Enthesis cells are maintained with stable expression of HIF1-alpha following Achilles tendon injury

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Disclosures: NONE.

INTRODUCTION: Achilles tendon ruptures are one of the most common tendon injuries.[1] Biopsies from patients with chronic Achilles insertional tendinopathy and mid-portion rupture show increased fibrosis (i.e., excessive deposition of extracellular matrix, ECM) and increased vascular ingrowth.[2] Hypoxia inducible factor 1-alpha (HIF1a) is elevated in tendinopathy and is also associated with fibrosis.[3] The role of HIF1a has predominantly been associated with disease progression in tendinopathy[3]; however, if and how HIF1a regulates tendon remodeling and healing following injury remains unknown. Recently, we showed that stable expression of HIF1a leads to an expansion of the enthesis during postnatal growth. Additionally, *Hif1a* is required for enthesis maintenance during postnatal growth. We have also observed that, following Achilles laceration, enthesis cells are no longer present after 3wks of healing. Because HIF1a promotes cell survival, in part, by modulating vascularization, we hypothesized that stable expression of HIF1a in tendon stromal cells would improve healing, promote angiogenesis, and maintain cells at the enthesis following injury.

METHODS: All animal procedures were approved by IACUC. We first identified the time course of cell maintained at the tendon enthesis following Achilles tendon laceration using C57BL6/J mice (12-16wks of age, n=6 male and n=9 female). We generated mice for stable (non-degradable) expression of HIF1a by breeding loxP-stop-loxP (LSL)-HIF1dPA (proline to alanine substitutions) mice (JAX stock 009673) in Scx-lineage cells (HIF1dPA; LSL, Cre+ mice) and wildtype (Cre-negative or Cre-positive heterozygous LSL mice). We then investigated if and how stable expression of HIF1a influenced tendon and enthesis remodeling following injury (HIF1dPA and wildtype mice; 12-16wks of age, n>3 per genotype). All mice received either unilateral or bilateral Achilles tendon lacerations (full width injury). Mice were euthanized at late inflammatory (7 days post injury, dpi), proliferative (14dpi), or remodeling (21-28dpi) stages of healing. We evaluated cellular number and collagen organization on C57BL6/J mice using paraffin histology inclusive of the injury region, tendon stump, and enthesis. We evaluated if and how stable expression of HIF1a influenced tendon healing and enthesis remodeling following injury, using bilateral tendon lacerations on HIF1dPA mice (n=3 at 14dpi; n=2 at 28dpi) and age-matched wildtype littermates (n=3 at 14dpi; n=3 at 28dpi). At time of euthanasia, one distal hindlimb from each mouse was dissected, fixed, and decalcified for paraffin histology. The Achilles tendon and injury/scar from the second injured limb was carefully dissected and immediately flash frozen for protein assays. Histology sections were stained using Hematoxylin and Eosin (for cell counting), Picrosirius red (to visualize collagen organization), and immunohistochemistry (CD31 to visualize vascularization). Slides were imaged using an automated slide scanner and cells in the enthesis were counted using the FIJI plugin, StarDist. For organization, slides were imaged using circular polarized light microscopy with a 10x objective on an epifluorescent microscope (Leica). Images were acquired using a polarization camera and ThorCam software (ThorLabs Inc.). The degree of linear polarization (DoLP) and angle of linear polarization (ALP) were analyzed at the tendon stump (injured) or midsubstance (intact) using the Math and SciPy Stats libraries in Python3. Differences in DoLP and AoLP of tendon and number of cells within the entheses were compared using one-way ANOVAs (Graphpad v10, Prism, LaJolla, CA). Frozen tendons for protein assays were pulverized using metal beads for mechanical dissociation in liquid nitrogen and protein was isolated using RIPA buffer with protease and phosphatase inhibitors. The amount of protein from each sample was quantified using bicinchoninic acid (BCA) assays (Pierce, ThermoFisher) and identical amounts of protein were used for array blots to identify differences in protein expression related to angiogenesis between wildtype and HIF1dPA mice at 14dpi (R&D Systems).

