# Dynamic Muscle Loading is a Key Regulator of Embryonic Tendon Cell Proliferation and Matrix Deposition

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**INTRODUCTION**: In the mouse embryo, *Scx+* tendon progenitor cells differentiate and condense to form distinct tendons at E13.5 with patterning largely complete by E14.5. From E14.5 onward, embryonic tendons continue to grow in length and thickness, which is likely fueled by a combination of cell proliferation and matrix deposition. Despite the importance of tendon as a load bearing tissue, little is known about the mechanisms regulating tendon response to loading during development. Previous work identified dynamic loading from muscle as a key regulator of embryonic tendon growth; in muscle paralyzed mutant embryos (muscular dysgenesis, *mdg*), tendons are normal at E14.5, but subsequent growth is impaired, as evidenced by thinner tendons by E16.5[1]. To determine the basis of this growth defect, we assessed tendon cell identity, proliferation, and matrix synthesis in the *mdg* mutant, spanning E14.5 to E18.5.

**METHODS:** Mice: Wild-type (WT) and *mdg* embryos were collected from timed matings. *ScxGFP* was crossed into the background to facilitate visualization of tendons. Proliferation: Pregnant dams were injected with 0.20mg EdU 2 hours prior to harvest. Proliferating cells were detected in transverse Achilles sections using ClickIT Plus EdU Imaging Kit (E14.5-E16.5, n=3). scRNA-seq: Fore- and hindlimbs were dissected from E14.5 and E16.5 embryos, digested to single cell (1.5 mg/mL collagenase V), and scRNA-seq carried out (10X Genomics). Analyses were performed using Seurat and differentially expressed genes (DEGs) were defined by a log2FC>0.25 and p.adj<0.05. *In Situ* Hybridization: RNAScope Multiplex Fluorescent V2 was carried out on transverse cryosections. Statistics were performed with student's t-tests with significance set at p<0.05. Male and female embryos were used for all assays and all mouse work was carried out under IACUC approval.

**RESULTS**: While no difference in proliferation was observed between WT and *mdg* embryos at E14.5 (prior to the onset of tendon phenotype), there was significantly less proliferation in *mdg* tendons at E15.5 and E16.5, suggesting that loss of dynamic loading from muscle contraction significantly reduced the proliferation of tendon cells (**Fig. 1A**). Although there was no difference in tendon cell density, the tendon cross-sectional area was reduced (**Fig. 1B**), suggesting defects in lateral growth may be due in part to defects in tendon cell proliferation. scRNA-seq revealed 11 major clusters, including a large cluster of musculoskeletal cells containing *Scx+* and *Sox9+* cells. Subcluster analysis of this population revealed 7 subclusters identified based on markers associated with: cartilage, proximal and distal patterning, dorsal and ventral patterning, skeletal progenitors, connective tissue progenitors, and tendon, with one cluster remaining unidentified (**Fig 2**). DEG analyses revealed no DEGs between E14.5 WT and *mdg* tendon cells. By E16.5, there were 15 DEGs between WT and *mdg* tendon cells. Interestingly, transcription factors associated with tendon cell fate were not different; many of the genes that were enriched in WT compared to *mdg* were related to tendon matrix components or matrix regulation (*Thbs4, Timp3, Col3a1, Col5a2, Col12a1, Vim*) and TGFβ signaling (*Tgfbi, Cd109*) (**Fig 2**). To confirm scRNA-seq results, we used RNAScope for *Thbs4* (matrix assembly) and *Timp3* (matrix turnover) and confirmed drastically lower expression of *Thbs4* in *mdg* mutant tendons (**Fig 3**). *Timp3* expression was localized to the epitenon surrounding individual WT tendons, which was completely lost in the *mdg* mutant. Collectively, these data indicate that tendon matrix development is also impaired in the absence of dynamic muscle loading.

