AWARD NUMBER: W81XWH-18-1-0352

TITLE: Neurotization to Improve Graft Reinnervation and Recovery Following Severe Muscle Injuries

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CONTRACTING ORGANIZATION: Virginia Commonwealth University, Richmond VA

REPORT DATE: August 2021

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
August 2021	Annual Report	01Aug2020-31Jul2021

4. TITLE AND SUBTITLE

Neurotization to Improve Graft Reinnervation and Recovery Following Severe Muscle Injuries

5a. CONTRACT NUMBER
W81XWH-18-1-0352

5b. GRANT NUMBER
DM170424

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Extremity soft tissue trauma can result in permanent loss of skeletal muscle mass and denervation, posing a significant clinical challenge in the military. Clinical options are to either neglect the wound, expecting fibrosis to develop, or to perform surgery and fill the muscle void with a local autologous muscle graft. The development of non-contractile tissue (mainly fibrosis) in the muscle injury is typically observed in cases where neural innervation is irrecoverable and muscle function is severely impaired. Interruption of the intramuscular neural connections in these devastating injuries is a serious regenerative obstacle that is rarely considered. We demonstrate here that muscle force is recovered only slightly through the use of DMM and autograft, confirming what typically occurs functionally in VML injuries. When we investigated histological data we indeed showed that our findings support the hypothesis that severing those intramuscular neural connections potentially impairs muscle regeneration. In this project, we determined that DMM and autograft are sufficient to support some new muscle fiber growth and satellite cell activity. Furthermore, we demonstrated positive AChR-gamma and NCAM staining in DMM treated sites and autograft treated sites. In addition, DMM sites had more intense staining for AChRs compared to autograft. Collectively, these data suggest that these intramuscular neural connections are important in maintaining contractile properties of muscle fibers and could potentially regulate muscle regeneration. Future studies will demonstrate whether improving innervation within the existing muscle or in the graft area will improve muscle regeneration.

15. SUBJECT TERMS Neurotization; Volumetric Muscle Loss; Denervation					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified	Unclassified	31	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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1. INTRODUCTION:

Extremity soft tissue trauma, in particular, VML injury, results in permanent loss of skeletal muscle mass, posing a significant clinical challenge. Treatments for these devastating injuries are to stabilize the wound by either allowing fibrosis to occur or to engraft autologous tissue. Although some autologous grafts can support limb salvage, functional recovery rates are often low (8%)[36,37]. As a consequence, these grafts typically remodel into non-contractile tissue after a long healing and reparative process [38,39]. Despite post-operative efforts to rehabilitate the muscle deficit using physical therapy, patients continue to experience functional losses. In total, these surgeries and rehabilitation cost the United States $600 billion (2016 data) each year [37,2].

The rapid degeneration of muscle fibers following traumatic muscle injury seems to be related to disuse and loss of trophic support and feedback normally provided by intact axons. Unlike most cells in the body, muscle fibers function as a syncytium of fused nucleated cells. In cases of muscle trauma, these multinucleated muscle fibers are lost in the injured area. Initially, muscle injury activates a pool of quiescent, regenerative satellite cells that migrate to the injured site, differentiate into myoblasts, and fuse with existing myofibers to bolster their reduced size. However, lack of muscle regeneration and re-innervation in the traumatized area subsequently negates these temporary mechanisms, leaving muscle to promote reparative features like fibrosis. With time, degeneration occurs, and fibrosis develops in the traumatized area. In addition, distally affected muscle fibers lose the intramuscular connections that are important in motor unit recruitment during contraction. Fibrosis, denervation, and lack of muscle regeneration collectively contribute to muscle weakness. Eventually, the injured muscle is completely replaced with fibrotic adipose tissue with no hope of recovery.

Reduced motor function is directly associated with the development of non-contractile tissue (mainly fibrosis) in the muscle injury area. This suboptimal reparative state can be ascribed to a combination of inflammatory mediators, overactive connective tissue cells, dysfunctional regenerative processes, and denervation both in the zone of injury and distal to the defect that limit de novo regeneration. As such, achieving functional gains will require treatment modalities that favor de novo muscle regeneration over fibrosis.

Skeletal muscle regeneration is strongly influenced by environmental factors such as extracellular matrix (ECM). We have developed a decellularized muscle matrix (DMM) that supports de novo fiber formation within the central region of the DMM graft in a rodent VML model. These types of models are harsh regenerative environments where few de novo muscle fibers form in the graft area. Instead, excessive collagenous matrix forms, which is theorized to impair muscle regeneration and subsequent innervation. Use of DMM provides a cell-free ECM with the proteins and structure necessary to regenerate skeletal muscle and has the potential to reduce the amount of fibrotic scar that develops. Considering the effects of denervation muscle atrophy, we sought to determine whether atrophy continued to be a contributing factor that abrogates muscle regeneration, and whether we could overcome this co-morbidity using neurotization strategies to re-innervate newly regenerating and denervated muscle.

2. KEYWORDS:

Volumetric Muscle Loss; Denervation; Atrophy; Neurotization; Regeneration

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The major goals of this project are to:
1. Examine denervation markers following a severe muscle defect created in a rat gastrocnemius (100% complete).
2. Semi-quantitatively assess muscle staining to determine degree of denervation following muscle graft surgeries (100% complete).
3. Neurotize muscle grafts using peroneal and tibial nerve grafts (100% complete).
4. Assess histology, immunostained sections, and protein levels in those animal studies (25% complete)
5. Test muscle function following neurotization (100% complete).
6. Determine ryanodine receptor and sarcoplasmic reticulum calcium ATPase levels (0% compete).

What was accomplished under these goals?

Specific Aim 2. Determine if muscle graft neurotization using peroneal or tibial axon sources affects re-innervation following injury.

Major goals: 3) Neurotize muscle grafts using peroneal and tibial nerve grafts. 4) Assess histology, immunostained sections, and Western blot. 5) Muscle function tests following neurotization. 6) RyR and SERCA protein assessment.

Neurotization Experiments

We report here our progress on assessing gene expression in neurotization studies which informs us with regard to Major Task 4 and even Major Task 6.

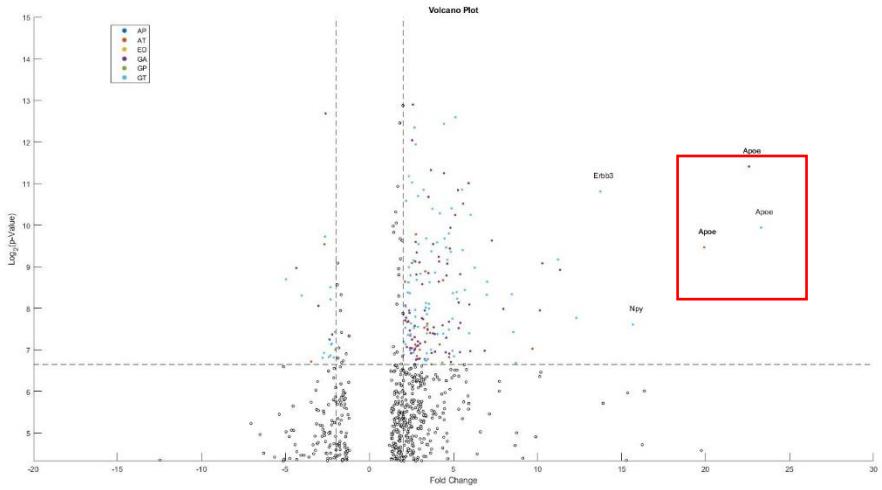


Figure 1. Volcano plot of differential gene expression for all neurotization samples.

