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

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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.		

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1. INTRODUCTION: *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

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: *Provide a brief list of keywords (limit to 20 words).*

Volumetric Muscle Loss; Denervation; Atrophy; Neurotization; Regeneration

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.

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 (100% complete)
5. Test muscle function following neurotization (100% complete).
6. Determine ryanodine receptor and sarcoplasmic reticulum calcium ATPase levels (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.

Specific Aim 1. Determine if decellularized and autologous muscle grafts are denervated in rat gastrocnemius.

Major Tasks: 1. Examine denervation markers following a severe muscle defect created in a rat gastrocnemius, and 2. Semi-quantitatively assess muscle staining to determine degree of denervation following muscle graft surgeries.

Significant Results: (Major findings, developments, and/or conclusions, and other achievements)

Major Task 1: Examine denervation markers following a severe muscle defect created in a rat gastrocnemius

Subtask 1: Replace 1.5 x 1.0cm muscle defect in rat gastrocnemius with decellularized or autologous muscle.

Methods: In vivo studies were performed with DMM autografts, and shams. Grafts were implanted into the gastrocnemius of a rat using a 1.5 x 1.0 cm defect in the lateral gastroc head. Implants were kept for 8 weeks and assessed for muscle physiology at the end of 8 weeks. Muscle physiology was performed as such:

Volumetric muscle loss surgery. Male Sprague-Dawley rats (250-300g) were obtained from Harlan Laboratories (Frederick, MD) and were divided into four groups (N=8 rats/group): sham, autograft, DMM, and empty graft. All surgical procedures were performed under an approved protocol at VCU and approved ACURO. Rats were anesthetized using 4% isoflurane/400ml/minute O₂. The surgical area was shaved with clippers and cleaned using 3x alternating swabs of isopropanol and chlorhexidine. Rats were transferred to the operating table, and anesthesia was continued at 1-3% isoflurane in O₂. An oblique anterolateral incision extending from the patella to the calcaneus was made, and scissors were used to insinuate deep to the common calcaneal tendon in order to separate it from the calcaneal tuber. After an incision in the biceps femoris muscle to expose the gastrocnemius was made, the left lateral gastrocnemius muscle was carefully separated posteriorly off the superficial flexor muscle toward the gastrocnemius origin in the lateral condyle. A 1.5 x 1 cm defect was cut in the lateral gastrocnemius, taking care to preserve the tibial nerve. At the end of surgery, biceps femoris was sutured closed using 5-0 nylon and skin was stapled closed thereafter.

Sham surgeries were performed as described except when the left lateral gastrocnemius was exposed, no incision was made to create a defect. Instead, biceps femoris and skin were closed without a defect. Rats that received a graft were either treated with autograft, DMM, or collagen plug. Autografts were taken from the same gastrocnemius tissue that was removed. These muscle tissue grafts were completely excised from the muscle and then sutured back in place in the same orientation. DMM grafts were sutured into muscle defects taking care to orient the anisotropic features in the direction of the muscle fibers. Collagen plugs were purchased (Resorbable Collagen PLUG, ACE Surgical Supply, Inc., Brockton, MA) and sutured into the defect dry. All Sham, Autograft, Collagen, and DMM rats survived and were harvested at 8 weeks.

Results: Animals grafts were implanted successfully and were patent throughout the course of the study. No animals died during the study, allowing us to maximize the study's statistical power. Muscle force analysis is currently ongoing and will be available in the next report. In addition, muscle samples are being prepared for a full genetic analysis using Nanostring technology (Neuropathology kit). This technology will measure over 700 genes that quantify axonal growth and synaptic vesicle activity in addition to providing data that assess immune response. We anticipate those data to be ready either for the next quarterly report or in Q3, depending on the rate of data analysis.

Conclusion: We implanted DMM and autologous grafts in Sprague Dawley rat VML models with no adverse effects as previously reported.

Subtask 2: Assess muscle force production to determine degree of deficit.

Muscle physiology. Peak force, time to peak force (contraction), time from peak force to baseline (relaxation), and total time of submaximal force-time curve following stimulation were measured. Rats were anesthetized using a vaporizer at 4% isoflurane/400ml/minute O₂. Following induction of general anesthesia, the sciatic nerve was isolated, and sural and peroneal branches ligated. Sciatic nerve was then stimulated using platinum electrodes connected to a Grass stimulator model SD9 (Astro-Med, Inc., Westwarwick, RI) at 2-msec duration and 2-msec delay at varying voltages and frequencies. The knee and ankle joints were immobilized, and the Achilles tendon was cut from its insertion and connected to a MLT500/A force transducer (ADInstruments, Inc., Colorado Springs, CO) with 2-0 silk sutures. Output was collected digitally using a Sony VAIO computer and LabChart 8 software (ADInstruments). Optimal muscle length, stimulating voltage, and tetanic frequency were determined. During muscle lengthening, muscle was stimulated to tetanus for 3 seconds at each interval. Once optimal length was determined, tetanic contraction was stimulated at 3 second intervals until peak tetanic force dropped, indicating fatigue. Immediately following this force drop, 3 separate submaximal stimulations were measured. Those stimulations were used for muscle physiology assessments. Peak force was measured at the peak of the submaximal force-time curve. Contraction was measured as the time to peak force from 0 Newtons (baseline) to the peak of the submaximal force-time curve. Relaxation was measured as the time from peak submaximal force to 0 Newtons (baseline). Contraction and relaxation time was measured as the total period of the force-time curve (baseline to baseline).

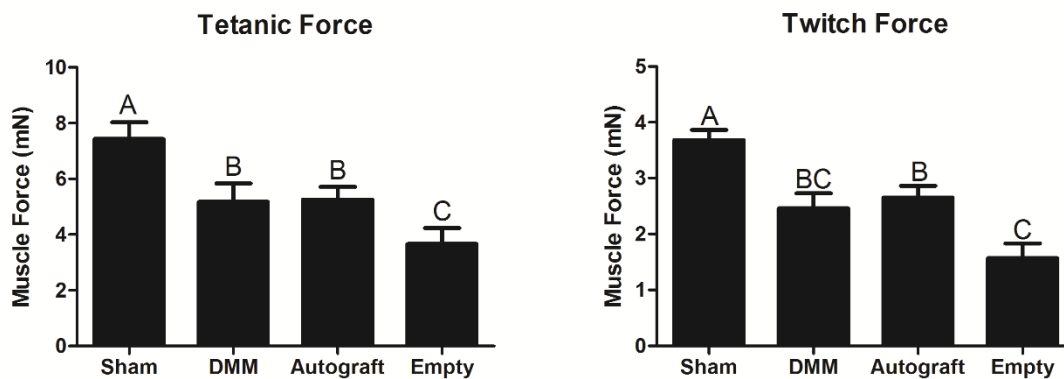


Figure 1. Muscle force tests.

Results: Muscle force analysis demonstrated a reduction in force when either DMM or autograft were compared to Sham animals. In addition, we also tested no repair animals to determine the degree of injury caused and whether functional deficits persisted at the end of 8 weeks.

Results presented in Fig. 1 demonstrate the amount of force reduced in DMM, autograft, and empty treated animals. Sham treated animals exhibited the highest force output. DMM and autograft displayed a significant reduction in force that amounted to ~27% reduction in maximal tetanic force compared to sham. In addition, VML animals that were not repaired showed ~40% reduction in

tetanic force output. When we assessed single muscle twitch force following a fatigue protocol, we showed fairly similar results with a near ~50% reduction in muscle force in empty groups, while DMM and autograft displayed no changes between each other but were reduced by 25-30% compared to sham.

Subtask 3: Harvest muscle and characterize histological morphometries.

Harvest muscle: Gastrocnemius muscles were harvested from rats immediately following physiology testing and immersed in formalin fixative. Regions of interest for injury sites were identified by position of the nylon suture and muscles were cut cross wise and longitudinally. Tissues were then dehydrated and embedded in paraffin wax for histology. Once sectioned the tissues were dried and placed in a slide box for future staining.

Major Task 2: Semi-quantitatively assess muscle staining to determine degree of denervation following muscle graft surgeries.

Subtask 1: Histomorphometry for stained muscle sections.

Histomorphometry. Histological sections stained with H&E or Masson’s trichrome were imaged using a 10x and 40x objective. Healthy muscle from sham operated animals was used as a positive control. Healthy muscle fibers were defined as having a polygonal shape with peripheral nuclei and homogenous fiber size distribution. Previously, we showed histologic evidence that autograft and DMM treated sites had newly regenerated fibers (centrally located nuclei). Regenerating muscles were defined as myofibers with centrally located nuclei with a small rounded appearance. Due to this previously published manuscript we did not measure centrally located nuclei again. Instead to begin to test our hypothesis for this study, we first quantified the percentage of muscle, fatty tissue infiltrate, connective tissue, and mixed muscle and connective tissue (sites of newly regenerating muscle fibers). To assess tissue composition, Masson’s trichrome stained sections were used and blue and red color were used to identify regions of muscle or connective tissue at 10x. Furthermore, histologic tissue analysis was performed at 10x to identify regions of interest for fat and mixed muscle and connective tissue. Tissue was analyzed (n = 8) using Zeiss imaging analysis software (Zen Blue, Oberkochen, Germany) with longitudinally cut tissue sections over the entire tissue sample, including both intact muscle fibers and the treated zone of injury using grafts.

