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Use of ankle immobilization in evaluating treatments to promote longitudinal muscle growth in mice

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Abstract

Introduction: Difficulty in modeling congenital contractures (deformities of muscle-tendon unit development that include shortened muscles and lengthened tendons) has limited research of new treatments.

Methods: Early immobilization of the ankle in prepuberal mice was used to produce deformities similar to congenital contractures. Stretch treatment, electrostimulation, and local intramuscular injection of a follistatin analog (FST-288, Acceleron Pharma) were assessed as therapeutic interventions for these deformities.

Results: Ankle immobilization at full plantarflexion and 90 degrees created tendon lengthening and muscle shortening in the tibialis anterior and soleus. Stretch treatment produced minimal evidence for longitudinal muscle growth and electrostimulation provided no additional benefit. Stretch treatment with FST-288 produced greater longitudinal muscle growth and less tendon lengthening, constituting the best treatment response.

Discussion: Ankle immobilization recapitulates key morphologic features of congenital contracture, and these features can be mitigated by a combination of stretch and pharmacological approaches that may be useful in patients.

Keywords

Contracture; myostatin inhibition; stretch; electrostimulation; follistatin

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Introduction:

Congenital contractures are abnormally short and stiff skeletal muscles presenting at birth that constitute a major and long-term source of disability in children. Causes of congenital contractures are situations where movement is insufficient or restricted *in utero*, and can include neurological diseases, neuromuscular junction disorders, connective tissue diseases, muscle diseases, space limitations within the uterus, intrauterine or fetal vascular compromise, or maternal diseases¹. These conditions lead to short muscle and long tendon components of the muscle-tendon units (MTUs), resulting in impairment of movement²⁻⁴. Approximately 1% of all live births show some sort of contracture⁵, which can range from single-joint reduced mobility (such as clubfoot) to severe contractures of several limb joints (such as arthrogryposis multiplex congenita)¹.

Despite the broad-ranging impact of contractures, difficulty in modeling these deformities has limited research in this area. The paucity of rodent models of congenital contracture has also slowed the development of alternative or additional therapeutic approaches. While “contracture” has been used to describe abnormal limb appearance in some rodent models of central nervous system or neuromuscular disease, these instances are not associated with the tendon lengthening and muscle shortening that we have attempted to model here. The overall goals of this study were 1) the establishment of a model of congenital contracture that was amenable to studies of both mechanism and treatment, and 2) the assessment of three potential treatment strategies in affecting muscle and tendon growth using this model. To generate the congenital contracture model, we hypothesized that differences in the timing of rodent and human muscle-tendon development were likely responsible for these difficulties, and that a model of immobilization-induced muscle shortening would allow the study of contracture therapies. The terminal stages of musculoskeletal maturation in rodents occur in the first few weeks of postnatal life, to the extent that the maturational state of neonatal human muscle most closely corresponds to mouse or rat muscle at 3 weeks of age^{6,7}. Assuming that a lack of movement or mobility at a late but critical developmental stage is sufficient for contracture production^{8,9}, we hypothesized that immobilization early in life could be used to study treatments affecting longitudinal muscle growth. Several classic experiments have demonstrated the potential for immobilization in young rodents to produce shortened muscles and lengthened tendons^{10,9,11-14}. More recent studies have used immobilization to study the later-onset contractures seen in cerebral palsy and other disorders^{15,16}, but treatment and longitudinal muscle growth were not key foci of these systems. This study expands on that work to develop an immobilization-based model that evaluates longitudinal muscle growth as a key endpoint for the evaluation of treatments. The development of a reproducible model of immobilization-induced muscle shortening provides a means for studying contractures in a genetically normal (CD-1) mouse background, which is highly desirable because the majority of patients with contracture do not suffer from genetic disease¹⁷.

