Optogenetic contraction of muscle leads to elevated gene expression of metabolic and inflammatory pathways in tendon and bone

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INTRODUCTION: Loading is essential for functional development and growth of the musculoskeletal system.[1] During embryonic development, tendon extends from the cartilage anlagen to guide load transfer from skeletal muscle.[2] While several studies have established the requirement of muscle contraction during skeletal growth, these studies have primarily focused on the absence or reduction of muscle loading rather than increased muscle contraction.[2–4] In mouse and rat studies of skeletal adaptation to mechanical loading, in vivo cyclic loading has commonly been used to induce adaptation of bone and tendons.[5–9] More commonly used models of tendon loading, such as forced treadmill running (uphill or downhill), are effective for inducing structural changes to the musculoskeleton but also have their drawbacks, with high variability,[10] anxiety and systemic inflammation,[11] and organismal metabolic conditioning induced by cardiovascular load.[12] To address these challenges, we developed the use of optogenetics as a non-invasive tool to induce muscle contraction for *in vivo* tendon loading in growing mice. Optogenetics facilitates controlled depolarization of activatable cells (e.g., neurons and muscle cells) with high spatial and temporal specificity.[13] We used channelrhodopsin-2 (ChR2) which, when present on the cell membrane, acts as a non-specific light-activated cation channel.[14] We and others have previously shown that optogenetic-mediated activation of skeletal muscle allows for precise and controlled contraction of specific muscle groups[15,16] and, therefore, loading of their associated tendon and bone. In a previous study, we showed that daily optogenetic loading leads to structural and morphological adaptation of the young Achilles enthesis. In this study, we examined how daily bouts of muscle loading, induced by optogenetic activation, can elicit differential gene expression in Achilles tendon and calcaneus in the adolescent mouse limb.

METHODS: All procedures were approved by the Institutional Animal Care and Use Committee at UMich. We generated optogenetic muscle-specific mouse lines, as previously described, that expressed a YFP-fused ChR2 light-sensitive (455nm) opsin in skeletal muscle when treated with tetracycline.[17] Dams were treated with doxycycline chow at the time of mating and maintained on chow until offspring were weaned. Offspring were genotyped using PCR. At 3wk of age, mice were anesthetized with isoflurane. The right limb was stabilized at the knee joint, and the right foot was placed on a foot pedal. For pulsed light modulation, a collimated LED light (455 nm, 900 mW, M455L3, Thorlabs) and an LED driver (DC2200, Thorlabs, Newton, NJ) were used.[17] Tricep surae muscles were stimulated using a 10Hz pulsed light (70ms on, 30ms off; 10 cycles) followed by 4 seconds rest for 20 minutes (240 loading cycles). We performed bulk RNA-seq from Achilles tendons and calcanei of optogenetically-loaded mice (loaded and contralateral limbs) at 3-4hr post-final loading bout. Mice were euthanized 3hr after the 5th or 12th consecutive day of loading and tendon and calcaneus from loaded and contralateral limbs were immediately isolated and separated under RNase-free conditions and snap frozen (n=3 male mice/time point). Tissues from age-matched mice with normal cage-activity were also collected (naïve; n=3 male mice). Tissues were pulverized in Trizol and total RNA was isolated. Poly-A mRNA libraries were prepared and sequencing was performed using Illumina NovaSeq Shared platform.[18] Differential gene expression was determined from count matrices with either paired design (loaded vs. contralateral) or unpaired design (loaded or contralateral vs. naive) in DESeq2 in R/Bioconductor.[19]

