How ACL Fibroblasts Sense Slow Growth Elongation Changes with Collagen Fiber Maturation: It's Not All FAK

Kelly Ott[1], Jennifer L. Puetzer[1, 2]
Departments of [1]Biomedical Engineering [2]Orthopaedic Surgery, Virginia Commonwealth University, Richmond, VA, USA
ottkr@vcu.edu

Disclosures: Kelly Ott (N), Jennifer Puetzer (N)

INTRODUCTION: Collagen fibers are the primary source of strength in tendons and ligaments.[1] Cells organize these fibers hierarchically, from nm-wide fibrils into larger fibers and fascicles.[1] These fibers largely do not regenerate after injury or with repair, thus there is a need to understand how cells form these fibers to better drive regeneration.[1,2] It is well established that mechanical cues, including slow growth elongation, are critical to cell-driven fiber formation, however the role of each cue is unknown.[3] Previously, we developed a static culture system that guides ligament (ACL) fibroblasts to produce native-sized hierarchical fibers over 6 weeks,[2] and we have shown that slow stretch at neonatal (1 mm/day)[4] and postnatal (0.1 mm/day)[5] ACL growth-rates further drives maturation in this system. Interestingly, we also found that the rate of stretch affected cells differentially depending on the degree of organization, with neonatal rates increasing maturation in unorganized collagen and postnatal rates driving improvements later in culture once cells were anchored on aligned fibers, suggesting a shift in mechanotransduction.[6] There is a need to understand how cells sense these subtle mechanical cues at different levels of collagen organization to better stimulate fiber regeneration after injury and in engineered replacements. It is well established that cells on collagen fibrils primarily sense tensile loads via focal adhesions with the extracellular matrix, regulated by focal adhesion kinase (FAK).[7] However, it is unknown whether slow stretch is dynamic enough to stimulate FAK or how cellular mechanotransduction changes once larger fibers and fascicles have formed. The objective of this study is to evaluate the role of FAK in cellular sensing of slow stretch at different degrees of collagen organization. We hypothesize that FAK is a primary mechanosensor for slow stretch and that inhibiting FAK during slow stretch will limit collagen maturation at all levels of hierarchical organization.

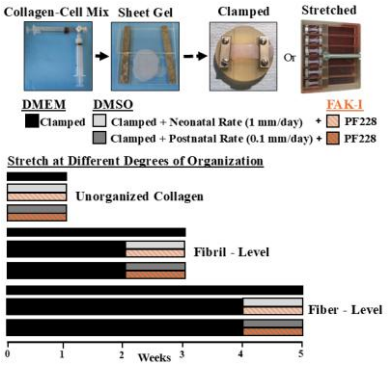

Fig 1: Depiction of experimental setup & timeline of culture

METHODS: Neonatal bovine ACL fibroblasts were mixed with type I collagen to form gels at 20 mg/mL collagen and $5x10^6$ cells/mL.[2] Rectangles (8x30 mm) were cut from gels and statically cultured in our clamping device to guide hierarchical fiber formation (Fig 1A). More specifically, constructs began with unorganized collagen at 0 weeks, which cells organized into aligned fibrils by 2 weeks and larger fibers at 4 weeks.[2] At 0, 2, or 4 weeks, after developing unorganized collagen, aligned fibrils, or fibers, constructs were transferred to a tensile bioreactor and stretched at neonatal (1 mm/day)[4] or postnatal (0.1 mm/day)[5] ACL growth rates for 1 week (Fig 1B).[6] To evaluate the role of FAK in cellular mechanosensing at different levels of collagen organization, FAK was inhibited with 10 µM PF-228[8] (FAK-I) for the duration of stretch (Fig 1B). Controls remained statically clamped throughout culture or were dosed with DMSO during stretch as vehicle controls. Post-culture, collagen organization was analyzed via confocal reflectance.[2] DNA, glycosaminoglycan (GAG), lysyl oxidase (LOX) activity, and collagen content were measured via PicoGreen, DMMB, LOX activity, and hydroxyproline (hypro) assays.[2] Mechanical properties were measured via tensile tests at 0.75% strain/sec to failure. Statistical analysis was performed by 2-way ANOVA with Tukey's post-hoc (p<0.05).

