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14. ABSTRACT

Follistatin is a possible anabolic treatment for denervation atrophy induced muscle weakness following prolonged denervation. Follistatin DNA treatment seemed to have both anabolic and neurotrophic effects when administered after nerve repair following prolonged (6-month) temporary denervation. Follistatin protein treatment was not associated with improved recovery though both protein and DNA treatments stimulated regenerative satellite cells. These observations are encouraging and warrant further investigation.

15. SUBJECT TERMS

FOLLISTATIN, ANABOLIC, DENERVATION ATROPHY, NERVE REPAIR, RODENT

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1. INTRODUCTION:

Functional recovery following major peripheral nerve injuries is often suboptimal despite adherence to well accepted nerve repair principles. Though a multifaceted problem, the poor muscle functional recovery often seen following nerve regeneration is in large part due to the progressive catabolic process affecting muscle fibers called “denervation atrophy.” While many researchers have approached this issue by attempting to improve axonal regeneration speed, efficiency, and accuracy (and thereby limiting the degeneration of the muscle), we have sought treatment options aimed at maximizing the potential of the muscle fibers that were able to achieve reinnervation. After experimenting with anabolic steroids (nandrolone), we determined that a more potent but safer anabolic agent would be a better option. Follistatin is a glycoprotein that both blocks the muscle inhibiting peptide myostatin and possesses remarkable independent muscle stimulating properties as well. We hypothesized that the administration of recombinant follistatin delivered to rodent muscles subjected to prolonged but temporary denervation periods (of either 3 or 6 months) would improve final muscle recovery and function. Most published studies have delivered the follistatin as recombinant DNA though some successful administration of recombinant protein has been demonstrated as well leading us to form two wings for our study—one exploring recombinant DNA administration and one exploring protein administration.

2. KEYWORDS:

Denervation atrophy, anabolic, Follistatin, nerve injury, nerve repair, rodent

3. ACCOMPLISHMENTS:

The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

- **Specific Aim 1**: Utilize an established rodent model of denervation atrophy
    - Regulatory Review and Approval Process- complete
    - Testing the Protein Stability- complete
    - Pilot Study (N=15; Follistatin recombinant DNA, Protein, and Alzet Pump Control Groups). Each group has 5 animals. (100% complete)
    - Denervation of hind limb muscles (3 and 6 months) Twelve groups (N=12; total of 144 rodents) were divided into control (sham surgery, sham treatment), sham surgery, sham treatment, and experimental groups (denervation surgery + treatment). Experimental and sham treatment groups underwent left tibial nerve transection to denervate left gastrocnemius muscle. Control and sham surgery groups underwent exposure of the nerve without transection. - (100% complete)
    - Re-innervation of hind limb muscles. (3 and 6 months) Denervation was reversed by repairing the transected tibial nerve using graft obtained from contralateral tibial nerve. Control rats underwent harvest of graft without repair. - (100% complete)

- **Specific Aim 2**: Treat re-innervated muscle with Follistatin:
    Recombinant DNA and AAV was provided by Vector BioLabs; BioVision provided the protein.
    - Treatment of re-innervated hind limb muscles (3-month and 6-month groups). All rats will undergo either injection of recombinant follistatin DNA packaged in AAV (into gastrocnemius muscle) or implantation of drug delivery reservoir (with either carrier or recombinant follistatin protein + carrier)- (100% complete)

- **Specific Aim 3**: Determine treatment effects utilizing strength testing, muscle morphology, electrophysiology nerve testing
    - Testing of muscle recovery/nerve regeneration (3 months). All rats underwent muscle morphology measurements, nerve conduction, and force generation studies of tibial nerve and gastrocnemius muscle. -(100% complete)
    - Immunohistology staining and histology of muscle (3 months). Fiber type analysis and satellite cell quantification to be determined for all specimens. – (fiber type analysis 100% complete, satellite cell analysis pending)
    - Measurement of Follistatin levels in muscle (3 months) immunoassay – (100% complete)
    - Testing of muscle recovery/nerve regeneration (6 months) All rats to undergo muscle morphology measurements, nerve conduction, and force generation studies of tibial nerve and gastrocnemius muscle. - (100% complete)
    - Immunohistology staining and histology of muscle (6 months) Fiber type analysis and satellite cell quantification to be determined for all specimens. - (fiber type analysis 100% complete, satellite cell analysis pending)
    - Measurement of Follistatin levels in muscle (6 months) immunoassay – (100% complete)

- **Specific Aim 4**: Histology (of nerve and muscle), Manuscript preparation, Presentation
    - Histology of muscle/nerve (3 and 6 months) Cross sections of muscle specimens will be stained and fiber size, axon numbers, and myelination measured. – (100% complete)
    - Data Analysis (3 and 6 months) – (100% complete)
    - Manuscript Preparation (3 and 6 months) – (100% complete)

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

- **Objective: Utilize an established rodent model of denervation atrophy**
  - Methodology:
    - Overview: twelve groups of male Sprague-Dawley rats (n=12; 144 rats total) were used in the study as outlined in Figure 1. Half of the rats underwent hind limb denervation (by tibial nerve transection) for either 3 months (Groups 1, 2, and 5) or 6 months (Groups 7, 8 and 11) before nerve repair, followed by twelve weeks of re-innervation (tibial nerve repaired).
    - The left sciatic nerve was exposed via a standard biceps femoris semi-tendinosis muscle splitting approach and the tibial nerve was transected just past the bifurcation. The two nerve ends were separated and buried in muscle bellies (held in place by a single 10-0 suture) to prevent inadvertent nerve regeneration
    - Sham denervation groups (3, 4, 6, 9, 10, and 12) underwent sham operations in which nerves were exposed but not transected.
    - At either 3 months (Groups 1-6) or 6 months (Groups 7-12), all rats underwent a second survival surgery. The left hind limb was reopened and the sciatic nerve and its divisions re-exposed. For denervated groups (1, 2, 5, 7, 8, and 11) the transected tibial nerve was dissected out and repaired using 1 cm of tibial nerve autograft harvested from the contralateral leg (to avoid tension) using standard microsurgical techniques (two or three epineural 10-0 nylon sutures per repair site).
  - Results:
    - One animal from the 3 month groups (group 1), and four animals in the 6 month groups (one rat each from group 7, group 8 group 9, and group 11) died and could not be included in the final analysis. One animal was euthanized (for a mass) and four expired in the peri-operative period.
    - Denervation for 3 and 6 months with subsequent repair resulted in significantly reduced force output when compared to matched sham denervation groups (Tables 1 and 2, $p<0.03$ for all 8 group comparisons).
    - Denervation for 3 and 6 months with subsequent repair resulted in significantly lower muscle weights when compared to matched sham denervation groups (Tables 1 and 2, $p<0.001$ for all 8 group comparisons).
    - Denervation for 3 and 6 months with subsequent repair resulted in significantly smaller fiber areas and diameters of type I, type IIA, and type IIB fibers when compared to matched sham denervation groups (Tables 1 and 2, $p<0.05$ for 22 of 24 group comparisons in 3 month groups and 20 of 24 group comparisons in 6 month groups) with no consistent differences in the proportion of fiber type ($p>0.05$ for 17 of 20 group comparisons).