Treatment of contractures is focused on the specific symptoms experienced by each individual and may include physical therapy, removable splints, exercise, and/or surgery. Therapy of severely-affected patients is generally long-term, with the potential to achieve some level of ambulation in many patients^{18,19}. Progressive resistance exercise has

demonstrated the potential to increase strength in the context of contractures associated with cerebral palsy (which are likely distinct from congenital contractures), which suggests a possible role for muscle growth in contracture treatment²⁰. Current therapeutic strategies for contracture are generally focused on restoring flexibility but have not been designed to improve strength²¹. Therapies that promote longitudinal muscle growth could dramatically improve therapeutic outcomes. A number of genetic and pharmacological studies have shown that inhibiting myostatin/ActRIIB signaling can produce marked myofiber hypertrophy to increase muscle mass and strength (Reviewed in²²). Follistatin, a potent antagonist of myostatin/ActRIIB signaling, has been found to increase muscle mass locally following intramuscular injection and is capable of producing axial myofiber growth^{23–25}. However, the capacity of follistatin to promote longitudinal muscle growth is unclear. We hypothesize that therapy for immobilization-induced muscle shortening is improved by combining physical and pharmacological therapies.

Materials and Methods:

Live Animal Studies:

All studies were performed with approval from the Institutional Animal Care and Use Committee at The Medical College of Wisconsin. Both male and female mice were used based on their equivalency in pilot experiments. CD-1 albino mice (Charles River Laboratories) without genetic abnormalities were used for all experiments. During the period of immobilization-induced muscle shortening generation and treatment, mice were monitored daily through visual inspection and measurement of body weight.

Ankle Immobilization to Create Muscle-Tendon Unit Abnormalities:

The overall experimental design is depicted in Figure 1. Proof-of-concept studies of ankle immobilization were performed in the plantarflexed position (illustrated in Supplemental Figure 1) without inversion or eversion of the foot. Additional experiments were performed to assess MTU development following immobilization at a 90-degree angle (tibial-plantar angle, without inversion or eversion of the foot) to allow stretch in either the plantarflexed or dorsiflexed positions. Position and condition of the splinted ankle were monitored daily, and additional bands of tape were added to maintain immobilization with limb growth. Splints were entirely removed at 3 weeks of life and animals were evaluated for contracture formation. Some cohorts of mice were then either exposed to therapeutic interventions for an additional 2 weeks or assessed for the persistence of contractures for an additional 8 weeks.

Ankle Splinting to Produce Progressive Stretch:

To assess the impact of progressive stretch on muscle-tendon unit growth in the tibialis anterior (TA) muscle, mice with immobilization-induced muscle shortening produced at 90 degrees had their ankles re-splinted in progressively plantarflexed positions over a 2-week period (from 3–5 weeks of age). Splinting to produce stretch was evaluated using 3 stages (at 120, 150 and 180 degrees, to evaluate stretch alone and in combination with electrostimulation) or 5 stages (at 108, 126, 144, 162, and 180 degrees, to evaluate in combination with FST-288). Some experimental groups also included additional therapeutic approaches beyond stretch treatment, as described below.

Assessment of Combined Stretch Treatment Plus Electrostimulation Treatment:

To determine whether increased isometric contractile activity would promote longitudinal muscle growth beyond what is seen with stretch treatment alone, awake mice with immobilization-induced muscle shortening produced at 90 degrees underwent leg electrostimulation for 30 min/day with concurrent progressive stretch in three stages over a 2-week period (from 3–5 weeks of age). Contraction of leg muscles was evoked by transcutaneous stimulation to the fibular and tibial nerves with a loop electrode (cathode) encircling the leg below the fibular head and a patch electrode (anode) against the shaved lower back. The procedure was developed in 13–15 day old awake mice and maximum current intensity was defined as the level that produced strong TA and gastrocnemius isometric contractions without activating thigh or tail muscles and causing vocalization and startle movements. At maximum, the contracting muscles of the splinted limb were palpably hard, and the leg, ankle and foot stiffened visibly from the opposing tensions developed by co contraction of the dorsi- and plantar-flexor muscles. Electrode (carbonized rubber) conductance was facilitated using Spectra 360 electrode gel (Parker Laboratories, Inc., Fairfield, NJ). The pattern and waveform of stimulation used were based on those employed in humans to build muscle strength with minimal fatigue²⁶ and were well tolerated 30 min/day by the awake mice constrained under soft nylon netting. Electrode impedance averaged 34 ± 4 kOhms. The stimuli were generated by a Grass S88 stimulator producing square pulses of 11–28 V (16.1 ± 2.1 V) at 50 Hz that were converted to biphasic pulses (400 μ sec duration) via a SIU 5 isolator. The stimulation, delivered in a duty cycle of 1 second on, 3 seconds off trains, was repeated continuously for 30 minutes. Maximum contractions were elicited by stimulating currents averaging 13.2 ± 1.7 mA (range 8.9–21.8 mA).

