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TITLE: Follistatin: A Potential Anabolic Treatment for Re-Innervated Muscle

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**CONTRACTING ORGANIZATION: Virginia Commonwealth University
Richmond, VA 23298**

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14. ABSTRACT Follistatin is a possible anabolic treatment for denervation atrophy induced muscle weakness following prolonged denervation. Adenovirus delivery of Recombinant FS-288 DNA isoform does not induce hypertrophy in normal rodent gastrocnemius muscle. The efficacy of Adenoassociated virus delivery of FS-288 DNA or direct delivery of recombinant FS-288 protein is currently not known.						
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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, muscle

- 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?

- **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. - pending
- Measurement of Follistatin levels in muscle (3 months) immunoassay - pending
- 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. - (pending)
- Immunohistology staining and histology of muscle (6 months) Fiber type analysis and satellite cell quantification to be determined for all specimens. - pending
- Measurement of Follistatin levels in muscle (6 months) immunoassay - pending
- **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. - pending
- Data Analysis (3 and 6 months) - pending
- script Preparation (3 and 6 months) – pending

What was accomplished under these goals?

All animals from 3-month denervation groups (1-6) underwent all surgical procedures. The terminal surgeries are underway for 6-month denervation groups (7-12). Muscle weight and muscle developed force data (from three month groups) have been collected but statistical analysis still incomplete. Below are key observations noted from the processed data (*see appendix 2* for the data):

Observations: ELISA, muscle histology, nerve histology, etc. are pending and no final conclusions can be made.

1. 3-month Protein:

- a. Muscle Weight: No positive effect seen in treatment groups: injured (Group 1 vs Group 5a) and non-injured (Group 3 vs Group 6a)
- b. Muscle Developed Force: No positive effect seen in treatment groups: injured (Group 1 vs Group 5a) and non-injured (Group 3 vs Group 6a)

2. 3-month Virus:

- a. Muscle Weight: No positive effect seen in treatment groups: injured (Group 1 vs Group 5a) and non-injured (Group 3 vs Group 6a)
- b. Muscle Developed Force: No positive effect seen in treatment groups: injured (Group 1 vs Group 5a) and non-injured (Group 3 vs Group 6a)

3. 3-month Protein vs 3-month Virus:

a. Muscle Weight:

- i. Injured Groups (Group 1 vs Group 2): Virus treatment seems to be slightly better than protein
- ii. Non-injured Groups (Group 3 vs Group 4): There seems to be no difference between virus or protein treatment.

b. Muscle Developed Force:

- i. Injured Groups (Group 1 vs Group 2): Virus treatment seems to be slightly better than protein
- ii. Non-injured Groups (Group 3 vs Group 4): There seems to be no difference between virus or protein treatment.

What opportunities for training and professional development has the project provided?

Training Activities: Mark Feger, an MD student, as part of summer research rotation, helped procure the protein and virus needed for the study and also helped in Pilot Study-ELISA. He presented the following abstract in VCU School of Medicine Student Research Symposium-2017. (*see appendix 4*)

Evaluation of human recombinant follistatin and adeno-associated viral vector delivery methods in rodents

Mark A. Feger, Gaurangkumar Patel, Satya Mallu, Jonathan Isaacs

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

- Complete remainder of animal surgeries from 6-month denervation groups (7-12)
- Process and prepare following data for analysis:
 - 3-month denervation groups (1-6):
 - Follistatin ELISA – Run the assay, collect data, and prepare to present data
 - Muscle Histology – Process tissue, image the slides, analyze the slides, and prepare to present data
 - Compound Nerve Action Potentials – Review the recorded signal, extract data from the signal, prepare to present data
 - Nerve Histology – Process tissue and prepare slides to image and analyze
 - 6-month denervation groups (7-12):
 - Muscle Weight – Prepare to present the data
 - Muscle Developed Force – Review the recorded signal, extract data from the signal, prepare to present data
 - Compound Nerve Action Potentials – Review the recorded signal, extract data from the signal, prepare to present data
 - Follistatin ELISA – Run the assay, collect data, and prepare to present data
 - Muscle Histology – Process tissue and prepare slides to image and analyze
 - Nerve Histology – Process tissue and prepare slides to image and analyze.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Nothing to report.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

See below.

Actual or anticipated problems or delays and actions or plans to resolve them

Situation 1:

Due to unpredicted and unforeseen circumstances, the final surgeries (surgery 4) for 3-month denervation protein groups (1, 3, 5a, and 6a) were delayed from 4 weeks to 6 weeks post start of the treatment (surgery 3). To ensure we can still compare the results within 4 major groups in the study (3-month denervation protein groups (1, 3, 5a, and 6a), 3-month denervation virus groups (2, 4, 5b, and 6b), 6-month denervation protein groups (7, 9, 11a, and 12a), and 6-month denervation virus groups (8, 10, 11b, and 12b)), the final surgeries (surgery 4) for all animals in the study was decided to be done 6 weeks post start of the treatment (surgery 3).

