Transcriptomic Analysis of Late Tendon Development in Normal and Paralyzed Chick Embryos

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Disclosures: We have nothing to disclose.

INTRODUCTION: While the transmission of muscle forces by tendons is essential for joint movement, how tendons acquire their mechanical capabilities during development is unclear. The load-bearing capability of tendons is generated during embryonic and neonatal stages[1-3]. In particular, chick tendons experience a rapid increase in tissue mechanics during late embryonic development, characterized by an increase in collagen content and crosslinking as well as an increase in collagen fibril diameter and length[4-6]. Previous work shows that this transition is impaired when chick embryos lose the ability to contract their muscles and mechanically stimulate their tendons[7]. While these findings suggest that mechanobiology is essential for tendon maturation, the specific mechanotransduction signaling pathways that drive late tendon development are still unknown. Therefore, the objective of this study was to identify the mechanobiological mechanisms driving late tendon development in normal and paralyzed chicken embryos. We hypothesized that during late development mechanotransduction signaling transitions from cell-cell to cell-matrix interactions and that this will be inhibited in paralyzed embryos.

METHODS: *Sample Collection:* Fertilized White Leghorn eggs were used for all experiments. To study normal development, embryos were sacrificed at embryonic days 16 (E16) and 20 (E20). To study the effects of paralysis, embryos were windowed at embryonic day 3 (E3) and treated to induce either rigid or flaccid paralysis with decamethonium bromide (DMB) or pancuronium bromide (PB), respectively. Treatment began at embryonic day 13 (E13) with 100 uL of 0.2% DMB or PB in an HBSS and antibiotic-antimycotic solution, followed by daily 50 uL doses from E14 to E19. Control samples were treated with vehicle only, and embryos were sacrificed at E20. For all samples, flexor digitorum longus (FDL) and flexor digitorum brevis (FDB) digit II tendons were harvested from the hindlimbs. Each sample (n=3 for normal development, n=4 for paralysis) consisted of eight tendons (two FDL and two FDB from two embryos). Development samples were flash-frozen in liquid nitrogen (LN), stored at -80°C, and shipped on dry ice for analysis. Paralysis samples were placed in Trizol, flash frozen in LN, and stored at -80C until RNA extraction and shipment. *RNA Sequencing:* Samples were sequenced on an Illumina platform (2x150bp, ~50M reads/sample). Reads were quality checked, trimmed, aligned to the *Gallus gallus* reference genome (GRCg7b), and transcript abundances quantified. DESeq2 was used to identify differentially expressed genes (DEGs) between E16 and E20, DMB and CTRL, and PB and CTRL. DEGs with a Benjamini-Hochberg adjusted p-value < 0.05 and absolute log2 fold change > 1 were considered significant. *Downstream Analysis:* Significant DEGs were converted to human orthologs using g:Profiler. For gene ontology (GO) and transcription factor (TF) analyses, DEGs were separated into upregulated and downregulated groups. Enriched GO terms and pathways were identified using DAVID and QIAGEN Ingenuity Pathway Analysis (IPA), respectively, and limited to an adjusted p-value < 0.05. TF activity was evaluated with ChEA3 and limited to a score < 0.05.

RESULTS: For normal development, GO analysis revealed enrichment of various terms related to extracellular matrix (ECM) remodeling, cell adhesion, development, signal transduction, and Wnt signaling between E16 and E20 (**Fig. 1A**). Similarly, IPA identified numerous pathways related to ECM remodeling and mechanotransduction signaling (integrins, Rho GTPase, Wnt, TGF-β, AKT) as well as other signaling pathways (activin, IGF, S100) (**Fig. 1D**). Furthermore, TF analysis suggested an increased activity of mechanosensitive regulators such as EGR1, TEAD, TCF, SRF, and SMAD (data not shown). With rigid (DMB) paralysis, GO showed enrichment of ECM organization and cell adhesion (**Fig. 1B**). IPA revealed perturbation of numerous ECM remodeling pathways and many pathways related to mechanotransduction and calcium signaling (integrins, gap junctions, Rho GTPase, FAK, Wnt, S100) (**Fig. 1E**). TF analysis suggested differential activation of TCF, LEF, SRF, TEAD, SMADs and EGR1 (data not shown). Finally, with PB treatment, there were fewer perturbed GO terms and pathways. However, they still included ECM remodeling and mechanotransduction, including FAK and Wnt (**Fig. 1C,F**). TF analysis showed altered activity of TCF, SRF, SMAD, STAT3, EGR1, and LEF (data not shown).