According to Figure 1, it appeared that unique gene profiles correlated with treatments. For instance, we found that *Apoe* expression was significantly higher in tibial nerve and graft alone treatments compared to empty defect, healthy controls, and peroneal treated injury sites. This led us to investigate further into unique gene profiles for all groups tested, and we confirmed that gene quality using a principal component analysis, Figure 2.

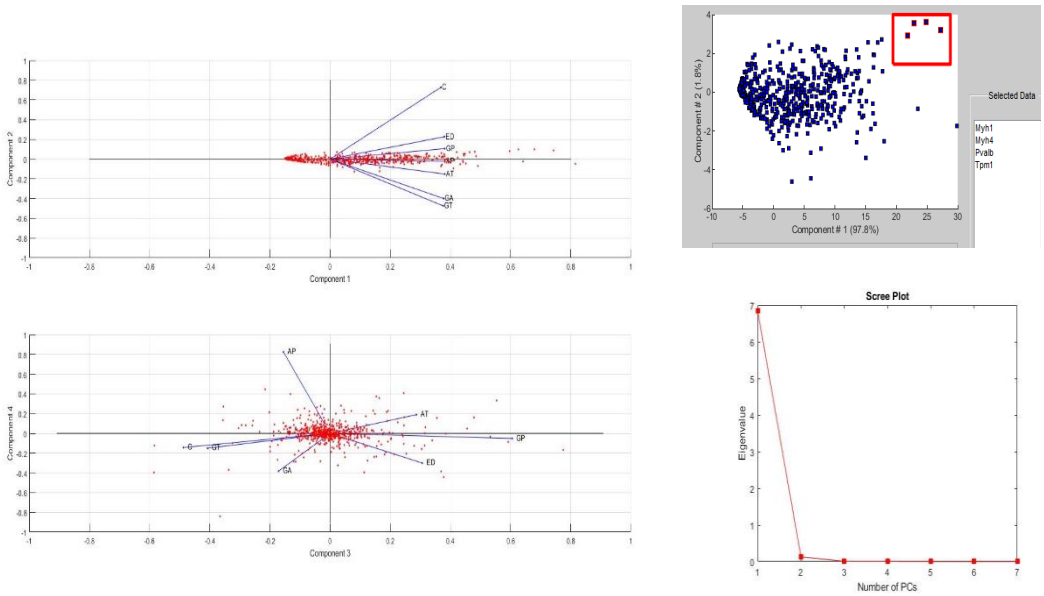


Figure 2. Two-dimensional principal component analysis (PCA) with Scree plot confirms quality of the samples tested.

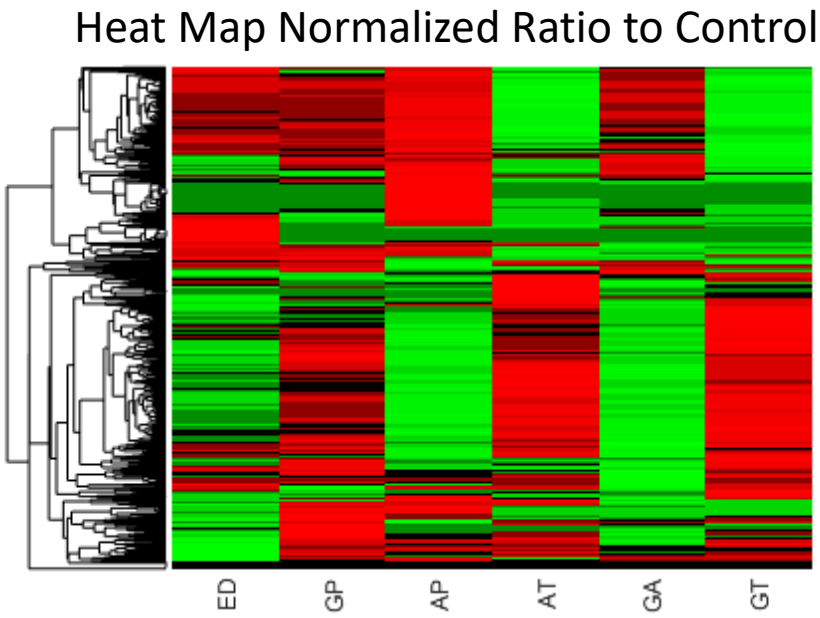


Figure 3. Heat map normalized to healthy control muscle showing all expressed genes. Red indicates upregulated genes compared to control while green represents downregulated genes compared to control.

We represented our data using heat maps to identify whether gene profiles were dependent on neurotization treatment. Based on our data, it appeared that unique profiles were ascribed to each type of treatment suggesting that neurotization had a unique effect depending on whether graft used was decellularized muscle or an autograft.