- 3 months of denervation consistently resulted in lower satellite cell counts when compared to sham denervation; 6 months of denervation consistently resulted in lower satellite cell counts when compared to sham denervation ($p=0.045$ in sham protein groups and $p=0.031$ in sham DNA groups) (Tables 1 and 2, $p<0.001$ in both sham groups).
- denervation for 3 and 6 months with subsequent repair resulted in consistently smaller axon inner diameters (Table 3, $p<0.001$ for all 8 group comparisons) and g-ratios (Table 3, $p<0.001$ for all 8 group comparisons) with a relatively consistent reduction in the number of axons (Table 3, $p<0.01$ for 6 of 8 group comparisons) compared to sham denervation.
- Increasing the temporary denervation period from 3 to 6 months resulted in smaller muscles (Figure 5, $p=0.007$) but had no effect on muscle force (Figure 6). Increasing the sham denervation period from 3 to 6 months had no effect on muscle weight or force production. Three additional months of temporary denervation and sham denervation consistently resulted in smaller individual type 2B, type 2A, and type 1 muscle fiber areas and diameters (Figure 7, $p<0.05$ in 17 of 24 group comparisons) with a shift from type 2B to type 2A and type I fiber types ($p<0.05$ in 9 of 12 analyses). There was no change in satellite cell counts between short and long denervation periods. The only significant change in nerve histology was a smaller g-ratio with 6 months of denervation compared to 3 months of denervation ($p=0.003$) but there was a trend toward smaller inner diameters as well ($p=0.059$). There was no effect of increased length of temporary denervation or sham denervation with regards to axon counts ($p>0.05$ for all analyses).

- Conclusions: Nerve repair alone was unable to fully reverse the progressive atrophic effects of prolonged denervation.
  - Denervated gastrocnemius muscles from the tibial nerve autograft donor limbs were not extensively analyzed but, similar to other reports on chronic denervation[1], suffered a greater than 80% loss of mass compared with age matched controls.
  - With reinnervation some mass was restored though unlike the findings of Kobayashi et al. who demonstrated that after 3 months of temporary denervation, muscle loss stabilized[1], gastrocnemius muscles temporarily denervated for 6 months were statistically smaller than those denervated for only three months.
  - Force generation, while again less than control muscles, did not seem to further degenerate between short and long-term denervation periods. Force generation is dependent on cross sectional muscle fiber size[2] and muscle fiber organization[1]. Though we saw a general decrease in muscle fiber size (between 3 and 6 month temporary denervation groups), this did not translate to a detectable reduction in force generation.
  - Muscle fiber organization and muscle belly fibrosis were not evaluated.

- Axon counts, axon diameters, and G-ratios (as a measure of myelination) were all decreased in untreated temporarily denervated groups though only G-ratios and to a lesser extent, axon diameters seemed to further diminish with longer denervation periods. Negative effects on myelination and axonal growth are reflective of the poor neurotrophic environment within the chronically denervated distal nerve stumps[3]. Fu and Gordon have demonstrated time dependent blunting of axonal regeneration[4] as well as a gradual degradation of neurotrophic support in the distal nerve stump[5] following periods of denervation.
- In general, our data shows that six months of temporary denervation seemed to create greater physiological and functional deficits than three months of denervation.

- **Objective: Treat re-innervated muscle with follistatin**
  - Methodology:
    - Overview: after twelve weeks of re-innervation, Groups 1 and 7 were administered recombinant follistatin protein (isoform FS-288) and groups 2 and 8 were administered an adeno-associated viral (AAV) vector expressing follistatin DNA (isoform FS-317). Groups 5 and 11 received a representative sham treatment (vehicle only). The other half of the rats underwent sham surgeries and 3 months (Groups 3, 4, and 6) or 6 months (Groups 9, 10, and 12) of matched 'denervation' periods followed by a sham repair surgery. After twelve weeks of sham 're-innervation', Groups 3 and 9 were treated with recombinant follistatin protein, groups 4 and 10 were treated with recombinant follistatin DNA, and groups 6 and 12 received a representative sham treatment (vehicle only).
    - After 12 weeks of nerve regeneration time (to allow axons to regenerate to the muscles), all rats underwent a third survival surgery.
    - A subcutaneous osmotic pump drug delivery system with 200 microliter reservoir and delivery rate of 0.25 microliters/ hour (Figure 2) (model 2ML4 Alzet , Durect Corporation,Cupertino, CA, USA) was placed in the lumbar area to administer a continuous infusion of recombinant follistatin protein suspended in phosphate-buffered saline (PBS) (groups 1,3, 7 and 9) or PBS carrier only (1/2 of the rats in groups 5, 6, 11, 12). All treated rats received 90µg of commercially available recombinant follistatin isoform FST-288 (BioVision Incorporated, Milpitas, CA, USA) suspended in 200µL of PBS.

- Recombinant DNA groups received three evenly spaced injections (100µL x 3 locations for total 300µL) into the ipsilateral gastrocnemius muscle of suspended AAV vector (groups 2, 4, 8, 10) or equivalent PBS injection only (1/2 of the rats in groups 5, 6, 11, 12). The AAV vector consisted of a cytomegalovirus (CMV) promoter and it delivered follistatin isoform FST-317 (AAV1-CMV-h-FST317, Vector Biolabs, Malvern, PA, USA). FST 317 is FST-288 combined with a 29 amino acid signaling peptide that is cleaved prior to activation. All injections provided $>1.0 \times 10^{12}$ vg/ml. Animals in the recombinant DNA groups were anesthetized as if undergoing a surgical manipulation to ensure controlled injections.
- **Follistatin Protein Quantification:** Soluble protein was isolated from muscle tissue with a mammalian tissue lysis and extraction reagent (CelLytic™, Sigma-Aldrich, Saint Louis, MO, USA). Muscle follistatin levels were measured with a human follistatin immunoassay kit (Quantikine ELISA Kit, R&D Systems, Minneapolis, MN, USA). A total of 100 µg of protein was analyzed and prepared according to manufacturer specifications. Muscle follistatin concentrations were determined by comparison to a standard curve created from recombinant human follistatin using a Tecan Sunrise OEM Microplate Absorbance Reader.

- Results:
  - All osmotic pumps were empty at final testing
  - Follistatin protein quantification: In 3 and 6 month sham treatment groups, there were no differences in the level of follistatin protein between temporary denervation groups and their respective sham denervation groups (Table 4, $p>0.05$ for 4 of 4 analyses). In follistatin DNA treatment groups, temporary denervation resulted in significantly higher levels of follistatin protein compared to sham denervation groups that were also treated with follistatin DNA ($p<0.001$ for both 3 and 6 month denervation periods). Following 3 and 6 months of temporary denervation and subsequent repair, treatment with follistatin DNA resulted in significantly increased follistatin levels when compared to treatment with sham DNA (3 Month: FST DNA=10994±9789pg/mg vs. sham DNA=2898±1830pg/mg, p=0.001 and 6 Month: FST DNA=2772±2762pg/mg vs. sham DNA=108±37pg/mg, p=0.003). All 6 month groups treated with follistatin protein or representative sham protein resulted in follistatin levels that were below detection threshold and thus were unable to be quantified or utilized for group comparisons.

- Conclusions:
  - Successful viral vector delivery of follistatin DNA (and continuous follistatin protein production within the "infected" muscle fibers) was verified by elevated levels of follistatin protein detected within the target muscle tissue.
  - Undetectable levels of protein were measured in recombinant protein treated muscles after the longer denervation period.
    - could indicate: a failure of delivery, inadequate dosing, or a loss of protein potency due to gradual degradation within the implanted reservoir.
    - focal follistatin protein delivery would only elevate local levels of follistatin during the course of administration and our analysis of protein level was completed 2 weeks after the implanted reservoir had completely administered the recombinant protein.

- **Objective: Determine treatment effects utilizing strength testing, muscle morphology, electrophysiology nerve testing, histology (of nerve and muscle), and immunohistology**
  - Methodology:
    - All outcome measures were recorded after four and a half months of regeneration (nerve repair or sham repair) which corresponded to one and a half months post initiating "treatment" (follistatin protein, follistatin DNA, or representative sham). At the conclusion of testing all animals were euthanized with an intraperitoneal injection of 150mg Euthasol and were disposed of according to our institutional policy.
    - **Muscle function:** Terminal muscle strength for all groups consisted of exposure of the tibial nerve and isolation of the medial gastrocnemius muscle and tendon (for all groups). The hind limb was secured to a platform via placement of Kirschner wires through the femoral condyle and the distal tibia, and the medial gastrocnemius tendon was transected and coupled to a MLT500/A force transducer (AD Instruments, Inc., Colorado Springs, CO) using 4-0 silk suture. Strength testing was performed with a Grass stimulator (Model SD9, Astro-Med Inc., West Warwick, RI) and platinum electrodes. Stimulation was performed using 2 ms duration and 2 ms delay at varying voltages. Stimulus intensity and muscle fiber length were optimized as previously described and as recommended by Shin et al.[6] After optimization of muscle parameters, 3 supramaximal stimulations (5V, 1 Hz) were delivered to the sciatic nerve with 2-minute rest intervals between stimulations. Contraction strength was converted to digital data using ADI Instruments Power Lab system (ADInstruments, Inc., Colorado Springs, CO) and recorded using a Sony VAIO laptop computer (Sony Corporation, Tokyo, Japan). At the conclusion of muscle testing, medial gastrocnemius muscles were harvested and weighed.

