HIF1a Regulates Cell Survival and Vascularization in the Mouse Achilles Tendon Enthesis

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INTRODUCTION: The tendon enthesis is essential for transmission of muscle loads to the skeleton, and its maturation takes place during postnatal growth concomitant with independent mobility.[1] The enthesis maintains a hypoxic microenvironment during postnatal growth[2] and its maturation is marked by the establishment of Gli1+ fibrochondrocytes.[3] While hypoxia is essential for embryonic development, its role during postnatal enthesis maturation is not well understood. Recently, we have shown that the postnatal enthesis relies on hypoxia inducible factor-1a (Hif1a) to form a fibrocartilage interface between tendon and bone.[2] Specifically, tendon and enthesis progenitors which constitutively lack expression of Hif1a exhibit profound focal cell death and extracellular matrix disruption, leading to significant impairments in enthesis structure and function.[2] Yet, if and how Hif1a regulates maintenance of enthesis progenitors is not well known. We studied the effect of Hif1a gain-of-function (GOF) in Scx+ enthesis progenitors as well as inducible loss-of-function (LOF) in Gli1+ fibrochondrocytes *in vivo* to identify the critical timing and cell-dependent role of Hif1a during postnatal enthesis maturation.

METHODS: All procedures were approved by the Unit for Laboratory Animal Medicine at the University of Michigan. To establish the time course for Gli1CreERT2 expression in the postnatal Achilles enthesis, we crossed homozygous reporter mice with the NuTRAP allele (Nuclear tagging and Translating Ribosome Affinity Purification) with Gli1-CreERT2 mice. Pups were injected subcutaneously with tamoxifen (Tam) (75mg/kg; in corn oil) at postnatal day (P)4, P7, and P14. The presence of the NuTRAP allele allows for Cre-mediated expression of mCherry (mRANGAP1, to label nucleus) and EGFP (L10a, to label polysomes)[4]. We generated Hif1a conditional knockout mice (Hif1cKO, inducible: Hif1a$^{fl/fl}$; Gli1CreERT2)[5,6] by breeding Hif1a$^{fl/fl}$; Gli1CreERT2 male sires to Hif1a$^{fl/fl}$ females. Pups were injected with a single dose of Tam in corn oil subcutaneously at either P7 or P14. We also generated Hif1a overexpression mice (HIF1dPA-ScxCre) by generating Hif1dPA;ScxCre+ heterozygous male sires with Hif1dPA females. The Hif1dPA mouse harbors a hemagglutinin (HA)-tagged human *HIF1A* (hypoxia inducible factor 1, alpha subunit; HIF1α)[7]; this GOF insertion includes a loxP-flanked neomycin resistance cassette followed by hemagglutinin-tagged human HIF1A cDNA modified with two proline to alanine substitutions (P402A, P564A) that evade degradation in the mouse cytoplasm. Mice were maintained on mixed C57BL/6J/;FVB/NJ background (n ≥ 3/sex/genotype). Control mice included ScxCre+ heterozygous floxed mice (Cre+ control) as well as Cre-negative floxed mice (Controls for ScxCre and Gli1CreERT2 strains). At P28, Hif1dPA and Control mice were weighed and assessed for forelimb grip strength (Bioseb, Pinellas Park, FL). For lineage tracing, all mice were euthanized at P21. All Hif1dPA-ScxCre and littermate control mice were euthanized at P56. Hif1cKO and Control mice were euthanized at P21 (for the P14 Tam dosing group only, n = 2 per genotype) or P56 (for both P7 [n=1 cKO and n=1 Control] and P14 [n=3 cKO and n=5 Controls] Tam dosing groups). Mice were weighed after euthanasia and radiographs were obtained using digital X-ray (Kubtec Xper40). Distal hindlimbs from all mice were skinned and fixed in 4% paraformaldehyde for 24-48hr after dissection followed by decalcification using 14% ethylenediamine-tetraacetic acid (EDTA). For lineage tracing, Achilles entheses were sectioned following cryo-embedding at 14µm thickness and imaged using a Nikon Eclipse Ni E800 with DS-Ri2 camera. Hif1cKO, Hif1dPA, and control entheses were paraffin embedded and sectioned at 7 µm thickness. Slides were also stained using H&E or Silver stain and enthesis area was measured using ImageJ/FIJI[8] (for Control and HIF1dPA sections only). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was used to identify cell death (for Hif1cKO and Control entheses at P21 or P56; after P14 Tam injections only).