Assessment of Stretch Treatment Plus FST-288 Injection:

We evaluated longitudinal muscle growth using FST-288 (Acceleron Pharma, Cambridge, MA), a locally-distributing follistatin analog that was not expected to have therapeutic effects outside the injected leg²³. Mice with immobilization-induced muscle shortening produced at 90 degrees underwent intramuscular injections with FST-288 concurrently with progressive stretch over a 2-week period (from 3–5 weeks of age). Stretch was performed in five stages to allow injections and splint changes to be performed at the same time. Mice received semiweekly, intramuscular 20–30 μ l injections of drug or vehicle into the lateral compartment (fibularis muscles). Additional control groups in this study included 1) mice without immobilization-induced muscle shortening or stretch treatment, but with intramuscular injection of FST-288 at the same location and 2) mice with immobilization-induced muscle shortening and five-stage stretch treatment, but with injection of vehicle (Tris-buffered saline) instead of FST-288.

Bone, Muscle, and Tendon Measurements:

Depending on the experiment involved, animals were euthanized for anatomical evaluation and tissue collection at 3 weeks (proof of concept experiments), 5 weeks (treatment experiments), or 11 weeks (persistence experiments) of life. Following euthanasia, hindlimbs were removed above the knee and fixed in phosphate buffered saline containing 4% paraformaldehyde, pH 7.2 and stored at 4°C. The tibiae and leg muscles with complete

tendons were removed from the treated and control sides. Lengths were measured with the aid of dissecting microscope magnification and a ruler graduated in 0.5 mm units.

The tendon arising distally in the TA was cut at the insertion on the 1st metatarsal. Proximally, TA muscles were freed from their origin in the tibial periosteum to preserve full length myofibers. Muscle (belly) length was taken from the most proximal fiber origin to the furthest distal fiber attachment to the tendon. Extensor digitorum longus (EDL) tendons were dissected at the single proximal tendon originating from the lateral epicondyle of the femur and the distal tendon attached to the middle (3rd) digit. The soleus tendons were cut at the calcaneal insertion and the lateral tibia below the knee joint. Plantaris tendons were cut at the lateral femoral epicondyle and the calcaneal insertion. When calculating the percentage of total tendon length to muscle belly length in the EDL, soleus and plantaris, the lengths of the proximal and distal tendons were summed.

Determination of Sarcomere Number:

Experiments evaluating the effects of stretch treatment, electrostimulation, and FST-288 injection also used the number of sarcomeres in series to quantify longitudinal muscle growth. Following measurement of muscle belly and tendon lengths using a dissecting microscope, the TA muscles were further dissected. The total length of single fibers was the distance between the attachment on distal tendon to the proximal origin on the periosteum. A 1–2 mm mid- fiber segment was then cut from the myofibers whose length had been measured. The segment was mounted in Fluoromount aqueous medium (Millipore Sigma, St. Louis, MO) on a glass slide and coverslipped. The cross striations of individual myofibers were brought into focus and photographed with a Spot RT3 digital camera (SPOT Imaging, division Diagnostic Instruments, Inc. Sterling Heights, MI) attached to a Nikon E600 light microscope using the 40× objective and the substage condenser closed down to enhance sarcomere cross striations. The images were opened in MetaMorph Imaging software 7.8 (Diagnostic Instruments, Inc., Sterling Heights, MI). The distance morphometry program was employed to measure the distance covered by 10 sarcomeres in series in 30 different myofibers per muscle. The total number of sarcomeres per myofiber was calculated by dividing the measured myofiber length (prior to dissection) by the average length of a single sarcomere for the same myofibers.