Situation 2:

Based on the muscle weight and muscle developed force data from 3-month denervation protein groups (1, 3, 5a, and 6a), the protein treatment did not have any positive effect on muscle recovery post denervation and nerve repair.

Based on the muscle weight and muscle developed force data from 3-month denervation virus groups (2, 4, 5b, and 6b), the virus treatment did not have any positive effect on muscle recovery post denervation and nerve repair.

The observed results to date (which still need to be validated by further analysis) suggest:

- 1) Either the hypothesis is not supported and follistatin has no beneficial effect on improving muscle recovery
- 2) Or there has been a failure of methodology-the lack of hypertrophy in control groups is concerning and in direct contrast to the pilot study results (which showed a hypertrophic effect).

Possible explanations for the differences observed in pilot study and the control groups include:

1. Follistatin only asserts a hypertrophic effect on growing muscle. In the pilot study, immature (3 months old) rats were treated with protein/virus. In the main study, at time of treatment the rats were either 9 months old (3-month denervation groups) or 12 months old (6-month denervation groups).
2. Protein treatment is very sensitive to changes in volume/concentration. In pilot study, 2 mL reservoir pumps were used. In main study, 0.2 mL reservoir pumps were used (due to irritation observed in the rodents with the bigger pumps). The same amount of protein was delivered in both studies by weight though the concentration of protein delivered was different. Of course, this does not explain the findings in the virus group.
3. Hypertrophic effect of follistatin is temporally limited. In pilot study, animals underwent final surgeries 4-weeks post treatment surgery. In main study, animals underwent final surgeries 6-weeks post treatment surgery.

ELISA muscle analysis is still pending to confirm follistatin deliver but the protein solution and viral preparation was the same used in the pilot study. Further study may be necessary to explain our unexpected findings. Pending final analysis, will discuss with science officer possible future steps.

Changes that had a significant impact on expenditures

Nothing to report.

6. PRODUCTS:

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

Accepted for *MHSRS 2017 meeting presentation. (see appendix 3)*

Abstract ID is: *MHSRS-17-1469*

Abstract Title: *Comparison between different Follistatin delivery methods (AAV, AV, and Protein) to enhance motor recovery post peripheral nerve injury and regeneration*

Research Topic: *Extremity Regeneration*

Journal publications.

Nothing to report.

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers and presentations.

Nothing to report.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

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?

Example:

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: 1.5

Contribution to project: Regulatory process, supervising the study.

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

Name: Satya Mallu, M.D.

Project Role: Co-investigator

Nearest person month worked: 4.5

Contribution to project: Assisted with regulatory process, performed pilot and main study surgeries.

Funding support: VCU salary, Cook Biotech, Inc. industry grant, Flow-through funding from NIH: 1R34NS097113-01, AFSH grant

Name: Gaurangkumar Patel, B.S.

Project Role: Lab technician

Nearest person month worked: 10.5

Contribution to project: Pilot and Main study, assisted with pilot and main study surgeries.

Funding support: VCU salary

Name: Mary Shall, PhD

Project Role: Co-PI

Nearest person month worked: 2.5

Contribution to project: Pilot and Main study

Funding support: VCU salary

Name: Scott Vota, DO

Project Role: Co-investigator

Nearest person month worked: 1.65

Contribution to project: Pilot study

Funding support: VCU salary and MCV physicians salary for clinical work

Name: Jeffery Dupree, PhD

Project Role: Co-investigator

Nearest person month worked: 0.25

Contribution to project: Pilot study

Funding support: VCU salary and NIH grants

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Orthopaedic Surgery now has the equipment that Neurology was using to perform nerve conduction studies, and has taken over that portion of the Scope of Work. Dr. Vota's salary remaining salary support is now allocated to lab staff. There was no change to the Scope of Work or timeline when this change was made.

What other organizations were involved as partners?

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:

Nothing to report.

QUAD CHARTS:

Attached. (*see appendix I*)

9. APPENDICES:

1. Quad Chart
2. Preliminary, 3-month group data analysis report
3. MHSRS Presentation Abstract
4. VCU Student Research Symposium Abstract

Follistatin: A Potential Anabolic Treatment for Re-Innervated Muscle

Proposal #11231008



PI: Jonathan Isaacs, MD

Org: Virginia Commonwealth University

Award Amount: \$705,041

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).