RESULTS SECTION: We used principal component analyses (PCA) to evaluate in an unsupervised approach the quality and structure of our bulk gene expression data (Fig1A). To avoid any compensatory effects of the contralateral limb and because we saw discrete separation of naïve from loaded (5 and 12day) tendons in PCA, we used naïve data to measure fold change between loaded and cage activity tendons and bones. We found optogenetic loading resulted in downregulation of numerous genes associated with tendon stem cells, including *Tppp3* and *Fbn1*, which were down at both 5 and 12 days. Of the top 20 downregulated genes in tendon following loading, we identified genes associated with extracellular matrix synthesis at one or both time points, including *Mtn3*, *Acan*, *Col8a1*, *Thbs1*, *Col9a2*, *Bglap*, and *Mmp9*. In bone, daily bouts of loading resulted in increased expression of *Il1ra1*, *Ccl21a*, and *Ccl19* at one or both time points. We used Ingenuity Pathway Analysis (IPA, Qiagen, Germany) to predict upstream regulators and determine loading-induced activated or inhibited pathways (Fig1B). In tendon, we identified loading elicited activation at both 5 and 12 days of eukaryotic initiation factor 2 (EIF2) signaling, glycolysis, and Rho GDP-dissociation inhibition signaling (RHOGDI). Mechanical loading of tendon resulted in suppressed mechanobiology pathway activation. In bone, loading activated genes associated with inflammation (e.g., NFkB).

DISCUSSION: The role of increased loading on mineralization of the growth plate during postnatal maturation was elucidated in our bulk RNAseq studies, and we identified key pathways that are transcriptionally activated in the growing calcaneus as well as tendon. Activation of RHOGDI signaling and downregulation of mechanosensitive pathways (e.g., RAC, actin cytoskeleton) in tendon was surprising, considering the robust structural and morphological changes we previously observed in the loaded enthesis.[20]

SIGNIFICANCE/CLINICAL RELEVANCE: Increased activity during periods of rapid growth, such as in children and during adolescence, can lead to overuse induced pain and dysfunction, such as Sever disease. Basic science approaches are needed to better understand tendon and bone adaptation to loading.

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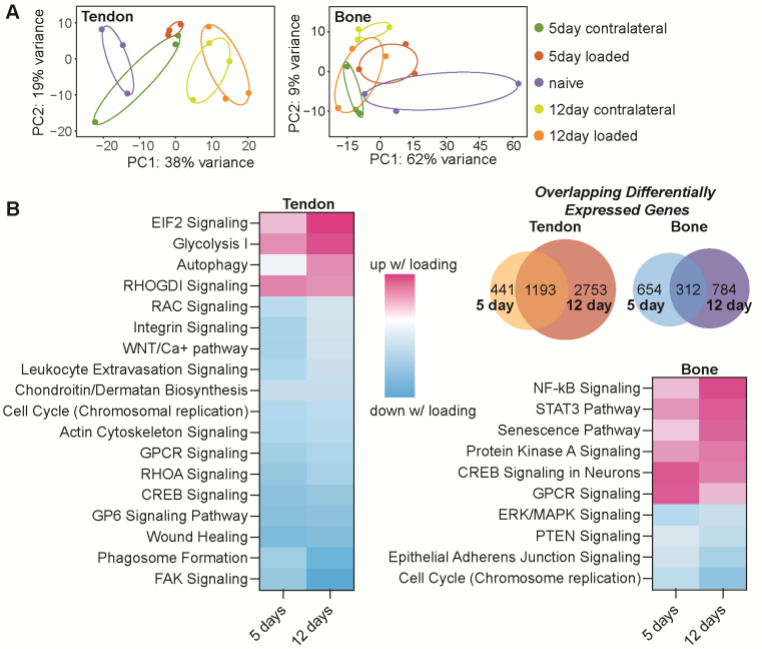

Figure 1. (A) Principal component analysis shows discrete separation of naïve from loaded (5day and 12day) tendons. (B) Loading induced upregulation of EIF2 signaling, glycolysis, and Rho GDP-dissociation inhibition signaling and downregulation of FAK, actin cytoskeletal, and integrin signaling pathways. With repeated loading and in bone, inflammatory pathways such as NFkB and STAT3 signaling, senescence and CREB signaling are upregulated, while cell cycle (replication) and ERK/MAPK are inhibited. After 5 and 12 days of loading, 1,193 and 312 genes in tendon and bone, respectively, were differentially expressed.