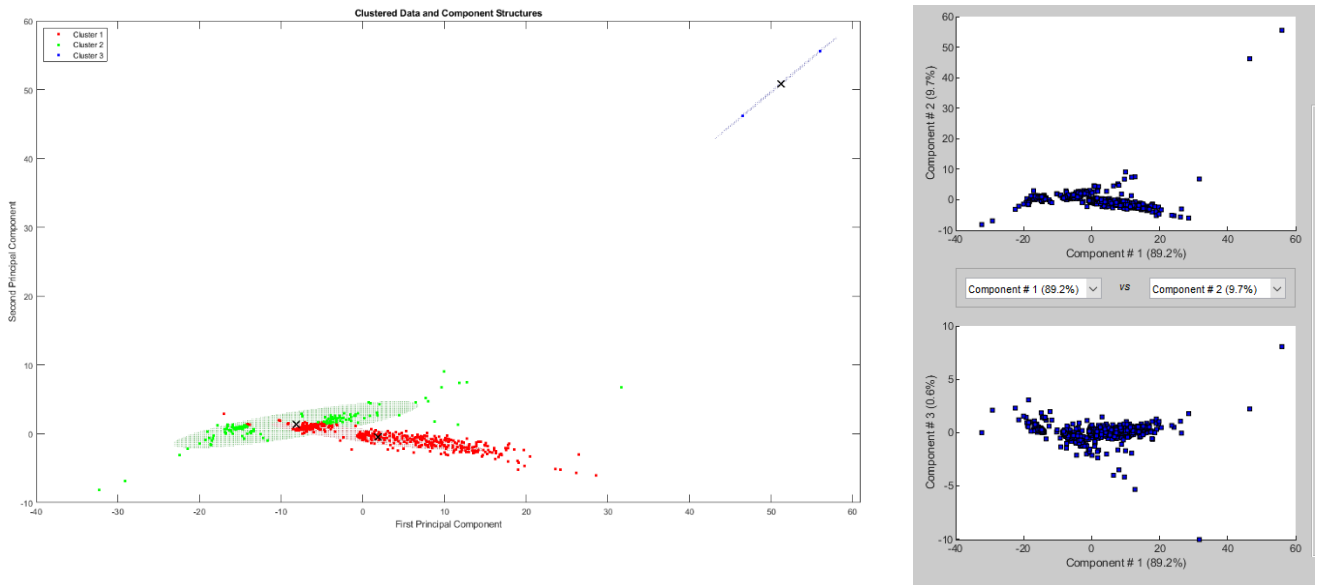


Figure 4. PCA plots with Gaussian clustering to identify unique gene clusters embedded within treatment groups.

According to these data in Figure 4, the Gaussian clustering of the principal components showed which genes are associated with the 2 dominant groups observed from the parallel plot coordinates. This produced a list of genes in two different clusters providing us with a starting point to explore further.

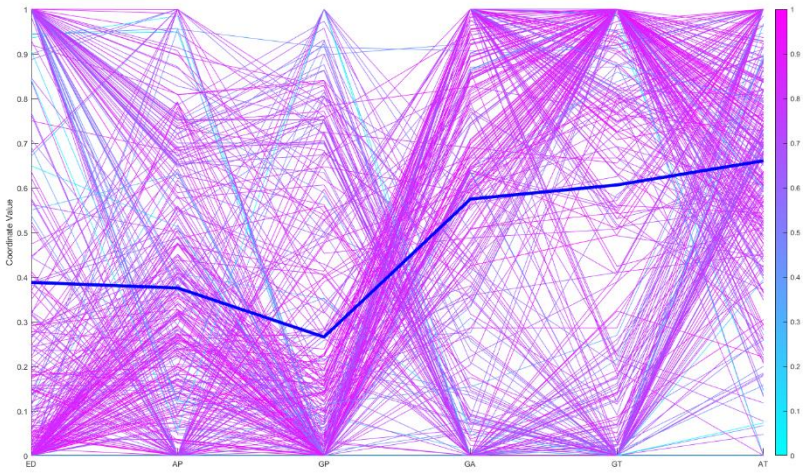


Figure 5. Parallel coordinate plots demonstrate further that gene clusters were unique. The dark blue line represents the mean expression across all genes and acts as "line of best fit" across all gene expression. Gene expression was normalized between 0 and 1 for each gene for all groups.

The dark blue line in Figure 5 demonstrates that decellularized muscle grafts and autografts neurotized with the peroneal nerve were significantly different from all other treated injury sites.

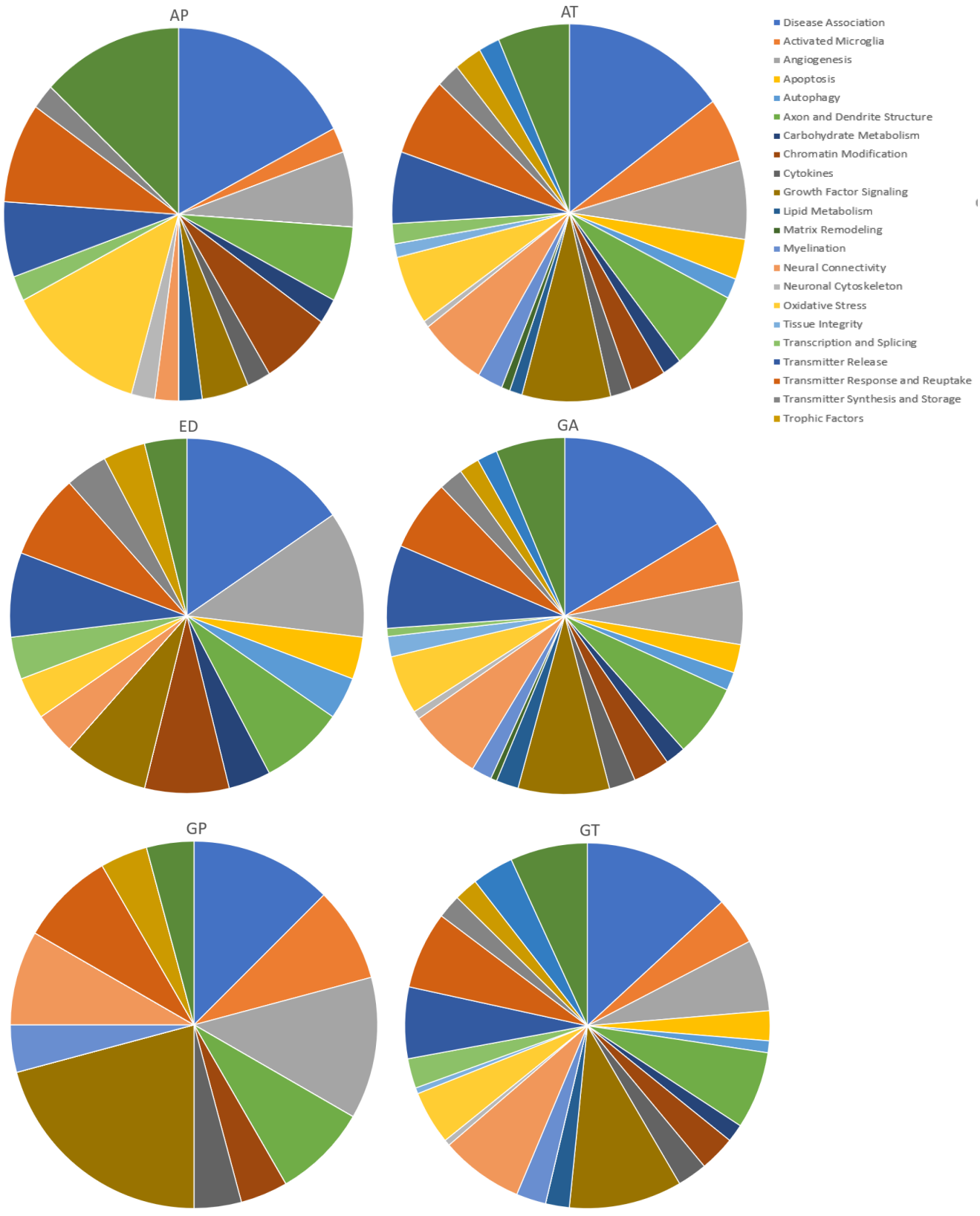
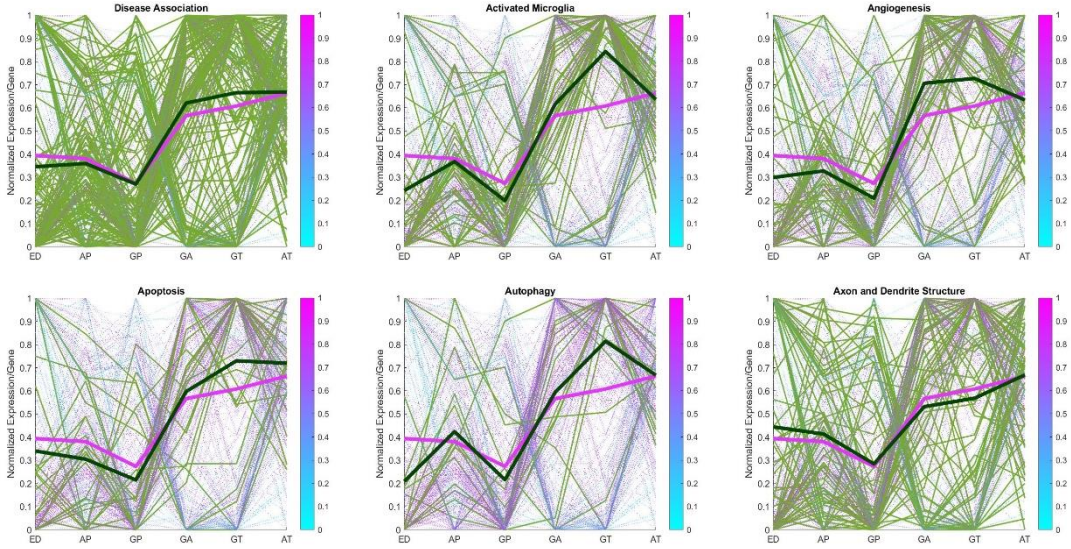