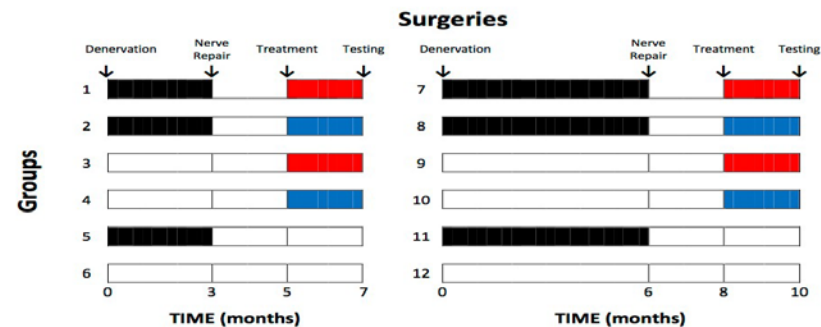
Approach

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 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	

Follistatin Project Experiment Timeline



Note: EXP and SHAM TREATMENT undergo DENERVATION Surgeries; SHAM SURGERY and CTRL have "NO" DENERVATION Surgeries.

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 Project Surgeries Completed

✓ Denervation of hind limb muscles

CY17 Goal – Treat re-innervated muscle with Follistatin, Determine treatment effects utilizing strength testing, muscle morphology, electrophysiology nerve testing; 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-ONGOING

☐ Immunohistology staining and histology of muscle-ONGOING

☐ Measurement of Follistatin levels in muscle-ONGOING

☐ Histology of muscle/nerve-ONGOING

☐ Data Analysis-ONGOING

☐ 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 CY17 reflecting the delays occurred.

Budget Expenditure to Date

Projected Expenditure: \$705,041

Actual Expenditure: \$603043

Follistatin Main Study Results

3-month Protein:

Group	n	Group Description
Group 1*	12	Three-month denervation (surgery 1) followed by nerve repair (surgery 2) and then protein treatment (surgery 3) twelve weeks post-surgery 2.
Group 3	12	Sham denervation (surgery 1) followed by sham nerve repair (surgery 2) and then protein treatment (surgery 3) twelve weeks post-surgery 2.
Group 5a	6	Three-month denervation (surgery 1) followed by nerve repair (surgery 2) and then saline treatment (surgery 3) twelve weeks post-surgery 2.
Group 6a	6	Sham denervation (surgery 1) followed by sham nerve repair (surgery 2) and then saline treatment (surgery 3) twelve weeks post-surgery 2.

Table 1: 3-month Protein Experimental Groups (*One animal from Group 1 died during the course of the study. Therefore, the following data only includes n=11 for Group 1.)

	Ipsilateral Limb (Left)	Contralateral Limb (Right)
Group 1	0.64 ± 0.18	0.49 ± 0.18
Group 3	2.22 ± 0.17	2.08 ± 0.26
Group 5a	0.93 ± 0.17	0.37 ± 0.04
Group 6a	2.42 ± 0.19	2.14 ± 0.22

Table 2: 3-month Protein Muscle Weight Descriptive Statistics (value = average ± standard deviation, units = grams)

