SMAD4 Signaling Underlies Neuromuscular Contractures: A Target for Localized Drug Eluting Hydrogel Therapy

Elaine A. Nagahara[1,2], Peter T. Shyu[1], X. Edward Guo[1], Alice H. Huang[2], Varun Arvind[2]
[1]Department of Biomedical Engineering, Columbia University, New York, NY [2]Department of Orthopedic Surgery, Columbia University, New York, NY

DISCLOSURES: Elaine A. Nagahara (N), Peter T. Shyu (N), X. Edward Guo (N), Alice H. Huang (N), Varun Arvind (N)

INTRODUCTION: Neuromuscular contractures (NC) are debilitating and affect an estimated 17 million people worldwide, costing $2.2 billion in the U.S. annually.[1-3] During post-natal development, NC arise in patients with neuromuscular disease due to mismatch in skeletal and soft tissue growth resulting in asymmetric tensioning across the joint. While recent studies have demonstrated development of muscle from neuromuscular disease, [4-8] *in vivo* studies indicate the role of extra-muscular tissues in NC formation.[5, 9] In previous studies, we established a model of NC formation following sciatic nerve transection (SNT) of neonatal mice and identified a novel role for the myotendon elongation in NC formation.[10] Here we utilize this model to uncover cellular and molecular drivers of NC formation and test inhibition of this pathway using small molecule delivery methods with the goal of developing a therapeutic for treating patients with NC.

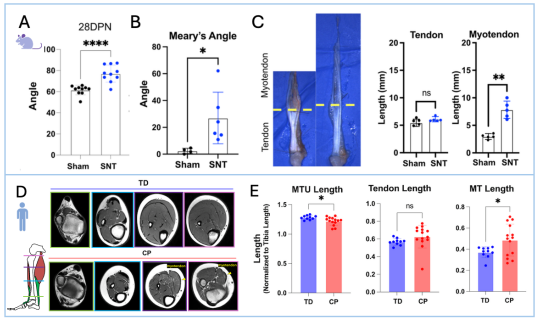

Fig. 1. Preclinical model reproduces functional impairments of NC and reveals myotendon elongation as seen in human patients with CP. SNT resulted in **a)** fixed ankle equinus contracture, **b)** cavus foot deformity, and **c)** myotendon elongation. **d)** MRI images of typically developing (TD) and CP patients show **e)** myotendon with increased length in CP patients.

METHODS: Neurotomy: Neonatal mice (P5, male and female) underwent sciatic nerve transection. Sham surgeries were performed on contralateral limbs. Ankle range of motion (ROM) was assessed at 28 days post neurotomy (DPN), skeletal deformity (Meary's angle) was assessed at P120 using microCT. Tendon and myotendon length were quantified at 28 DPN from stereomicroscope imaging. Bulk-RNA seq was performed at 14 DPN. Gene expression via qPCR was measured at 14 DPN. Immunostaining was carried out against pSMAD4 with DAPI counterstaining at 14 DPN. BMP-SMAD4 signaling was inhibited through daily IP administration of LDN 193189 at 2.5 mg/kg/day from P5 to P21. All procedures were carried out under approved IACUC guidelines. Patient MRI: MRI images were obtained from typically developing (TD) and patients with NC due to cerebral palsy from Bolsterlee et al. under IRB approval (ages 6-14, male and female). Thermogel: Hydrogels were made with PLGA-PEG-PLGA triblock copolymers (Mw ~1,100:1,000:1,100 Da; LA:GA 75:25). Noggin or BSA (25 and 10% w/v). Supernatant was collected at time points 1 hr, 1, 2, 4, 7 days after gelation. Protein release was calculated by microBCA quantitation (Pierce). To test *in vitro* SMAD4-BMP signaling inhibition, adult tendon cells (maintained in 10% FBS, 1% P/S in DMEM) were cultured with either noggin-loaded gels or empty gels in Transwell inserts. Cell viability and gene expression were measured at d0, 3, and 7. Statistics were determined by ANOVA or Student's t-tests with significance set at $p<0.05$.