AP
AT
ED
GA
GP
GT
Disease Association
Activated Microglia
Angiogenesis
Apoptosis
Autophagy
Axon and Dendrite Structure
Carbohydrate Metabolism
Chromatin Modification
Cytokines
Growth Factor Signaling
Lipid Metabolism
Matrix Remodeling
Myelination
Neural Connectivity
Neuronal Cytoskeleton
Oxidative Stress
Tissue Integrity
Transcription and Splicing
Transmitter Release
Transmitter Response and Reuptake
Transmitter Synthesis and Storage
Trophic Factors

Figure 6. Pie chart category counts represent how associated gene groups changed with treatment.
Of note, trophic signaling genes were far higher in peroneal and tibial nerve treatments for decellularized muscle grafts than were for autografts. We are investigating further into the which genes are of most interest based on their categorical association and their significance level compared to all treatments.

Major goals: 3) Neurotize muscle grafts using peroneal and tibial nerve grafts. 4) Assess histology, immunostained sections, and Western blot. 5) Muscle function tests following neurotization. 6) RyR and SERCA protein assessment.

Neurotization Experiments

We report here continued progress on Major Task 4 and 6.



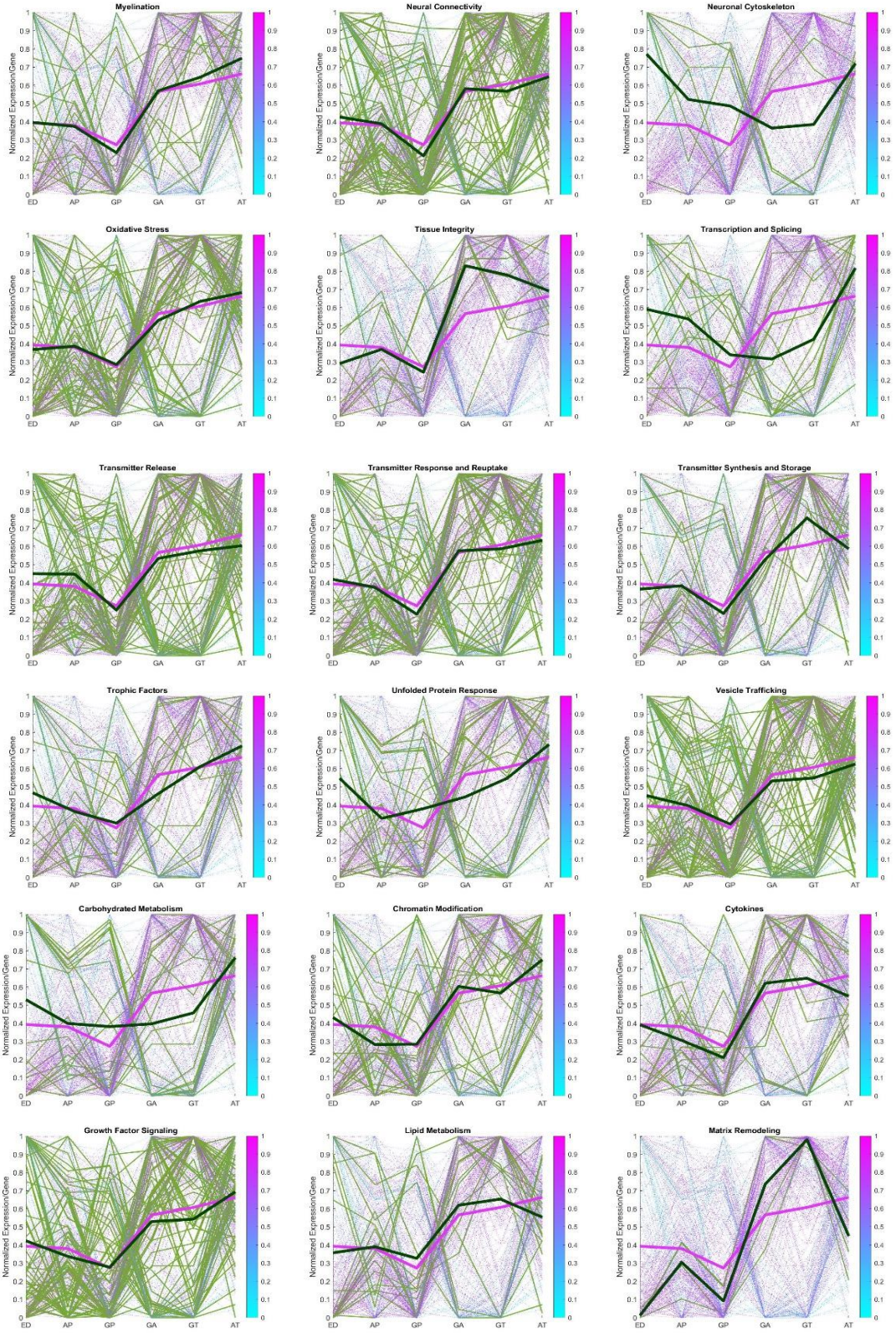
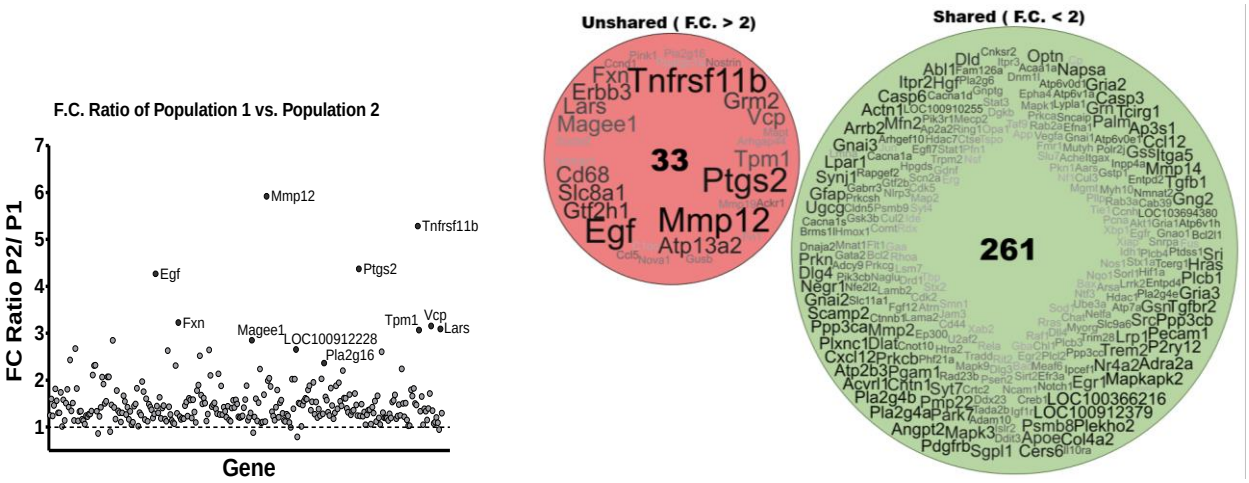