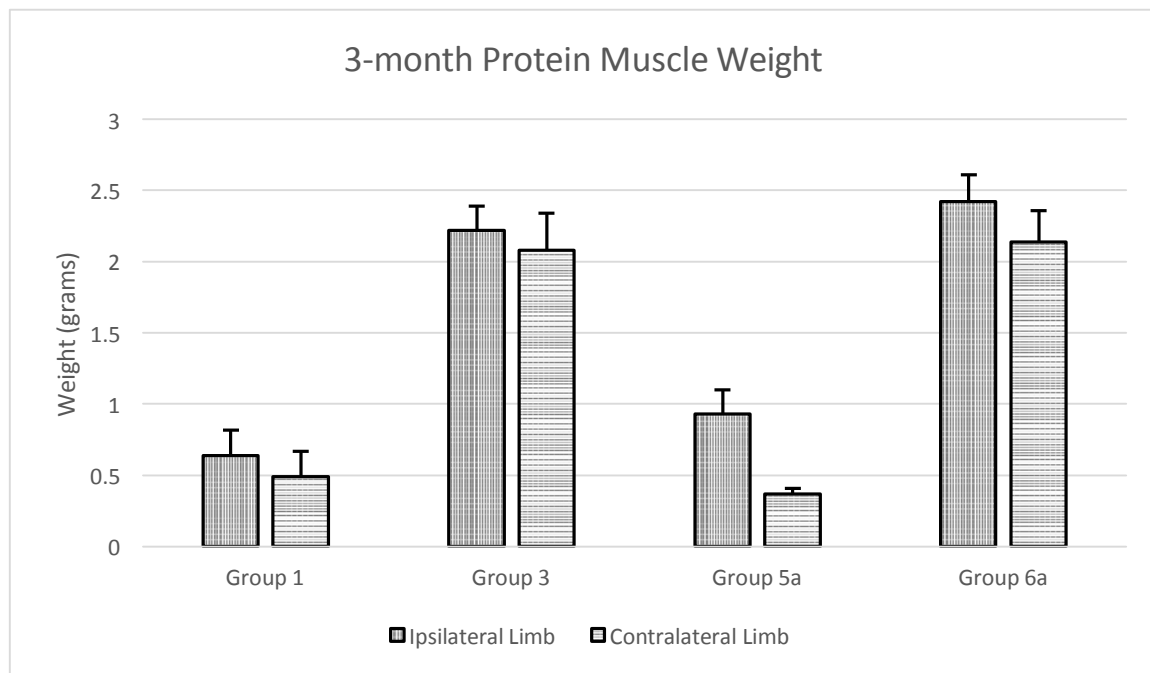


Figure 1: 3-month Protein Muscle Weight (error bars = standard deviation)

	Ipsilateral Limb (Left)	Contralateral Limb (Right)
Group 1*	0.373 ± 0.187	Not Recorded
Group 3	0.979 ± 0.467	1.437 ± 0.633
Group 5a*	0.451 ± 0.248	Not Recorded
Group 6a	1.745 ± 0.597	2.012 ± 0.692

Table 3: 3-month Protein Muscle Developed Force Descriptive Statistics (value = average ± standard deviation, units = Newtons) (*Muscle developed force not recorded from the contralateral limb for Group 1 and 5a as the nerve on contralateral limb was used for nerve repair on ipsilateral limb.)

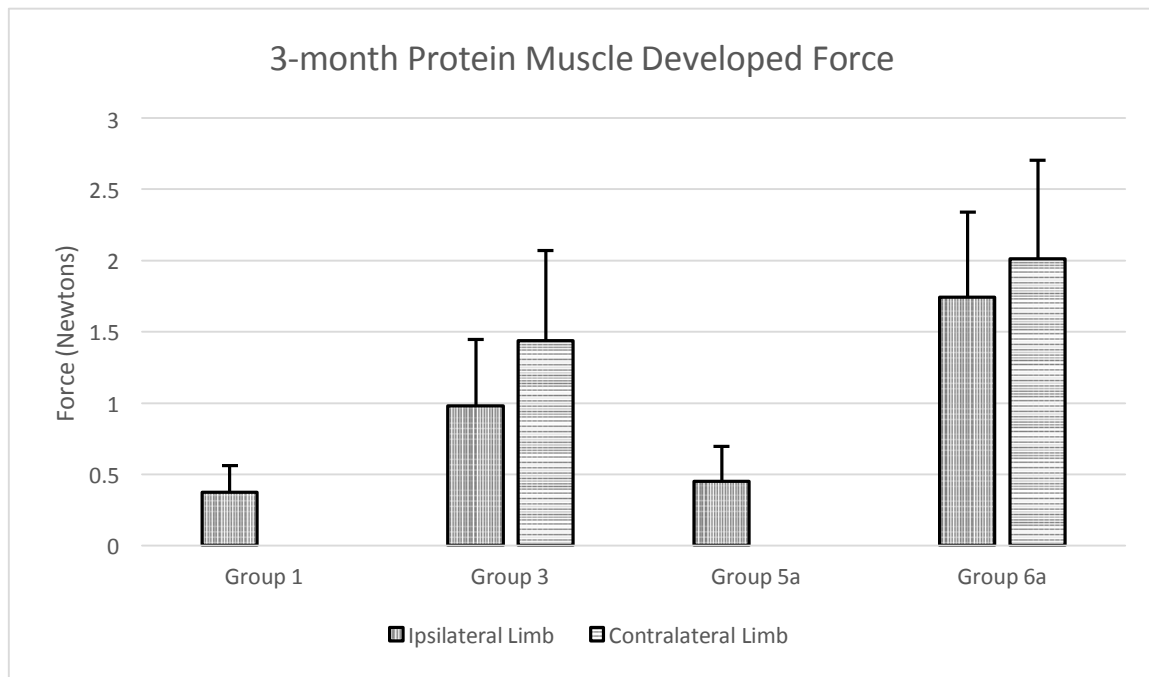


Figure 2: 3-month Protein Muscle Developed Force (error bars = standard deviation)

3-month Virus:

Group	n	Group Description
Group 2	12	Three-month denervation (surgery 1) followed by nerve repair (surgery 2) and then virus treatment (surgery 3) twelve weeks post-surgery 2.
Group 4	12	Sham denervation (surgery 1) followed by sham nerve repair (surgery 2) and then virus treatment (surgery 3) twelve weeks post-surgery 2.
Group 5b	6	Three-month denervation (surgery 1) followed by nerve repair (surgery 2) and then saline treatment (surgery 3) twelve weeks post-surgery 2.
Group 6b	6	Sham denervation (surgery 1) followed by sham nerve repair (surgery 2) and then saline treatment (surgery 3) twelve weeks post-surgery 2.