- **Muscle histomorphometric analysis**: The harvested gastrocnemius muscles were immersed in TBS Tissue Freezing Medium (Medical Sciences, Inc., Durham, NC), maintaining medial and lateral orientation and in vivo length in a plastic histology mold (TedPella, Inc. Redding, CA). The embedded muscle was rapidly frozen by plunging the entire mold into isopentane cooled in liquid nitrogen for about 1 minute and stored in a freezer at -70° F. A one centimeter block was cut transversely from each muscle about five mm distal to its origin. Serial 10 µm transverse cryostat sections were prepared from each muscle block, collected on Fisher Superfrost Plus microscope slides (Fisher Scientific, Suwanee, GA) and air dried before being returned to the -70° freezer. Mouse monoclonal antisera was used for immunostaining of myosin heavy chains (MHC) and skeletal muscle satellite cells, including rat MHC I antibody (WB-MHs) (Vector labs, Burlingame, CA), MHC 2a antibody (SC-71) (Developmental Studies Hybridoma Bank, Univ. Iowa), MHC 2b antibody (BF-F3) (Developmental Studies Hybridoma Bank, Univ. Iowa), and Satellite Cell Pax7 antibody (sc-81975) (Santa Cruz Biotechnology, Inc. Dallas, TX). Muscle sections were placed in 10mM phosphate buffered saline (PBS, pH 7.5). Non-specific binding was blocked by a 20-minute incubation period in 10% normal blocking serum. The sections were then incubated with diluted (1:50) primary antibody for 1 hour, rinsed with PBS and incubated with diluted (1:1000) biotinylated secondary antibody for 30 minutes. After rinsing in PBS, the sections were reacted with Vectastain® Elite ABC Reagent (Vector Laboratories, Inc., Burlingame, CA) for 30 minutes, followed by another wash in 10mM PBS. A diaminobenzadine solution was used for visualization (Vector DAB kit, Vector Laboratories). The stained sections were dehydrated in ascending alcohols, cleared in xylene and mounted in permount. An Image-Pro®Plus image analysis system (v. 7.0) with a Nikon Microphot-7xA Microscope and a Q Imaging digital camera (Media Cybernetics®, Silver Spring, MD), and Image-Pro v. 7.01 image analysis software was used to analyze the minimum diameter and area of muscle fibers. One hundred positive fibers were analyzed from random areas from each muscle section.

- **Electrophoresis of Myosin Heavy Chains:** The remainder of the medial gastrocnemius muscle was used for differential myosin heavy chain (MHC) analysis. Frozen muscles were lyophilized, minced with scissors and homogenized with a pellet pestle in ice-cold extraction buffer (0.3 M NaCl, 0.15 M Na_2HPO_4, 10 mM EDTA, pH 6.5). The solution was agitated and stirred at 4ºC for 60 minutes and centrifuged (10,000 X gravity) for 10 minutes. The total protein concentrations of the supernatants were determined using the Bio-Rad protein assay for microtiter plates (Bio Rad Laboratories, Hercules, CA), based on the Bradford dye-binding procedure. The supernatants were diluted to 0.25 mg/ml in extraction buffer and stored at -70ºC. MHC isoforms were separated using a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) technique. This technique enables the separation of the adult MHC isoforms typically expressed in rat skeletal muscles.Gel slabs (0.75 mm thick) consisted of a 13.5 cm 8% separating gel and a 4 cm 4% stacking gel. All gels were made from the same stock solutions and all chemicals were of electrophoresis grade. A 2X Laemmli sample buffer was added to the muscle samples to yield a final protein concentration of 0.125 mg/ml. Samples were boiled for 5 minutes to denature the protein. Each lane on a gel was loaded with 20 µl of a muscle sample. Tris-glycine-SDS running buffers cooled to 4ºC were used and electrophoresis performed using a vertical slab gel unit (Protean II xi Cell, Bio Rad Laboratories) run at 275 V for 30 hours at 4ºC.Separating gels were silver stained using the Silver Stain Plus Kit (Bio Rad Laboratories). Images of silver-stained gels were obtained using an AGFA Duoscan HiD scanner (AGFA Corporation, Ridgefield Park, NJ). Relative proportions of MHC isoforms were determined using Gel-Pro® Analyzer (Media Cybernetics®, Silver Spring, MD), image analysis software.
- **Nerve Histomorphometric Analysis:** The tibial nerves (repaired and sham nerves) were harvested en bloc and fixed in 4% paraformaldehyde at 4° C prior to rodent euthanization. Nerve sections were obtained 5 mm distal to the repair site and stained with toluidine blue. Histologic specimens underwent axon counting and morphologic measurements - 9 high powered fields (40X) and multiplied by the appropriate factor as determined by the cross sectional area of the specimen using a 10X image.

- Results:
  - Three rats from the 6 month groups (one each in group 7, group 8, and group 11) had force data excluded for technical reasons (poor muscle response during nerve stimulation) but these animals were included in other analyses.
  - Muscle force: In the 3 month sham denervation groups, treatment with follistatin protein resulted in significantly reduced force production when compared to sham protein treatment (FST protein=0.979±0.467N vs. sham protein=1.745±0.596N, p<0.001). After 6 months of denervation and subsequent repair, treatment with FST protein trended towards increased force production when compared to sham protein treatment (FST protein=0.630±0.448N vs. sham protein=0.201±0.180N, p=0.066). There were no other significant differences in force output between treatment and sham treatment groups. (Tables 1 and 2)
  - Muscle weight: Following 3 months of denervation and subsequent repair, treatment with follistatin protein resulted in significantly lower muscle weights when compared to sham protein (FST protein=0.636±0.179gm vs. sham protein=0.927±0.167gm, p=0.019). In the 3 month sham denervation groups, treatment with follistatin DNA trended towards higher muscle weight when compared to sham DNA (FST DNA=2.427±0.205gm vs. sham DNA=2.202±0.254gm, p=0.063). After 6 months of denervation and repair, treatment with follistatin DNA resulted in significantly larger muscles when compared to the sham DNA (FST DNA=0.908±0.473gm vs. sham DNA=0.394±0.071gm, p=0.014). There were no other significant differences in muscle weight between treatment and sham treatment groups. (Tables 1 and 2)
  - Muscle histology (Tables 1 and 2):
    - In follistatin protein treatment groups, 3 months of denervation with subsequent repair did result in an increased proportion of type IIB fibers (denervation=51.0±8.6% vs. sham denervation=40.4±6.1%, p=0.009) and a decreased proportion of type IIA (denervation=28.7±7.1% vs. sham denervation=43.4±6.8%, p<0.001) when compared to sham denervation.
    - However, in follistatin protein treatment groups, 6 months of denervation and subsequent repair resulted in an increased proportion of type IIA fibers when compared to sham denervation (denervation=24.9±4.7% vs. sham denervation=20.9±3.2%, p=0.041).
    - Following 3 months of denervation and subsequent repair, treatment with follistatin protein decreased the diameter size of type 1 fibers (FST protein=37.4±10.1*um* vs. sham protein=48.8±8.5*um*, p=0.003) and the area (FST protein=1371.9±554.4um^2 vs. sham protein=3237.2±779.1um^2, p<0.001) and diameter (FST protein=29.2±7.5*um* vs. sham protein=48.5±6.0*um*, p<0.001) of type IIA fibers when compared to sham protein treatment.

