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Collagen XII is a Critical Regulator of Tendon Function: Development of a Conditional Mouse Model

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Introduction

Collagen XII is a fibril-associated collagen with interrupted triple helices (FACIT) that regulates collagen fibril assembly, and mutations in *Col12a1* result in myopathic Ehlers-Danlos Syndrome (mEDS). Patients with mEDS experience excess weakness at birth, hypermobile distal joints, and an absence of deep tendon reflexes[1], indicating impaired tendon function due to the absence of collagen XII. Tendons in a global *Col12a1*$^{-/-}$ knockout mouse model demonstrated disrupted grip strength and tendon fiber structure as well as disordered tenocyte organization[2]. However, secondary effects due to involvement of bone and muscle may occur in this model, and the isolated role of collagen XII in tendon has not been elucidated. To address this limitation, the objective of this study was to create and characterize a conditional *Col12a1*-null mouse model to target collagen XII knockout in tendons using a Scleraxis-Cre driver. We hypothesized that tendon-targeted knockout of *Col12a1* expression would impair tendon function.

Methods

Model Development

A promoter-driven knockout embryonic stem (ES) cell line was obtained from the KOMP Repository (ID: CSD29388, *Col12a1*$^{tm2a(KOMP)Wtsi}$). ES cell clones were injected into wild-type C57BL/6-Albino blastocysts, and resulting chimeric mice were backcrossed to produce mice with the targeted allele, *Col12a1*$^{+/ta}$. *Col12a1*$^{+/ta}$ mice were bred with FLPe mice (B6; SJL-Tg(ACTFLPe) 9205Dym/J, Jackson Labs) to excise the FRT flanked neo sequences. The resulting offspring were crossbred with C57BL/6 mice for ± generations and then intercrossed to obtain conditional knockout mice, *Col12a1*$^{flox/flox}$. *Col12a1*$^{flox/flox}$ mice were bred with Scleraxis-Cre (*Scx-Cre*) mice to obtain tendon-targeted heterozygous (Het, *Col12a1*$^{+/\Delta ten}$) and homozygous (KO, *Col12a1*$^{\Delta ten/\Delta ten}$) collagen XII knockout mice.

Gene & Protein Expression

Col12a1 expression and collagen XII content were assessed in flexor digitorum longus (FDL) tendons from mice at day 10 using qPCR and Western blots, respectively.

Immunofluorescence

FDLs were dissected, fixed in 4% paraformaldehyde, embedded in optimal cutting temperature compound, and sectioned in the transverse plane at 5 μm thickness. Immunofluorescence staining of collagen XII was performed using a rabbit anti-mouse Col XII antibody (KR33, 1:500 dilution) with a donkey anti-rabbit Alexa Fluor 568 (1:200 dilution) secondary antibody.

Grip Strength

Using a grip strength meter, mice were lowered toward the grip platform and upon grasping, mice were pulled away steadily until the grip was broken. The force applied just before the mouse lost its grip was recorded as the peak force.

Tendon Mechanics

FDL tendons from day 60 mice were dissected from the foot, cleaned of excess tissue, and mechanically evaluated as described[3]. Tensile testing was performed using the following protocol: preconditioning, stress relaxation at 5% strain, and a ramp to failure at a rate of 0.5%/s.

Statistics

One-way ANOVAs with Tukey post-hoc tests were conducted. Significance was set at $p \leq 0.05$.

Results

Col12a1 expression was reduced in *Col12a1*$^{\Delta ten/\Delta ten}$ KO mice compared to Cre-littermate control (Ctrl) mice though baseline expression, determined from traditional collagen XII knockout mice[2], was not reached in KO mice (Figure 1A). Furthermore, the α1(XII) chain was present at comparable levels in the control group: Cre-, *Scx-Cre* and *Col12a1*$^{flox/flox}$ mice (data not shown). Collagen XII content was lower in Het mice and just above background in KO mice compared to Ctrl (Figure 1B). Collagen XII immunofluorescence localization demonstrated efficient knockdown in the tendon proper but