Myelination
Neural Connectivity
Neuronal Cytoskeleton
Oxidative Stress
Tissue Integrity
Transcription and Splicing
Transmitter Release
Transmitter Response and Reuptake
Transmitter Synthesis and Storage
Trophic Factors
Unfolded Protein Response
Vesicle Trafficking
Carbohydrated Metabolism
Chromatin Modification
Cytokines
Growth Factor Signaling
Lipid Metabolism
Matrix Remodeling
Normalized Expression/Gene
ED
AP
GP
GA
GT
AT

Figure 7. Parallel Coordinate Plots-MODE coloring to demonstrate changes in expression patterns amongst the groups.

Neurotized grafts outperformed injury control samples, where matrix remodeling was reduced suggesting that fibrosis is also reduced in these injury sites. Interestingly, neuronal cytoskeletal gene expression levels were elevated in peroneal neurotization injuries compared to control graft injury sites.



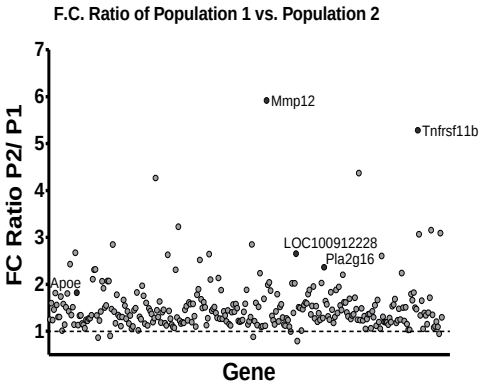


Figure 8. F.C. ratio of two different populations (Pop1 and Pop2). Pop1 consisted of empty defect, autograft peroneal, DMM peroneal and Pop2 consisted of autograft tibial, DMM tibial, and DMM alone. Pictured on the right are shared (green) and unshared (red) genes between the two populations.

Several genes of interest were extrapolated from these data and tested using quantitative real time PCR.

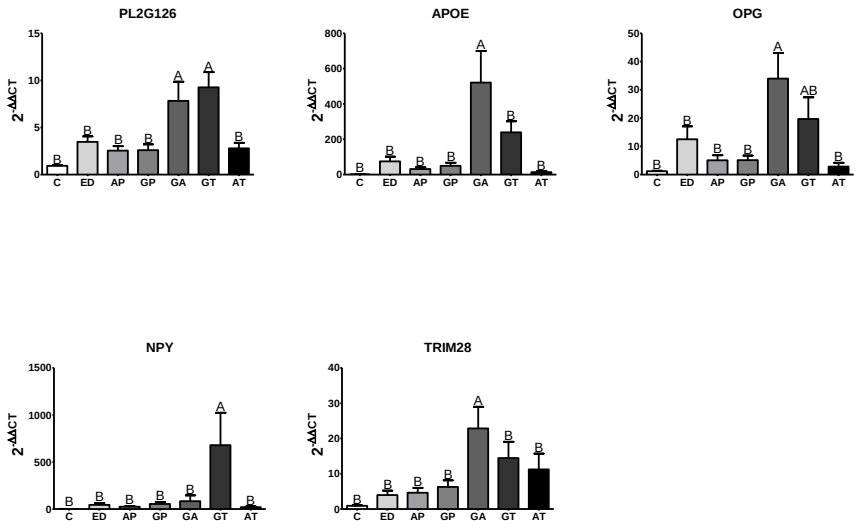


Figure 9. Isolated mRNA from muscle lysates was assessed using real time qPCR to detect fold change in gene expression. Primers tested were Pl2g126, Apoe, Spp1 (OPG), Npy, and Trim28.

Apoe, Spp1 (Opg), Trim28, Npy, and Pl2g126 demonstrated a significant increase in injury sites treated with DMM alone. When neurotization was added to the treatment, Apoe, Spp1, Npy, and Trim28 were reduced. More, when tibial neurotization was used there was a continued elevation in gene expression levels for Pl2g126 and Spp1. These differentially regulated genes could suggest additional markers of fibrosis. More we wanted to determine if these genes were regulated during myogenic differentiation.