DISCUSSION: Consistent with our hypothesis, our results demonstrate that multiple mechanotransduction signaling mechanisms are differentially regulated with development and are perturbed with paralysis. Our combined analyses specifically suggest that Wnt, TGF-β, YAP/TAZ, and MRTF/SRF may play a role in driving tendon development. Furthermore, each of these signaling pathways are also perturbed with muscle paralysis (with DMB treatment in particular), which is consistent with their role in mechanotransduction. While we also observed differential regulation of integrin interactions, cell and cell-cell adhesions, Rho GTPase activity, and FAK signaling with normal development and paralysis, the directionality of these changes was mixed. That is, during normal development, Rho signaling decreased while integrin signaling increased. Similarly, FAK signaling decreased with DMB treatment but increased with PB treatment. This suggests that mechanotransduction during development may be more complicated than our hypothesized transition from cell-cell to cell-matrix interactions. Additionally, our results suggest that flaccid paralysis affects tendon development differently than rigid paralysis, which may be due to the distinct mechanisms of the drugs. DMB irreversibly binds to acetylcholine receptors, causing sustained muscle contraction that could provide static stretch to tendons, whereas PB blocks acetylcholine binding altogether, preventing contraction. Further work is needed to identify which mechanotransduction pathways drive the structural and mechanical changes that are critical for proper tendon development.

SIGNIFICANCE/CLINICAL RELEVANCE: These findings identify the potential pathways that underlie the biological mechanisms driving tendon development and the production of a robust tensile load-bearing soft tissue. Understanding these mechanobiological mechanisms may improve tissue engineering strategies for functional tendon replacements and guide future treatments for tendon injuries and disease.

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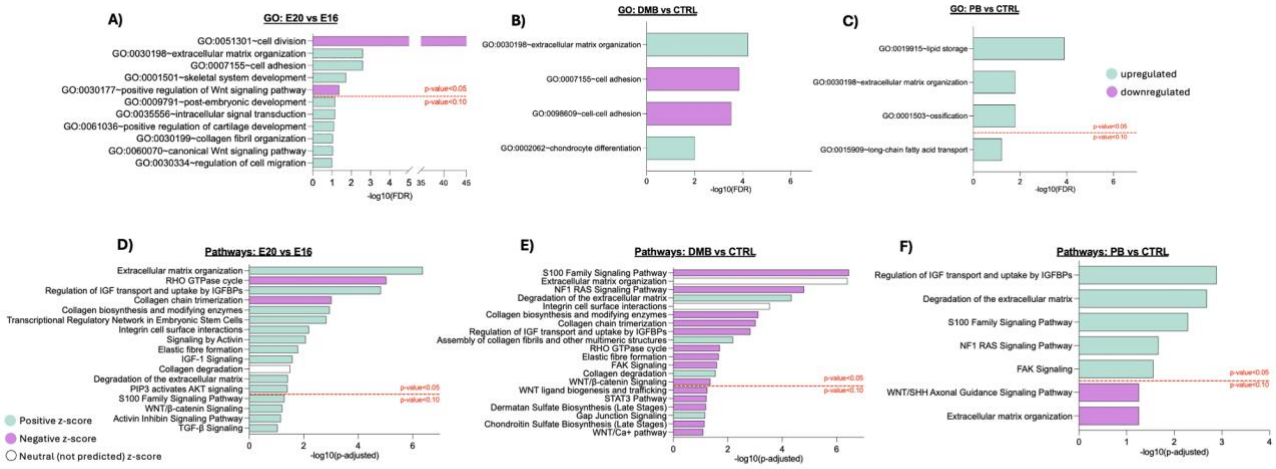

Figure 1: A-C) Enrichment analysis of GO biological process terms using significant upregulated and downregulated DEGs in DAVID across developmental and paralysis treatment comparisons. **D-F)** Pathway enrichment analysis of significant DEGs using IPA for all comparisons. Positive z-score = upregulated pathway, negative z-score = downregulated pathway.