revealed that **(C)** collagen density was higher, and **(D)** the matrix was more disorganized in **(E)** KO tendons compared to **(F)** CTRL. **(G)** Cell density was higher and **(H)** nuclei were rounder in KO tendons. **(I-J)**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

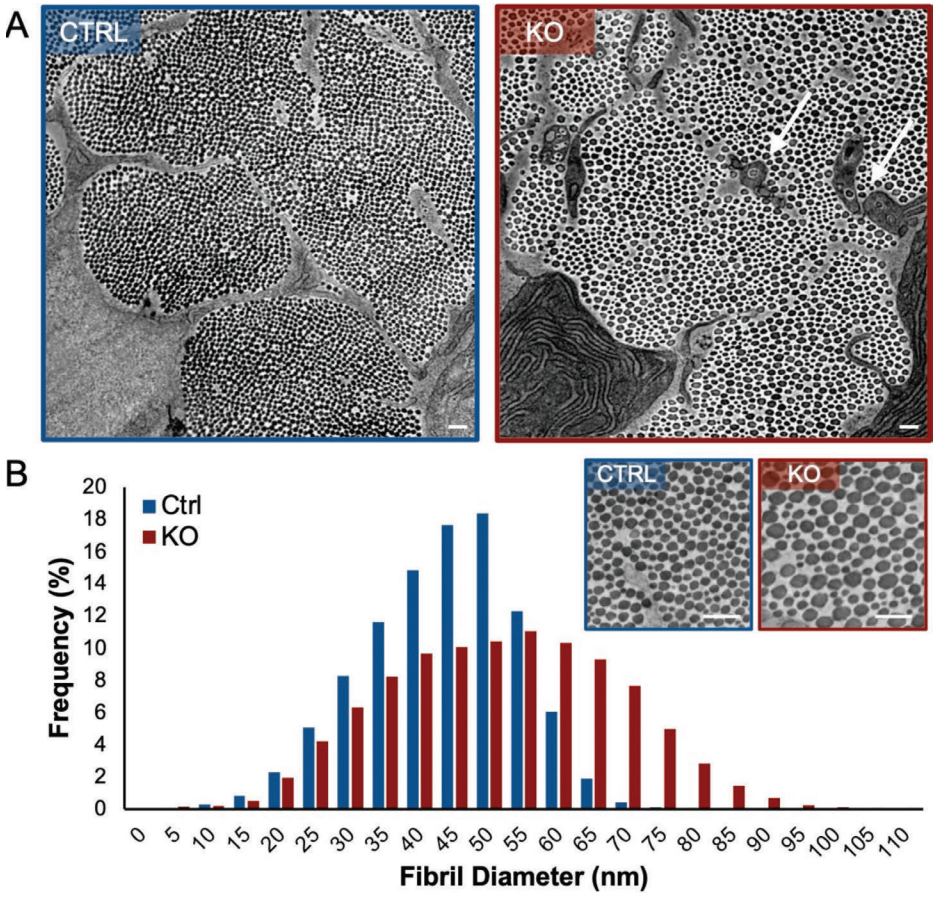

Figure 2. (A) Cell protrusions in CTRL tendons interact with neighboring cells and form fibril bundles, while those in KO tendons are fragmented and contain several fibripositors (white arrows); **(B)** KO tendons have a heterogenous collagen fibril diameter population with a greater percentage of larger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

The collagen fibril diameter distribution in KO tendons was more heterogeneous with a greater percentage of larger diameter fibrils compared to CTRL (Figure 2B). Gene expression: As expected, Col12a1 expression was significantly reduced (Figure 3A), while expression of *Fn1, Mmp2, and Serpine1* (Figure 3B-D) was increased in KO tendons. Expression of tendon-related genes (*Col5a2, Dcn, Bgn, Tnc, Tnmd*) and those associated with cell-cell and cell-matrix interactions (*Cdh11, Cdh2, Cd44, Itgb1*) were also increased in KO tendons (data not shown).

Discussion

During tendon development, proper cell and matrix organization is essential for establishing tendon hierarchical structure and function, and our findings indicate that collagen XII is critical in this process. In the absence of collagen XII, postnatal day 10 patellar tendons have disrupted matrix and cell organization, altered cell and nuclear shape, increased fibril diameter, and increased expression of tendon and cell-matrix related genes.

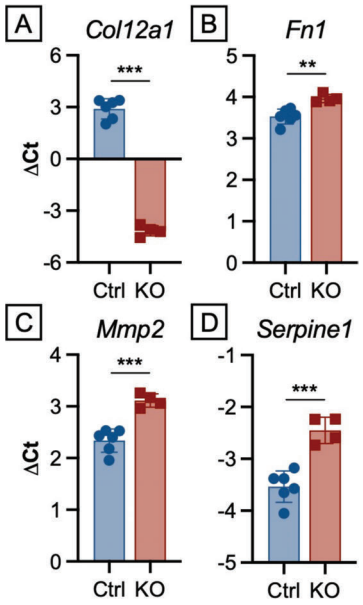

Figure 3. (A) *Col12a1* expression is significantly reduced in KO mice, while expression of **(B)** Fn1; **(C)** *Mmp2*; **(D)** *Serpine1* is increased. (**p < 0.01, ***p < 0.001).

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

Significance

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

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