- Following 3 months of denervation and subsequent repair, treatment with follistatin DNA resulted in an increased proportion of type IIB fibers when compared to sham DNA (FST DNA=59.3±11.3% vs. sham DNA=48.5±6.1%, p=0.035) and trended towards a decreased type IIb fiber area (p=0.056).
- In the 3 month sham denervation groups, treatment with follistatin DNA resulted in larger type IIA fiber areas when compared to sham DNA treatment (FST DNA=4499.9±912.2um^2 vs. sham DNA=3594.8±1328.5um^2, p=0.040).
- Following 6 months of denervation and repair, treatment with follistatin protein resulted in larger diameters of type I muscle fibers (FST protein=39.0±14.2*um* vs. sham protein=28.6±12.2*um*, p=0.035) and trended toward larger type I fiber areas (p=0.054).
- After 6 months of denervation and repair, treatment with follistatin DNA resulted in larger diameters of type IIB muscle fibers (FST DNA=37.5±13.6*um* vs. sham DNA=24.5±12.3*um*, p=0.019) and trended toward larger type IIB fiber areas (p=0.074).
- In 6-month sham denervation groups, treatment with follistatin protein resulted in larger areas and diameters of type I (p=0.030 and 0.046, respectively) and type IIB (p=0.001 and 0.009, respectively) fiber types with a trend towards larger type IIA fiber areas (p=0.051) when compared to sham protein.
- In 6 month sham denervation groups, treatment with follistatin DNA resulted in significantly lower proportion of type IIB fibers (Figure 3, FST DNA=59.2±7.3% vs. sham DNA=65.8±1.8%, p=0.018) with a trend towards a higher proportion of type IIA fibers (p=0.085) when compared to sham DNA. There were no other significant differences in muscle fiber type area, diameter, or proportion.

- Satellite cell counts (tables 1 and 2):
    - In groups treated with follistatin protein, 3 months of denervation resulted in significantly higher satellite cell counts than sham denervation groups that were also treated with follistatin protein (p=0.002), but this same interaction was not seen after 3 months of denervation in FST DNA groups (p=0.489). In the 3 month denervation and repair groups, treatment with follistatin protein (FST protein=157.9±29.0 vs. sham protein=114.8±12.1, p<0.001) and follistatin DNA (FST DNA=129.1±9.3 vs. sham DNA=110.2±17.6, p=0.041) both significantly increased satellite cell counts compared to respective sham treatments. However, in sham denervation groups treatment with follistatin protein significantly decreased (FST protein=134.8±17.0 vs. sham protein+153.0±16.7, p=0.037) and treatment with follistatin DNA trended towards lower (FST DNA=133.9±7.3 vs. sham DNA=150.8±17.3, p=0.067) satellite cell counts.

- In groups treated with follistatin protein or follistatin DNA, 6 months of denervation resulted in significantly higher satellite cell counts than sham denervation groups that were also treated with follistatin protein or DNA (Figure 4, p=0.012 in follistatin protein groups and p=0.026 in follistatin DNA groups). Following 6 months of denervation and repair, treatment with follistatin protein (FST protein=179.1±40.0 vs. sham protein=104.0±12.2, p<0.001) and follistatin DNA (FST DNA=170.4±32.7 vs. sham DNA=110.5±22.9, p<0.001) both significantly increased satellite cell counts compared to respective sham treatments. There were no other significant differences between treatment groups for the number of satellite cells.

- Nerve histomorphometery (Table 3):
  - In the 3 month denervation and repair group (FST DNA=1196±360 vs. sham DNA=1773±694, p=0.013) and in 3 month sham denervation group (FST DNA=3055±153 vs. sham DNA=3501±221, p=0.050), treatment with follistatin DNA resulted in significantly lower axon counts. In the 3 month sham denervation group, follistatin DNA significantly increased axon inner diameters (FST DNA=8.65±0.25*um* vs. sham DNA=7.89±0.47*um*, p=0.024).
  - Following 6 months of denervation and subsequent repair, treatment with follistatin DNA resulted in significantly larger g-ratios (FST DNA=0.462±0.036 vs. sham DNA=0.347±0.112, p=0.002) and inner diameters (FST DNA=2.92±0.28*um* vs. sham DNA=2.10±0.81*um*, p=0.015) when compared to sham DNA treatment. In 6 month sham denervation groups, treatment with follistatin protein resulted in significantly smaller inner diameters (FST protein=7.73±0.43*um* vs. sham protein=8.63±0.57*um*, p=0.006) and treatment with follistatin DNA resulted in significantly higher axon counts (FST DNA=7636±2945 vs. sham DNA=4694±3783, p=0.023) compared to respective sham treatments. There were no other significant differences in measures of nerve histology (inner diameter, g-ratio, or axon counts) between treatment groups.
- Conclusions:
  - In normal muscle: FS-288 DNA (but not protein) treatment did appear to have a mild anabolic effect as evidenced by increased type IIA muscle fiber size and a trend towards increased muscle weight.

  - Others have shown a much more profound effect in normal muscle- Winbanks et al. doubled the size of the tibialis anterior and increased force production 40% compared with controls 28 days post AAV-FST 288 injection in 8 week old mice[7]; Gilson et al. reported a 37% increase in mice tibialis anterior muscle mass, 17 days post FS-288 plasmid injection; Yaden et al. found a similar (37%) increase in mice gastrocnemius muscle mass 24 days post FS-288 plasmid injection[8].
  - Indwelling catheter may have caused muscle tissue irritation or damage (Figure 2)

- In normal muscle: satellite cell counts were decreased with treatment
  - Viguie et al. found an initial increase in satellite cells preceded a gradual progressive decline over several months[9]. Depletion of this pool of regenerative cells has been implicated in the establishment of irreversible denervation atrophy[5,9,10].
- In reinnervated muscle: FS-288 DNA had an anabolic effect as evidenced by increased muscle weight, increased type IIb muscle fiber size
  - No improvement in muscle force may be explained by underpowered statistical analysis or yet unquantified changes in muscle fiber physiology (others have reported poor correlation between muscle strength and mass[11])
- In reinnervated muscle: FS-288 DNA and protein increased satellite cells
  - satellite cell activation is generally considered an essential component of muscle hypertrophy[12,13].
  - a follistatin induced bolstering of the satellite cell population might have a more profound impact with even longer periods of denervation[14].
- In short term (3 month) denervation: follistatin protein treatment had a negative effect and resulted in lower muscle weights and smaller type I and type IIA fibers when comparing short temporary denervation groups.
  - Gangopadhyay noted an analogous decreased in type I and IIA fibers though with an increase in type IIB fibers following systemic administration of recombinant FS-288 in a mouse model[15].

                - It is conceivable that a temporary spike in follistatin triggered an upregulation of myostatin, and increased myostatin levels have been shown to cause muscle atrophy[16].
                    - we did not have concurrent levels of myostatin to completely understand how this interaction may have influenced our results in opposing manners as the length of denervation changed.
        - In long term (6 month) denervation: follistatin protein treatment had a mild anabolic effect and resulted in larger type I fibers and a trend towards increased muscle force generation
        - FS-288 DNA may have a stimulatory effect on chronically denervated nerve tissue
- Other observations:
    - Timing of follistatin treatment and chronicity of denervation may affect outcomes
        - Follistatin treatment improved muscle recovery if administered prior to denervation (i.e. to innervated muscle) but not afterwards[17]
        - The 12 week delay following nerve repair in this study to allow reinnervation prior to initiating treatment may have been too early and the muscles may have been inadequately reinnervated at the initiation of therapy. Since the muscles injected with follistatin DNA would continue producing follistatin protein as reinnervation progressed, this may have biased the results towards this treatment group.
        - The sedentary environment did not allow us to evaluate if follistatin could enhance the ability of muscle to adapt to the progressive loading that is commonly utilized during recovery of muscle strength after periods of temporary denervation.
        - Timing of muscle testing relative to recombinant protein could have affected data positively or negatively
            - Muscle hypertrophy may have peaked during protein administration and the muscle may have regressed at the time of formal testing.
            - Muscles may have been gradually getting stronger and a later testing point may have revealed a more profound treatment effect
                - Increased muscle strength plateaued at around 10 weeks post FS-315 DNA administration in mdx mice[18], though target muscle mass increased between 70 and 100% at 4 weeks and 100 and 135% at 8 weeks post injection (FS-288 DNA) in an unrelated study[17]

Table 1. Muscle histomorphometry means, standard deviations (in parentheses), and statistical results for short (3 month) temporary denervation groups.