Statistical testing:

Data were first evaluated by the Kolmogorov-Smirnov test for normality, and Barlett's test for equal variances. Student's t-test or one-way ANOVA with post hoc Tukey-Kramer testing was utilized for those passing. For those failing and that did not achieve normality with Box Cox transformation (sarcomere number, tendon length and tendon%/MTU), nonparametric testing (Mann-Whitney or Kruskal-Wallis with post hoc Dunn's multiple comparison test) was utilized. Statistics were conducted with GraphPad Prism 7, and significance was accepted at $p < 0.05$. See figure legends for specific statistical analysis of each data set.

When comparing within a group, the splinted side was compared with the contralateral nonsplinted limb. The values for each mouse were normalized by the ratio of the left (L)

splinted to (R) right nonsplinted side. Group data compared in this manner included: L/R sarcomere number, L/R tendon length, sarcomere number/tendon length, L/R percent tendon length/MTU, and L/R sarcomere number/tendon length.

Results:

Animal Condition Assessments:

Splinted mice were generally very tolerant of the ankle immobilization procedure, and animals were not removed from the study due to distress. The ratio percent of immobilized to non-immobilized tibia length across all groups averaged $99.6 \pm 0.9\%$, consistent with preservation of tibial growth. Overall length of the MTU was also assessed in all experiments, and data are reported in figure infographics. Overall, changes in total MTU length (either lengthening or shortening) were within 5% of normal/nonsplinted values. Contralateral controls were not different from nontreated controls conducted in parallel.

Ankle Immobilization in the Plantarflexed State:

Ankle immobilization in the fully plantarflexed splinting position produced a demonstrable deformity of the ankle (Figure 2) including shorter muscles and longer tendons (soleus: splinted 12.1 ± 0.1 , unsplinted 12.6 ± 0.3 ; TA: splinted 16.0 ± 0.4 , unsplinted 16.6 ± 0.4 mm), which can be expressed as the percentage of the total MTU that corresponds to the TA tendon (Figure 3). Measurement of the MTUs of the TA, soleus, plantaris, and EDL muscles at 3 weeks of life revealed longer tendons and shorter muscles in the soleus and TA muscles that cross only the ankle (soleus tendon/total length ratio increased $9 \pm 2\%$ ($p=0.002$) and TA tendon/total length increased $17 \pm 2\%$ ($p=0.002$) relative to the unsplinted side (Figure 3). The plantaris and EDL muscles that crossed both the ankle and non-immobilized knee joints did not display tendon lengthening (plantaris tendon/total length increased $1 \pm 3\%$ and EDL tendon/total length increased $0 \pm 1\%$ after immobilization). Light microscopic comparison of epoxy-embedded normal and immobilized muscles had intact sarcomeres and myofibrils without evidence of endomysial fibrosis or muscle fiber degeneration (Figure 2D). To evaluate the potential for contractures to persist long-term, a subset of animals with ankle immobilization during 1–3 weeks of life were then followed without further immobilization or intervention until 11 weeks of life. Despite the lack of imposed ankle immobilization between 3–11 weeks of life, the deformities of the ankle and the tendon lengthening consistent with contracture persisted in the TA (TA tendon/total length greater by $8 \pm 2\%$; $p=0.016$) on the side that had been immobilized; Figure 3). A summary infographic of these data is shown in Figure 3B.

Ankle Immobilization at 90 Degrees:

Mice with immobilization-induced muscle shortening due to immobilization at 90 degrees showed significant tendon lengthening when evaluated at 5 weeks of life without further immobilization or other interventions (TA tendon/total length increased $13 \pm 2\%$ in comparison to non-immobilized TA, $p=0.012$; Figure 4). Decreased longitudinal muscle growth was further demonstrated by a decrease in TA serial sarcomere number ($30 \pm 2\%$ decrease in immobilized limbs in comparison to non-immobilized limbs, $p=0.026$) and the ratio of sarcomere number/tendon length (decreased by $48 \pm 3\%$ when comparing the TA's of

immobilized vs. non-immobilized limbs, $p=0.0079$) in animals with immobilization and no treatment in comparison to their non-immobilized limbs.