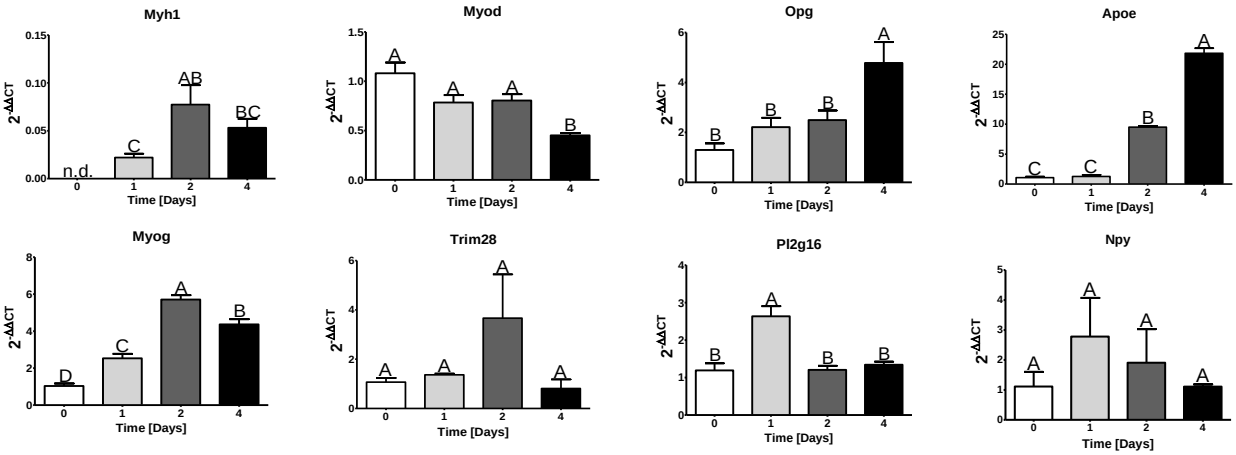


Figure 10. Myogenic differentiation in C2C12 mouse myoblasts over a time course of 0, 1, 2, and 4 days. Myogenic factors confirm successful differentiation of the cells, and Spp1 (Opg)

and Apoe are clearly regulated during differentiation, suggesting a role in myogenesis and regeneration.

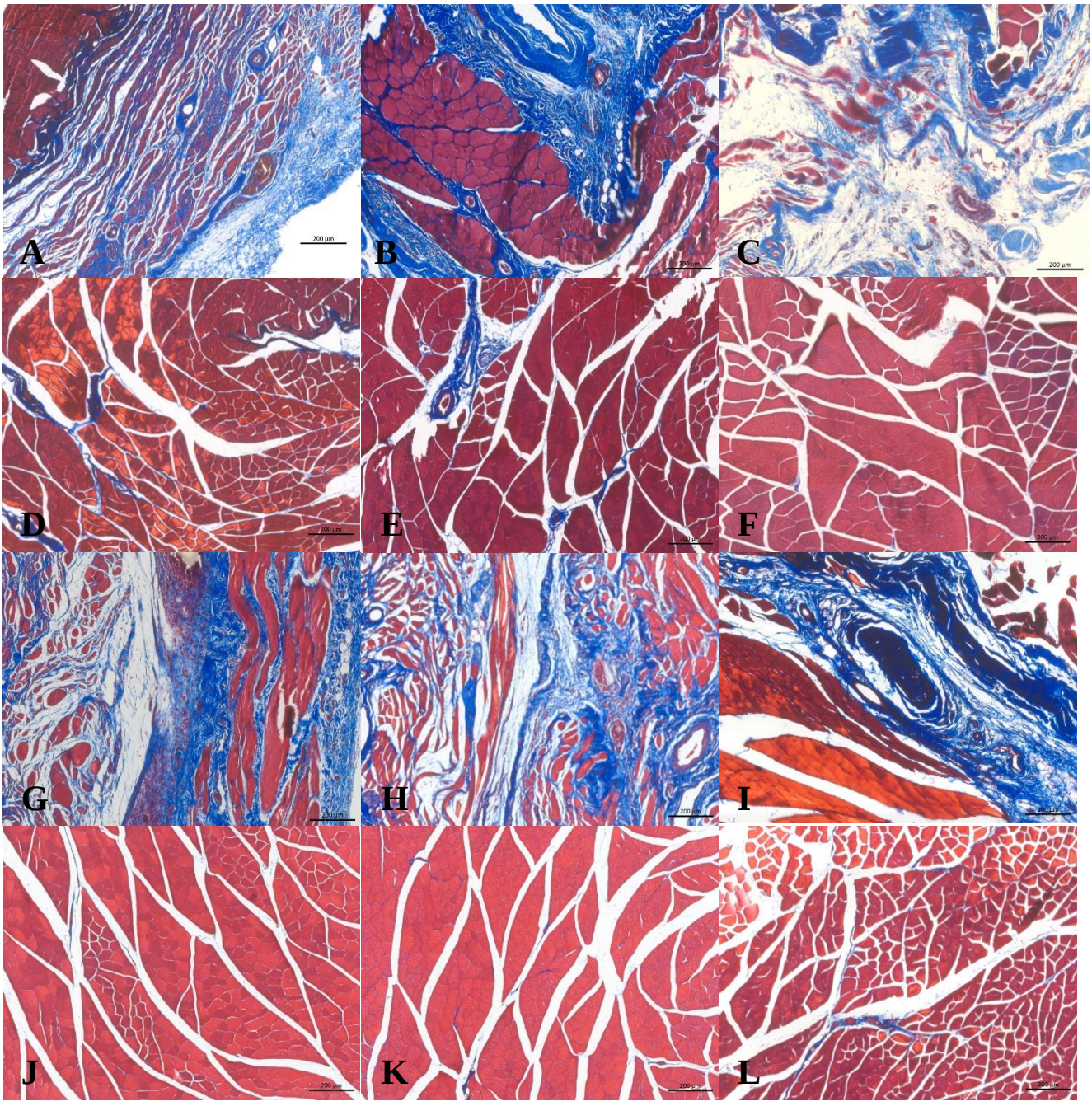


Figure 11. Neurotization improves muscle development and augments the appearance of fiber maturation in DMM treated animals. Images are representative of overall outcomes in each animal group. (A) Autograft with a peroneal neurotization, (B) DMM with peroneal neurotization, (C) DMM no neurotization, (D) Autograft with peroneal neurotization control, (E) DMM with peroneal neurotization control, (F) DMM no neurotization control, (G) Autograft with tibial neurotization, (H) DMM with tibial neurotization, (I) Empty defect, (J)

Autograft with tibial neurotization control, (K) DMM with tibial neurotization control, (L) Empty defect control.

Neurotization augmented DMM treated muscle defects where peroneal neurotization demonstrated the most muscle regeneration in the injury site. This is depicted in Figure 1B where the entire image is within the injury site. Muscle fibers that did regenerate were healthy with a mature morphology. In contrast, while tibial neurotization (Figure 1H) showed increased fiber regeneration within the injury site compared to DMM without neurotization, fiber morphology appeared more dystrophic than in peroneal neurotized DMM. We did confirm that DMM without neurotization supported de novo muscle fiber development in the injury site (Figure 1C), and that a significant portion of the injured area was connective tissue. Furthermore, our autograft neurotized injuries (Figure 1G,H) also supported muscle fibers, but appeared dystrophic with intense alinine blue staining for collagen between muscle fibers. Finally, empty defect muscle injuries were consistent with no repair injuries in rodents where there is a clear distinct "wall" of fibrosis versus intact muscle tissue (Figure 1I). Control muscles show that no effects were detected on the contralateral leg (Figure 1D-F, J-L).

We have also made additional progress on understanding denervation-induced muscle atrophy and its relation to denervation in VML injury sites. We report here progress on that study which provides additional information in relation to Major Task 1.