	Muscle Force (N)	Muscle Weight (gm)	Type I			Type IIA			Type IIB			Satellite Cell Count
			Area (um^2)	**Diameter (*um*)**	**Proportion (%)**	**Area (um^2)**	**Diameter (*um*)**	**Proportion (%)**	**Area (um^2)**	**Diameter (*um*)**	**Proportion (%)**	
FST Protein and Denervation	0.37 (0.19)	0.64* (0.18)	1988.8 (803.1)	37.4 * (10.1)	20.3 (6.0)	1371.9* (554.4)	29.2* (7.5)	28.7 (7.1)	1958.8 (992.0)	37.4 (10.1)	51.0 (8.6)	157.9* (29.0)
Sham Protein and Denervation	0.45 (0.25)	0.93* (0.17)	3222.5 (1068.9)	48.8* (8.5)	16.8 (3.7)	3237.2* (779.1)	48.5* (6.0)	33.5 (13.5)	2726.7 (474.2)	44.4 (3.5)	49.7 (10.6)	114.8* (12.1)
FST Protein and Sham Denervation	0.98* (0.47)	2.22 (0.17)	4379.5 (709.1)	55.1 (6.4)	16.5 (4.6)	4417.3 (624.3)	55.9 (5.6)	43.4 (6.8)	6404.0 (1220.3)	66.5 (6.4)	40.4 (6.1)	134.8* (17.0)
Sham Protein and Sham Denervation	1.75* (0.60)	2.42 (0.20)	4434.2 (750.2)	57.9 (5.2)	19.4 (7.1)	4673.8 (404.0)	58.3 (3.7)	32.9 (6.6)	5592.9 (1355.8)	62.6 (7.2)	47.7 (6.7)	153.0* (16.7)

Abbreviations: FST=follistatin, N=newton, gm=grams, um=micrometers, %=percent
* denotes significant differences between treatment and matched sham treatment at $p<0.05$

Table 2. Muscle histomorphometry means, standard deviations (in parentheses), and statistical results for long (6 month) temporary denervation groups.

	Muscle Force (N)	Muscle Weight (gm)	Type I			Type IIA			Type IIB			Satellite Cell Count
			Area (um^2)	**Diameter (*um*)**	**Proportion (%)**	**Area (um^2)**	**Diameter (*um*)**	**Proportion (%)**	**Area (um^2)**	**Diameter (*um*)**	**Proportion (%)**	
FST Protein and Denervation	0.63 (0.45)	1.00 (0.53)	2268.9 (1410.1)	39.0* (14.2)	9.2 (2.9)	1942.7 (1200.2)	35.6 (12.1)	24.9 (4.7)	2106.1 (1120.7)	37.7 (12.1)	65.9 (3.8)	179.1* (40.0)
Sham Protein and Denervation	0.20 (0.18)	0.81 (0.33)	1312.0 (953.2)	28.6* (12.2)	12.1 (2.2)	1419.9 (590.4)	29.5 (7.9)	20.5 (3.5)	1702.4 (1010.4)	32.2 (11.8)	67.3 (2.2)	104.0* (12.2)
FST Protein and Sham Denervation	1.03 (0.40)	2.33 (0.36)	4207.3* (1112.0)	55.6* (9.9)	9.9 (3.3)	3178.6 (675.6)	45.8 (6.0)	20.9 (3.2)	4836.4* (1322.8)	56.8* (8.5)	69.2 (5.2)	147.9 (31.8)
Sham Protein and Sham Denervation	1.29 (0.63)	2.17 (0.49)	3130.2* (426.9)	45.8* (4.2)	8.2 (4.3)	2419.1 (435.5)	39.5 (4.0)	24.1 (3.3)	2666.8* (326.6)	42.2* (2.9)	67.7 (2.9)	137.5 (8.8)

Abbreviations: FST=follistatin, N=newton, gm=grams, um=micrometers, %=percent
* denotes significant differences between treatment and matched sham treatment at $p<0.05$

Table 3. Nerve histology means, standard deviations (in parentheses), and statistical results for short (3 month, left) and long (6 month, right) temporary denervation groups.

3 Month Groups	**Inner Diameters (*um*)**	**G-ratios**	**Axon Counts**
FST Protein and Denervation	2.40 (0.31)	0.39 (0.05)	1211.6 (232.9)
Sham Protein and Denervation	2.40 (0.37)	0.38 (0.05)	1316.2 (140.9)
FST Protein and Sham Denervation	8.34 (0.64)	0.67 (0.04)	3204.4 (305.6)
Sham Protein and Sham Denervation	8.33 (0.83)	0.70 (0.05)	3428.6 (365.1)

6 Month Groups	**Inner Diameters (*um*)**	**G-ratios**	**Axon Counts**
FST Protein and Denervation	2.50 (0.49)	0.44 (0.05)	2420.0 (979.9)
Sham Protein and Denervation	2.40 (0.45)	0.41 (0.05)	1629.2 (873.2)
FST Protein and Sham Denervation	7.73* (0.43)	0.67 (0.04)	2268.6 (415.1)
Sham Protein and Sham Denervation	8.63* (0.57)	0.70 (0.02)	2558.8 (1361.3)

Abbreviations: FST=follistatin, um=micrometers
* denotes significant differences between treatment and matched sham treatment at $p<0.05$

Table 4. Follistatin level means, standard deviations (in parentheses), and statistical results for short (3 month, left) and long (6 month, right) temporary denervation groups.

3 Month Groups	**Follistatin Protein Level (pg follistatin/mg of protein)**
FST Protein and Denervation	2678.8 (2183.6)
Sham Protein and Denervation	2649.8 (608.7)
FST Protein and Sham Denervation	2282.0 (1516.9)
Sham Protein and Sham Denervation	3301.4 (715.3)

6 Month Groups	**Follistatin Protein Level (pg follistatin/mg of protein)**
FST Protein and Denervation	Not detectable
Sham Protein and Denervation	Not detectable
FST Protein and Sham Denervation	Not detectable
Sham Protein and Sham Denervation	Not detectable

Abbreviations: FST=follistatin, pg=picograms, mg=milligrams
* denotes significant differences between treatment and matched sham treatment at $p<0.05$