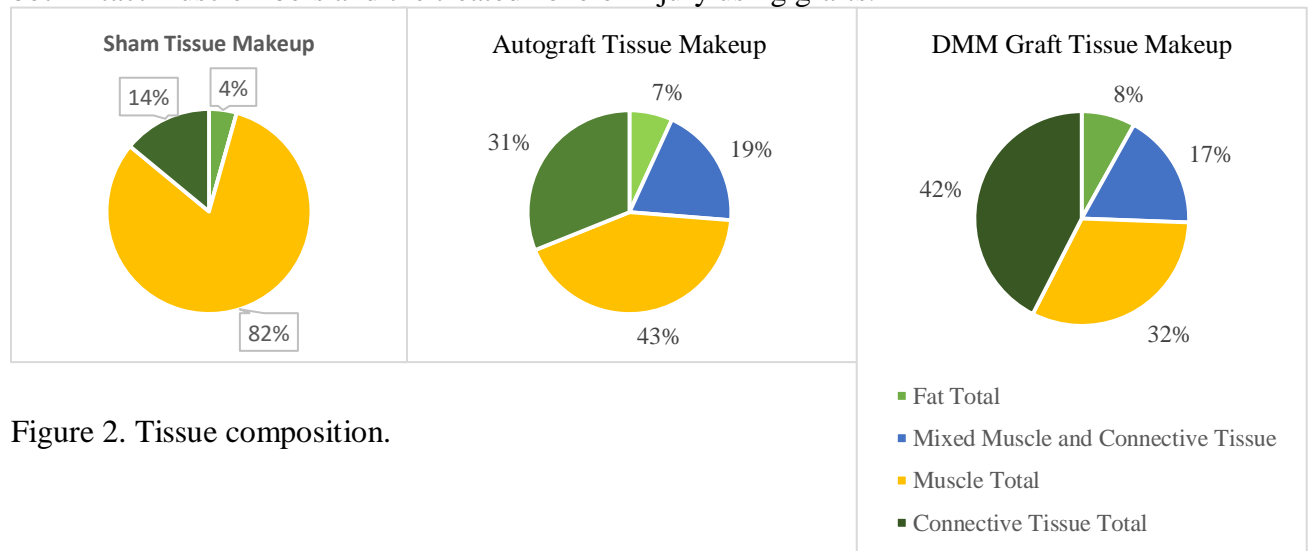


Figure 2. Tissue composition.

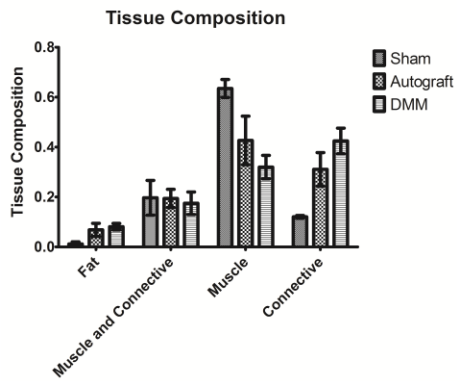
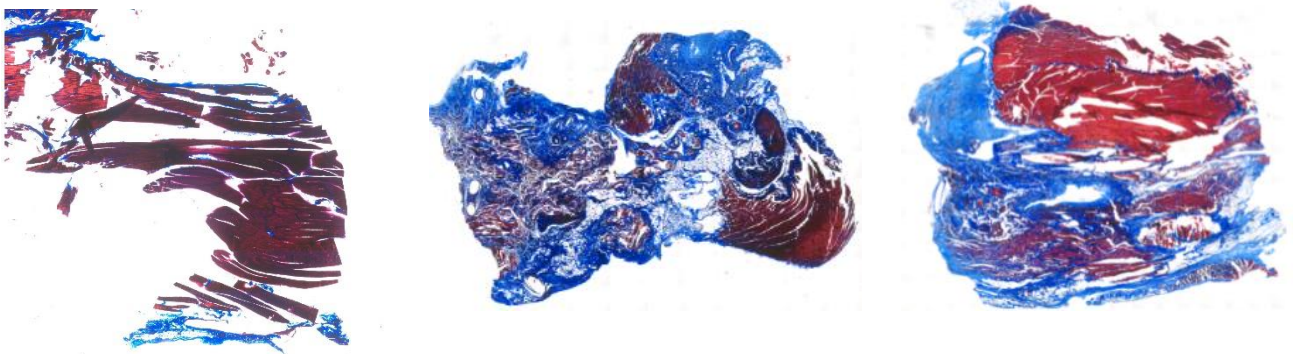


Figure 3. Tissue Composition.

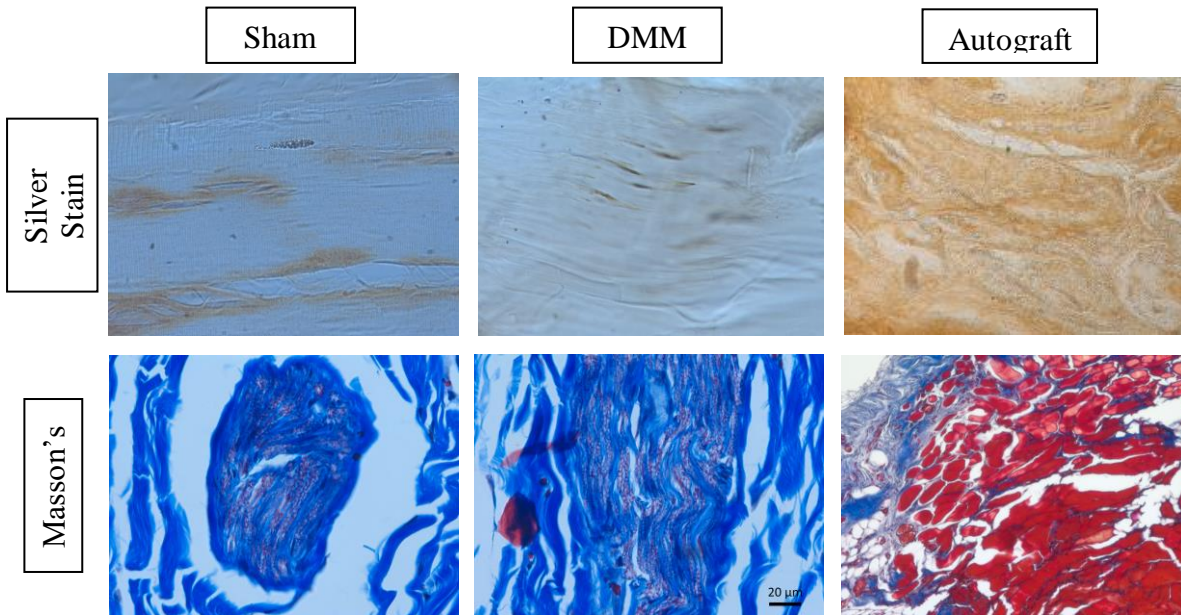


Figure 4. Silver staining and Masson's Trichrome stain to investigate whether nerve is able to grow into DMM without neurotization.

Results: Histomorphometry indicated an average of 42% connective tissue at and 17% mixed tissue groups in the DMM graft group. These data suggested that DMM was sufficient to support some new muscle fiber growth within the graft. Moreover, 17% of the area contained newly regenerating muscle fibers within a connective tissue area, an improvement over the previous data which demonstrated far less within the graft area. Similarly, autograft results showed 31% connective tissue and 19% mixed tissue groups, indicating that DMM and autograft had similar effects on injury sites. The fibrotic tissue formation at the injury site is further corroborated by histology due to the low structural organization and lack of developing fibers in graft groups. While de novo muscle fibers were present in the autograft and DMM, a majority of analyzed regions in the autograft displayed degrading fibers. The Bielschowsky's silver stain showed positive areas of neuromuscular junctions in Sham. Those NMJs were undetected in autograft samples and there were some positively stained neural cells within DMM; however, their morphology did not indicate NMJ formations. These data indicate a lack of significant neural connections, and those results are further corroborated in fluorescently stained tissues. Despite these lack of data, Masson's trichrome also demonstrated nerve bundles within the graft area of DMM and these were comparable to nerve bundles stained in Shams. Lastly, we did not detect any nerve bundles in autograft samples.

Subtask 2: Identify neuromuscular junctions and fiber types using MyHCs, NCAM, AChRs to assess their quality.

Immunohistochemistry. Immunohistochemistry was used to stain for neural cell adhesion molecule (NCAM), nicotinic acetylcholine receptor-epsilon (AChR- ϵ), nicotinic acetylcholine receptor-gamma (AChR- γ), and Pax7. Two AChR receptors were chosen to distinguish between mature adult acetylcholine receptors (AChR- ϵ) and fetal receptors (AChR- γ). Sections were deparaffinized and rehydrated prior to staining. To remove methylated crosslinks from antibody binding sites, slides were incubated at 95°C for 5 minutes in 10mM sodium citrate with 0.05% Tween-20. Slides were washed and then incubated with blocking serum (1.5% goat serum in PBS, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour. Samples were washed with PBS/0.6% Tween-20, and incubated for one hour with primary antibody. Primary antibodies used in this experiment were diluted to 2 μ g/ml in PBS with 1% BSA and 0.3% Tween-20. Samples were washed again with PBS/0.6% Tween-20 and incubated for one hour with secondary antibody diluted 1:200 in PBS/1% BSA/0.3% Tween-20. Secondary antibodies used in this experiment were from Invitrogen (Carlsbad, CA): Alexa Fluor 594 (goat anti-mouse, A11005; goat anti-rabbit, A11012) and Alexa Fluor 488 (goat anti-mouse, A11001; goat anti-rabbit, A11008). Slides were washed three times for five minutes each with PBS, then incubated with DAPI (R37606, Invitrogen). Cover slips were mounted with anti-fade mounting media (Invitrogen) and imaged using a Zeiss laser confocal scanning microscope.