Table 4: 3-month Virus Experimental Groups

	Ipsilateral Limb (Left)	Contralateral Limb (Right)
Group 2	0.99 ± 0.37	0.40 ± 0.09
Group 4	2.43 ± 0.20	2.16 ± 0.20
Group 5b	1.08 ± 0.24	0.43 ± 0.11
Group 6b	2.20 ± 0.26	2.21 ± 0.27

Table 5: 3-month Virus Muscle Weight Descriptive Statistics (value = average ± standard deviation, units = grams)

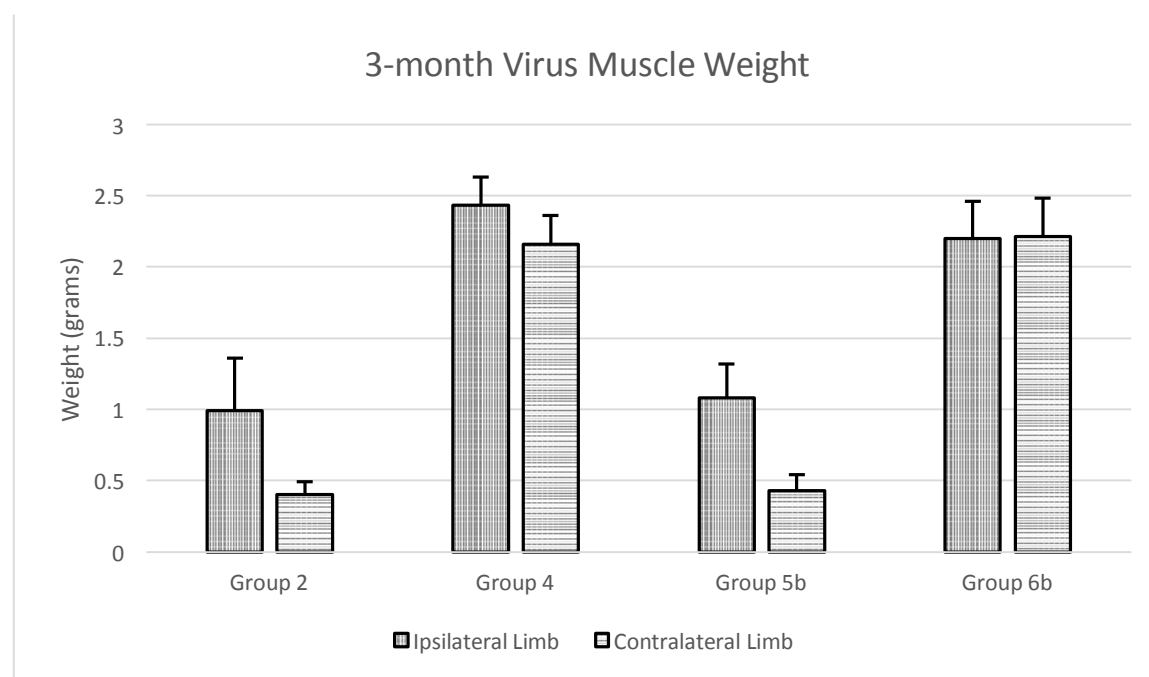


Figure 3: 3-month Virus Muscle Weight (error bars = standard deviation)

	Ipsilateral Limb (Left)	Contralateral Limb (Right)
Group 2*	0.495 ± 0.285	Not Recorded
Group 4	1.088 ± 0.438	1.393 ± 0.328
Group 5b*	0.487 ± 0.302	Not Recorded
Group 6b~	1.070 ± 0.380	1.599 ± 0.456

Table 6: 3-month Virus Muscle Developed Force Descriptive Statistics (value = average ± standard deviation, units = Newtons) (*Muscle developed force not recorded from the contralateral limb for Group 2 and 5b as the nerve on contralateral limb was used for nerve repair on ipsilateral limb.) (~Damage to nerve during final surgery for one of the animal in Group 6b, disabled from doing muscle force recording from the contralateral limb. Therefore, n=5 for the contralateral limb in Group 6b.)

