Focal Adhesion Kinase Regulates Mechanosensitive Gene Transcription and Tendon Maturation

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Introduction

Throughout development and postnatal growth, resident tendon cells respond to mechanical cues from the nascent tendon extracellular matrix (ECM) to regulate tissue properties. Focal adhesion kinase (FAK, gene: *Ptk2*) is an intracellular protein kinase that regulates the actin cytoskeleton and cell-ECM adhesions. In *in vitro* tendon cell culture, FAK inhibition alters cell morphology and attenuates the cell's tenogenic gene expression response to growth factor stimulation and mechanical stretching.[1–4] In addition, FAK inhibitor treatment of explanted tendons attenuates ECM to nuclei strain transmission, indicating that FAK plays an active role in regulating cell-ECM attachment within the native tendon ECM.[5] Finally, tendon-targeted FAK conditional knockout mice demonstrate altered development of tendon structure compared to wildtype (WT) mice at 30 days of age (P30), specifically exhibiting reduced cross-sectional area (CSA) yet mostly normal mechanical properties.[4] Despite these known roles for FAK in tendon, the degree to which FAK regulates mechanotransduction within the native tendon ECM as well as the regulatory role of FAK-dependent mechanotransduction in maintaining tendon homeostasis throughout postnatal growth remain unknown. Therefore, the objectives of this study were to define the role of FAK in (1) regulating mechanotransductive gene expression in response to de-tensioned free-floating explant conditions and (2) regulating tendon homeostasis during postnatal growth. We hypothesized that (1) FAK inhibition will attenuate the tendon gene expression response to free-floating explant conditions and that (2) tendon-targeted FAK conditional knockout will negatively impact tendon maturation and maintenance of homeostasis during postnatal growth, specifically by eliciting a reduction in tendon CSA and mechanical properties.

Methods

Free-Floating Tendon Explant

Flexor digitorum longus (FDL) tendons from male and female P30 WT mice were freshly dissected and randomized to receive FAK-inhibitor (FAK-I; 10 M PF-573228; Tocris; Minneapolis, MN) or control (DMSO) media (DMEM supplemented with 5% FBS and 25 mM HEPES). Tendons were maintained in explant culture conditions at 37°C for 1.5, 4, or 12 hours to assess the initial mechano-response to acute de-tensioning (n = 5/treatment group/timepoint). *Gene Expression:* Following explant treatment, RNA was immediately isolated from all tendons to quantify mechanotransductive gene expression using Taqman assays (*Acta2*, *Cyr61*, *Mmp3*, *Mmp13*, *Ptk2*; Housekeeper control: *Abl1*).

In Vivo Mouse Model

We utilized tendon-targeted FAK knockout (Scx-Cre;FAK$^{F/F}$; FAK-KO) mice,[6] in which we validated reduced *Ptk2* expression.[4] Achilles tendons (ATs), FDL tendons, and patellar tendons (PTs) from P60 FAK-KO and WT littermate controls were used for viscoelastic mechanical testing (males only) and histology (males and females). *Viscoelastic Mechanics:* Tendon CSAs were measured (n = 11-13/genotype), 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 ankle and knee joints were fixed, decalcified, paraffin embedded, and sectioned in the sagittal plane (n = 3-5/genotype). Additional knee joints were sectioned in the transverse plane to visualize the PT cross-section (n = 4-7/genotype). Overall tissue morphology was visualized via toluidine blue staining.

Results

Free-Floating Tendon Explant

In DMSO-treated tendons, we observed substantial increases in *Mmp3* (~90-fold) and *Mmp13* (~15-fold) gene expression with time under de-tensioned free-loading conditions, while expression of genes involved in cell contractility (*Acta2*, the Yap/Taz target gene *Cyr61*, and *Ptk2*) were not dramatically affected at these timepoints (Figure 1A-

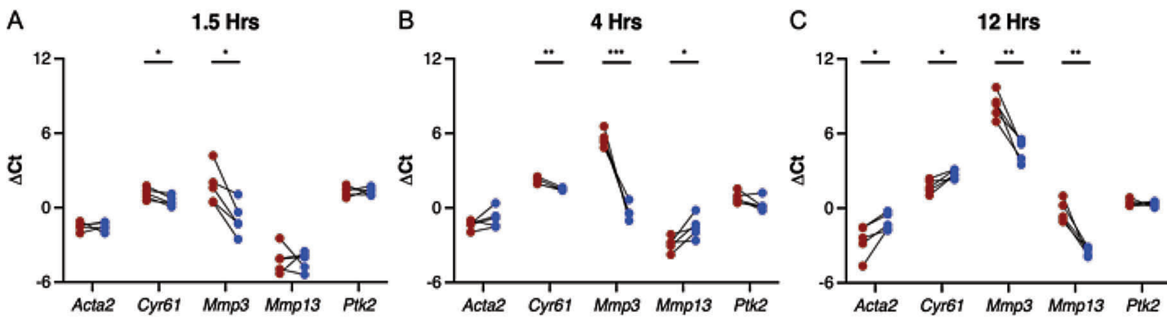

Figure 1. Mechanotransductive gene expression response to de-tensioning was significantly affected by FAK-I treatment. Gene expression levels (represented as "ΔCt") for DMSO and FAK-I treated tendons following **(A)** 1.5; **(B)** 4; and **(C)** 12 hours of free-floating explant conditions. Data represented as mean "±" standard deviation with plotted individual datapoints. Connected datapoints indicate contralateral FDLs. Bars indicate significant differences (t-test: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

C). Strikingly, FAK-I treatment resulted in differences in mechanotransductive gene expression at all timepoints evaluated. Specifically, compared to DMSO treated tendons, FAK-I treated tendons demonstrated reduced Mmp3 expression at all timepoints and reduced Mmp13 expression at the 12 hour-timepoint (Figure 1A-C). In addition, FAK-I treatment modulated the expression levels of Acta2 and *Cyr61* throughout the experiment (Figure 1A-C).