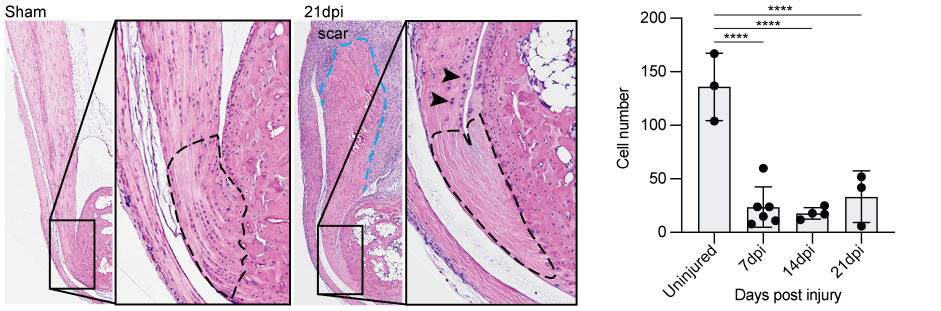

Figure 1. Achilles tendon laceration leads to rapid loss of enthesis resident cells in C57BL6/J mice. Dots represent biological replicates.**** p<0.0001.

RESULTS: In our initial pilot study, we found cells in the enthesis were gone by 21dpi. We then evaluated how early the cells disappear, and found that cell numbers were already reduced by 7dpi (Fig 1). DoLP of the tendon did not differ between control and injured groups, however AoLP was higher at 7dpi and 14dpi compared to intact controls ($p<0.01$), indicated higher variation with injury. During healing, angiogenic markers (e.g., endostatin, platelet factor 4, serpin F1) were increased with HIF1a activation (Fig 2). Angiogenic targets modulated during healing by stable expression of HIF1a included coagulation factor III, FGF1, IGFBP-2, and osteopontin (Fig 2). Stable expression of HIF1a led to increased CD31+ cells in the enthesis (Fig 3) and maintained cells in this region for most, but not all, injured Hif1adPA tendons compared to controls.

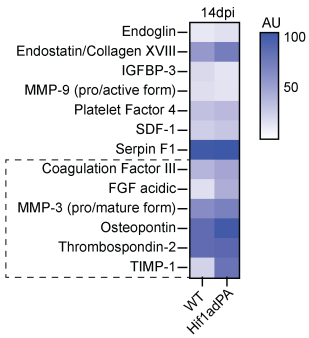

Figure 2. Expression of several angiogenic markers following Achilles tendon injury were modulated by Hif1adPA. Highlighted in dashed box include factors associated with matrix remodeling (MMP3, TIMP-1) and osteopontin which were elevated with overexpression of HIF1a (Hif1adPA). n = 2 injured tendons per group. Protein expression normalized to reference dots on Proteome Profiler blots for mouse angiogenesis markers.

DISCUSSION: In this study, we showed that stable expression of HIF1a in stromal cells leads to elevated levels of osteopontin, MMP3, and TIMP1 following injury which may accelerate collagen deposition and remodeling as well as recruitment and maintenance of inflammatory cells in the healing tendon. Hif1adPA mice had increased vascular ingrowth following injury, which may explain how the enthesis cells are maintained following injury. While this work provides insight into tendon remodeling and the role of HIF1a, it remains unclear if and how HIF1a influences functional changes of the tendon following injury. Future studies are needed to identify functional outcomes of tendon healing modulated by stromal cell expression of Hif1a, as well as to identify the cell specific contributions of HIF1a during tendon repair and enthesis maintenance.

SIGNIFICANCE: The discovery of druggable targets, such as HIFs, may provide new avenues for therapeutic treatment of tendon healing and regeneration. Basic science studies investigating the cell-specific mechanisms of HIF1a in tendon repair will provide foundational support for drug discovery approaches.

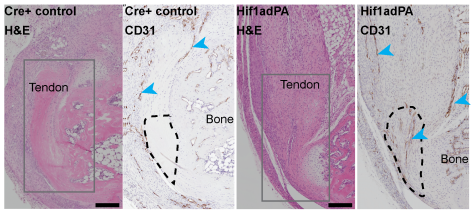

Figure 3. Enthesis cells were maintained after proximal tendon injury in Hif1adPA mice. ScxCre+ control mice lost enthesis cells by 28dpi (dashed outline; representative female from n=3 mice). However, Hif1adPA entheses maintained cells following tendon injury (representative female from n=3 mice). Additionally, injured Hif1adPA tendons had more CD31+ vessels (blue arrow). Scale = 200 micrometers.

REFERENCES: 1. Pedowitz, CRMM 2013; 2. Giordano Cureus 2022; 3. Millar, Ann Rheum Dis 2012.

ACKNOWLEDGEMENTS: NSF CAREER Award 1944448 (MLK); NIH R01AR079367 (MLK); NIH P30 AR069620; NSF GRFP (LH); Frankel Cardiovascular Center Summer Undergraduate Research Fellowship (MKJ)