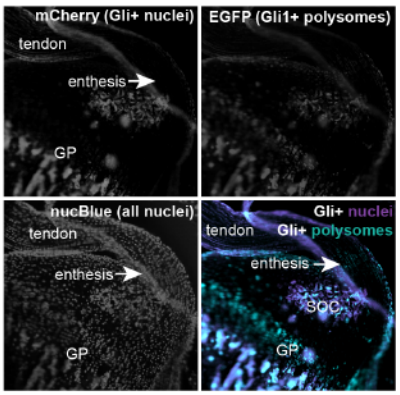

Figure 1. Pulse-chase of Gli1+ cells using the NuTRAP reporter in the mouse Achilles enthesis. Pups were injected with tamoxifen SC at P14 and euthanized at P21. All images show the same enthesis with different fluorescent reporters; mCherry labeled nuclear envelope of Gli1+ cells; EGFP labeled translating polysomes of Gli1+ cells; and nucBlue labeled all nuclei. Cyan and magenta overlay shows Gli1+ cells only (no nucBlue). Gli1+ cells populate the Achilles enthesis as well as most of the growth plate (GP). SOC = secondary ossification center.

RESULTS: Lineage tracing at P4, P7, and P14 (Fig 1) all showed effective labeling of resident cells of the Achilles tendon enthesis when using NuTRAP; Gli1CreERT2. All three time points labeled the secondary ossification center, growth plate, and enthesis. Inducible Hif1a LOF in Gli1+ cells at P14 did not affect the morphology or cell viability of the postnatal enthesis compared to Cre- controls (data not shown). However, skeletal defects, including shortened tibiae and articular joint (e.g., knee) deformities, were prominent in adult (P56) Hif1cKO mice after Tam delivery at either P7 or P14 (Fig 2). No significant difference in weight was measured between Control and Hif1cKO mice (Fig 2). Overexpression of Hif1a in ScxCre progenitors (Hif1dPA) led to increased enthesis area at P56 compared to controls (Fig 3) with no notable skeletal deformities on X-ray and no difference in grip strength (data not shown).

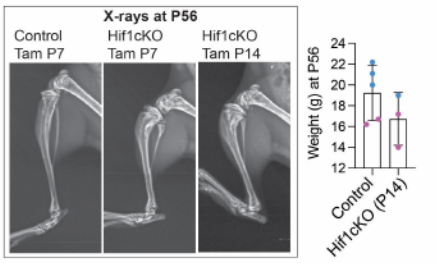

Figure 2. Inducible knockdown of Hif1a in Gli1+ progenitors (Hif1cKO) leads to disruption in long bone growth. Hif1cKO mice were generated following induction of Cre-recombinase at either postnatal day 7 (P7) or P14 following a single tamoxifen injection and mice were euthanized and X-rayed at P56. Tibia length was shorter in Hif1cKO mice when Hif1a loss of function was induced at either P7 (Tam P7) or P14 (Tam P14), with no remarkable differences between the two delivery time points. No significant difference in weights were observed between Control and Hif1cKO (P14 tamoxifen) mice by P56. All X-rays to scale.

DISCUSSION: HIF1A is a crucial transcription factor that mediates cellular responses, including survival, under hypoxic conditions. In this study, we found that inducible Hif1a LOF resulted in impaired bone elongation. Previously, we showed constitutive Hif1a LOF (ScxCre) led to significant enthesis deformities including cell death.[2] Unlike this constitutive LOF model, we did not observe increased cell death when induced knocked down of Hif1a at P14 in enthesis-progenitor Gli1+ cells. Current experiments are measuring cell death following inducible LOF of Hif1a at P7. We have previously shown the enthesis is hypoxic during postnatal growth[2], and constitutive overexpression of HIF1A led to an enlarged enthesis, suggesting a potential expansion of enthesis progenitors in response to sustained HIF1A. This expansion may be associated with increased vascularization, which we are currently investigating. Future work will study how stable expression of HIF1A influences enthesis vasculature as well as tendon/enthesis healing following injury.

SIGNIFICANCE/CLINICAL RELEVANCE: Understanding the role of Hif1a during enthesis maturation will help inform the use of HIF-targeting therapeutics for treatment of diseased tendon attachments throughout the lifespan.

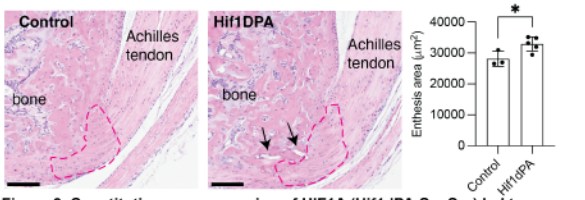

Figure 3. Constitutive overexpression of HIF1A (Hif1dPA-ScxCre) led to expansion of the enthesis and increased enthesis vascularization. Hif1aDPA mice had larger entheses compared to control littermates (overlaid from Silver-stained images). Dashed lines = enthesis region. Arrowheads highlight large vessels found adjacent to the enthesis in HIF1DPA but not control mice. H&E stained, paraffin sections. Scale bar = 100µm. *, p = 0.034, unpaired t-test.

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