Paralysis dysregulates extracellular matrix- and actin cytoskeleton-associated genes during tendon development

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INTRODUCTION: Tendons are connective tissues that transmit forces from muscle to bone to facilitate movements, such as walking, running, jumping, chewing, and talking. To perform these mechanically demanding functions, tendons develop specialized material properties that depend on the tendon extracellular matrix (ECM) and are regulated by fetal movements[1-3]. Reduced fetal movements (paralysis) are associated with musculoskeletal (MSK) birth disorders (e.g., clubfoot, arthrogryposis, joint laxity) in which tendons have developed abnormally. These tendon disorders can significantly impair daily life functions and can be debilitating[4]. Fetal paralysis can be flaccid or rigid, with both types of paralysis leading to abnormal tendons[5]. Current treatment strategies for MSK disorders do not improve tendon material properties, which is implicated in the recurrence of limb deformities or predisposition of the tendon to future injury[6]. Addressing the critical knowledge gap in how paralysis results in abnormal tendons is needed to develop treatment strategies that promote or restore tendon material properties. Here, building on our previous findings that embryo movement regulates tendon material properties[3], we aimed to identify potential mechanisms by which tendon development is altered during paralysis. We used flaccid and rigid paralysis chick embryo models to determine how lack of movement, independent of paralysis type, leads to abnormal tendons. We performed tensile testing to demonstrate that both paralysis models lead to inferior tendon material properties, and RNA-sequencing (RNA-seq) analysis to identify specific genes and potential biological mechanisms involved in aberrant tendon development. We hypothesized that fetal paralysis dysregulates genes involved in cell-ECM interactions and ECM synthesis and remodeling that could influence tendon material properties.

METHODS: Animal procedures received prior IACUC approval. Fertilized White Leghorn chicken eggs were treated daily from developmental days (D) 13-16 with pancuronium bromide or decamethonium bromide to induce flaccid or rigid paralysis, respectively, or saline vehicle treatments for normal movement controls. At 6h post-injection, embryo movements were counted via candling over 3 min. On D17, embryos were euthanized and Achilles tendons were dissected using our previously established marking protocol[2] for tensile testing, histology, or bulk RNA-seq. Tensile testing was performed as previously described[2]. For histological staining, hindlimbs were paraffin-sectioned transversely at 6μm thickness, Alcian blue (AB) staining was performed, and regions of interest at the same length of the tendon were analyzed for glycosaminoglycans (GAG). Differences in material properties or AB intensity were compared between conditions using one-way ANOVA with Tukey's post hoc with $p<0.05$ considered significant. We tested N=4-5 and N=3 biological replicates, consisting of one tendon per embryo for each N, for tensile testing and histology, respectively. For RNA-seq, differentially expressed genes (DEGs) were determined using DESeq2[7]. DEGs with $|\log2 \text{ fold change (L2FC)}|\geq1$ and false discovery rate<0.05 were considered significant. Gene ontology (GO) analysis was performed using DAVID, and ClueGO was used to consolidate GO terms[8,9]. N=3 (each N was 2 tendons from one embryo) were analyzed for RNA-seq. Sex as a biological variable was not studied as chick embryo sex cannot be ascertained during experimentation due to lack of external gonads.

RESULTS: Embryo movement frequencies were reduced during flaccid and rigid paralysis compared to normal movement (**Fig. 1A**). Increases in Achilles tendon elastic modulus (**Fig. 1B**) and ultimate strength (not shown), but not ultimate strain (not shown), were inhibited by flaccid and rigid paralysis, relative to normal movement. Tendon gene expression clustered by condition (flaccid paralysis, rigid paralysis, normal movement), based on the principal component analysis (PCA) plot of PC1 versus PC2 (**Fig. 2A**). Flaccid and rigid paralysis led to more downregulated than upregulated DEGs (**Figs. 2B, C**) and similarly dysregulated 251 genes, of which 39 were upregulated and 212 were downregulated (**Fig. 2D**). Specific DEGs that were similarly regulated by flaccid and rigid paralysis included *COL20A1, SPARC, LMOD2, TMOD4,* and *ACTN2* (**Fig. 2E**). GO term analysis showed that flaccid and rigid paralysis similarly dysregulated 168 biological processes, 45 cellular components, and 26 molecular functions. After GO term consolidation, 'chondrocyte differentiation' and 'muscle structure development' were among the top 5 upregulated and downregulated biological processes, respectively (**Fig. 3A**). AB staining intensity was not significantly different between Achilles tendons of normal movement, flaccid paralysis, and rigid paralysis (**Fig. 3B**).