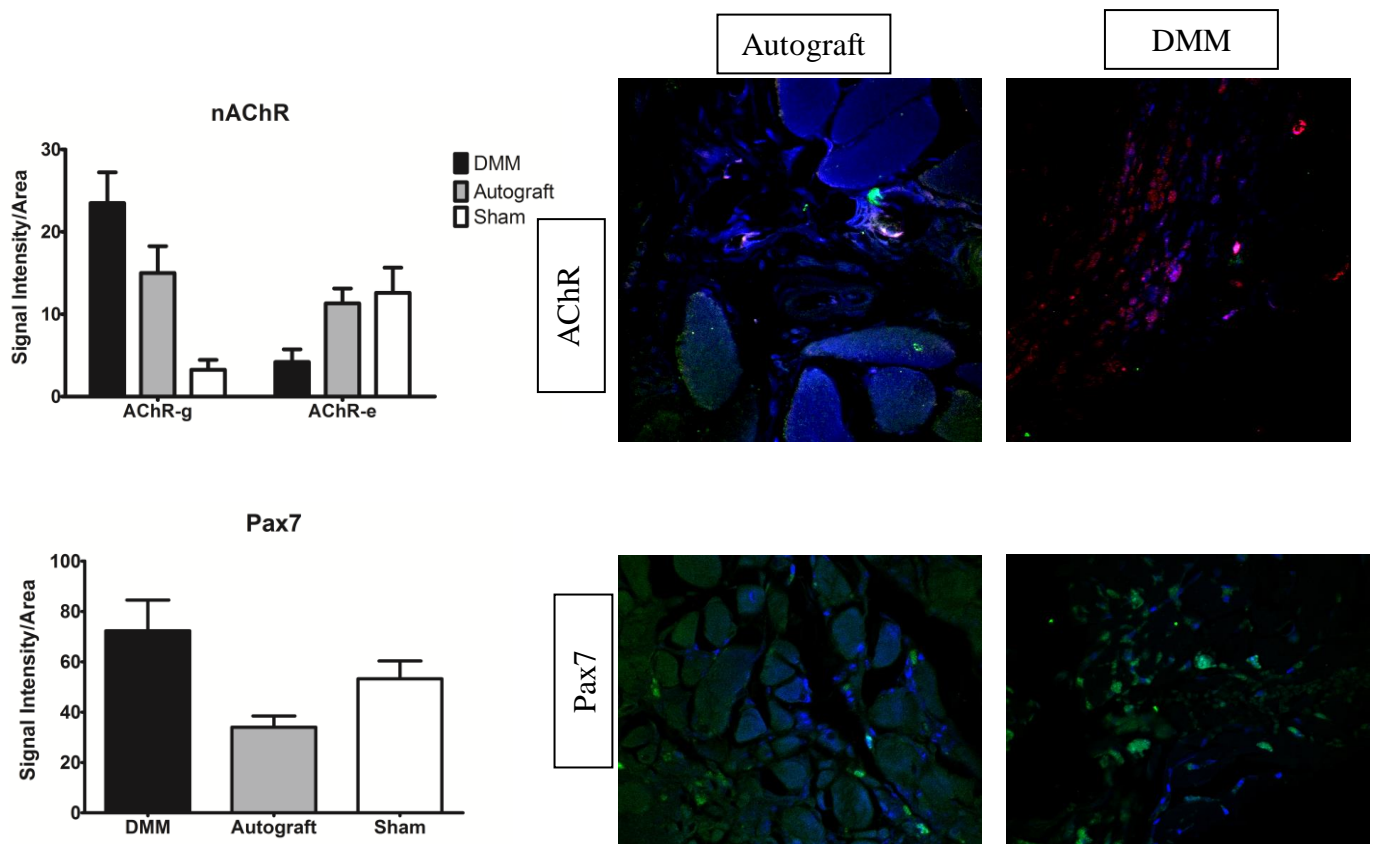


Figure 5. Acetylcholine receptor and Pax7 staining.

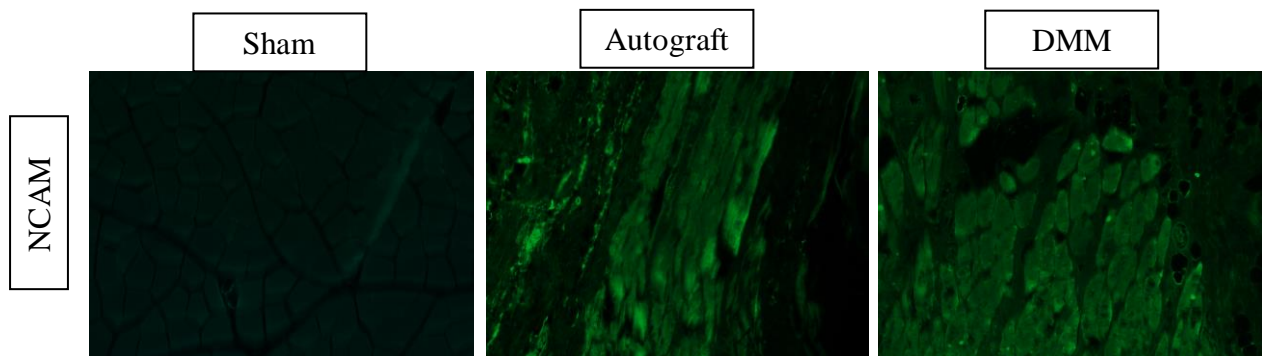


Figure 6. NCAM staining.

Results: Immunohistochemical staining was used to determine whether DMM and autograft samples were denervated or showed some levels of innervation. AChRs were separated by their isoforms that indicate either the fetal phenotype (gamma) or the mature phenotype (epsilon). Indeed, we confirmed what we observed previously which was increased gamma staining in DMM samples compared to sham and autograft. We then quantified those data and demonstrated DMM had greater signal intensity for gamma stained tissue and was virtually absent for epsilon stained tissue. In contrast, autograft showed more staining for epsilon compared to DMM which could be remaining NMJs that are intact or degenerating. Lower gamma stained autograft supports these findings. We further characterized Pax7 staining for satellite cells and confirmed our previous findings that DMM supported what appeared to be far more satellite cells compared to autograft. We quantified those results and determined that DMM indeed support more Pax7 positive cells while autograft was reduced compared to sham. Whether the differences shown in these graphs are significant still needs to be determined. Lastly, we stained for NCAM and showed a lack of NCAM staining in Sham controls, but positive NCAM staining was present in both DMM and autograft in the graft and near injury margins.

Prior work demonstrated that skeletal muscle defects repaired with decellularized muscle matrix improved muscle force output compared to empty defects, and there was no evidence of re-innervation in newly regenerated muscle fibers within the graft area or around the graft area. From those studies performed in Aim 1 of this proposal, we determined that no de novo muscle fibers that formed due to decellularized muscle or autologous muscle treatments were innervated.

Gene analysis using Nanostring

In addition to performing histology and immunohistochemistry, we assessed gene expression using lysates from VML decellularized muscle and autograft treatments. Quality RNA was successfully obtained from graft sites, and those RNA were sent off for Nanostring analysis. Results from that analysis are shown below, which included both muscle and neural gene markers. We report full genetic analysis using those gene panels and we will focus on some select differentially expressed genes within those cohorts.

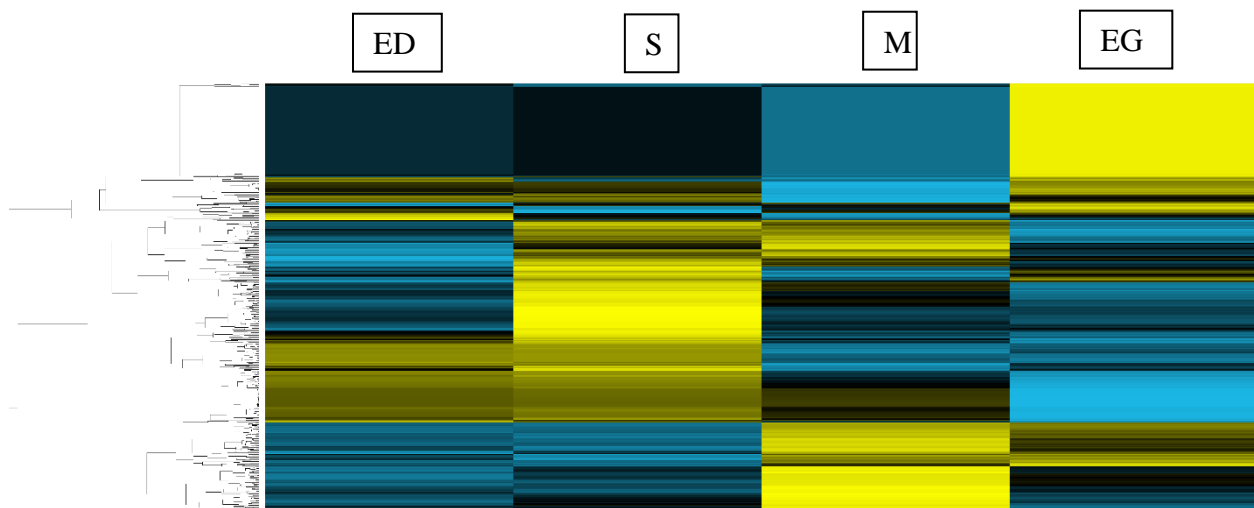


Figure 6. Nanostring analysis of VML injuries versus sham where VML injuries were treated with DMM (EG – empty graft) or autograft (M – muscle). Those animals were compared to no treatment (ED – empty defect) and sham (S).

mRNA levels in VML animals appeared different than sham animals. Furthermore, VML animals treated with decellularized muscle grafts (EG) showed some unique differences from VML animals that received no treatment (ED). More, VML animals that received an autograft (M) were similar to EG groups as well, but also displayed some unique differences from ED. These data according to the heat map showed that treating a VML injury resulted in different expression levels from leaving the injury to undergo fibrosis.