The overall goal of these studies was to further determine whether differences in muscle markers were distinctly different when nerves were cut and trophic signaling was lost (denervated, SFN), or muscle was paralyzed but trophic signaling was intact (botox, BTX).

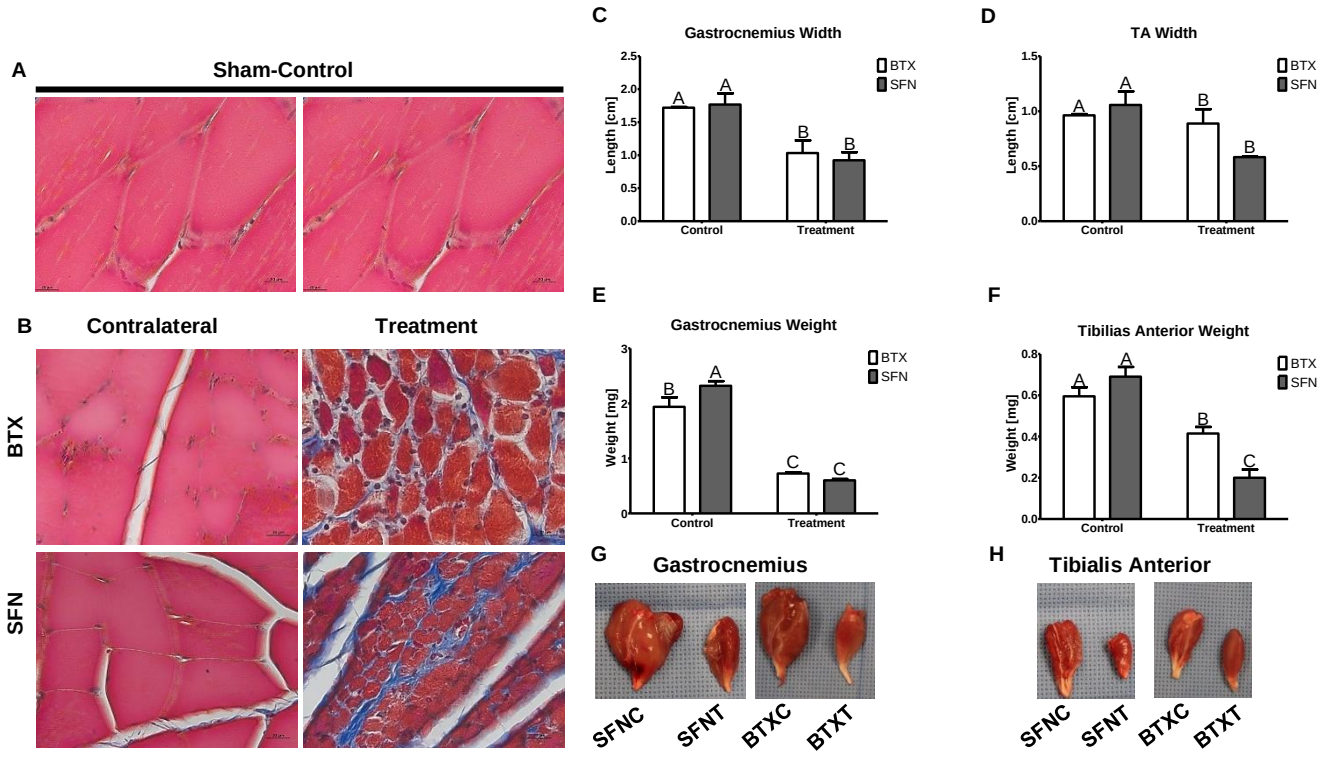


Figure 12. Denervation-induced muscle paralysis and botox muscle paralysis demonstrate similar mass loss in gastrocnemius and tibialis anterior muscles.

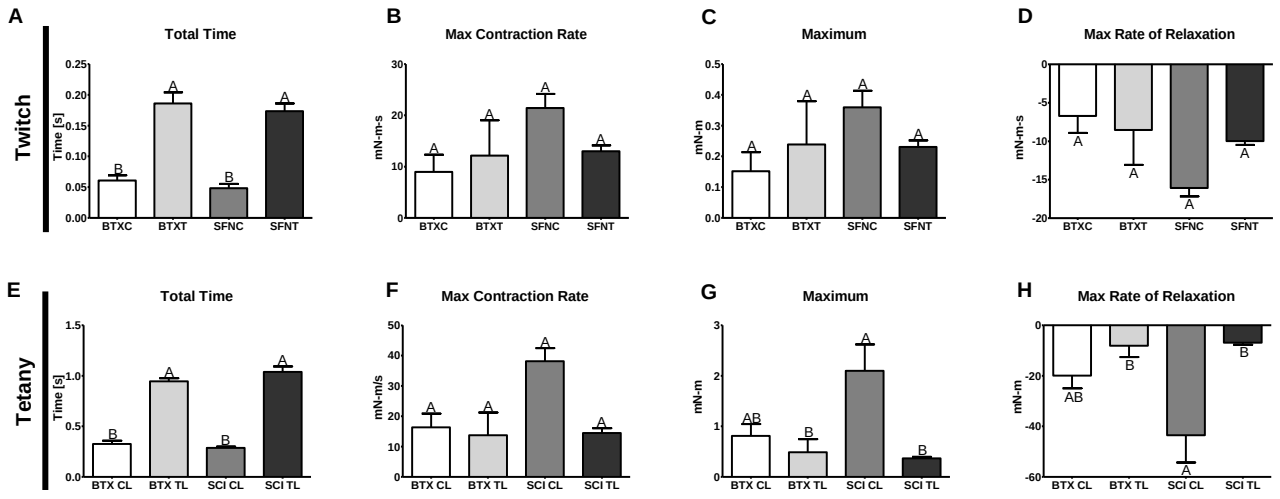


Figure 13. Muscle function tests showed that muscle force was impaired in both cases of paralysis. Interestingly, relative to control legs botox was no different for max contraction rate, peak force, and max rate of relaxation.

Functionally, denervated muscle exhibited similar characteristics to VML injury models where the total time for contraction increases, max contraction rate decreases, peak force is reduced, and max rate of relaxation is slower.

