Type III Collagen Supports Recovery of Matrix Structure and Tissue Mechanics in Early Neonatal Tendon Healing

Margaret K. Tamburro[1], Miranda K. Doro[1], Susan Volk[2], Louis J. Soslowsky[1]
[1]McKay Orthopaedic Research Laboratory, University of Pennsylvania, Philadelphia, PA
[2]School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA
Margaret.Tamburro@pennmedicine.upenn.edu

Disclosures: Margaret K. Tamburro (N), Miranda K. Doro (N), Susan W. Volk (N), Louis J. Soslowsky (N)

INTRODUCTION: Injured mature tendons heal poorly with type III collagen (COL3)-rich scar. Compared to mature tendon, neonatal tendon heals with improved speed and quality via recruitment of intrinsic, *Scleraxis* (*Scx*)-expressing tenocytes.[1-2] Other models of improved healing have increased COL3 in the healing matrix[3-4] and the neonatal tendon is COL3-rich,[5] but contributions of COL3 to superior neonatal tendon healing are unknown. Thus, our objective was to define the regulatory contributions of COL3 produced by *Scx*-expressing tenocytes to the reparative cell activity, matrix formation, and mechanical recovery characteristic of neonatal healing. We hypothesized that intrinsic tenocytes mount a robust COL3 healing response to support early cell recruitment and provisional matrix formation and late cell and matrix maturation to quickly restore healthy tendon structure and function after neonatal tendon injury.

METHODS: Mice with tendon-targeted *Col3a1* knockdown (KD, *Scx*-Cre$^{+/-}$; *Col3a1*$^{Flox/Flox}$) and wildtype littermate controls (WT, *Scx*-Cre$^{-/-}$; *Col3a1*$^{Flox/Flox}$) received excisional patellar tendon biopsy punch injury (0.5 mm diameter) at postnatal day 7 (p7, IACUC approved). After sacrifice during early (1-week post-injury (wpi), p14) and late (3-wpi, p28) healing, hindlimbs were harvested bilaterally for cell, matrix, and mechanical assessment; uninjured, age- and genotype-matched hindlimbs were harvested as developmental controls at p14 and p28 ($n \geq 7$ per genotype per timepoint for each group and assay, mixed sex). Gene expression was assessed with multiplexed qPCR (96.96 Dynamic Array IFC) for a panel of genes related to tendon healing. Cell infiltration and matrix structure were assessed with whole tendon imaging; tendons were stained with SYTOX^{TM} Green, optically cleared with a modified form of SeeDB,[6] and imaged with forward second harmonic generation (fSHG) imaging. Cell density, nuclear shape, matrix disorganization (circular standard deviation of fSHG signal), and matrix density (intensity of fSHG signal) were measured in Fiji (150 µm x 150 µm region of interest in healing matrix). Matrix properties were considered relative to WT uninjured developmental controls at each timepoint. For assessment of elastic tendon mechanics, patella-patellar tendon-tibia complexes were prepared[7] and tested with a quasistatic ramp to failure (0.1% strain/s) with image capture (2 Hz). After outlier removal (2.2 IQR), groups were compared with one-way ANOVAs at each timepoint with Šídák's multiple comparison tests for injured WT vs KD, uninjured developmental control WT vs KD, and WT and KD injured vs uninjured developmental control ($\alpha = 0.05$). Gene expression data were compared with principal component analysis.

RESULTS: *Scx*-Cre-driven excision reduced *Col3a1* expression in healing KD compared to WT tendons at both 1- and 3-wpi (Fig. 1A). As expected, compared to uninjured, age- and genotype-matched developmental controls, *Col3a1* expression was induced after injury in WT but not KD tendons (Fig. 1A). Cell density and nuclear shape at 1- and 3-wpi were not different based on *Col3a1* reduction but were influenced by injury (data not shown). Gene expression at 1- and 3-wpi was not markedly altered by *Col3a1* reduction (Fig. 1B-C). At 1-wpi, gene expression was substantially different from uninjured developmental controls (Fig. 1B), but, by 3-wpi, gene expression was more similar to developmental controls (Fig. 1C). Healing matrix architecture was influenced by COL3 loss. KD tendons had increased matrix disorganization, indicated by increased circular standard deviation of fSHG signal, at 1-wpi (Fig. 2A-A'). This discrepancy resolved by 3-wpi when matrix disorganization was high compared to uninjured developmental controls but not different between healing KD and WT samples (Fig. 2A, C). Matrix density was low compared to uninjured developmental controls for both KD and WT tendons at 1- and 3-wpi (Fig. 2B). Interestingly, matrix density was increased in KD compared to WT for uninjured developmental controls at both p14 and p28, but this pattern was not observed in healing KD compared to WT tendons at 1- or 3-wpi (Fig. 2B-B', C). At the level of tissue mechanics, healing KD tendons had reduced stiffness compared to WT at 1-wpi (Fig. 3A-A') despite comparable cross-sectional areas (data not shown). Compared to uninjured developmental controls, KD tendons had reduced stiffness at 1-wpi (avg. ↓ 46%) and WT tendons had reduced stiffness at 3-wpi (avg. ↓ 31%, Fig. 3A). Midsubstance modulus was not different between healing KD and WT samples at 1- or 3-wpi but was reduced for both genotypes compared to developmental controls (Fig. 3B-B').