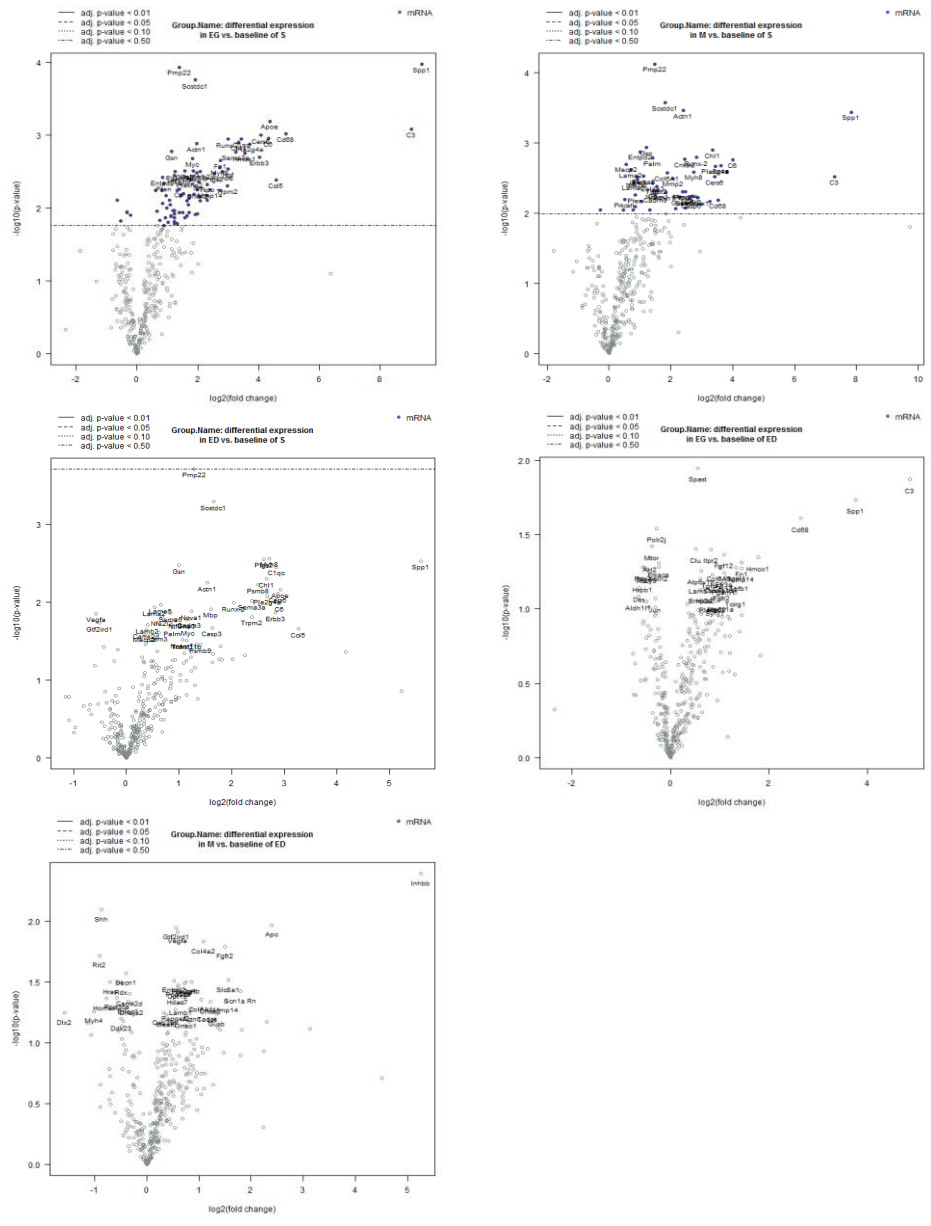
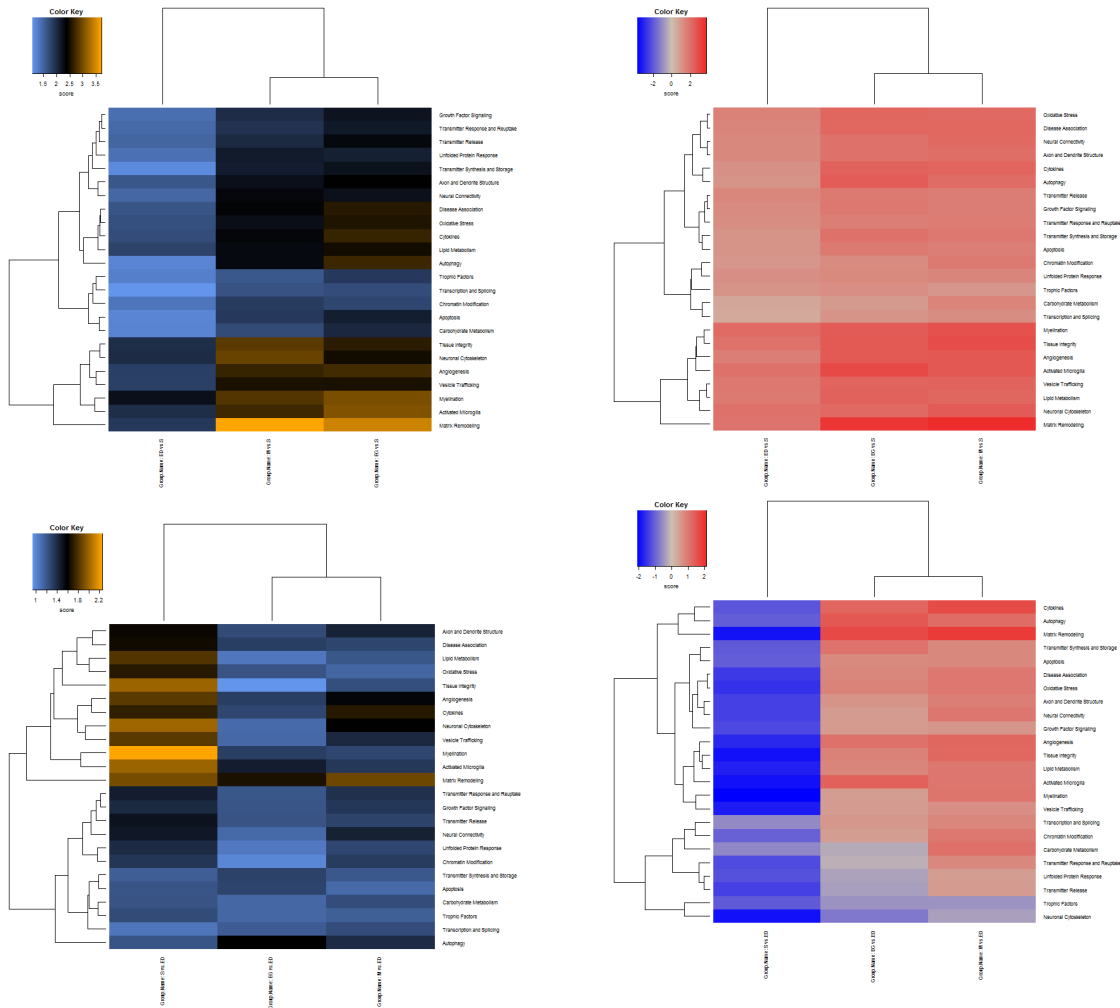


Figure 7. Volcano plots of differentially expressed genes in DMM, autograft, untreated, and sham animals.

Analysis of fold changes in mRNA levels were compared to sham, where we determined that both DMM and autograft groups shared most differentially expressed genes. However, some of the levels

for those shared genes were different between treatments. When we compared VML treatments to no treatment, we determined that DMM and autograft shared less genes, indicating that the different treatment modalities elicited different responses. Importantly, we determined that both DMM and autograft were pro-myogenic, demonstrating elevated levels of Myh8, a gene that encodes for new



muscle fiber formation, and Pax7, a gene marker of satellite cells. In addition, other extracellular matrix conditions that would favor regeneration were elevated in DMM treated animals such as Col6a1 and Col4a1 which encode for basal lamina proteins and are known to stimulate muscle regeneration. *More, we determined that Ncam1 gene levels in DMM and autograft were elevated compared to sham, confirming our immunohistochemical staining and that muscle graft treatment sites remained denervated.*

Figure 8. Global significance scores to summarize differentially expressed genes and the extent of differential expression in VML animals compared to sham.

When we examined neural markers, we determined that genes encoding for axon and dendrite structure like Apoe, Spp1, and Ch11 were upregulated in DMM and autograft treated animals. In addition, genes encoding for myelination were high such as Pmp22 in both DMM and autograft animals, and trophic signaling for neurotrophin-3 was upregulated in DMM treated sites.

Interestingly, global significance scores in Figure 3 showed that neural connectivity, vesicle trafficking, myelination, and angiogenesis related genes were higher in VML treated animals compared to sham. In addition, those same gene sets in untreated animals were downregulated when compared to sham, suggesting that treatment alone aided in improving the neural microenvironment. Not surprisingly, matrix remodeling expression was highest in treated animals. When we explored global significance scores VML treated animals and sham versus untreated animals we determined that neural connectivity and myelination were highest in autograft animals compared to no treatment. In addition, transmitter release genes were higher in autograft than when DMM was compared to no treatment. Taken together these data indicate that while DMM displays a pro-myogenic and pro-neurogenic environment it remains less so than when host muscle is used.

Denervation Atrophy Studies

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).

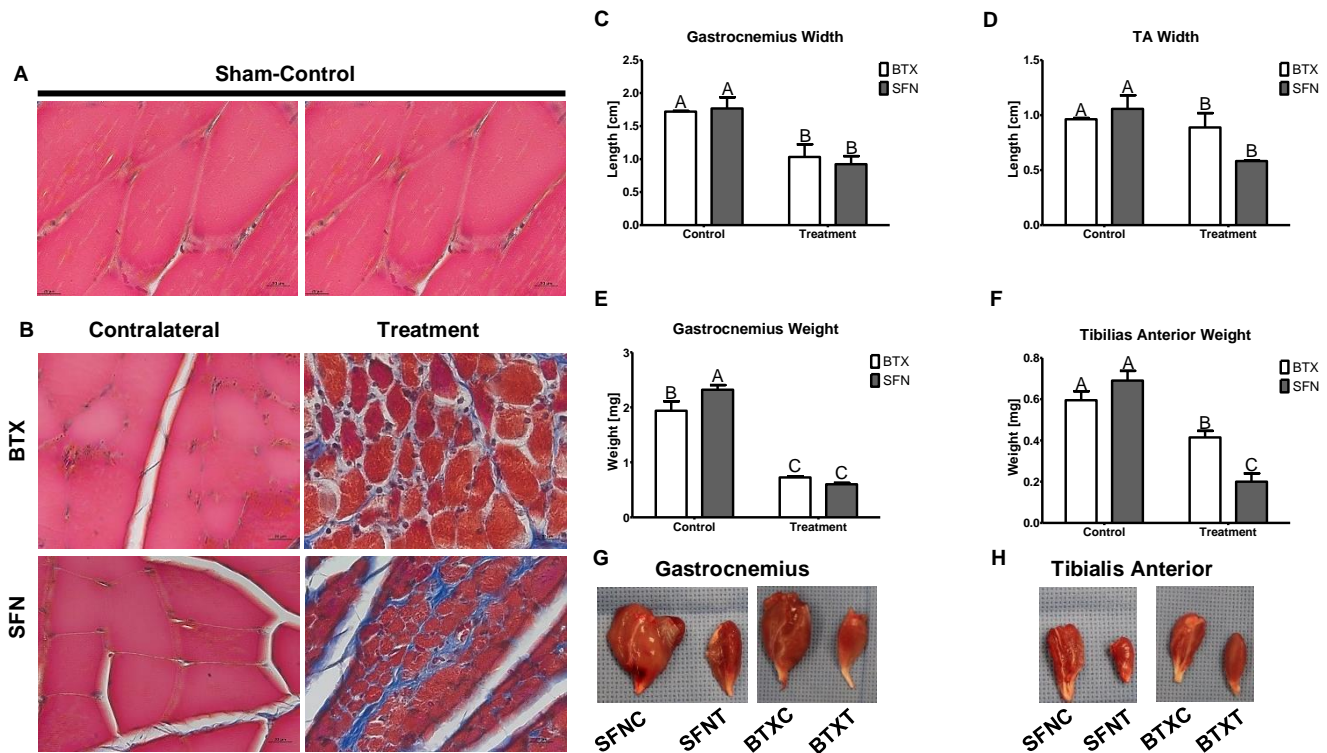


Figure 9. Denervation-induced muscle paralysis and botox muscle paralysis demonstrate similar mass loss in gastrocnemius and tibialis anterior muscles.

