# Eccentric Muscle Loading Induces Positive Alterations to the Transcriptome in a Murine Model of Tendinopathy

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**INTRODUCTION:** Achilles tendinopathy (AT) is a common disease that causes pain, reduced mobility, and diminished quality of life[1]. Eccentric loading is an effective physical therapy treatment for AT that improves tendon biomechanical properties and clinical symptoms[2]. However, a gap in knowledge remains regarding a functional understanding of the transcriptomic adaptations in response to eccentric loading protocols. The objective of this study was to investigate transcriptomic changes in response to rehabilitative muscle loading using a mouse model of Achilles tendinopathy.

**METHODS:** Under IACUC approval, tendinopathy was induced in 12-week old, male C57Bl/6J mice using 2 sets of paired intratendinous injections of rHuTGFβ-1given 48 hours apart[3, 4]. Mice were randomly assigned to 1 of 4 groups: muscle loading at 50 or 100 Hz to replicate 50% or 100% body weight (BW), age-matched injured/untreated (IU) control, or a naïve (uninjured) control. Mice in the injured groups were treated twice weekly for 1, 2, or 4 weeks with 3 sets of muscle loading repetitions per session. The naïve group consisted of mice that were the median age of the other treatment groups. Starting 48 hours following the completion of tendinopathy induction, each mouse assigned a loading therapy (50% BW, 100% BW, or IU) was anesthetized, the hind right limb was secured to the pedal of a dual-mode servo motor transducer, and electrodes were inserted into the plantar flexor muscles to stimulate the tibial nerve[4]. Under computer control, the foot was rotated through the entire ankle joint range of motion (±18°) while the plantar flexors simultaneously contracted to achieve the desired BW loading[5]. For age-matched IU groups, mice were anesthetized, loaded into the footplate and the electrodes placed, but no stimulation delivered. Mice were euthanized 24 hours following the final muscle loading treatment and Achilles tendons were harvested for transcriptomics[6]. A total of 12 mice per group was used for each loading magnitude and duration. Isolated tendon samples were pooled in groups of 2 tendons per sample, resulting in a total of 6 samples per group. A total of 56 tendons (28 mice) were pooled to create an n of 6 samples for the naïve group. Samples were homogenized, RNA column purified and quality control performed to select samples with an RNA integrity number $\geq$ 4.0. RNA-Seq was performed using Illumina NovaSeq 6000 S4, reads were trimmed, mapped, and read counts and expression values were determined using gene length corrected trimmed mean of M-values (GeTMM), differentially expressed genes (DEG) identified and Principal Component Analysis (PCA) performed. Functional analysis of transcriptomic adaptations was performed using weighted gene co-expression network analysis (WGCNA) using the entire transcriptome to identify associations between gene modules. Traits were tested by correlating module eigengenes to traits of interest (injury; muscle loading). Gene ontology was used to analyze the list of genes within each cluster and interpreted using the database for annotation, visualization, and integrated discovery to identify biological processes. Ingenuity Pathways Analysis (IPA) was used to identify pathways, upstream regulators, and their network interactions[6].

**RESULTS:** Principal component analysis of the differentially expressed genes revealed distinct pattens of gene expression between specific groups across all time points and diverged over time **(Fig. 1)**. The 4-week 50% and 100% BW loading groups are clustered together the 1- and 2-week IU and 100% BW groups are all loosely clustered together in the same quadrant. The naïve group is very tightly clustered together and also with the 4 week IU group. WGCNA using DEG identified 8 independent modules, 3 of which (Red; $R^2$=0.83, Yellow $R^2$=0.87, and Brown; $R^2$=0.96) were positively associated ($R \geq 0.7$; $p \leq 0.05$) with exercise. The Yellow and Red modules were associated with 100% BW loading at 2 weeks and contained 39 and 82 significantly overrepresented biological processes, respectively **(Fig. 2)**. The Brown module was associated with 50% BW loading at 2 weeks; the identified genes were associated with general biological process. The biological processes within the Yellow module were associated with response to prostaglandin E, cell fate, extracellular matrix orientation, and inflammatory response. The Red module contained biological processes responsible for response to prostaglandin F, cell-cell signaling, cytoskeleton organization, response to hypoxia, and positive regulation of cell migration. The Grey module was negatively correlated with the unexercised group. IPA revealed greater numbers of activated pathways in exercised groups when compared to unexercised groups, which had increased instances of inhibited pathways. Notably, IPA revealed the 50% BW group showed significant activation of oxidative phosphorylation, phagosome activation, extracellular matrix organization, collagen biosynthesis pathways and inhibition of neutrophil granulation, mTOR, mitochondrial dysfunction, and inhibition of metalloproteinase pathways.