DISCUSSION: We investigated cellular, matrix, and mechanical consequences of reduced *Col3a1* expression by *Scx*-expressing intrinsic tenocytes during neonatal tendon healing. Our excisional patellar tendon injury model replicated the improved healing speed and quality previously reported in neonatal compared to adult tendons.[1-3,8] Further, COL3 loss hindered the early neonatal healing process, reducing matrix organization and tissue stiffness at 1-wpi. We did not detect substantial gene expression changes to explain these matrix findings, but untargeted transcriptomic and proteomic approaches may reveal COL3-mediated mechanistic changes. Interestingly, by 3-wpi, matrix organization and tissue stiffness were no longer impacted by COL3 loss, which may indicate a switch to COL1-driven matrix formation. Neonatal findings contrast with young adult tendon healing where COL3 loss did not influence matrix and mechanical properties at any phase of tendon healing,[9] offering the prospect that unique aspects of the neonatal COL3 response enhance early healing. Investigation of alternative methods of COL3 modulation free from confounding influences inherent to constitutive knockdown models, including inducible *Col3a1* knockdown and therapeutic COL3 biomaterial application, will complement this work. In conclusion, consistent with the established role of COL3 as the primary structural component of the early healing matrix, COL3 supports matrix formation and tissue stiffness in early neonatal tendon healing.

SIGNIFICANCE: We demonstrated the importance of COL3 in the enhanced speed and quality of early neonatal tendon healing which lays the foundation for translational investigations of therapeutic COL3 modulation to improve mature tendon healing through biomimicry of neonatal healing mechanisms.

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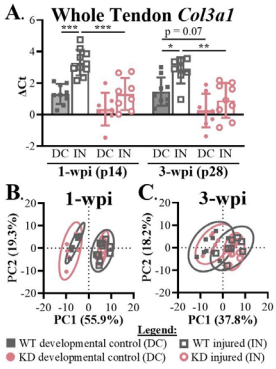

Figure 1. In KD tendons, injury did not increase *Col3a1* expression compared to uninjured developmental controls (DCs, A). Principal component analysis showed grouping of 95% confidence ellipses based on injury status at 1-wpi (B) but not 3-wpi (C). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

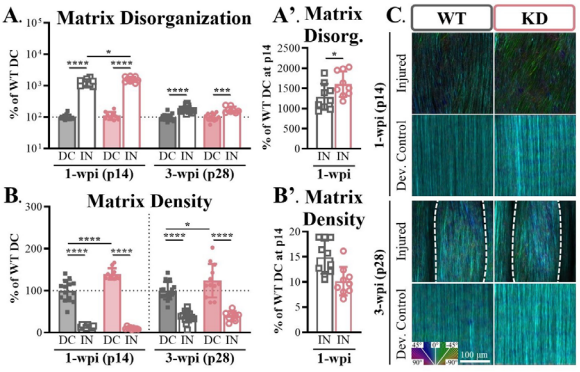

Figure 2. Healing KD tendons had more matrix disorganization than WT at 1-wpi. At 3-wpi, both KD and WT tendons had more disorganization than uninjured developmental controls without genotype-specific effects (A, A'). Matrix density was reduced by injury at 1- and 3-wpi without genotype-specific effects (B, B'). KD developmental controls had increased density compared to WT at p14 and p28 (B). In representative images, signal was enhanced for healing matrix (entire field of view for 1-wpi, middle portion marked with outline for 3-wpi) visibility (C). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

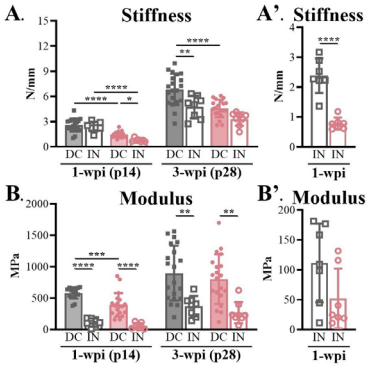

Figure 3. KD tendons had reduced stiffness compared to WT at 1-wpi (A, A'). Stiffness was reduced in KD tendons at 1-wpi and WT tendons at 3-wpi when compared to uninjured developmental controls (A). Midsubstance modulus was reduced compared to developmental controls at both timepoints for both KD and WT tendons (B, B'). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.