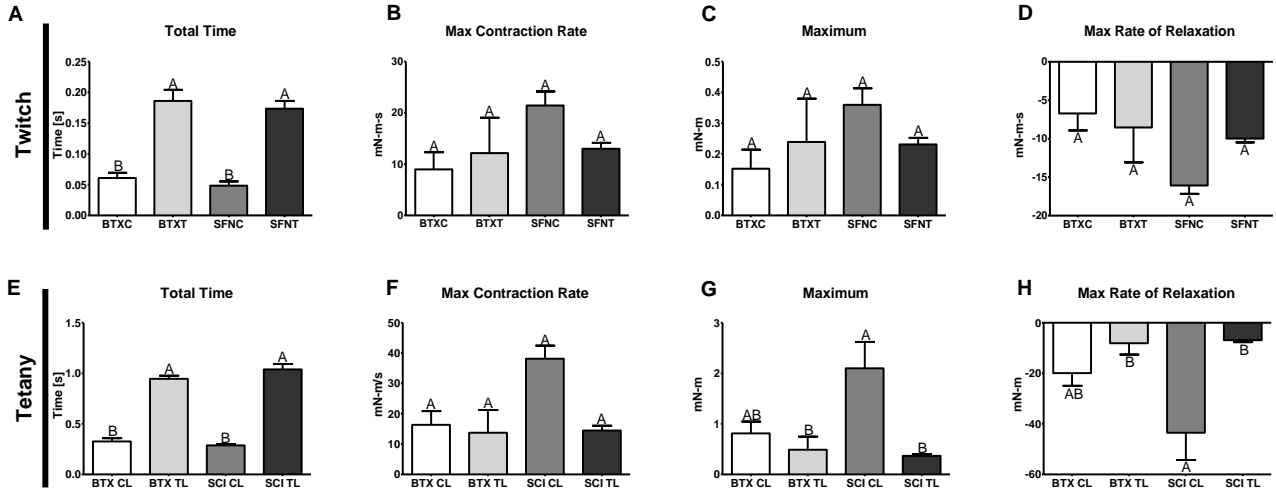


Figure 10. Muscle function tests showed that muscle force was impaired in both cases of paralysis. Interestingly, relative to control legs botox was no different for max contraction rate, peak force, and max rate of relaxation.

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.

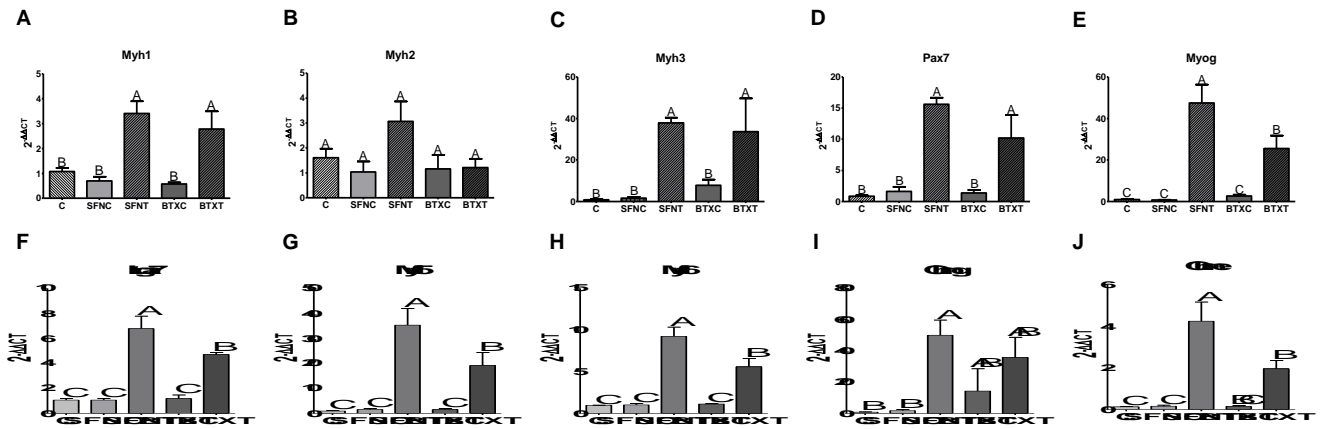


Figure 11. Gene expression levels are elevated in paralyzed muscle. All paralyzed muscle increased gene expression levels except Myh2 where only denervated muscle increased Myh2 expression. In addition, botox treated muscles showed lower myogenic expression levels for Myog, Itga7, Myf6, and Myf5.

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.

Milestone #1: We composed a manuscript that demonstrated the degree of denervation, but when we assessed it for submission it appeared weaker and we decided to include it in our manuscript using neurotization.

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

Major Tasks: 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.

Subtask 1: Neurotize muscle grafts using peroneal nerve grafts

Subtask 2: Neurotize muscle grafts using tibial nerve grafts

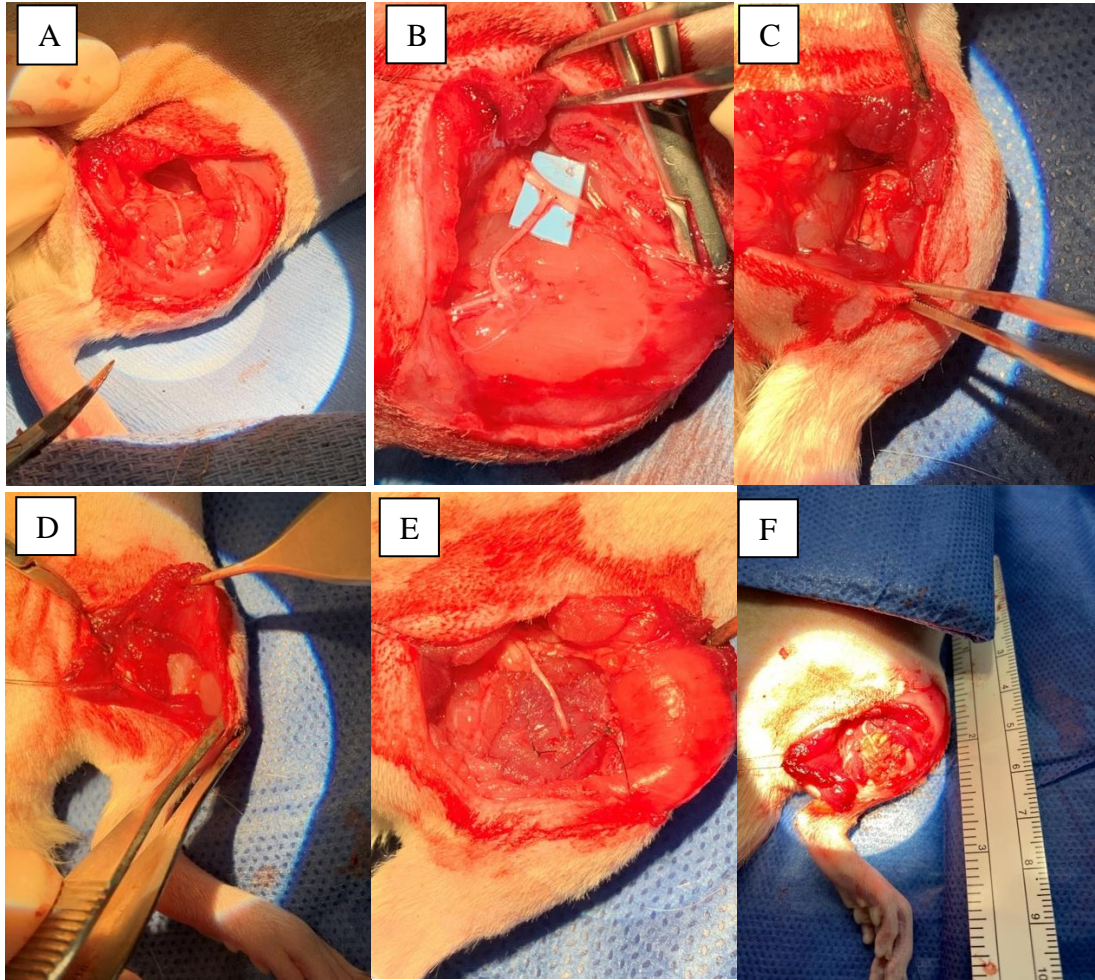


Figure 12. Neurotization surgeries completed.

Figure 12 demonstrates example images from neurotization surgeries where either DMM or autograft were implanted into the muscle defect. Neurotization was performed by either using a peroneal swing over graft or an end-to-side coaptation procedure using the peroneal nerve as the graft and the tibial nerve as the nerve used to donate axons to the peroneal graft. (A) shows a peroneal swing over prior to graft implantation, (B) shows an end-to-side procedure prior to graft implantation, (C) shows the graft alone with the peroneal nerve cut and embedded into the wall of the biceps femoris, (D) shows an empty defect with the same nerve embedment procedure as in C, (E) shows a peroneal swing over after autograft implantation, and (F) shows a peroneal swing over after DMM implantation.