**DISCUSSION:** Our analysis clearly supports a transcriptomic signature specific to exercise and highlighted the unique cellular responses associated with the different muscle loading protocols. Many were pathways critical in improving tendon healing. Loading at 50% and 100% BW had distinct expression profiles at 1 week based on the PCA. The 100% BW group is grouped with the IU group at 1 week, suggesting that this level of loading may be excessive early in healing, hindering the healing process. The overrepresented biological processes identified in the Yellow module suggest that 100% BW at 2 weeks increases expression crucial for wound healing and improving tendon function post-injury. From the IPA, 50% BW loading activated pathways beneficial to tendon health, supporting the conclusion that lower loading magnitude may be more conducive to tendon healing, at least early in the healing process. The results from this study, combined with our previously reported biomechanical and histological outcomes, suggest a substantial benefit from eccentric muscle loading in our murine model of tendon injury[4]. Based on these findings, implementing 50% BW loading in the first few weeks of treatment, then transitioning to 100% BW loading might be the most advantageous method to promote robust tendon healing.

**SIGNIFICANCE/CLINICAL RELEVANCE:** The transcriptomic alterations seen in response to eccentric muscle loading coupled with our previously reported improvements to the biomechanical properties and histochemical architecture of injured Achilles tendons support the benefits of eccentric loading exercises in treating Achilles tendinopathy. Ongoing investigation of the key biological processes and pathways identified in this study will deepen our understanding of the healing process and enable further refinement of physical therapy protocols.

**REFERENCES:** [1] Ceravolo M. et al. Clin J Sport Med, 2020; [2] Frizziero A et al. J Sports Med Phys Fit, 2016; [3] Rezvani S et al. J Applied Physiol, 2021; [4] Easley D et al., *Trans ORS* 49:2672, 2024; [5] Hamm SH et al. Mol Ther Methods Clin Dev, 2021; [6] Menarim B. et al. Front Immunol, 2021.

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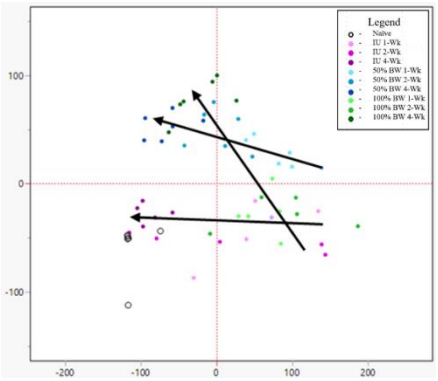


**Fig. 1**: Principal Component Analysis from entire transcriptome showing diverging patterns of expression in response to muscle loading over 4 weeks compared to controls. Each dot represents an individual sample.

| YELLOW MODULE | | |
|---|---|---|
| **Biological Process** | **Genes** | **Fold Enrichment** |
| Response to prostaglandin E | TGFBR3, CCL19-PS3, CCL21A | 16 |
| Positive regulation of neutrophil chemotaxis | CCL19-PS3, DNM1L, CCL21A | 8 |
| Neutrophil chemotaxis | CCL24, CXCL9, CXADR, DPEP1, CCL21A | 6 |
| Osteoblast differentiation | SOX2, TGFBR3, BMP4, WNT11, BGLAP, GLI1 | 5 |
| Cell fate commitment | SOX2, BMP4, WNT11, HEY2 | 5 |
| Extracellular matrix organization | MMP12, ADAMTS5, MMP27, SPOCK2, COL4A4, KAZALD1 | 3 |
| Inflammatory response | GBP5, CCL24, TPSB2, CXCL9, CFH, RARRES2, PRKCZ, C4B, IL2RA, DPEP1, CCL21A, IL18R1, LAT | 3 |

| RED MODULE | | |
|---|---|---|
| **Biological Process** | **Genes** | **Fold Enrichment** |
| Response to prostaglandin F | EDN1, HMGCS2 | 137 |
| Central nervous system myelination | MAG, PLP1, MAL, SOX10, ASPA | 45 |
| Cell-cell signaling | EDN1, GJC3, GJB1, DHH | 10 |
| Cytoskeleton organization | STRIP2, CCL11, UGT8A, SYNE2 | 7 |
| Cellular response to hypoxia | EDN1, KCNMB1, BCL2, FMN2 | 6 |
| Homophilic cell adhesion via plasma membrane adhesion molecules | CADM2, AMIGO2, PLXNB3, L1CAM, CDH19 | 6 |
| Positive regulation of cell migration | EDN1, CCL11, PLP1, CLDN1, SYNE2 | 4 |

**Fig. 2:** WGCNA identified the yellow and red modules to be positively associated with 100% BW loading at 2 weeks with 29 and 82 biological processes significantly overrepresented, respectively. The top ranked overrepresented biological processes and their corresponding fold enrichment are shown above.