In Vivo Mouse Model

For tendon viscoelastic mechanical properties, we observed reduced CSA in all FAK-KO tendons, with the greatest effects in the ATs and PTs (Figure 2A). Structurally, FAK-KO resulted in reduced stiffness in the ATs (Figure 2B) and reduced maximum load in the FDLs (Figure 2C) compared to WT tendons. Interestingly, material properties including modulus and maximum stress were increased in FAK-KO ATs and PTs (Figure 2D-E). Finally, dynamic moduli were increased in the FAK-KO ATs and PTs at all frequencies evaluated (Figure 2F).

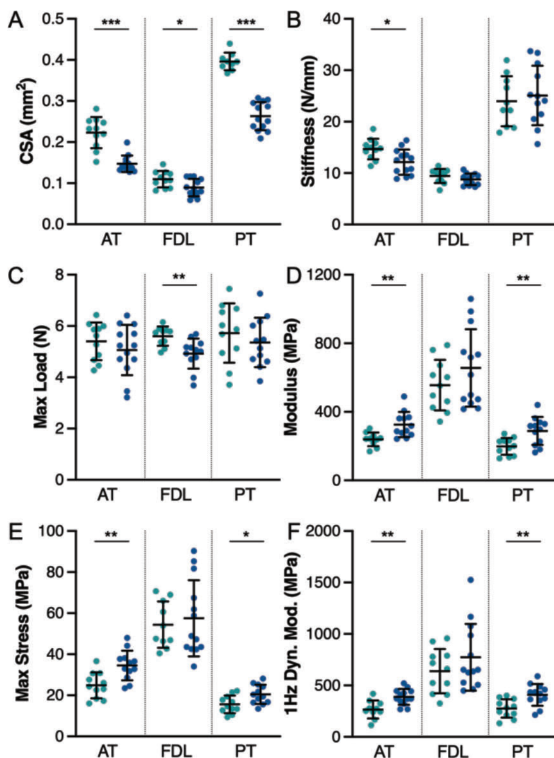

Figure 2. FAK-KO tendons demonstrated reduced size yet increased material properties at P60. **(A)** CSA; **(B)** stiffness; **(C)** max load; **(D)** modulus; **(E)** max stress; and **(F)** 1 Hz (shown as representative of all frequencies) dynamic modulus values for WT and FAK-KO tendons. Data represented as mean "±" standard deviation with plotted individual datapoints. Bars indicate significant differences (t-test: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

Discussion

This study investigated the regulatory roles of FAK on cell mechanotransduction within the native tendon ECM and on maintaining tendon homeostasis during postnatal growth. Consistent with our first hypothesis, FAK inhibition affected mechanotransductive gene expression levels in response to de-tensioned, free-floating explant conditions. Previous experiments demonstrated that tendons respond to de-tensioning by increasing expression of matrix remodeling genes such as *Mmp3* and *Mmp13* and decreasing expression of mechanotransductive genes such as *Acta2* and *Cyr61*.[7–10] In the present study, we observed similar severe increases in *Mmp3* and *Mmp13* with increased duration of explant treatment. Interestingly, FAK-I treatment suppressed this catabolic response to de-tensioning. In addition, we observed that FAK-I alters *Acta2* and *Cyr61* expression throughout our experimental timepoints. Taken together, these results indicate that FAK regulates the sensation of and response to changes in mechanical tension in the tendon *in situ* ECM.

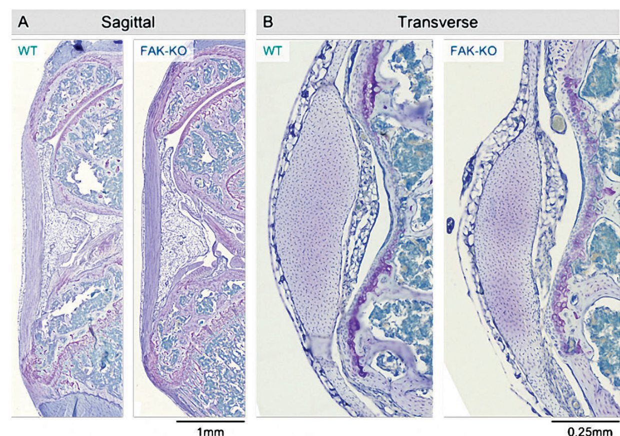

Figure 3. Paraffin histology confirmed the reduced size of FAK-KO tendons at P60. **(A)** Sagittal; **(B)** Transverse sections of patellar tendons from WT and FAK-KO mice.

Consistent with our second hypothesis, we observed substantially reduced CSA with FAK-KO in all tendons evaluated. Interestingly, despite their reduced size, FAK-KO tendons generally demonstrated increased material properties compared to WT tendons. Collectively, these results indicate that FAK regulates tendon size throughout postnatal development and that FAK-KO ultimately yields a tendon with increased material properties. Ongoing work will define the mechanism by which FAK regulates tendon size during development and postnatal growth. Specifically, we will investigate the regulatory role of FAK on tendon cell proliferation and ECM deposition. In addition, future work will investigate how mechanical loading regulats FAK-dependent mechanotransduction and tendon response *in vivo*.

Acknowledgements

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