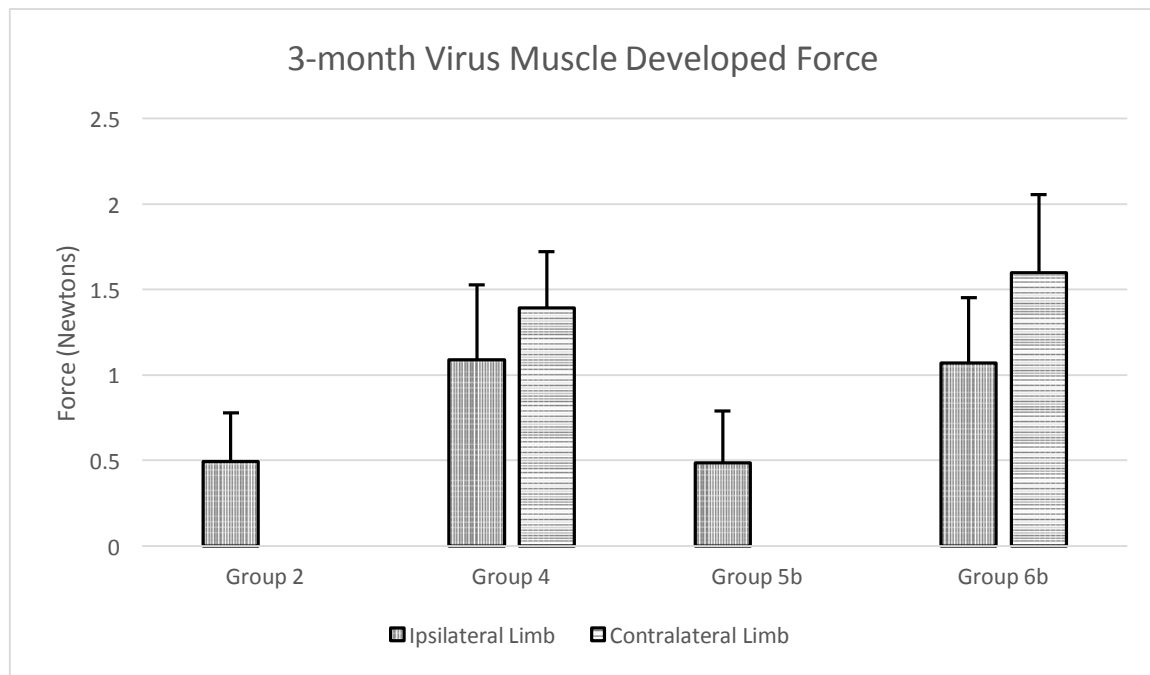


Figure 4: 3-month Virus Muscle Developed Force (error bars = standard deviation)

Observations: (No statistical analysis conducted thus far as other data points (ELISA, muscle histology, nerve histology, etc.) are pending. Therefore, no conclusions can be made from the data presented in this report. Nonetheless, observations on the data presented are shared below.)

1. 3-month Protein:

- a. **Muscle Weight:** Treatment seems to impact both injured (Group 1 vs Group 5a) and non-injured (Group 3 vs Group 6a) groups negatively.
- b. **Muscle Developed Force:** Treatment seems to impact both injured (Group 1 vs Group 5a) and non-injured (Group 3 vs Group 6a) groups negatively.

2. 3-month Virus:

- a. **Muscle Weight:** Treatment seems to impact both injured (Group 2 vs Group 5b) and non-injured (Group 4 vs Group 6b) groups naturally (neither negatively or positively).
- b. **Muscle Developed Force:** Treatment seems to impact both injured (Group 2 vs Group 5b) and non-injured (Group 4 vs Group 6b) groups naturally (neither negatively or positively).

3. 3-month Protein vs 3-month Virus:

- a. **Muscle Weight:**
 - i. **Injured Groups (Group 1 vs Group 2):** Virus treatment seems to impact more positively than protein treatment.
 - ii. **Non-injured Groups (Group 3 vs Group 4):** There seems to be no difference between virus or protein treatment.
- b. **Muscle Developed Force:**
 - i. **Injured Groups (Group 1 vs Group 2):** Virus treatment seems to impact more positively than protein treatment.
 - ii. **Non-injured Groups (Group 3 vs Group 4):** There seems to be no difference between virus or protein treatment.