RESULTS: Similar to previous work,[6] confocal revealed neonatal stretch (1 mm/day) drove accelerated fiber development compared to static-clamped and postnatal constructs when applied to unorganized collagen (Fig 2). This fiber development was reduced but still present in FAK-I neonatal constructs at 1 week and little-to-no alignment was observed in FAK-I postnatal constructs. Interestingly, when stretch was applied at 3 weeks once fibrils had formed, FAK-I appeared to have little effect with all groups formed aligned fibers, and when applied at the fiber level, FAK-I constructs at both stretch rates appeared to have improved organization compared to DMSO controls (Fig 2). Compositionally, FAK-I largely blocked stretch-driven increases in DNA and GAG accumulation at all levels of organization (Fig 3A). Conversely, the effect of FAK-I on LOX activity and collagen content varied with degree of organization. Most notably, FAK-I when postnatal stretch was applied to the fiber level resulted in reduced LOX activity and increased collagen accumulation (Fig 3A, week 5). The effect of FAK-I on tensile properties also varied with degree of organization. Similar to confocal, FAK-I reduced tensile properties when stretch was applied to unorganized collagen, had no effect at the fibril level, and with neonatal stretch actually increased properties at the fiber level (Fig 3B). Conversely, FAK-I with postnatal stretch reduced tensile properties, rather than increased them, when applied at the fiber level (Fig 3B).

DISCUSSION: Overall, FAK-I differentially affected collagen maturation based on stretch rate and degree of organization. FAK-I blocked gains in collagen organization and tissue strength when stretch was applied to unorganized collagen. This correlates with previous work,[7] suggesting FAK is highly involved in cellular sensing of load during fibril formation. However, FAK-I had little effect on cellular response to neonatal or postnatal stretch at 3 weeks, suggesting a reduced role of FAK in mechanotransduction once aligned fibrils have formed. Further, FAK-I increased collagen organization, concentration, and tissue strength when stretch was applied at the fiber level, suggesting other mechanosensors, such as stretch ion channels or primary cilia, are being activated by slow stretch elongation at these later stages of development. Future work will evaluate the role of other mechanosensors in responding to slow elongation.

SIGNIFICANCE: Slow growth plays a key role in fiber maturation, but it is difficult to replicate in adult tissues, so understanding the mechanotransduction pathways involved could help regenerate collagen fibers after injury. This data suggests that integrin-mediated adhesions play a key role in cells sensing slow stretch while fibrils are forming, but other mechanosensors may dominate once larger fibers have formed.

REFERENCES: 1.Galloway + *J Bone Joint Surg Am* 2013 2.Puetzer + *Biomat* 2021; 3. Killian + *J Shoulder Elbow Surg* 2012 4. Kalson + *Dev Dyn* 2011; 5.Cone + *PLoS One* 2019; 6.Ott + *TERMIS* 2024; 7.Lavagnino + *JOR* 2015; 8. Rajshankar + *J Cell Physiol* 2020

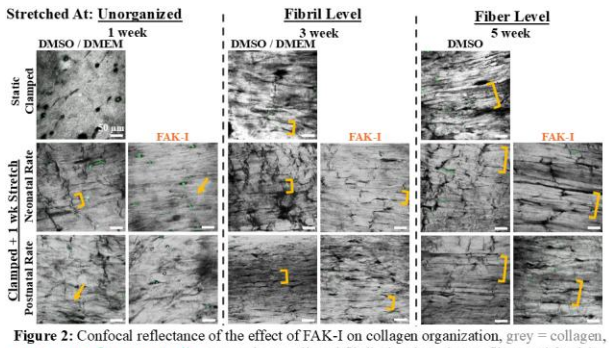

Figure 2: Confocal reflectance of the effect of FAK-I on collagen organization, grey = collagen, green = auto-fluorescent cells, arrows denote aligned fibrils, brackets denote fibers and fascicles

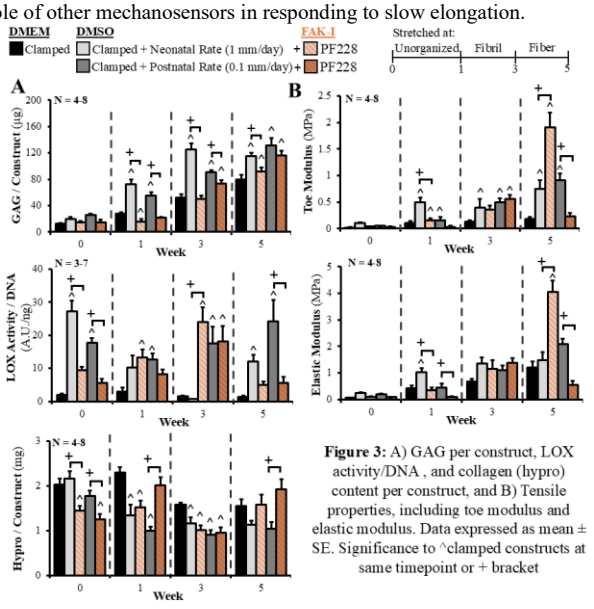

Figure 3: A) GAG per construct, LOX activity/DNA , and collagen (hypro) content per construct, and B) Tensile properties, including toe modulus and elastic modulus. Data expressed as mean ± SE. Significance to ^clamped constructs at same timepoint or + bracket