Inhibition of Focal Adhesion Kinase in Ligament Fibroblasts Reduces Fibril Organization, But Not Fiber or Fascicle

Tristan Strayer[1], Leia Troop[1], Jennifer Puetzer[1]
[1]Virginia Commonwealth University, Richmond, VA
strayert@vcu.edu

Disclosures: Tristan Strayer,(N), Leia D. Troop (N), Jennifer L. Puetzer (N)

INTRODUCTION: Collagen fibers are the primary source of strength in tissues throughout the body, particularly in tendons and ligaments. Cells organize these fibers hierarchically, assembling them from nanometer-wide fibrils to larger fibers and fascicles, growing in size with mechanical demand.[1,2] These fibers do not regenerate after injury, nor in engineered replacements, resulting in limited repair options.[1,2] There is a need to better understand how cells form hierarchical fibers to better engineer functional repairs and replacements. It is well established that mechanical cues, including cellular contraction and external loads, are critical to cell-driven hierarchical fiber development.[2] While cellular contraction forces, regulated via integrins, focal adhesion kinase (FAK), and the actin-cytoskeleton network are established to play a major role in fibril alignment,[3,4] their role at the fiber and fascicle length-scales are largely unknown.[4,5] Recently, we developed a novel culture system that guides anterior cruciate ligament (ACL) fibroblasts in high-density collagen gels to produce aligned collagen fibrils by 2 weeks, which mature into native-sized fibers and early fascicles by 4 and 6 weeks (Fig 1A&C DMEM).[1] This system provides a unique ability to study cellular mechanotransduction during hierarchical fiber formation so to better understand how cells form organized collagen fibers. The objective of this study is to evaluate how mechanical cues, translated via integrin-cytoskeletal contraction, regulate cellular development of hierarchical fibers by inhibiting FAK temporally while cells develop aligned fibrils, fibers, and fascicles in our system. We hypothesize cellular sensing via FAK plays a significant role at all levels of collagen organization and inhibiting it while fibrils, fibers, and early fascicles are forming will reduce collagen organization and mechanics.

METHODS: To form constructs, rat tail type I collagen and neonatal bovine ACL fibroblasts were mixed and cast into 1.5 mm thick sheet gels at 20 mg/mL collagen and $5x10^6$ cells/mL, as previously described.[1] To confirm FAK inhibition, 4 mm biopsies were cut from gels and cultured for 2 weeks with 0, 0.1, 1, or 10 µM PF-573228 (FAK-I). Similar to previous studies,[3] 10 µM FAK-I reduced focal adhesion formation via reduced vinculin staining, while maintaining cell viability (data not shown). To investigate the role of FAK during hierarchical fiber formation, rectangles (8 x 30 mm) were cut from cell-seeded sheet gels and cultured clamped in our device for up to 6 weeks (Fig. 1A) and FAK activity was inhibited with 10 µM FAK-I for 2 weeks during fibril, fiber, and fascicle formation (Fig 1B). More specifically, to investigate the role of FAK during fibril formation, constructs were treated with 10 µM FAK-I from 0-2 weeks of culture. To investigate the role of FAK during fiber formation, constructs were cultured in standard DMEM media[1] for 2 weeks to allow aligned fibrils to form, and then treated with 10 µM FAK-I from 2-4 weeks during fiber formation. Lastly, to investigate the role of FAK during early fascicle formation, constructs were cultured in DMEM for 4 weeks to allow for fiber formation, then treated with FAK-I from 4-6 weeks (Fig 1B). Controls consisted of a vehicle DMSO group for each condition and constructs cultured in standard DMEM for the duration of culture. Media was changed every 2-3 days and collected to track matrix turnover. Post culture, confocal reflectance was performed to analyze collagen organization. Tissue mechanics were analyzed via tensile tests at 0.75% strain/sec to failure. DNA, glycosaminoglycans (GAGs), collagen content, and LOX activity in constructs and media were measured via Picogreen, DMMB, hydroxyproline, and LOX activity assays and are reported per construct or normalized to DNA to account for differences in contraction.[1] All data are mean ± SD. Statistical analysis was performed by 1 or 2-way ANOVA with Tukey's post-hoc (p<0.05 significant, p<0.1 trending).

RESULTS: Confocal analysis revealed FAK-I during fibril formation (0-2 weeks) reduced cellular organization with little-to-no fibril alignment at 2 weeks compared to DMEM and vehicle controls (Fig 1C red box). However, FAK-I during fiber (2-4 weeks) and fascicle (4-6 weeks) formation had little effect on cell-driven organization, with 4 and 6 week FAK-I constructs developing fibers and early fascicles similar to controls (Fig 1C). Mirroring organization, tensile properties were reduced in 2wk FAK-I constructs, with a trending decrease in elastic modulus (Fig 2A) and significantly lower UTS (Fig 2B), toe modulus and transition stress compared to controls, while FAK-I during fiber and fascicle formation had no effect on mechanics, with all groups having similar increases in properties (Fig 2A&B, 4&6 wk data). Similarly, FAK-I had little effect on DNA, GAG, and collagen composition when applied during fiber and fascicle formation (Fig 2C-D 4&6 wk, GAG not shown), however when applied during fibril formation (2 wk data), DNA did not increase compared to 0 week (Fig 2C), suggesting reduced proliferation, and collagen significantly decreased compared to vehicle controls (Fig 2D). This was further confirmed by significantly higher accumulation of collagen in the media in 0-2 week FAK-I cultures compared to controls (Fig 2E). Interestingly, LOX activity in constructs nearly doubled with FAK-I during fibril formation, and significantly increased with FAK-I during fiber formation compared to controls (Fig 2F).

DISCUSSION: This work suggests cell-ECM interactions via FAK-cytoskeletal signaling plays a major role in fibril formation, but has less of a role later in hierarchical fiber development. Specifically, confocal and mechanical analysis revealed FAK inhibition significantly inhibited fibril formation, but had little effect during fiber and fascicle formation. Interestingly, FAK-I also significantly increased collagen degradation and LOX activity during fibril formation. This may suggest a loss of cellular tensional homeostasis, shifting cells to a more injurious, catabolic state.[6] However, once cells were anchored on aligned fibrils, FAK inhibition no longer had this effect and fiber formation continued similar to controls, suggesting other mechanosensors, such as stretch activated ion channels,[4,7] may play a larger role at the fiber and fascicle length-scale. On-going work is evaluating organization with SEM and picrosirius red staining, as well as evaluating changes in gene expression to confirm a catabolic shift.

SIGNIFICANCE: Due to the key role of mechanical cues in cell-driven fiber formation, understanding the mechanotransduction pathways that regulate collagen organization is critical to regenerate tissues after injury or in engineered tissues. This data suggests other mechanosensitive pathways besides integrin-mediated adhesions are key to hierarchical fiber formation beyond the fibril level.

REFERENCES: 1.Puetzer+*Biomat* 2021; 2.Galloway+*J Bone Surg Am* 2013; 3.Rajshankar+*J Cell Physiol* 2020; 4.Lavagnino+ *JOR* 2015; 5.Han+*Eur Cell Mater* 2014; 6.Nguyen+*AJP-LUNG* 2021; 7 Du+*Exp Biol Med* 2020

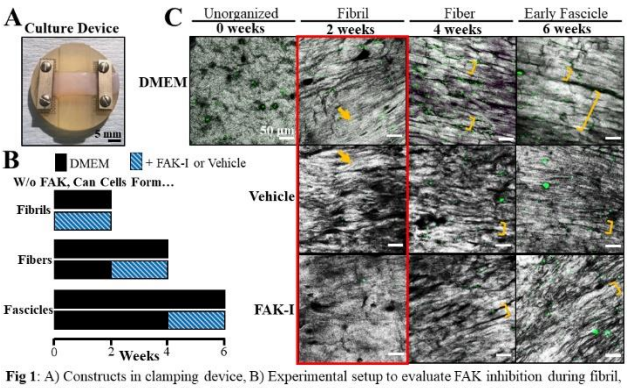

Fig 1: A) Constructs in clamping device, B) Experimental setup to evaluate FAK inhibition during fibril, fiber, and early fascicle formation, C) Confocal analysis of collagen organization. grey = collagen, green = auto-fluorescent cells, arrows denote aligned fibrils, brackets denote fibers & fascicles

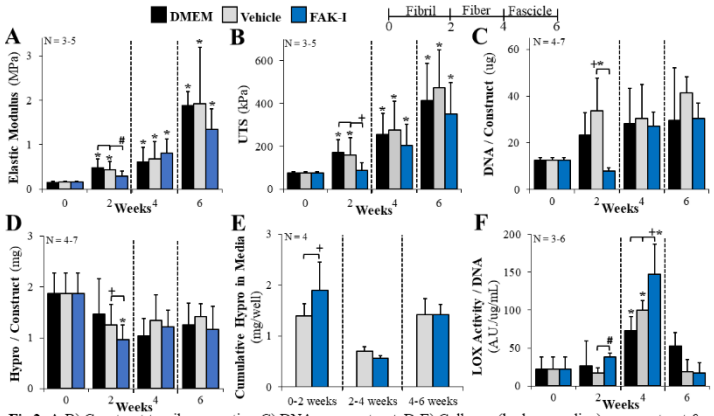

Fig 2: A-B) Construct tensile properties, C) DNA per construct, D-E) Collagen (hydroxyproline) per construct & accumulated in media over 2 weeks, F) LOX activity in constructs. Significance to *0wk & +bracket, #trending