Effect of Stretch Treatment on Immobilization-Induced Muscle Shortening Induced at 90 Degrees:

Stretch in 3 stages (120, 150, and 180 degrees) or 5 stages (108, 126, 144, 162, and 180 degrees) were evaluated and elicited essentially identical results (Figure 4). Measurement of sarcomere numbers and sarcomere number/tendon length calculations were suggestive of a modest benefit to longitudinal muscle growth as a result of stretch treatment. Sarcomere number decreased $17\pm4\%$ ($p=0.026$) in TA's from immobilized limbs in comparison to non-immobilized limbs following stretch treatment (Figure 4). Sarcomere numbers were not significantly different from the $30\pm2\%$ decrease seen in immobilized TA's without treatment. When viewed as the ratio of sarcomere number/tendon length, TA's from immobilized limbs showed decrease of $38\pm4\%$ ($p=0.0022$, Figure 4) in comparison to non-immobilized limbs. These values are consistent with a modest benefit in comparison to the $48\pm3\%$ decrease observed in untreated animals with immobilization-induced muscle shortening ($p=0.0079$)

Effect of Electrostimulation and Stretch on Immobilization-Induced Muscle Shortening at 90 Degrees:

Evaluation of TA muscle and tendon lengths after 2 weeks of stretch + electrostimulation revealed that there was no additional benefit to tendon length (increased by $13\pm1\%$ with stretch+electrostimulation in comparison to $13\pm2\%$ with no treatment and $10\pm1\%$ with stretch alone). Similarly, decreases in sarcomere number ($23\pm2\%$), and sarcomere number/tendon length (decreased by $42\pm2\%$) were not indicative of longitudinal muscle growth beyond that observed with stretch treatment alone (Figure 4).

Effect of FST-288 and Stretch on Immobilization-Induced Muscle Shortening at 90 Degrees:

In mice without ankle immobilization and lacking immobilization-induced muscle shortening, FST-288 exposure did not alter longitudinal muscle growth, as indicated by similar TA tendon/total lengths (similar within $1\pm1\%$), sarcomere numbers (similar within $3\pm4\%$), and sarcomere number/ tendon length (similar within $7\pm5\%$) on injected in comparison to non-injected sides (Figure 5). Control mice receiving 5 stages of stretch with vehicle injections showed similar degrees of tendon lengthening in comparison to our earlier stretch treatment studies (TA tendon/total MTU lengths greater by $17\pm1\%$ ($p=0.005$), sarcomere numbers were lower by $30\pm3\%$ ($p=0.0022$), sarcomere number/tendon length were lower by $49\pm3\%$ ($p=0.0022$); Figure 5). Treatment of animals with immobilization-induced muscle shortening with FST-288 in addition to stretch treatment produced a significant improvement by producing less of an increase in tendon/total MTU length (increased by $10\pm1\%$ in comparison to non-immobilized TA) while producing less of a decrease in sarcomere number (decreased by $18\pm1\%$ in comparison to non-immobilized TA, $p=0.0331$). Sarcomere number/tendon length was also dramatically improved (decreased by $30\pm2\%$ in comparison to non-immobilized TA, $p=0.0073$; Figure 5). When comparing stretch treatment alone to stretch+FST288, there was significant improvement in the ratio of

unsplinted to splinted limbs with respect to sarcomere number/tendon length values ($p < 0.01$). A summary of these results can also be found in Supplemental Table 1.

Discussion

The results of our study demonstrate the potential for ankle immobilization as a model for the study of longitudinal muscle growth, particularly in the study of potentially useful therapeutic approaches. As suspected, the ability to induce muscle shortening and tendon lengthening was highly dependent on whether the muscle-tendon unit was capable of even limited movement. While immobilization at the full ankle plantarflexion position produced tendon lengthening and muscle shortening in the soleus and TA muscles (which only cross the ankle joint), there was no evidence of muscle-tendon unit abnormality in the plantaris and EDL muscles (which cross both the ankle and knee joints and were thus not entirely immobilized by the ankle splinting).

The primary discovery from our study was that the intervention with the greatest impact on longitudinal muscle growth in contractures was a combination of stretch treatment and FST-288 injection. Stretch treatment alone had little impact on promoting longitudinal muscle growth in the TA muscle of developing mice. Stretch treatment alone may, however, be a more useful therapeutic option at later ages. The use of stretch therapy in the management of children with muscle contractures is based on its established effects in the adult population, where the stretch stimulus does not lead to tendon lengthening^{27,13}. In preadolescent experimental animals, however, stretch can produce tendon growth that outpaces the longitudinal growth of the muscle to generate a poorly functional muscle-tendon unit^{13,10,8}. Fortunately, stretch treatment did not negatively impact muscle-tendon unit development in this study. However, our findings also indicate that stretch alone may not have the positive impact on longitudinal muscle growth that has been described in adults. Electrostimulation treatment used here did not alter the growth of the muscle-tendon unit. While it is possible that longer or more frequent/involved electrostimulation treatment could be of benefit, the protocol used here was designed with clinical feasibility in mind and the ability to translate such protocols to the clinical setting may be challenging.

Progressive stretch using serial casting is similar to the Ponseti casting strategy currently used for the treatment of contractures. Ponseti casting involves a series of castings, Achilles tenotomy, and foot bracing to produce correction and avoid more extensive correction²⁸. It is, however, likely that this treatment can be improved with the integration of strategies to promote longitudinal muscle growth. The current duration of post-casting splinting is highly variable (from 2–5 years) due to uncertainty over the duration necessary to avoid reversion of the structural abnormalities. Additional study in this area may allow improvements in cast design and a clearer understanding of the necessary duration of casting treatments.

The most promising finding here is the evidence of additional benefit to longitudinal muscle growth when stretch treatment is combined with FST288. A number of genetic and pharmacological studies have shown that inhibiting myostatin/ActRIIB signaling can produce marked myofiber hypertrophy to increase muscle mass and strength (Reviewed in²²). In our prior work, we have demonstrated the capacity of ActRIIB-mFc (Acceleron

Pharma), a soluble activin type IIB receptor, to increase muscle size and strength in healthy mice²⁹. In treating immobilization-induced muscle shortening, however, local delivery and efficacy of a therapeutic agent was more desirable, and FST-288 better fit that profile. In this study, the effects of follistatin on MTU growth were assessed in immobilization-induced muscle shortening also treated with stretch treatment, as we believed that stretch would be necessary to stimulate longitudinal muscle growth. FST-288 plus stretch treatment promoted longitudinal muscle growth with decreased tendon lengthening over the two-week treatment period. The promotion of longitudinal muscle growth was mild but significant, and future work will focus on determining the functional impact of longitudinal muscle growth and whether treatment responses can be improved by extending the period of treatment. The pilot experiments reported here, however, offer interesting new directions in researching innovative therapeutic approaches for contractures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

EDL	extensor digitorum longus
MTU	muscle-tendon unit
TA	tibialis anterior

References

1. Kalampokas E, Kalampokas T, Sofoudis C, Deligeoroglou E, Botsis D. Diagnosing arthrogryposis multiplex congenita: a review. *ISRN obstetrics and gynecology* 2012;2012:264918. [PubMed: 23050160]
2. Flinchum D Pathological anatomy in talipes equinovarus. *J Bone Joint Surg Am* 1953;35-A(1):111–114. [PubMed: 13022712]
3. Ippolito E, De Maio F, Mancini F, Bellini D, Orefice A. Leg muscle atrophy in idiopathic congenital clubfoot: is it primitive or acquired? *Journal of children's orthopaedics* 2009;3(3):171–178.
4. Sharrard WJ. Paralytic deformity in the lower limb. *J Bone Joint Surg Br* 1967;49(4):731–747. [PubMed: 4864171]
5. Rink BD. Arthrogryposis: a review and approach to prenatal diagnosis. *Obstetrical & gynecological survey* 2011;66(6):369–377. [PubMed: 21851751]
6. Agbulut O, Noirez P, Beaumont F, Butler-Browne G. Myosin heavy chain isoforms in postnatal muscle development of mice. *Biology of the cell / under the auspices of the European Cell Biology Organization* 2003;95(6):399–406.

7. Dubowitz V Enzyme histochemistry of skeletal muscle. *Journal of neurology, neurosurgery, and psychiatry* 1965;28(6):516–524.
8. Crawford GN. The growth of striated muscle immobilized in extension. *J Anat* 1973;114(Pt 2):165–183. [PubMed: 4719655]
9. Williams PE, Goldspink G. The effect of immobilization on the longitudinal growth of striated muscle fibres. *J Anat* 1973;116(Pt 1):45–55. [PubMed: 4798240]
10. Williams PE, Goldspink G. Changes in sarcomere length and physiological properties in immobilized muscle. *J Anat* 1978;127(Pt 3):459–468. [PubMed: 744744]
11. Gardiner PF. Influence of reduced neuromuscular activity on the development of neonatal rat hindlimb muscle. *Developmental neuroscience* 1981;4(5):382–388. [PubMed: 6459921]
12. Bruce-Gregorios J, Chou SM. Core myofibers and related alterations induced in rats' soleus muscle by immobilization in shortened position. *J Neurol Sci* 1984;63(2):267–275. [PubMed: 6707693]
13. Tardieu C, Tabary JC, Tabary C, Huet de la Tour E. Comparison of the sarcomere number adaptation in young and adult animals. Influence of tendon adaptation. *J Physiol (Paris)* 1977;73(8):1045–1055. [PubMed: 615249]
14. Walton KD, Lieberman D, Llinas A, Begin M, Llinas RR. Identification of a critical period for motor development in neonatal rats. *Neuroscience* 1992;51(4):763–767. [PubMed: 1488121]
15. Trudel G, Uhthoff HK, Brown M. Extent and direction of joint motion limitation after prolonged immobility: an experimental study in the rat. *Arch Phys Med Rehabil* 1999;80(12):1542–1547. [PubMed: 10597804]
16. Lake SP, Castile RM, Borinsky S, Dunham CL, Havlioglu N, Galatz LM. Development and use of an animal model to study post-traumatic stiffness and contracture of the elbow. *J Orthop Res* 2016;34(2):354–364. [PubMed: 26177969]
17. Hall JG, Aldinger KA, Tanaka KI. Amyoplasia revisited. *Am J Med Genet A* 2014;164A(3):700–730. [PubMed: 24459070]
18. Fassier A, Wicart P, Dubousset J, Seringe R. Arthrogryposis multiplex congenita. Long-term follow-up from birth until skeletal maturity. *Journal of children's orthopaedics* 2009;3(5):383–390.
19. Bevan WP, Hall JG, Bamshad M, Staheli LT, Jaffe KM, Song K. Arthrogryposis multiplex congenita (amyoplasia): an orthopaedic perspective. *Journal of pediatric orthopedics* 2007;27(5):594–600. [PubMed: 17585274]
20. Damiano DL. Progressive resistance exercise increases strength but does not improve objective measures of mobility in young people with cerebral palsy. *J Physiother* 2014;60(1):58. [PubMed: 24856945]
21. Harvey LA, Katalinic OM, Herbert RD, Moseley AM, Lannin NA, Schurr K. Stretch for the treatment and prevention of contractures. *Cochrane Database Syst Rev* 2017;1:CD007455. [PubMed: 28146605]
22. Elliott B, Renshaw D, Getting S, Mackenzie R. The central role of myostatin in skeletal muscle and whole body homeostasis. *Acta physiologica* 2012;205(3):324–340. [PubMed: 22340904]
23. Gangopadhyay SS. Systemic administration of follistatin288 increases muscle mass and reduces fat accumulation in mice. *Scientific reports* 2013;3:2441. [PubMed: 23942549]
24. Nakatani M, Takehara Y, Sugino H, Matsumoto M, Hashimoto O, Hasegawa Y, Murakami T, Uezumi A, Takeda S, Noji S, Sunada Y, Tsuchida K. Transgenic expression of a myostatin inhibitor derived from follistatin increases skeletal muscle mass and ameliorates dystrophic pathology in mdx mice. *FASEB J* 2008;22(2):477–487. [PubMed: 17893249]
25. Winbanks CE, Weeks KL, Thomson RE, Sepulveda PV, Beyer C, Qian H, Chen JL, Allen JM, Lancaster GI, Febbraio MA, Harrison CA, McMullen JR, Chamberlain JS, Gregorevic P. Follistatin-mediated skeletal muscle hypertrophy is regulated by Smad3 and mTOR independently of myostatin. *J Cell Biol* 2012;197(7):997–1008. [PubMed: 22711699]
26. Doucet BM, Lam A, Griffin L. Neuromuscular electrical stimulation for skeletal muscle function. *The Yale journal of biology and medicine* 2012;85(2):201–215. [PubMed: 22737049]
27. Szoke G, Lee SH, Simpson AH, Prescott J. Response of the tendon during limb lengthening. *The Journal of bone and joint surgery British volume* 2005;87(4):583–587. [PubMed: 15795216]
28. Desai L, Oprescu F, DiMeo A, Morcuende JA. Bracing in the treatment of children with clubfoot: past, present, and future. *Iowa Orthop J* 2010;30:15–23. [PubMed: 21045966]