Major Task 4: Assess histology, immunostained sections, and Western blot

Subtask 3: Determine histomorphometry

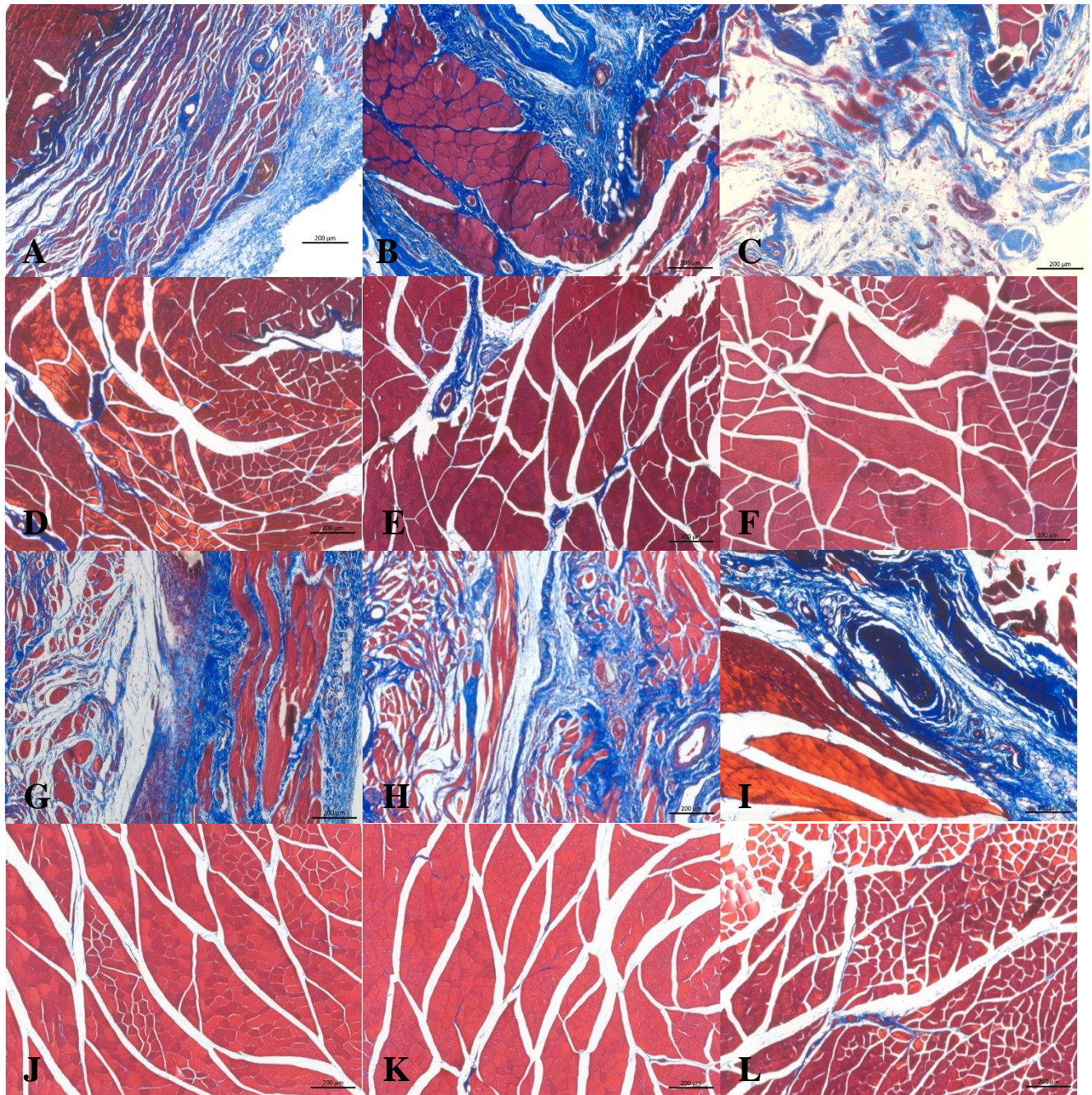


Figure 13. Neurotization improves muscle development and augments the appearance of fiber maturation in DMM treated animals. Images are representative of overall outcomes in each animal group. (A) Autograft with a peroneal neurotization, (B) DMM with peroneal neurotization, (C) DMM no neurotization, (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 13B 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 13H) 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 13C), and that a significant portion of the injured area was connective tissue. Furthermore, our autograft neurotized injuries (Figure 13G,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 13I). Control muscles show that no effects were detected on the contralateral leg (Figure 13D-F, J-L).

NOTE: Histomorphometry measurements are currently being assembled using immunohistochemical stained sections and the Akoya Inform program. This setup allows us to distinguish between fast and slow twitch muscle fibers along with their size. Moreover, we will also be able to distinguish centrally located nuclei and their fiber type stain.

Subtask 4: Examine immunohistochemical staining for innervation markers.

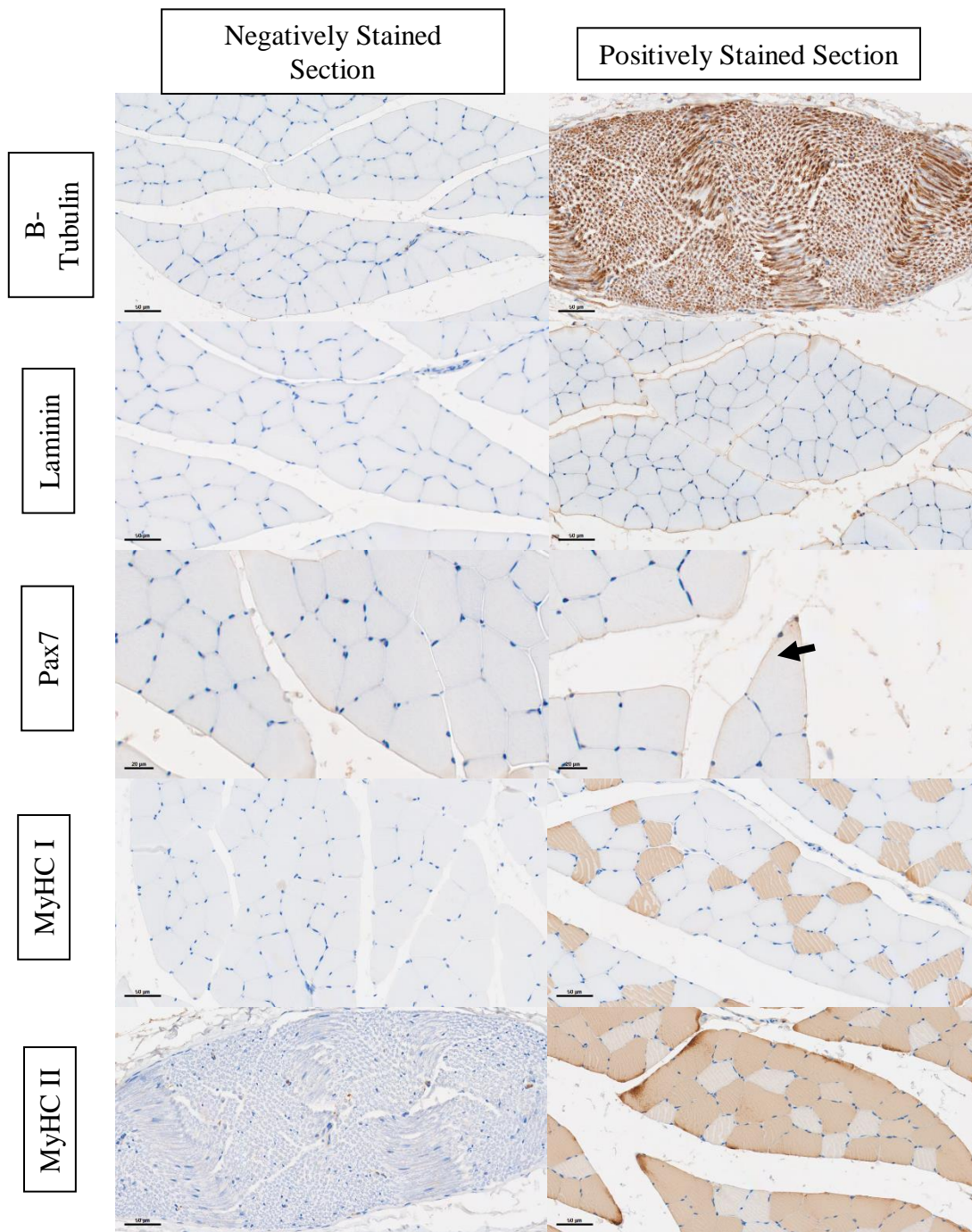
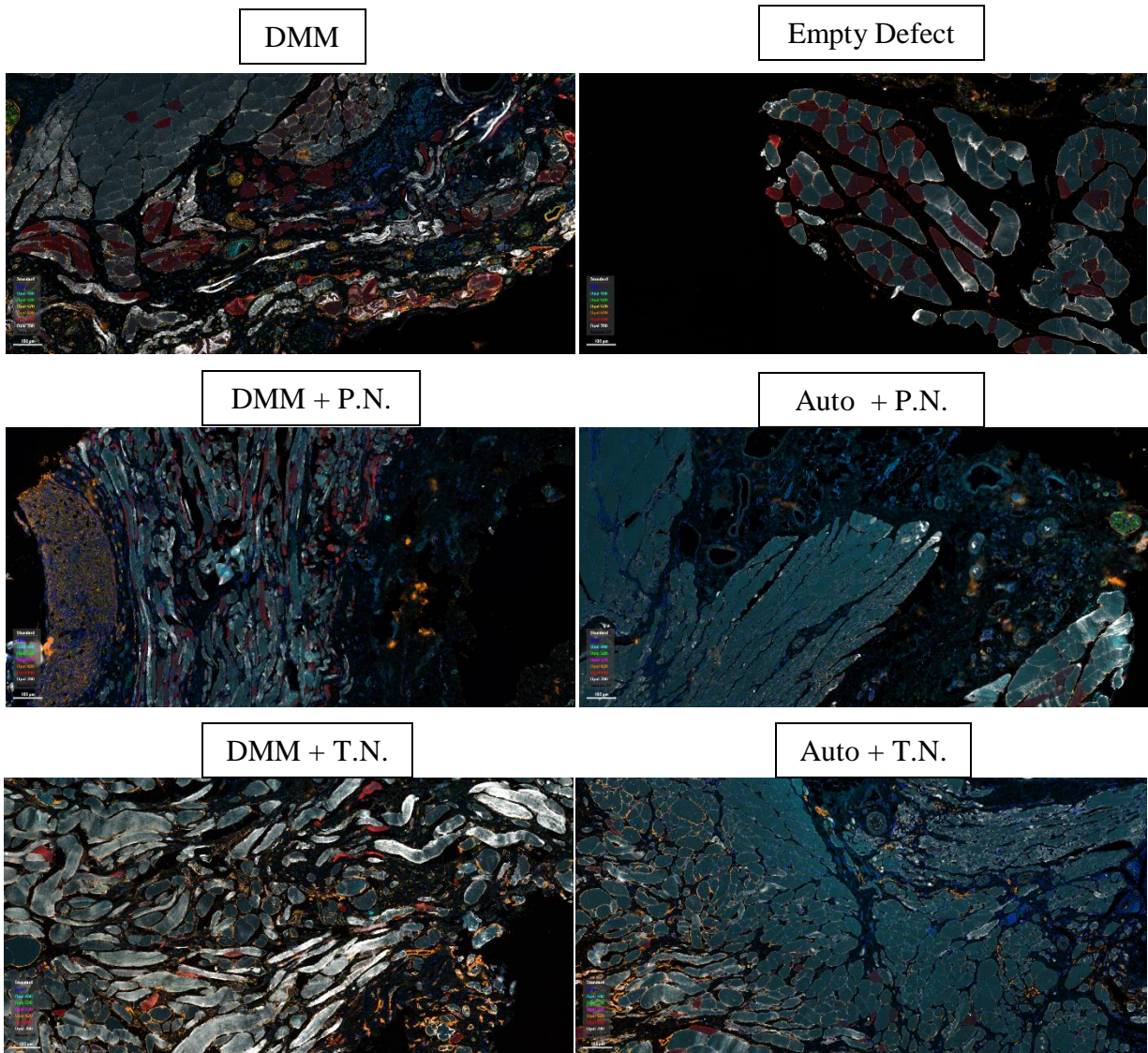