RESULTS: At 28 DPN in mice, SNT resulted in fixed ankle equinus contracture and cavus foot deformity (**1A, B**). Additionally, while there was no difference in tendon length following SNT, myotendon length increased (**Fig. 1C**), consistent with MRI sequences from CP patients with NC (**Fig. 1D, E**).[11, 12] To study transcriptional differences between tendon and muscle following SNT, bulk RNA-seq was carried out at 14 DPN. PCA analysis revealed samples clustered with greater separation between sham and denervated tendon groups compared to muscle, indicating higher transcriptomic variance in tendon (**Fig. 2A**). GSEA analysis on DEGs from mouse tendon following SNT showed enrichment for genes associated with "BMP response" (**Fig. 2B**), which was then confirmed by qPCR showing enhanced expression of *Bmpr1a* and *Smad4* in the myotendon compared to tendon and muscle (**Fig. 2C**). Supporting gene expression results, there was an increase in nuclear pSMAD4+, ScxGFP+ tenocytes found at the MTJ compared to sham controls (**Fig. 2D**). Analysis of CP patient myotendon similarly demonstrated nuclear pSMAD4 (**Fig. 2E**). To determine whether inhibition of BMP signaling could improve NC phenotype, we systemically delivered a small molecule inhibitor and found rescue of myotendon elongation and fixed ankle equinus contracture at 28DPN (**Fig. 2F, G**). Since systemic BMP inhibition may lead to off-target effects in patients, developed a thermogel system to test local, sustained delivery to the myotendon site. Pilot studies demonstrated gradual release of BSA from gels over time with continuing elution at d7 (**Fig. 3A**). *In vitro* testing of tenocytes stimulated with BMP-2 with noggin-loaded gels reduced BMP-responsive gene expression over 7 days (**Fig. 3B**).

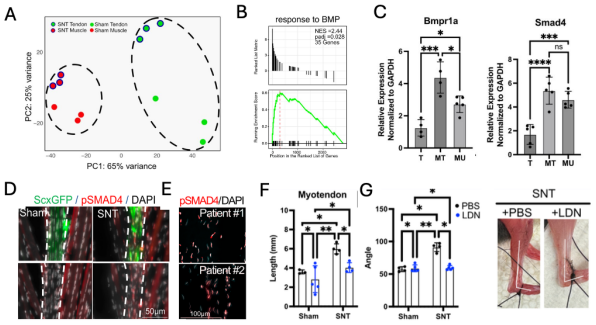

Fig. 2. Preclinical model shows BMP-SMAD4 as critical molecular regulator. SNT resulted in **a)** greater separation between sham and denervated groups in tendon, **b)** enriched BMP response associated gene signature, **c)** enhanced Bmpr1a and Smad4 expression, **d)** increased nuclear pSMAD4+, ScxGFP+ tenocytes at the MTJ **e)** in accordance with the increase seen in CP patients. LDN administration after SNT rescued **f)** myotendon elongation and **g)** fixed ankle equinus.

DISCUSSION: Here we show NC results from myotendon elongation with similar findings in human patients with NC supporting by MRI imaging. Our model recapitulates clinically relevant findings of ankle equinus and cavus deformity. Moreover, results from this study demonstrate a role for BMP signaling as a molecular regulator underlying myotendon elongation that induces limb contracture. Remarkably, given that we observed rescue of NC phenotype following systemic BMP inhibition in our pre-clinical model of NC formation, in this study we present pilot data for a novel myotendon therapeutic with effective long-term (7d) inhibition of BMP signaling in tenocytes that may be used to prevent NC formation while minimizing repeat injections and systemic toxicity. Ongoing studies will assess the efficacy of gels *in vivo* using our preclinical NC model. Although inhibition of BMP-SMAD4 signaling reduced myotendon elongation and improved joint contracture, it remains uncertain whether the effects were from direction inhibition of signaling in the myotendon or from indirect inhibition of muscle. Future studies will analyze myofibers to determine if muscle effects are observed.

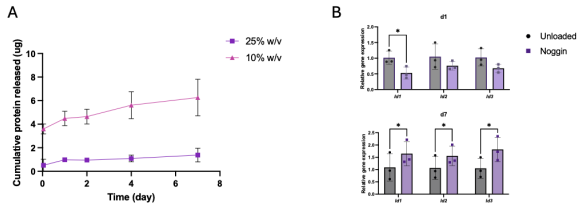

Fig. 3. Loaded thermogels demonstrate tunable gradual drug release and ability to inhibit SMAD4-BMP signaling with noggin. a) Noggin-loaded gels gradually release BSA, and increasing % w/v reduces release rate. **b)** Loaded gels result in reduced BMP-responsive gene expression at d1 and increased expression at d7.

SIGNIFICANCE/CLINICAL RELEVANCE: NC formation imposes restricted motion and soft tissue growth. Current research focuses on muscular causes that fail to prevent long-term progression, highlighting the importance of approaching this challenge from a tendon-specific mechanism.

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