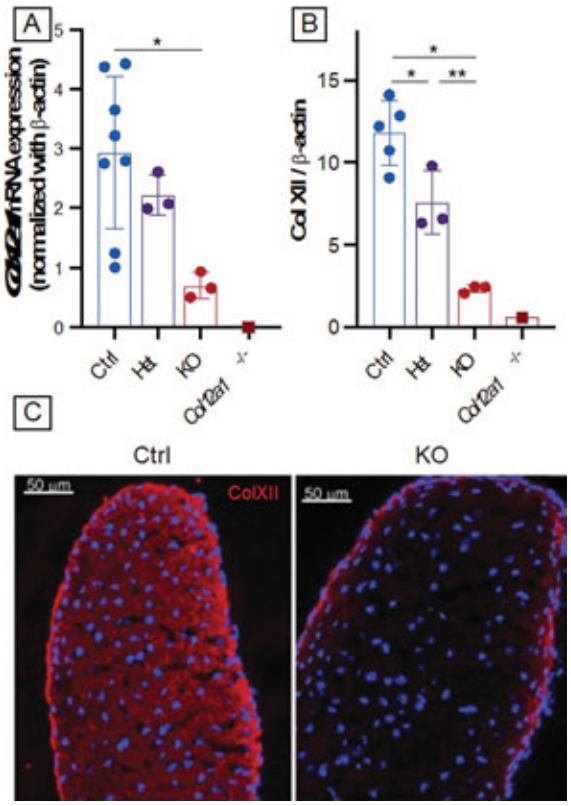

Figure 1. (A) *Coll2a1* and **(B)** collagen XII expression were significantly reduced in *Col12a1*$^{\Delta ten/\Delta ten}$ KO tendons compared to Ctrl though still above the baseline level established from conventional *Col12a1*$^{-/-}$ mice. **(C)** Efficient collagen XII knockdown was achieved in the tendon proper of KO tendons but not the surrounding peritenon. (*p ≤ 0.05 **p ≤ 0.01).

not in the surrounding peritenon as expected (Figure 1C). For joint function, female KO mice had reduced forelimb grip strength compared to Het (Figure 2A) while male KO mice had reduced strength compared to Ctrl mice (Figure 2B). At the tendon level, FDLs from day 60 male and female KO mice exhibited a reduction in mechanical properties. There was no difference in cross-sectional area (data not shown), but stiffness and modulus were both decreased in KO FDLs compared to Ctrl (Figure 2C, D).

Discussion

The overall goal of this study was to create a conditional *Col12a1*-null mouse model and target collagen XII knockout to tendons using a scleraxis-Cre driver. In FDLs of tendon-targeted *Col12a1*$^{\Delta ten/\Delta ten}$ KO mice, both mRNA and protein expression levels were decreased but did not reach the baseline levels of global collagen XII knockout mice. This suggests that cells from a non-tendon lineage are not targeted as expected, and collagen XII immunofluorescence indicates that the surrounding peritenon population likely contributes to the above baseline expression levels.

Furthermore, in the absence of *Col12a1* expression and therefore collagen XII, *Col12a1*$^{\Delta ten/\Delta ten}$ KO mice have impaired mechanics, as evidenced by reduced forelimb grip strength and FDL tendon mechanical properties. Reduced grip strength is consistent with joint function in the global *Col12a1*$^{-/-}$ knockout model, but interestingly, FDL tendon mechanical properties deviated from previous findings. In the global *Col12a1*$^{-/-}$ knockout model, FDLs had larger cross-sectional area and greater stiffness with no difference in tendon material properties[2]. In this study, however, there were no differences in FDL cross-sectional area in KO mice, but stiffness was significantly decreased, resulting in inferior tendon elastic modulus.

Differences in mechanical properties suggest that collagen XII is a critical regulator of tendon structure-function, and the contrasting findings from the global knockout model may be a result of secondary effects, such as those due to muscle and bone. Additionally, collagen XII knockout did not exhibit sex-specific effects with similar trends in grip strength and tendon mechanics for both male and female mice.

Future studies are necessary to elucidate sex-specific roles of collagen XII in tendon structure and determine the biological mechanisms underlying changes in tendon structure-function. In conclusion, grip strength and tendon mechanical changes in the tendon-targeted *Col12a1*$^{\Delta ten/\Delta ten}$ model support that collagen XII is a critical regulator of tendon function.

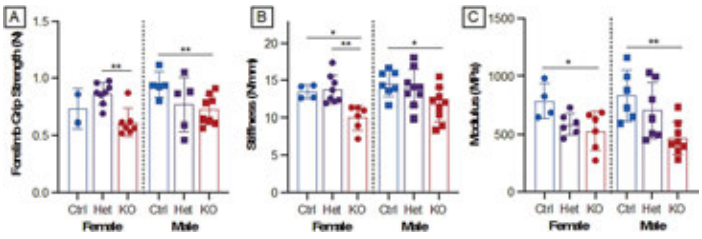

Figure 2. Forelimb grip strength was significantly reduced in **(A)** female *Col12a1*$^{\Delta ten/\Delta ten}$ KO mice compared to *Col12a1*$^{\Delta ten/+}$ Het and in male $^{-}$ KO mice compared to Ctrl. FDL tendon **(B)** stiffness and **(C)** modulus were significantly reduced in *Col12a1*$^{\Delta ten/\Delta ten}$ KO compared to Ctrl in both female and male mice. (*p ≤ 0.05, **p ≤ 0.01).

Significance

Through development of a tendon-targeted collagen XII knockout mouse model, this study demonstrates the critical role of collagen XII in regulating joint and tendon function. Elucidating guiding mechanisms will provide the foundation to leverage the role of collagen XII in therapeutic strategies, providing support for treatments that address conditions such as myopathic Ehlers-Danlos syndrome.

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