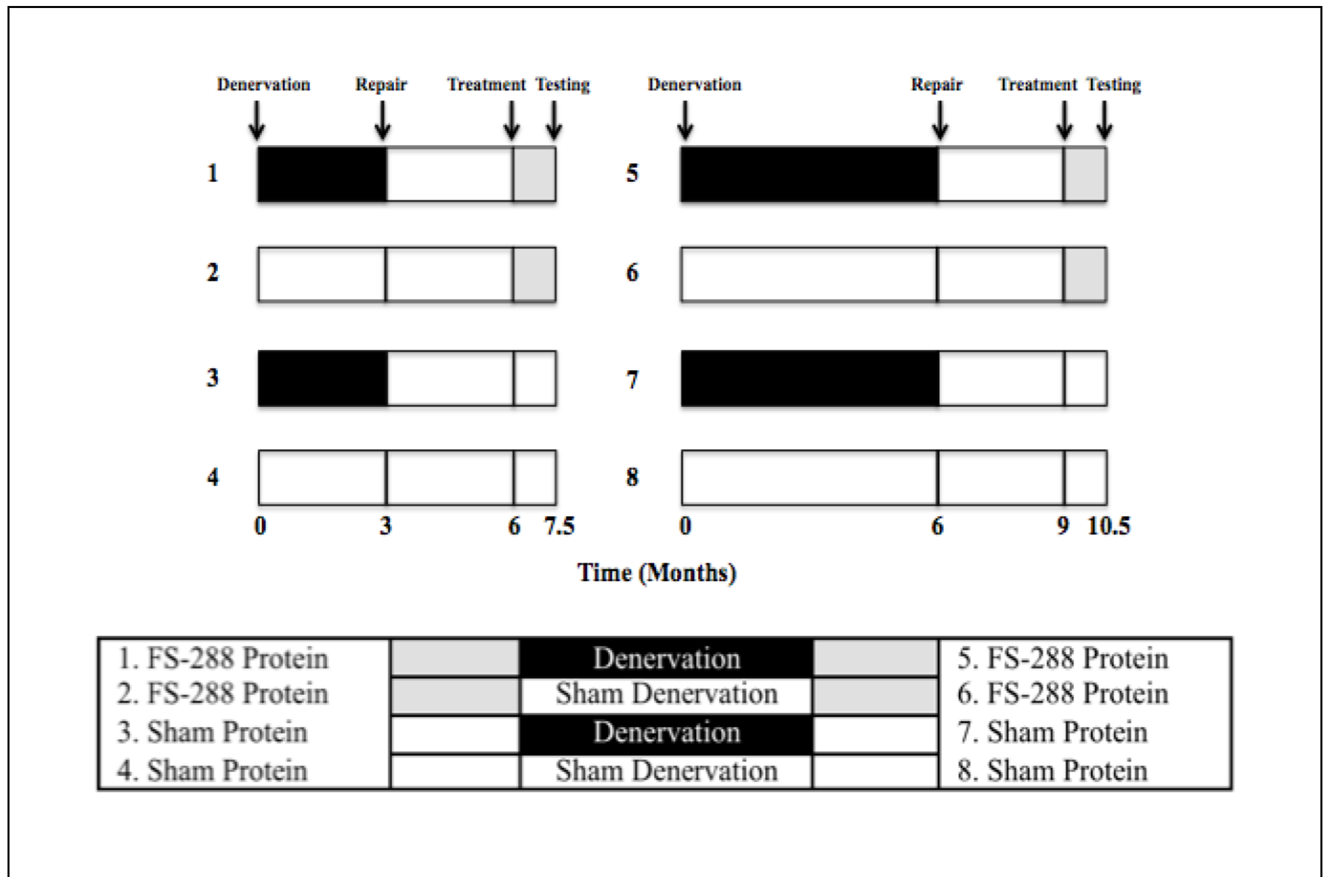

Figure 1. Overview of study design.

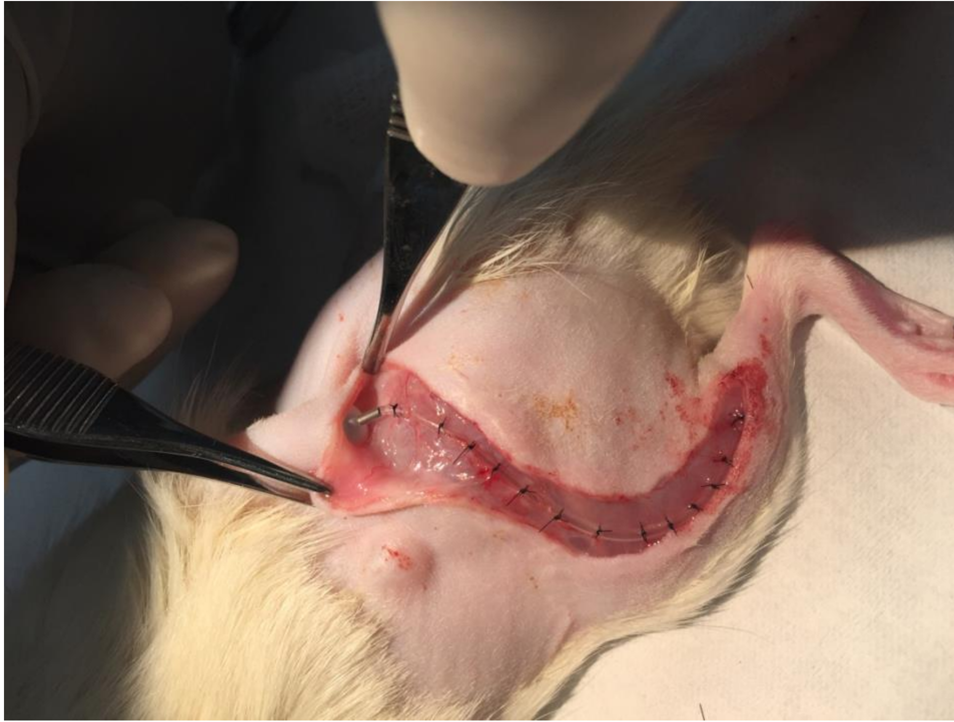

Figure 2. Subcutaneous osmotic pump delivery system utilized for recombinant follistatin protein and sham protein treatment groups.

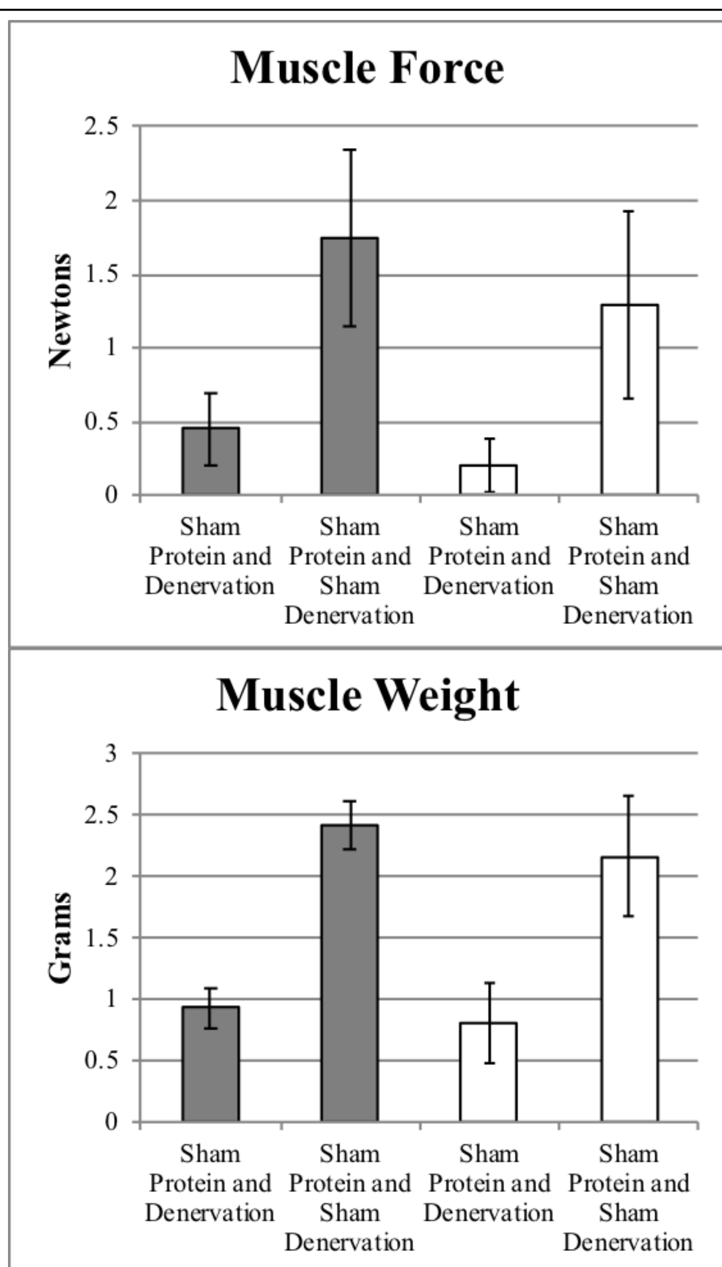

Figure 3. Effect of denervation vs. sham denervation (comparisons within same color) and effect of short (3 month - grey) and long (6 month - white) denervation (comparisons between colors) on muscle force (left) and muscle weight (right) in sham treatment groups.