Figure 14. Antibody optimization for muscle immunostaining. Negatively stained areas of the same section were imaged alongside positively stained sections to demonstrate positive staining. Arrow points to positively stained nucleus for Pax7.

To move forward on our multiplex immunofluorescent staining alongside immunostained quantification, we optimized each antibody using either a citrate or EDTA antibody unmasking for

10, 15, 30, and 60 minutes. In addition, we used varying concentrations of antibody in order to determine the most optimal staining conditions. Our most optimally stained sections are shown in Figure 1. Note the absence of b tubulin in the negatively stained section while in the nerve b-tubulin staining is intense. For laminin sections, note specific staining to the endomysium and perimysium. We also noted that laminin stains were weaker than other stains. Pax7 staining was considered positive when the staining aligned with the nucleus. Pax7 optimization was the most time consuming. MyHC I and MyHC II staining was specific and intense. Note the limited specific staining for MyHC I since gastrocnemius muscles are mostly MyHC II. In addition, note that MyHC II staining was prevalent but also specific where MyHC I fibers appeared unstained. Due to the prevalent nature of MyHC II,



our negatively stained section was of the nerve.

Figure 15. Opal stained sections demonstrate differences in muscle graft response to neurotization. All images obtained were within the grafted region. Images were stained using antibodies against Pax7 (480, cyan), alpha-bungarotoxin (570, pink), Laminin (620, orange), MyHC I (690, red), MyHC II (780, white/gray), and beta-

tubulin (520, green). Nuclei were stained using DAPI (400, blue). Linear unmixing was used to distinguish each channel in the image and background autofluorescence was subtracted. Scale bars are 100 um.

Empty defects were demonstrated an ability to regenerate new muscle fibers at the edges of the defect which had a significant number of MyHC I positively stained fibers compared to the medial head in a similar region of interest. When VML injuries were treated with DMM we observed typical regenerative features that had been previously seen in prior studies. Initial observations showed that VML injuries treated with DMM showed abundant fibrosis with de novo fiber formation within the graft area. DMM were cellularized mostly with single nucleated cells that had a spindle shaped appearance. While those cells were not stained specifically for fibroblast markers, their shape and absence of muscle markers led to the conclusion they were fibroblasts. DMM also showed the appearance of MyHC I and II positively stained muscle fibers. MyHC I fibers were observed near injury margins while MyHC II fibers were found away from the margins, within the grafted region. Pax7 positive cells were observed within DMM grafted regions, supporting our previous studies that consistently observed DMM to support Pax7 cells. We did not observe alpha-BTX staining.

Neurotized grafts responded differently. DMM grafts neurotized with peroneal nerve (P.N.) showed far more MyHC I positive fibers stained within the grafted region juxtaposed to MyHC II fibers. DMM + P.N. also showed the presence of Pax7 cells within the graft, and beta-tubulin stained cells outlined by laminin were also found between fibers, suggesting the presence of axons. In addition, alpha-BTX positive sites were observed. This differed from autografts neurotized with P.N. Autografts showed few MyHC I fibers stained within the injury site. This was compounded with a significant degree of fibrosis and the appearance of degenerating fibers. However, near the margin were an abundant number of small newly regenerating muscle fibers suggesting that while autografts + P.N. were more fibrotic they could still be regenerating new muscle. Several Pax7 positive cells were observed within the graft in addition to evidence of neurons within the area, and alpha-BTX positive cells were observed. Tibial nerve treated grafts appeared different from peroneal treated grafts. DMM + T.N. showed evidence of larger, mature muscle fibers within the injury site. Fibers stained positively for a mixture of MyHC I and II fiber types, and there was evidence of alpha-BTX stained areas in addition to beta-tubulin in same area, suggesting innervation. Autograft + T.N. also showed larger, mature fibers with a similar mixture of MyHC I and II fibers in the injury site. There was evidence of small regenerating fibers as well in addition to Pax7 stained cells within the area of newly regenerating fibers. Lastly, we also observed alpha-BTX stained fibers adjacent to beta-tubulin stains.

Subtask 5: Run gel electrophoresis to determine protein via Western blot. (NCAM, Syp, SV, AChR)

Nanostring analysis was used on our samples to provide a higher throughput and more unbiased approach to our data assessment. These studies informed us about neural and muscle markers including those listed in Subtask 5.

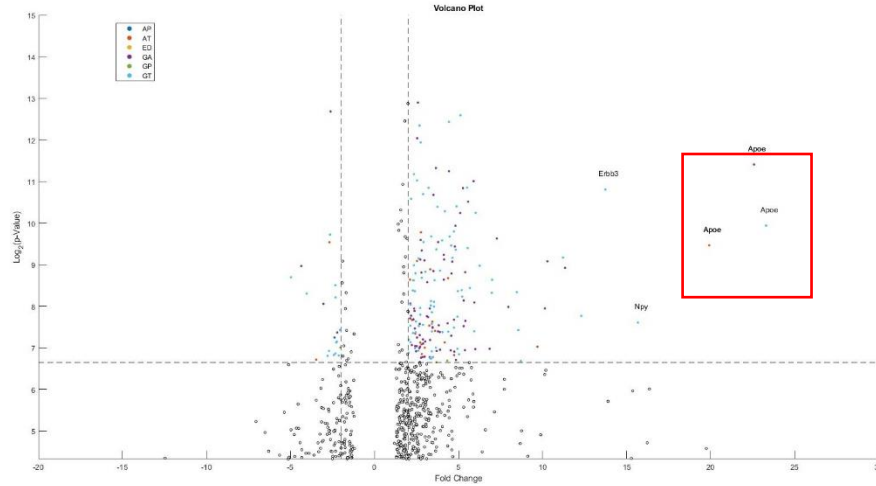


Figure 16. Volcano plot of differential gene expression for all neurotization samples.

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.

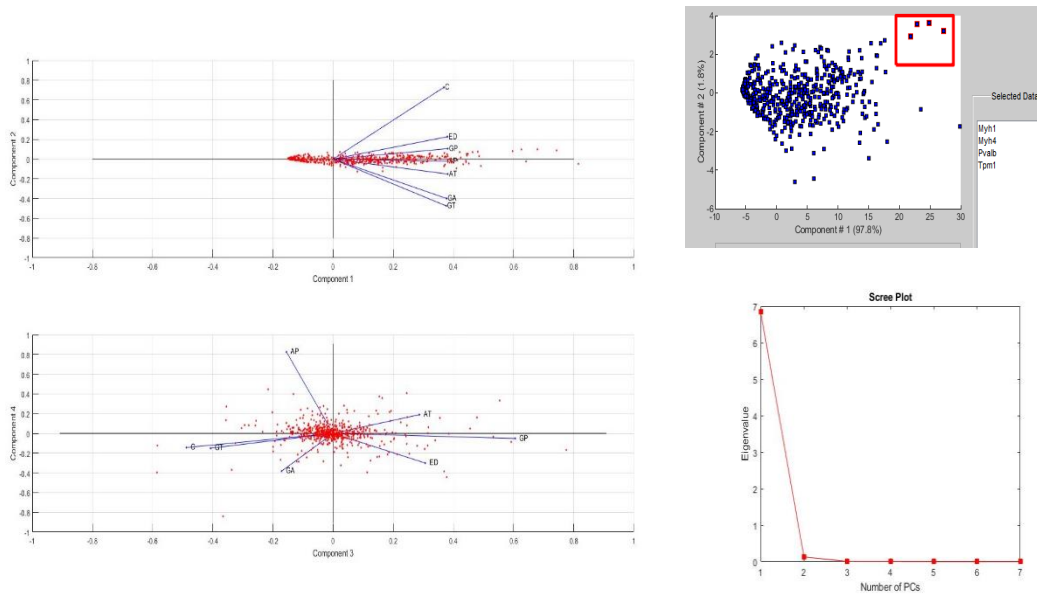


Figure 17. Two-dimensional principal component analysis (PCA) with Scree plot confirms quality of the samples tested.