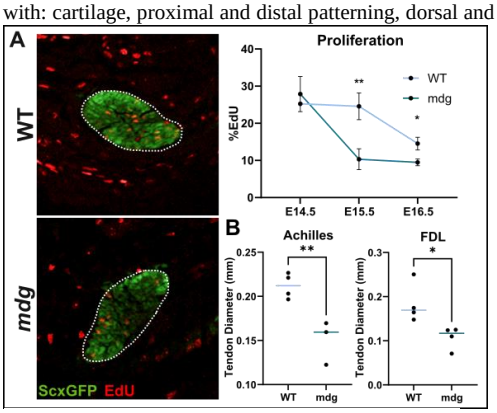


**Fig. 1:** *mdg* tendons are smaller (**1B**) and less proliferative (**1A**) than WT embryonic tendons. (n=3-4) t-test *p<0.05, **p<0.01.

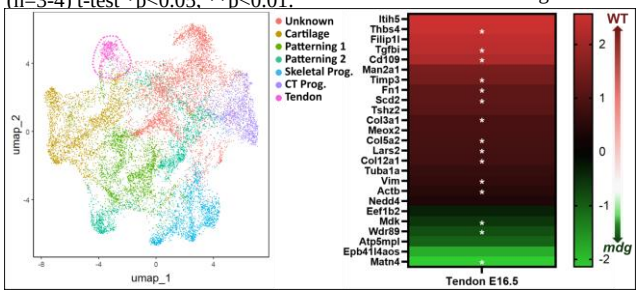


**Fig. 2:** scRNA-seq revealed 7 unique musculoskeletal populations in E14.5 and E16.5 developing mouse limbs. WT and *mdg* tendons are transcriptionally distinct by E16.5. *log2FC>0.25 and p.adj<0.05.

**DISCUSSION**: In this study, we evaluated the role of dynamic muscle loading on embryonic tendon growth and found that tendon cell proliferation and matrix development depended on loading. Surprisingly, we found that maintenance of tendon cell identity did not depend on dynamic loading, as there were no differences in the expression of mature tendon markers (*Scx, Tnmd, Mkx*) in *mdg* tendons at any stage (confirmed also by bulk RNA-seq of dissected tendons at E18.5, not shown). Interestingly, we observed *Timp3* (a metalloproteinase inhibitor) was expressed primarily in the epitenon of E16.5 WT tendons and was notably absent in *mdg* tendons. These intriguing findings indicate a requirement for muscle loading for epitenon maintenance and overall tendon organization. While it is not clear whether the epitenon has any developmental role for tendon in the embryo, a few tendons that normally split at E15.5 remain fused in *mdg*[1]. It is possible that Timp3 secreted by epitenon is required for individuation of these tendons. Current analyses aim to understand how unloading affects different tendon cell populations (epitenon vs. tendon body) and to identify the mechanotransduction pathways critical for embryonic tendon development.

**SIGNIFICANCE**: A better understanding of tendon development and mechanobiology will inform future tissue engineering and regenerative efforts.

**REFERENCES:** [1]Huang+ *Dev. 2015*

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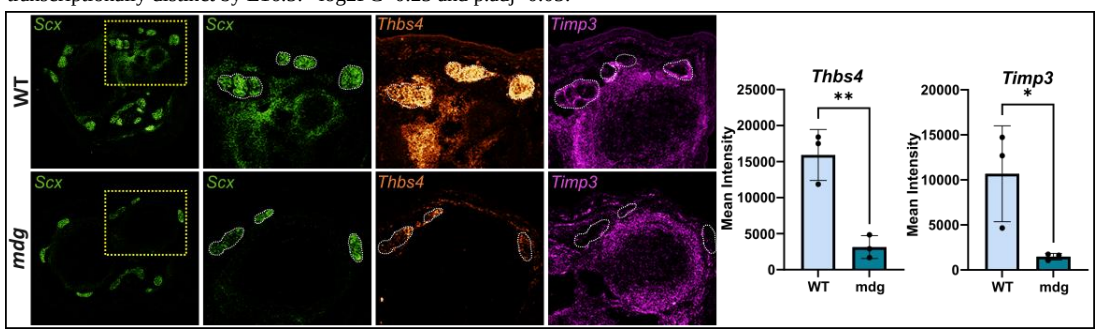


**Fig. 3:** *In situs* confirm lower expression of tendon matrix assembly gene *Thbs4* and matrix metalloproteinase inhibitor *Timp3*, but not tendon marker *Scx*. (n=3) t-test *p<0.05, **p<0.01.