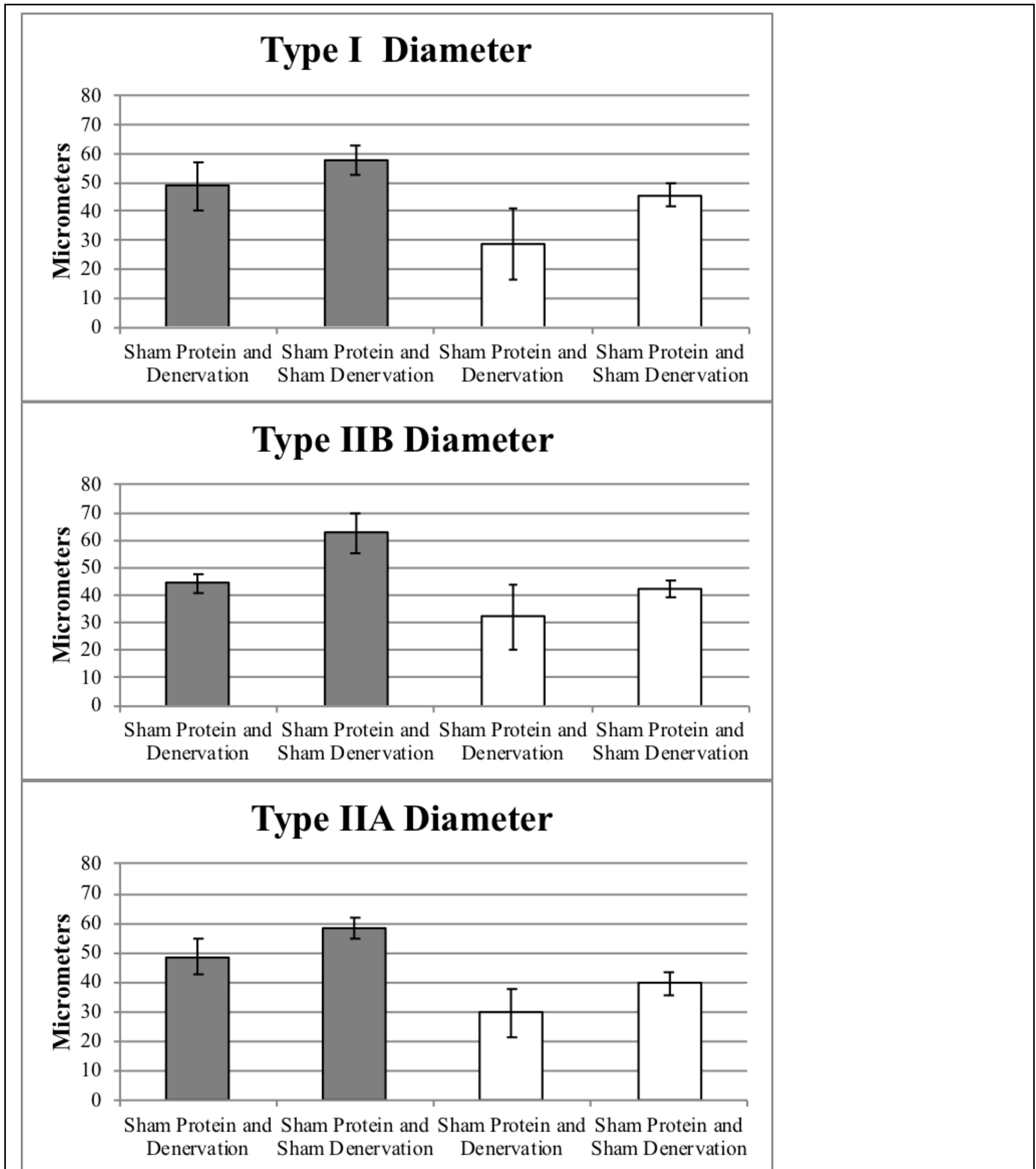

Figure 4. Effect of denervation vs. sham denervation (comparisons within same color) and effect of short (3 month) and long (6 month) denervation (comparisons between colors) on Type I, IIA, and IIB fiber diameters in 3 (grey) and 6 month (white) sham treatment groups.

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What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to Report.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Nothing to Report.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

At this point, there is not enough evidence to support a direct translation to clinic administration of follistatin to augment muscle recovery. The viral vector delivery seemed to have a legitimate positive effect and has therapeutic potential. In retrospect, our study may have been underpowered and while we could visually detect what appeared to be obvious differences in the bulk of muscles treated with Follistatin DNA, the standard deviation between groups prevented us from obtaining the strong statistical confirmation we had expected. In addition, the study revealed a variety of important questions that need to be considered regarding the optimal timing of treatment and the length of treatment necessary before a muscle response would be expected. Exercise or muscle stimulation might also influence the magnitude of response though this was not explored.

The paradoxical effect that we observed with follistatin protein treatment was unexpected and alludes to the complex nature of muscle atrophy and recovery as well as the yet incompletely appreciated myostatin/follistatin interplay in normal muscle healing. With the treatment delivery strategy utilized in this study, recombinant follistatin protein seems to have less clinical translational potential.

Satellite cells are felt by many researchers to have a key role in muscle recovery and failure of this system has been implicated in the development of irreversible denervation atrophy and failed muscle recovery in general. Increased satellite cells following follistatin treatment was a particularly interesting observation and worthy of future focused study.

Poor functional recovery following major peripheral nerve injury despite technically adequate nerve reconstruction remains a significant and unsolved problem for victims of military and civilian trauma. Partial recovery indicates either inadequate axon regeneration or insufficient preservation of contractile tissue to react to the re-established neural circuitry. In either scenario, augmenting and strengthening the available innervated muscle tissue remains an appealing and realistic therapeutic strategy. This study supports the continued pursuit of the most effective and practical clinical application of this treatment approach.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report.

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report.

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Nothing to Report.

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to Report.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to Report.

  Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

1. Viral Vector Delivery Of Follistatin Enhances Recovery Of Reinnervated Muscle
Gaurangkumar Patel, B.S., Mark Feger, Ph.D., Satya Mallu, M.D., Jonathan Isaacs, M.D.
Abstract ID: MHSRS-19-02170; Research Topic: Management & Treatment of Warfighter Neuromuscular Injuries
2019 Military Health System Research Symposium (MHSRS), at the Gaylord Palms Resort & Convention Center, Kissimmee, FL, Aug 19-22, 2019.

2. Follistatin Concentrations following Two Novel Recombinant Delivery Methods in Chronically Denervated Muscle
Satya Mallu, MD, Mark Feger, PhD, Gaurangkumar Patel, B.S., Mary Shall, PhD and Jonathan Isaacs, MD
Electronic poster at the 2018 ASPN Annual Meeting program, February 1-3, 2019 at the JW Marriott Hotel in Palm Desert, California.

3. Recovery of Chronically Denervated Muscle Enhanced with Follistatin Treatment.
Jonathan Isaacs, MD, Satya Mallu, MD, Mark Feger, PhD and Gaurangkumar Patel, B.S.,
Podium presentation at the ASPN Annual Meeting, February 1-3, 2019, at the JW Marriott Hotel in Palm Desert, CA.

4. Does Time make a Difference? The Effect of Follistatin on Reinnervated Skeletal Muscle Fiber Recovery after 3 months vs 6 months of Denervation.
M.S. Shall, J.E. Isaacs, S. Mallu, G. Patel
Poster presentation at Neuroscience 2018, November 3-7, San Diego, CA.