DISCUSSION: Our results confirm that material properties (elastic modulus, ultimate strength) are inhibited by reduced movement frequency, independent of paralysis type. Dysregulation of specific DEGs by both flaccid and rigid paralysis implicate *COL20A1, SPARC, LMOD2, TMOD4,* and *ACTN2* in the development of tendon material properties. *COL20A1* (downregulated by paralysis) and *SPARC* (upregulated by paralysis) encode collagen type XX and Secreted protein acidic and rich in cysteine (SPARC), respectively, which may be involved in regulating collagen fibrillogenesis during ECM development and could impact material properties[10,11]. In addition, studies in skin suggest that SPARC prevents integrin binding to collagen[12]. As such, upregulation of *SPARC* may also disrupt tendon cell interactions with the ECM. This is interesting as we have shown that cell interactions with the ECM can impact tendon material properties[13]. *LMOD2, TMOD4,* and *ACTN2* (downregulated by paralysis), encode Leidomodin 2, Tropomodulin 4, and Actinin alpha 2, respectively, which are all implicated in regulation of actin filament growth and stability[14-16]. These actin-associated genes are of interest as we have previously found the actin cytoskeleton also contributes to tendon material properties during early development[17]. While 'chondrocyte differentiation' was among the top upregulated biological processes, GAG staining intensity was similar between normal movement and paralysis, suggesting chondrogenesis did not occur during paralysis timepoints studied here. The most significantly downregulated biological process by paralysis was 'muscle structure development' which is consistent with reported downregulation of muscle-related biological processes during normal tendon development[18]. Future studies based on these findings could lead to the development of therapeutics to restore the material properties of abnormally developed tendons after fetal paralysis.

SIGNIFICANCE: Our study identified genes that are dysregulated by fetal paralysis during tendon development that may be used to inform future development of treatment strategies for tendons involved in musculoskeletal disorders.

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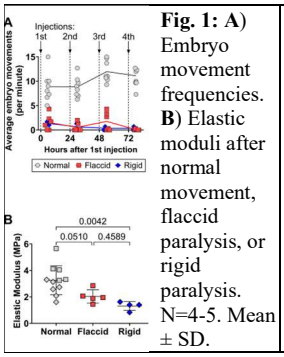

Fig. 1: A) Embryo movement frequencies. **B)** Elastic moduli after normal movement, flaccid paralysis, or rigid paralysis. N=4-5. Mean ± SD.

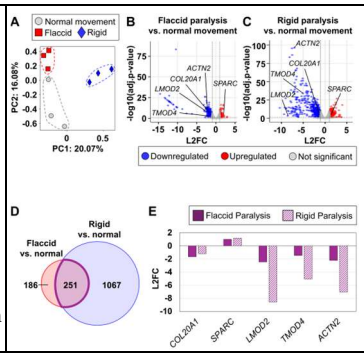

Fig. 2: A) PCA plot of gene expression of normal movement and flaccid and rigid paralysis (N=3). **B, C)** Volcano plots of DEGs by flaccid (**B**) and rigid (**C**) paralysis. **D)** Venn diagram showing 251 shared DEGs between paralysis types. **E)** L2FC of similarly regulated DEGs by both paralysis types.

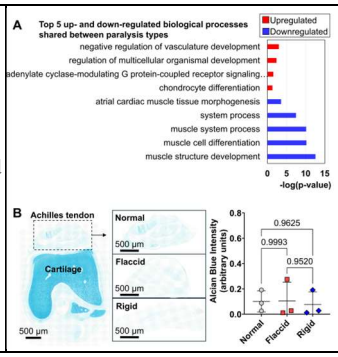

Fig. 3: A) Representative biological process GO terms. **B)** Representative AB staining of transverse sections and intensity quantification. N=3. Mean ± SD.