Muscle Tendon Crosstalk is an Essential Factor for Postnatal Tendon Growth in Murine

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INTRODUCTION: The tendon is critical for transmitting muscle-generated loads to joint movement. The tendon developmental process involves multiple biological organizations from the embryonic to the early postnatal period. Muscle has been suspected to play a central role in tendon maturation during the developmental process [1,2]. Muscle contraction provides both mechanical and chemical effects. Yet, little mechanistic work has considered the effects of muscle contraction on tendon development by distinguishing between mechanical and chemical influences. The first objective of this study was to explore the roles of muscle contraction in contributing to postnatal tendon maturation. The second objective was to elucidate which is the key regulator of the tendon maturation process, the mechanical and/or chemical effects of muscle contraction. In this study, by integrating muscle contraction effect for tendon tissue growth in sciatic nerve denervation model *in vivo* and tendon stimulation from muscle contraction and just mechanical stretch in postnatal mouse models *ex vivo*, we aimed to investigate whether mechanical force is an essential driver of tendon growth and to explore the mechanical effect and muscle-tendon crosstalk derived by muscle contraction.

METHODS: All animals and procedures were approved by the Animal Care Committee at SPU. *Postnatal surgery model*: Unilateral right sciatic nerve denervation model (SDN) was performed on postnatal day 7 (P1) (**Fig. 1A**). *qPCR Analysis:* Achilles tendon tissue sections (n=3/group) were collected at post-operative day (d) 7 and 21 for qPCR using a TaqMan™ Gene Expression Assays-TaqMan™ 96 well Array Plates. *Ex vivo experiments*: Achilles tendon and gastrocnemius/soleus muscle complex were harvested from P14 C57BL/6J mice and cultured in DMEM without FBS (starvation medium) in the ex vivo field (n=3/group) (**Fig.2A**). *Electrical Pulse Stimulation (EPS) Experiment*: To assess muscle contraction effect ex vivo, we invoked ex vivo muscle contraction by EPS. Non-stimulated AT were used as controls (**Fig.2B**). *Mechanical Stretch (MS) Experiment*: Explants were loaded at 1% cyclic loading in the mechanical stretch system device (MS) for 40 minutes. (**Fig.2D**). Mechanical Stretch groups were divided into two medium conditions: Control-Conditioned Medium (C-CM) and EPS-CM containing muscle contraction-derived factors. Non-stretch tendon cultured in DMEM without muscle contraction factor was used as a control. *Digital PCR analysis*: All *ex vivo* tendon samples were collected for Digital PCR using a miRCURY LNA miRNA PCR Assay. Target miRNAs were miR133a-3p and miR21-5p. U6 was used as a housekeeping target. *Statistical evaluation:* We performed a paired t-test for qPCR analysis. The dPCR analysis group was compared via Welch's t-test or one-way ANOVA with Tukey post-hoc tests, significance at $p<0.05$ (solid lines) and trends at $p<0.1$ (dashed lines).

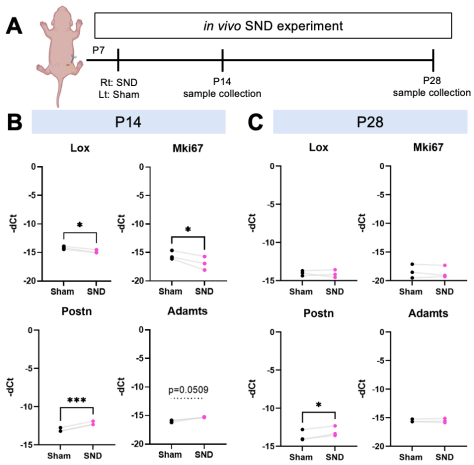

Fig.1. Gene expression of *Lox*, *Mki67*, *Postn*, and *Adamts*. (*) shows significant differences (p<0.05).

RESULTS SECTION: *Expression profile of SND tendons suggests decreased cross-link:* Gene expression analyses showed *Lox* and *Mki67* mRNA expression decreased in SND at P14. On the other hand, *Postn* expression increased in SND at both P14 and P28. *digital PCR results of Ex vivo tendon did not show a significant difference between interventions*.: We performed electrical stimulation to induce muscle contraction, and mechanical stretch to mimic the mechanical side of muscle contraction. We checked muscle and tendon-related miRNAs (miR133-3p and miR21-5p). However, there were no significant changes in miRNA expression between intervention methods.

DISCUSSION: In this study, we observed that muscle contraction increased *Lox* and *Mki67* activity. *Lox* is known to facilitate cross-link formation between adjacent collagen molecules. We previously showed that loss of muscle contraction decreases mechanical properties, especially the failure force of the tendon, but no significant difference *in Col1* mRNA expression in the SND model. Interestingly, *Postn* expression increased in the SND model. *Postn* regulates collagen fibrillogenesis in connective tissues [3]. Inhibiting muscle contraction may induce fibrillogenic-like degeneration reaction via the *Postn* cascade. Therefore, muscle contraction in postnatal mice tendons works to increase collagen cross-link and maturing mechanical properties. *Mki67* expression results provided further information about cell proliferation. A previous study showed tendon cell proliferations maintained at P14 [4]. However, the SND tendon showed decreased *Mki67* expression at P14 compared to Sham group, indicating that muscle contraction can sustain or induce the tendon cell proliferation activity. We hypothesized that an ex vivo model could elucidate in more detail the mechanisms in tendon mechanobiology. However, we could not conclude how mechanical force and chemical solution from muscle contraction affect tendon maturation.
We have some limitations. We check only two miRNAs and test just one time-point. Future research needs to clarify the detailed mechanism of the role of muscle contraction in tendon maturation. These knowledges give us the suggestion of science-based rehabilitation for disabled children.

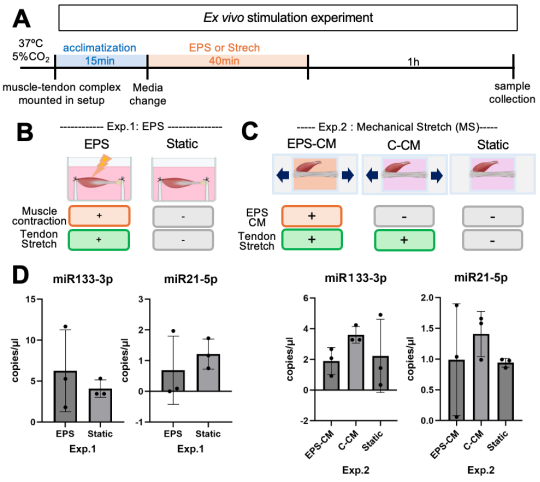

Fig.2. *Ex vivo* study design and results of dPCR.
(A) Overview of ex vivo experiment, (B) Electrical Pulse Stimulation (EPS) Experiment: EPS and Non-stimulated were used as controls. (C) Mechanical Stretch (MS) Experiment with/without EPS-CM. (D) Results of dPCR (n=3/each group)

SIGNIFICANCE/CLINICAL RELEVANCE: We showed that muscle tendon crosstalk is an essential factor for postnatal tendon growth. Our results indicate that collagen crosslinking is regulated by muscle contraction in the tendon growth phase.

REFERENCES: 1. Arvind et al., Ann N Y Acad Sci. 2018; 2. Havis E et al. Development. 2016. 3. R. A. Norris, J Cell Biochem. 2007 M.Grinstein et al., Elife. 2019
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