Title: Comparison between different Follistatin delivery methods (AAV, AV, and Protein) to enhance motor recovery post peripheral nerve injury and regeneration

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Background: Partial but functionally unsatisfactory motor recovery following major peripheral nerve injury and repair is common. Though a complex and multifactorial issue, temporal loss of muscle fiber size and contractile properties (known as denervation atrophy) within the target muscle is an important component of this problem. Most efforts to avoid or diminish this process have aimed at improving axonal regeneration rates and efficiency. We have been interested in alternative strategies focusing on reversing this process in the muscle itself utilizing anabolic treatments. Follistatin is a naturally occurring muscle growth and strength stimulator with substantial anabolic properties, and we are investigating its potential in reversing denervation atrophy. However, the optimal mode of protein delivery is not known. We have compared three different delivery methods: direct delivery of an active isoform to muscle tissue, delivery of recombinant Follistatin DNA to muscle cells utilizing Adenovirus (AV), and delivery of recombinant Follistatin DNA to muscle cells utilizing Adenoassociated virus (AAV).

Methods: Twenty (three month old female) Sprague-Dawley rats received one of the following treatment (n = 5 rats per group): Recombinant Follistatin Protein FS-288 (Group A), Recombinant Follistatin DNA FS-288 via AV vector (Group B), Recombinant Follistatin DNA FS-288 via AAV vector (Group C), and Sham Treatment (Group D). Placement of a subcutaneous osmotic pump in the lumbar area delivered Follistatin protein solution (Group A) directly to the right gastrocnemius muscle while the other groups underwent intramuscular injection to the right medial Gastrocnemius muscle (Group B = Follistatin AV vector, Group C = Follistatin AAV vector, Group D = Saline Solution). Animals were weighed and muscle force measurements obtained at 4 weeks post treatment. The bilateral gastrocnemius muscles were harvested, weighed, and stored at -80°C in OCT compound. A sample of muscle was removed for protein extraction and Follistatin quantification using Follistatin ELISA Kit (R&D Systems, INC.). One-Way Anova with post-hoc Tukey tests were performed to compare groups with an *a priori* level of significance set at $p < 0.05$.

Results: Animal weight was significantly highest ($p < 0.05$) in Group A (292.2 ± 14.1 gm) compared to all other groups (Group B = 256.7 ± 6.3 gm, Group C = 257.0 ± 5.7 gm, Group D = 255.9 ± 7.0 gm). Muscle weight and developed force were also highest in Group A (2.182 ± 0.073 gm and 1.847 ± 0.270 mV) compared to other groups (Group B = 1.955 ± 0.158 gm and 1.430 ± 0.357 mV, Group C = 2.160 ± 0.206 gm and 1.728 ± 0.425 mV,

Group D=2.083±0.174gm & 1.312±0.705mV) respectively. Follistatin protein levels measured using Follistatin ELISA kit were as follows: Group A = 757.6 pg/ml, Group B = 77.5 pg/ml, Group C = 420 pg/ml, and Group D = 65 pg/ml.

Conclusions: In rodents, a definite anabolic effect as demonstrated by increased muscle weight and strength was seen with direct delivery of Follistatin protein to muscle. Despite the solution being directed into a specific muscle, there did appear to be a systemic effect. Treatment delivery utilizing AAV appeared to produce high levels of Follistatin protein within the target muscle as well and a more modest anabolic effect is suspected. However, due to high standard of deviation and low sample size, no statistically significant effect could be demonstrated.

Evaluation of human recombinant follistatin and adeno-associated viral vector delivery methods in rodents

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INTRODUCTION

- Over 250,000 peripheral nerve injuries occur annually¹
- Treatment aimed at axonal regeneration often results in significant morbidity/loss of function²
 - Permanent disability is due to irreversible muscle denervation, atrophy and weakness³
- Enhancing the function of the remaining muscle fibers that are not irreversibly denervated may be a viable treatment approach
- By inhibiting myostatin, which is a negative regulator of muscle regeneration,⁴ we may be able to augment anabolic muscle recovery following nerve injury
- Follistatin is a potent inhibitor of myostatin⁵
- However, before follistatin can be applied to denervated rodent models, the efficacy of our planned delivery methods must be evaluated

Purpose

To evaluate gastrocnemius muscle mass, developed force, and rodent weight 4 weeks after administration of follistatin with 2 distinct delivery methods (viral vector and recombinant follistatin protein) compared to a sham injection group

METHODS

- 15 female Sprague-Dawley rats were utilized (n=5 per group)

Independent Variable = Group

- FST protein** = Human recombinant follistatin protein delivered continuously over 28 days via implantable drug delivery system (Alzet pump) and subcutaneous catheter over gastrocnemius



- AAV FST-317** = Adeno-Associated Viral Vector FST 317 isoform delivered via intramuscular injection
- Control** = Sham injection of standard buffer solution

Dependent Variables (each limb)