5. Does Follistatin augment skeletal muscle fiber recovery following moderate periods of denervation?
Shall M, Isaacs J, Mallu S, Patel G, and Feger MA
Poster Session Number: 502;
Session Title: Motor Neurons: Functional Relationships
Neuroscience 2017 at Walter E. Washington Convention Center, Washington, DC, Nov 11-15, 2017.

6. Comparison between different Follistatin delivery methods (AAV, AV, and Protein) to enhance motor recovery post peripheral nerve injury and regeneration.
Gaurangkumar Patel, B.S., Mark Feger, Ph.D., Satya Mallu, M.D., Jonathan Isaacs, M.D.
Abstract ID: MHSRS-17-1469; Research Topic: Extremity Regeneration
2017 Military Health System Research Symposium (MHSRS), at the Gaylord Palms Resort & Convention Center, Kissimmee, FL, Aug 27-30, 2017.

7. Evaluation of human recombinant Follistatin and adeno-associated viral vector delivery methods in rodents.
Mark A. Feger, Gaurangkumar Patel, Satya Mallu, Jonathan Isaacs
2017 Medical Student Research Poster Session, Virginia Commonwealth University School of Medicine, Richmond, VA, April 13, 2017.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

  Nothing to Report.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

  Nothing to Report.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

  Nothing to Report.

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

  Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

<u>*Example:*</u>

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name: Jonathan Isaacs, M.D.
Project Role: PI
Nearest person month worked average per annum: 1
Contribution to project: Regulatory process, supervising the study, Data Analysis and Manuscript Preparation.
Funding support: VCU salary, MCV physicians salary for clinical work, protocol no. ANG-CP-007, Cook Biotech, Inc. industry grant, Flow through funding from NIH: 1R34NS097113-01, Axogen, Inc., Polyganics

Name: Satya Mallu, M.D.
Project Role: Co-investigator
Nearest person month worked average per annum: 4
Contribution to project: Assisted with regulatory process, performed Pilot and Main study surgeries, Data Analysis.
Funding support: VCU salary, Cook Biotech, Inc. industry grant, Flow-through funding from NIH: 1R34NS097113-01, AFSH grant, Axogen, Inc.,

Name: Gaurangkumar Patel, B.S.
Project Role: Lab technician, Pilot Data Analysis.
Nearest person month worked average per annum: 8
Contribution to project: Main study, assisted with main study surgeries.
Funding support: VCU salary

Name: Mary Shall, PhD
Project Role: Co-PI
Nearest person month worked average per annum: 2.5
Contribution to project: ELISA and Muscle Fiber Type Analysis
Funding support: VCU salary

Name: Jeffery Dupree, PhD
Project Role: Co-investigator
Nearest person month worked average per annum: 0.25
Contribution to project: Pilot study, Study Consultant
Funding support: VCU salary and NIH grants

Name: Scott Vota, DO
Project Role: Co-investigator
Nearest person month worked average per annum: 0.5
Contribution to project: Pilot study, Study Consultant
Funding support: VCU salary and MCV physicians salary for clinical work

Name: Dorne Yager, PhD
Project Role: Consultant
Nearest person month worked average per annum: 0.5
Contribution to project: Immunohistology and Data Analysis
Funding support: VCU Salary

Name: Mark Feger, PhD
Project Role: Medical Student
Nearest person month worked average per annum: 0.5
Contribution to project: Pilot Study and Main Study Data Analyses, Assisting in Manuscript preparation.
Funding support: None.

Name: Omar Protzuk
Project Role: Medical Student
Nearest person month worked average per annum: 0.5
Contribution to project: Histology – Imaging and Data Analysis
Funding support: None.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

Attached. (See Appendix 1)

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

1. Quad Chart

Follistatin: A Potential Anabolic Treatment for Re-Innervated Muscle

Proposal #11231008

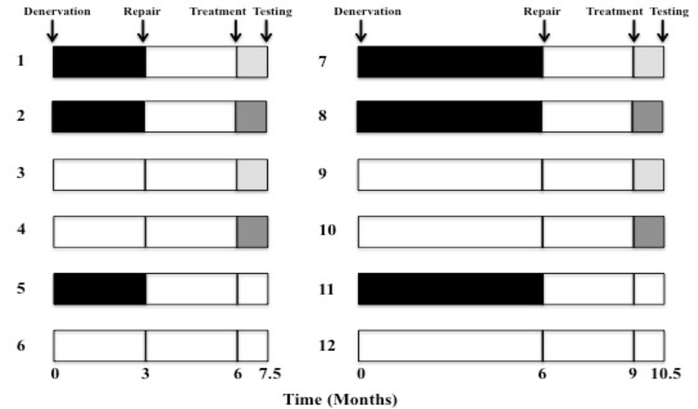
PI: Jonathan Isaacs, MD **Org: Virginia Commonwealth University** **Award Amount: $705041**

Study Aims

- To utilize an established animal model of denervation atrophy to determine if Follistatin treatment (administered either as a recombinant protein or as a recombinant DNA) will improve muscle recovery following re-innervation after prolonged periods of denervation.
- To determine Follistatin effects on nerve regeneration and intramuscular fibrosis (in re-innervated tissue).

Approach

Based on the pilot study result, rodents will undergo transection of one tibial nerve to denervate the hind limb muscles (including gastrocnemius). After a delay (of either 3 or 6 months) the nerve will be repaired and the muscles re-innervated. The re-innervated muscle will be treated with either recombinant follistatin protein (delivered thru an implantable drug delivery system) or recombinant follistatin DNA (delivered thru adeno-associated viral vectors injected into the reinnervated gastrocnemius muscle). After 8 weeks recovery, the effects of the follistatin treatment will be determined utilizing strength testing, muscle morphology, muscle histology, and muscle immunohistology (to determine muscle fiber type distribution and satellite, or regenerative cell, population pools). Nerve conduction testing will be performed to differentiate follistatin effects on nerve regeneration and function; muscle staining for collagen will determine effects on muscle fibrosis; and follistatin levels will be measured in treated muscle to confirm effective dosing and delivery of follistatin. Test results will be compared with sham surgery (plus FS treatment), re-innervation (without treatment), and control groups.



Timeline and Cost

Activities	CY 15	16	17	18
Regulatory Process & Pilot Project				
Denervation and re-Innervation of hind limb muscles, Treatment with FS and Testing				
Histology, Manuscript Preparation and Presentation				
Estimated Budget ($K)	**$10762**	**$390356**	**$303923**	

Updated: Richmond, Jun 15, 2019

Goals/Milestones

CY15 Goal – Utilize an established rodent model of denervation atrophy

✔ Regulatory Process – Received ACURO approved on Jan 19, 2016

CY16 Goals – Treat re-innervated muscle with Follistatin, Determine treatment effects utilizing strength testing, muscle morphology, electrophysiology nerve testing

☑ Pilot surgeries completed

☑ Denervation of hind limb muscles

CY17 Goal – Histology (of nerve and muscle), Manuscript preparation, Presentation

☑ Reinnervation of hind limb muscles

☑ Synthesis of Recombinant Follistatin DNA/Protein

☑ Treatment with Follistatin

☑ Testing of muscle recovery/nerve regeneration

☑ Immunohistology staining and histology of muscle

☑ Measurement of Follistatin levels in muscle

☑ Immunohistology of muscle/nerve

☑ Histology of muscle and nerve- Data Analysis

☑ Manuscript Preparation

Comments/Challenges/Issues/Concerns

- Pilot Project is added after consulting with GOR. Pilot project started with Follistatin DNA (with Adeno Virus) and Control Groups. FS-DNA with AV did not show any Follistatin Protein in muscle. We worked on Follistatin DNA Group with Adeno Associated Virus (AAV) vector delivery and the data analysis is complete. Some of the goals moved from CY16 to CY 17 reflecting the delays occurred.

Budget Expenditure to Date

Projected Expenditure: $705,041 Actual Expenditure: $705,041