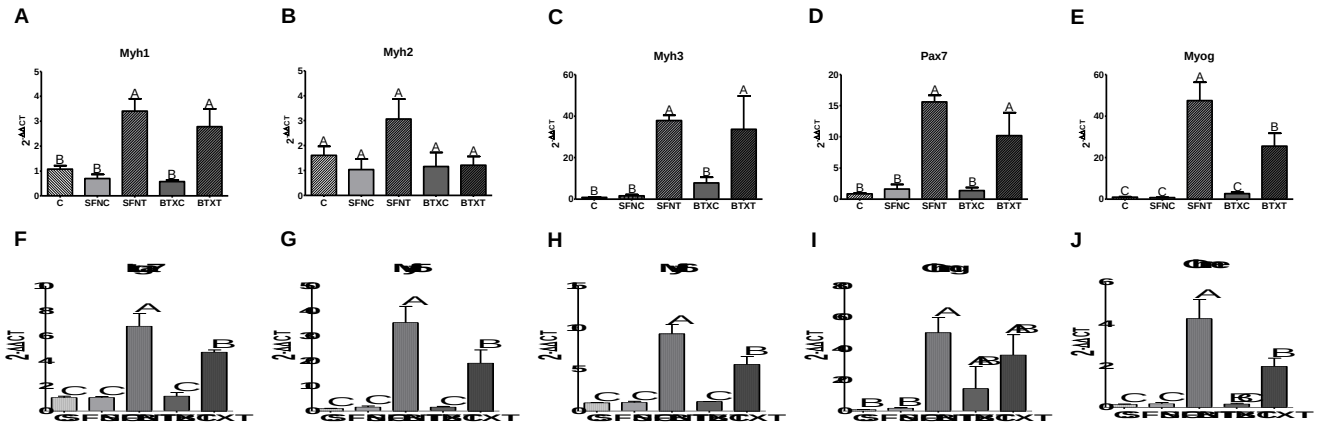


Figure 14. Gene expression levels are elevated in paralyzed muscle. All paralyzed muscle increased gene expression levels except Myh2 where only denervated muscle increased Myh2 expression. In addition, botox treated muscles showed lower myogenic expression levels for Myog, Itga7, Myf6, and Myf5.

Reparative mechanisms involving satellite cell activation and fusion into new fibers is a hallmark of denervation-induced muscle atrophy and a sign of disuse atrophy. More, data from VML injury sites shows similar trends where there is an extreme activation of regenerative genes. Yet, based on histological evidence there are clear differences in the regenerative landscape between the two models that still need to be discerned. We believe that our gene data previously shown in prior reports might uncover a key element.

Current progress and plans to complete all major tasks:

1. We ran into significant issues with tissue processing for immunohistochemical staining. Thus, we began using instead a core facility available at VCU to complete out this part of the project. In addition, the core facility will analyze and quantify all stained sections.
2. While IHC staining is being done and analyzed by the core facility, we are completing out all morphometric tests which include Feret diameter analysis, centrally located nuclei, and level of fibrosis. As of now, we have completed Feret diameters and are in process of assessing centrally located nuclei. Once that is complete fibrosis will be analyzed.

Stated goals not met:

1. Histomorphometry (estimated to complete in October)
2. Examine immunohistochemical staining (estimated to complete in November/December)
3. Milestone #2 and #3 will be completed following immunohistochemical staining and quantification (completion expected in December)

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

The project has provided the ability to train graduate students (both PhD and Masters), undergraduate students, and medical students. While training amongst these students varied depending on their specific contribution to the study, the ultimate goal was to expand their skillsets in multiple biological assessments and outcome measures such as surgery, animal care, force analysis, gene data and analysis, rodent anatomy, histological sectioning and staining, immunohistochemical staining, and protein analysis. Furthermore, while recently our department acquired funding to train undergraduates using an NSF-REU. These training opportunities will be used in the second summer of this project to better understand the interactions between muscle fibers and motor neurons.

This project also provided an ability to develop professionally as well. The PI attended several conferences using funds from both this grant and from startup money. Specifically, the PI attended Biomedical Engineering Society, Orthopaedic Research Society, Society for Biomaterials, and Military Health System Research Symposium. In addition, the PI became involved in grant workshops available at VCU during this project period, using data collected from this study to apply for future funding at both NIH and DoD.

How were the results disseminated to communities of interest?

The PI recently attended an event with Henrico County high school and middle school teachers to discuss the findings from these studies and inform them about the research occurring at VCU BME.

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

In the next reporting period (quarter 1 of year 3), we plan to finish histological assessment and be near completion of genetic results and analysis. We plan to finish milestone #1. We plan to have western blotting and immunohistochemical staining started but not fully complete.

4. IMPACT:

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

The impact of this project thus far indicates that intact, distal muscle fibers and fibers regenerated using decellularized muscle grafts are indeed denervated. The implications of this knowledge suggest that the effects of denervation muscle atrophy need to be considered when treating a volumetric muscle loss wound. Furthermore, gene data from freshly isolated tissue confirms this. When animals were treated using neurotization, there appeared to be improved muscle force output. This suggests that more motor units are being activated than under control conditions and suggests that innervation may play a role in these results.

What was the impact on other disciplines?

Initial results from neurotization suggests that clinical treatment of muscle injuries with allogenic or autogenic graft material may need to include neurotization strategies.

What was the impact on technology transfer?

Data from Aim 1 of this project demonstrates a possibility that any VML graft implanted into the muscle wound site will need to develop an additional strategy to address denervation. In addition, these findings could affect the way we prepare our grafts, possibly leading to a patent. More, our preliminary results appear favorable toward use of neurotization. Whether direct electrical stimulation is the main cause of increased motor unit recruitment, or trophic factors are playing a role in increased force production needs to be assessed. In addition, recent reports in the literature determined that motor end plates degenerate in VML injury sites. However, there was no test to determine if atrophy had also occurred.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

COVID-19 was problematic for efficient assessment of histological specimens. The Isaac lab shut down during the pandemic and recently opened back up in the beginning of August. Thus, we are making progress toward our additional tasks but were delayed due to the pandemic.

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

Delays in immunohistological staining occurred. We have since outsourced those samples to a VCU core facility that will perform IHC staining and analysis.

Changes that had a significant impact on expenditures

Nothing to report.

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

Significant changes in use or care of human subjects

No human subjects are used in this study.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Nothing to Report. Publication is in preparation and should be out for publication within the next 2 months.

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

Publication is in preparation.

Other publications, conference papers and presentations.

MHSRS, ORS, SfB

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

Nothing to report.

- **Technologies or techniques**

Nothing to report.

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

r any other invention reporting required under the terms and conditions of an award.

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: *Michael J. McClure*
Project Role: *PI*
Researcher Identifier (e.g. ORCID ID): https://orcid.org/0000-0002-5428-5270
Nearest person month worked: *0.3*
Contribution to Project: *Regulatory process, supervising studies, performed surgeries.*

Name: *Barbara D. Boyan*
Project Role: *Co-PI*
Researcher Identifier (e.g. ORCID ID): https://orcid.org/0000-0002-9642-0311
Nearest person month worked: *0.01*
Contribution to Project: *Involved in experimental design for animal studies and is actively engaged in data analysis.*

Name: *Jonathan E. Isaacs*
Project Role: *Co-PI*
Researcher Identifier (e.g. ORCID ID): https://orcid.org/0000-0002-7791-6725
Nearest person month worked: *0.01*
Contribution to Project: *Involved in experimental design for the animal studies.*

Name: *Geetanjali Bendale (replacement for Satya Mallu)*
Project Role: *Co-investigator*
Researcher Identifier (e.g. ORCID ID): Not available
Nearest person month worked: *0.05*
Contribution to Project: *Involved in muscle force tests and histology.*

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

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: