Focal Adhesion Kinase Regulates Physiological Tendon Development and Growth

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INTRODUCTION: Mechanical stimuli are known to impact tendon formation and homeostasis via cell mechanotransductive signaling. Focal adhesion kinase (FAK, gene: *Ptk2*) is an intracellular protein kinase that regulates cytoskeletal dynamics and transmission of mechanical strain to the cell nucleus from its surrounding extracellular matrix (ECM).[1] Pharmacological FAK inhibition alters cell morphology and tenogenic gene expression in monolayer cell culture and attenuates ECM to nuclei strain transmission and mechanotransductive gene expression in explant tendon culture.[2-7] Despite these known roles for FAK in tendon cells, the mechanism by which FAK regulates tendon physiology and the cell mechano-response throughout tendon development and postnatal growth remains unknown. Therefore, the objective of this study was to define the role of FAK in promoting cell proliferation and ECM deposition during the stage of rapid postnatal growth. We hypothesized that tendon-targeted FAK conditional knockout will reduce cell proliferation and impair matrix assembly, resulting in mechanically inferior tendons.

METHODS: To attenuate FAK expression *in vivo*, we utilized tendon-targeted FAK knockout (Scx-Cre;FAK$^{F/F}$; FAK-KO) mice,[8] in which we have previously validated reduced *Ptk2* expression.[6] Achilles tendons (ATs), flexor digitorum longus (FDL) tendons, and patellar tendons (PTs) from P10, P30, and P60 FAK-KO and WT littermate controls were used for viscoelastic mechanical testing, histology, and collagen fibril structure measures. In addition, we performed an EdU labeling experiment to quantify cell proliferation in P10 mice. Viscoelastic Mechanics: Tendon cross-sectional areas (CSAs) were measured, and tendons were subjected to a viscoelastic mechanical testing protocol (preconditioning, viscoelastic stress relaxation and dynamic frequency sweep, and a quasi-static ramp to failure). Histology: Whole knee joints from P10 mice were fixed, decalcified, paraffin embedded, and sectioned in the transverse plane to visualize the PT cross-section. Overall tissue morphology was visualized via toluidine blue staining. Cell Proliferation Analysis: Mice were injected with EdU (3μg/g bodyweight; Invitrogen A10044) at P0 and P2 and euthanized at P10. Knees joints were cut into sagittal sections and stained with Click-iT™ Cell Reaction Buffer Kit (Invitrogen C10269) to quantify EdU-positive nuclei within the PT. Collagen Fibril Structure: To quantify collagen fibril diameter distributions, PTs from all timepoints were fixed, embedded, sectioned at 85 nm, and imaged with transmission electron microscopy at 60,000x.

RESULTS: In our mechanical assessment, FAK-KO ATs and PTs exhibited reduced CSAs at P10 (**Fig. 1**). Despite this, there were few mechanical differences in structural or material properties in FAK-KO tendons at P10. This contrasts with tendons at P30 and P60 ages, in which FAK-KO tendons exhibited reduced size and structural properties (i.e., stiffness and max load) yet increased material properties (i.e., modulus and max stress) relative to WT tendons. Viscoelastic dynamic modulus values followed a similar trend to the other material properties (data not shown). Interestingly, while the reduced size of FAK-KO tendons was visible histologically at P10 (**Fig. 2A**), EdU labeling did not demonstrate a difference between proliferative cell behavior at this age (percent EdU-positive nuclei (Mean±SD); WT:7.0±2.5; FAK-KO:8.9±2.2). Finally, while the collagen fibril diameter distribution was not robustly altered between groups at P10 (**Fig. 2B**), the FAK-KO tendons demonstrated markedly smaller fibril diameters compared to WT tendons at both P30 and P60 (**Fig. 2C-D**).

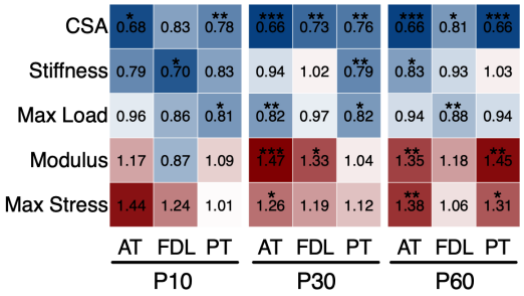

Figure 1. Viscoelastic mechanical testing datasets for all tendons evaluated in this study. Color and numbers within the cells indicate the ratio of the FAK-KO group mean relative to the WT group mean for that parameter. n=7-16/genotype/timepoint. Asterisks represent significant differences between WT and FAK-KO groups, which were compared with t-tests (*p<0.05; **p<0.01; ***p<0.001).

DISCUSSION: This study investigated the regulatory roles of FAK signaling on tendon physiology during postnatal growth. Consistent with our hypothesis, we observed reduced tissue size in FAK-KO tendons at all experimental timepoints. Interestingly, the differences in tissue mechanical properties were more drastic at the P30 and P60 timepoints compared to P10. Overall, these findings suggest that FAK regulates the generation of tendon size early in development, while altered ECM mechanical properties develop later during postnatal growth in FAK-KO mice. Given this finding, we hypothesized that FAK-KO led to altered tendon physiology by controlling cell proliferation and matrix deposition. While we did not observe a difference in EdU labeling or collagenous matrix deposition at P10, FAK ablation markedly reduced the size of collagen fibrils at P30 and P60, which suggests altered ECM deposition and remodeling behavior in FAK-KO tendons. Ongoing studies will further identify the effect of FAK-KO on the ECM structure by evaluating ECM-related gene expression and protein content. In addition, our future work will explore the effect of *in vivo* mechanical loading paradigms on FAK-dependent mechanotransduction and the tendon physiological response.

SIGNIFICANCE: Mechanical stimuli are essential for regulating tendon physiology, and defining the key signaling pathways that control tendon cell mechanotransduction will improve our understanding of disease and enable the development of improved therapies. Our results indicate that FAK signaling is important for tendon growth and the establishment of native structure/function.

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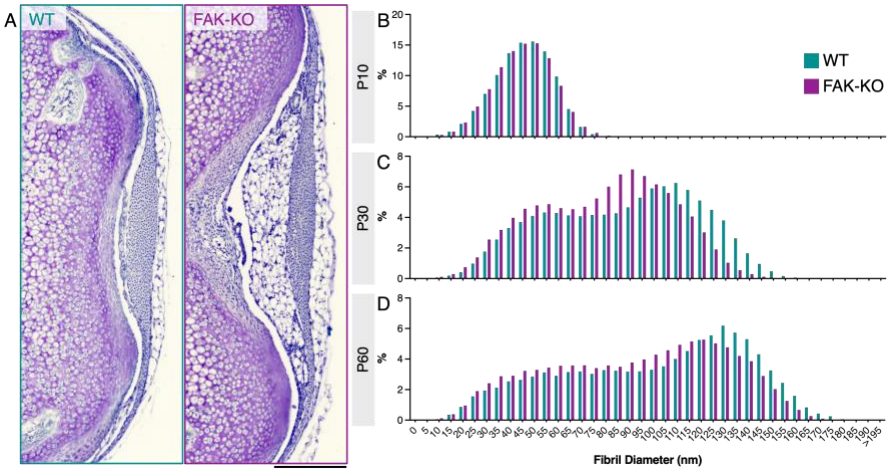

Figure 2. A) Representative images of transverse sections of P10 WT and FAK-KO tendons demonstrating reduced tendon CSA (n=4-5/genotype). B) P10, C) P30, and D) P60 collagen fibril diameter distribution quantifications (n=5-6/genotype/timepoint). Fibril diameter distributions were compared between groups using Kolmogorov-Smirnov tests, which demonstrated statistical significance (p<0.001) between groups at all experimental timepoints.