29. Lawlor MW, Read BP, Edelstein R, Yang N, Pierson CR, Stein MJ, Wermer-Colan A, Buj-Bello A, Lachey JL, Seehra JS, Beggs AH. Inhibition of activin receptor type IIB increases strength and lifespan in myotubularin-deficient mice. *Am J Pathol* 2011;178(2):784–793. [PubMed: 21281811]

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Figure 1: Diagram of Experimental Design.

This diagram illustrates the breakdown of experimental groups and timepoints for the experiments discussed in this manuscript. Colored boxes correspond to treatment group values in Figures 4 and 5.

Figure 2. Impact of Immobilization in the Plantarflexed Position on MTU Development (A, B) Casting of the ankle in the plantarflexed position at 1 week of life (A) results in a stable ankle deformity at 3 weeks of life (B). (C) Upon dissection of the tibialis anterior (TA) muscle, lengthening of the tendon in the immobilized limb is apparent. (D) Examination of epoxy-embedded, toluidine blue stained tissue did not detect histological abnormalities in the muscle or tendon tissue of mice following immobilization. Scale bars: 20 μ m.

Figure 3: Impact of Immobilization in the Plantarflexed Position on MTU Development.

(A) Tendon length (as calculated by the percentage of the MTU composed of tendon) is shown in the various lower-limb muscles in the presence and absence of immobilization in the plantarflexed position. Persistence of tendon lengthening can be evaluated by comparing results at 3 weeks of life (at the time of cast removal) and 11 weeks of life (8 weeks after cast removal). (B) Infographics of the soleus and TA muscle depict the relative proportion and location of muscle and tendon growth changes. * $p < 0.05$, ** $p < 0.01$, Mann-Whitney

Figure 4: Impact of Stretch and Electrostimulation on MTU Abnormalities Induced by Immobilization at a 90 Degree Ankle Position.

(A) Diagram of the studies comparing 3-stage stretch treatment, electrostimulation, and a combination of these treatments. A summary infographic of the average MTU length within each growth is shown at the bottom of the diagram. (B) Graphical depictions of tendon length (as % of the MTU; top), sarcomere number (middle), and sarcomere number/tendon length (bottom) measurements for the unsplinted (gray) and immobilized (colored) limbs in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Tendon length and sarcomere number: Mann-Whitney, sarcomere number/tendon length: ANOVA.

Figure 5: Impact of Stretch and FST-288 Treatment on MTU Abnormalities Induced by Immobilization at a 90 Degree Ankle Position.

(A) Diagram of the studies comparing 5-stage stretch treatment, FST-288 injection, and a combination of these treatments. A summary infographic of the average MTU length within each group is shown at the bottom of the diagram. (B) Graphical depictions of tendon length (as % of the MTU; top), sarcomere number (middle), and sarcomere number/tendon length (bottom) measurements for the unsplinted (gray) and immobilized (colored) limbs in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Tendon length and sarcomere number: Mann-Whitney, sarcomere number/tendon length: ANOVA.