- All dependent measures were recorded 4 weeks after initial injection or Alzet pump placement
 - Gastrocnemius muscle mass (g)
 - Developed force (N)
 - Rodent mass (g)

Statistical Analysis

- One-way ANOVA with post-hoc Tukey
 - Alpha set a priori @ 0.05

RESULTS

FST protein vs. Control

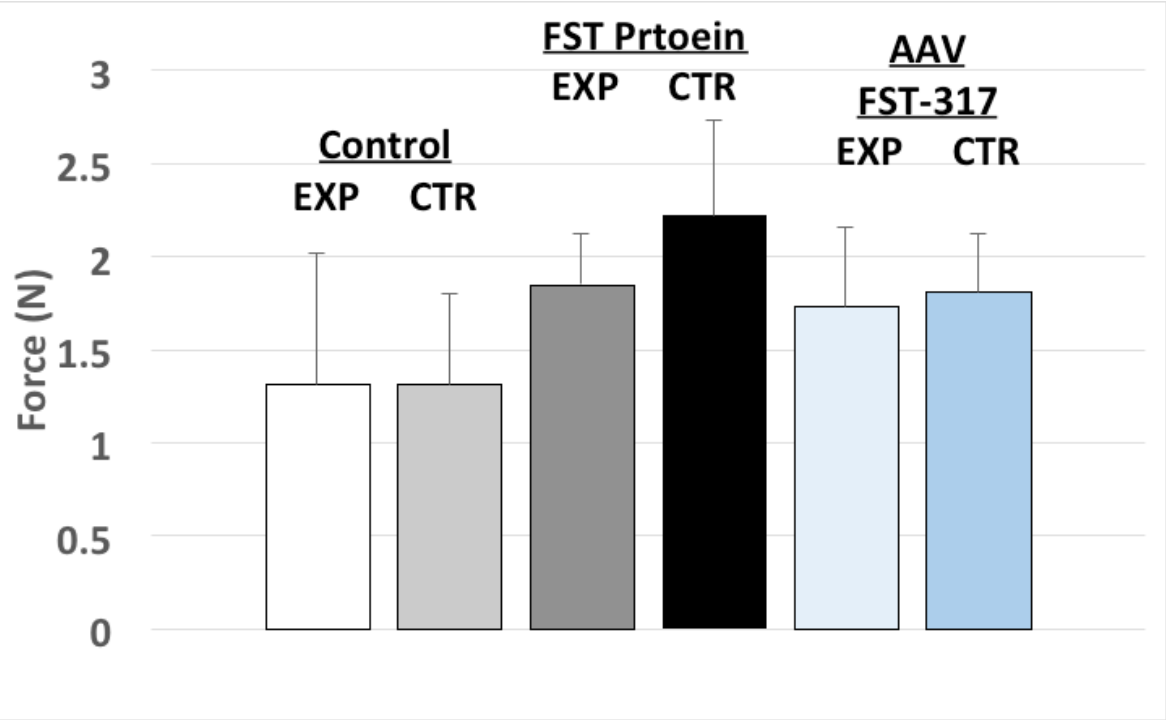
	Animal Weight (grams)	Muscle Weight (grams)		Developed Force (N)	
		EXP	CTR	EXP	CTR
Difference	+ 26.3	+ 0.099	+ 0.084	+ 0.535	+ 0.908
%	+ 10.3 %	+ 4.7 %	+ 3.9 %	+ 40.8 %	+ 69.0 %
Treatment Response	Positive	Positive	Positive	Positive	Positive

- Significant increase in rodent mass $p=.001$

AAV FST-317 vs. Control

	Animal Weight (grams)	Muscle Weight (grams)		Developed Force (N)	
		EXP	CTR	EXP	CTR
Difference	+ 1.12	+ 0.08	- 0.24	+ 0.416	+ 0.498
%	+ 0.44 %	+ 3.8 %	- 11.2 %	+ 31.7 %	+ 37.9 %
Treatment Response	Positive	Positive	Negative	Positive	Positive

- Significant increase in rodent mass $p=.002$



- Note - Additional data is being collected as we were underpowered to detect differences in muscle force

DISCUSSION

- Follistatin results in a 30-70% increase in force output after four weeks
 - Control and experimental limbs both increased in force output indicating a systemic effect after local delivery
- There were no differences between recombinant follistatin protein and follistatin viral vector delivery methods
- Preliminary data is sufficient for follow-up trial with implantable drug delivery system and adeno-associated viral vector delivery methods for follistatin protein in peripheral nerve injury rodent models

Conclusion

Administration of follistatin increases muscle force approximately 45% at four weeks and both implantable Alzet pumps and viral vectors appear to be appropriate delivery methods for rodent models in future research

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