Heat Map Normalized Ratio to Control

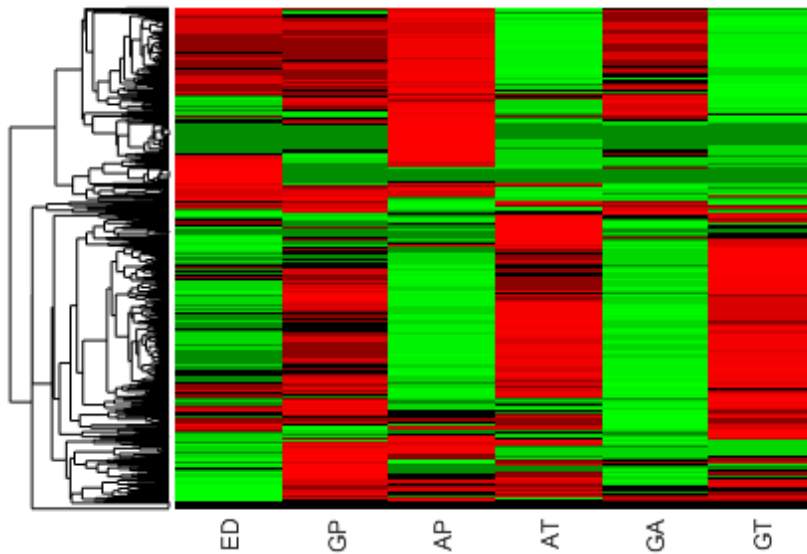
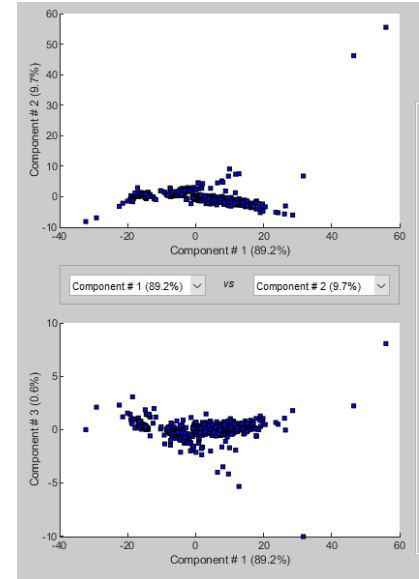
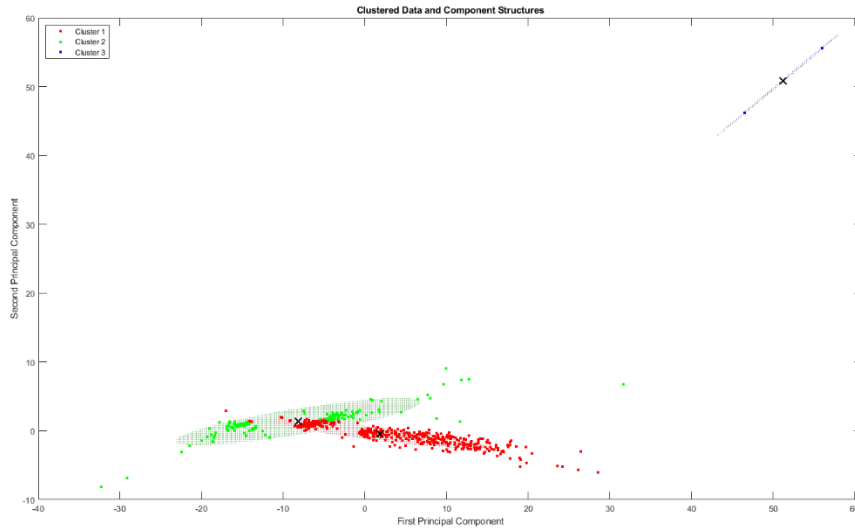


Figure 18. Heat map normalized to healthy control muscle showing all expressed genes. Red indicates upregulated genes compared to control while green represents downregulated genes compared to control.

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.



Group Expression Related to Gaussian Clusters

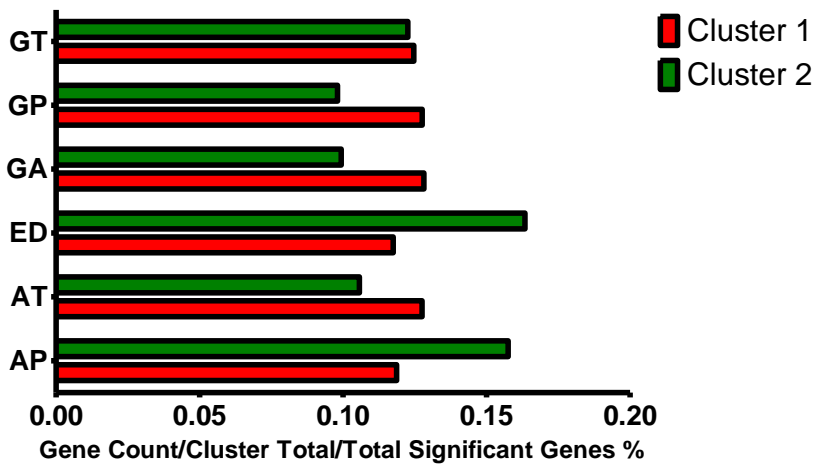


Figure 19. PCA plots with Gaussian clustering to identify unique gene clusters embedded within treatment groups.

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.

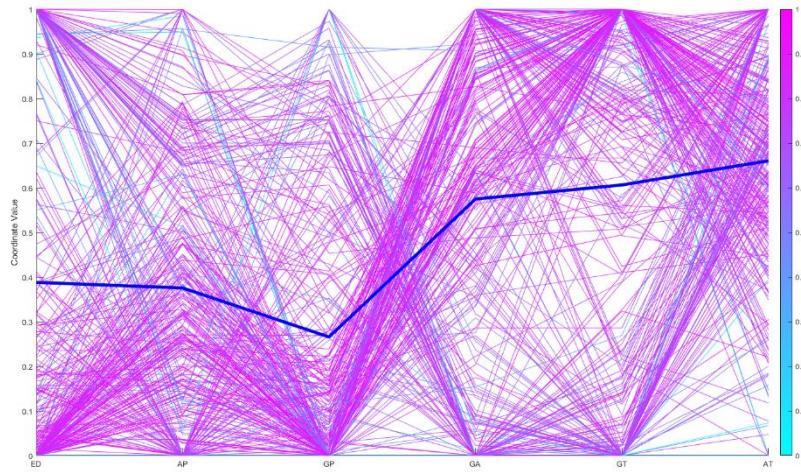


Figure 20. Parallel coordinate plots demonstrate further that gene clusters were unique. The dark blue line represents the mean expression across all genes and acts as “line of best fit” across all gene expression. Gene expression was normalized between 0 and 1 for each gene for all groups.

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.

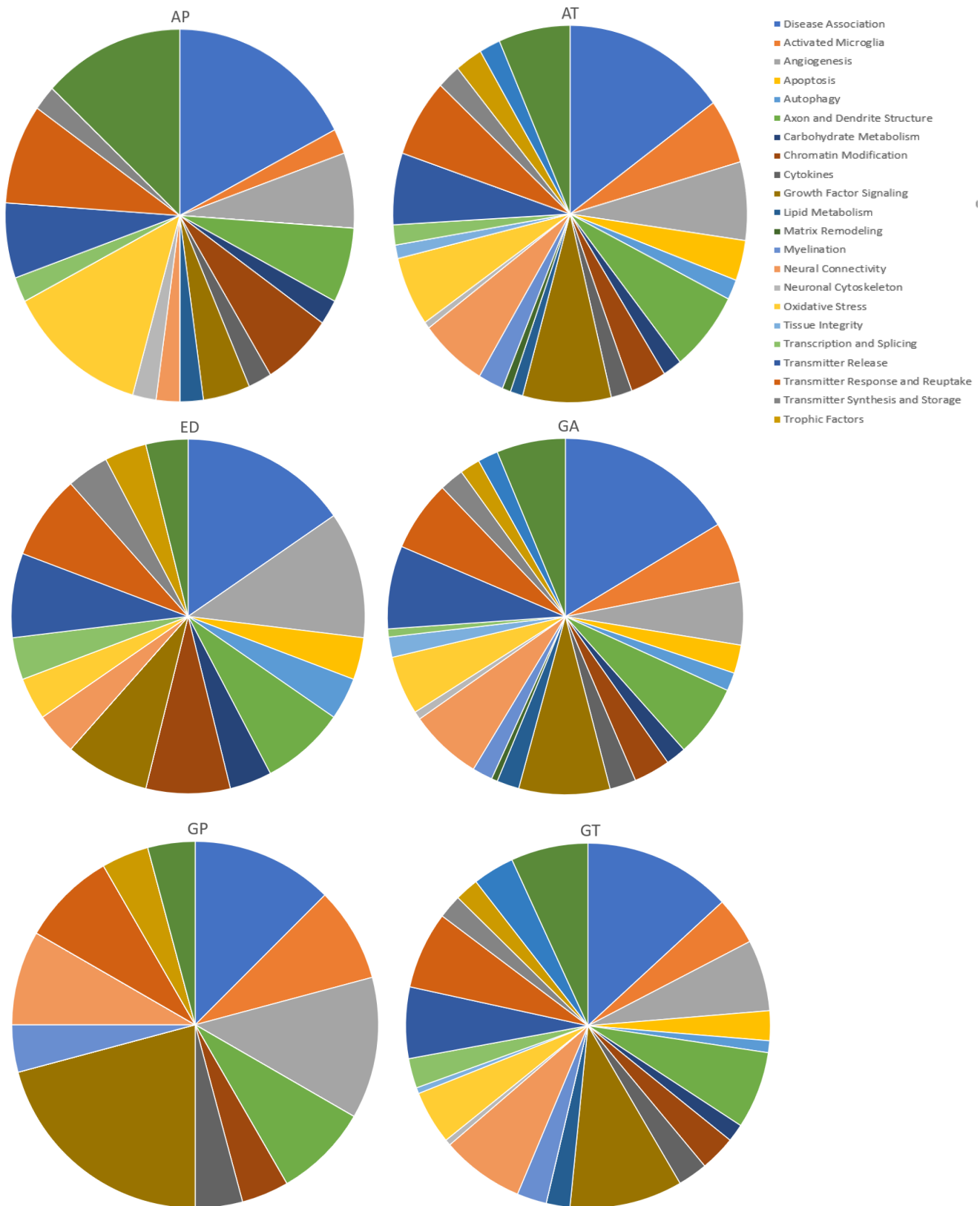
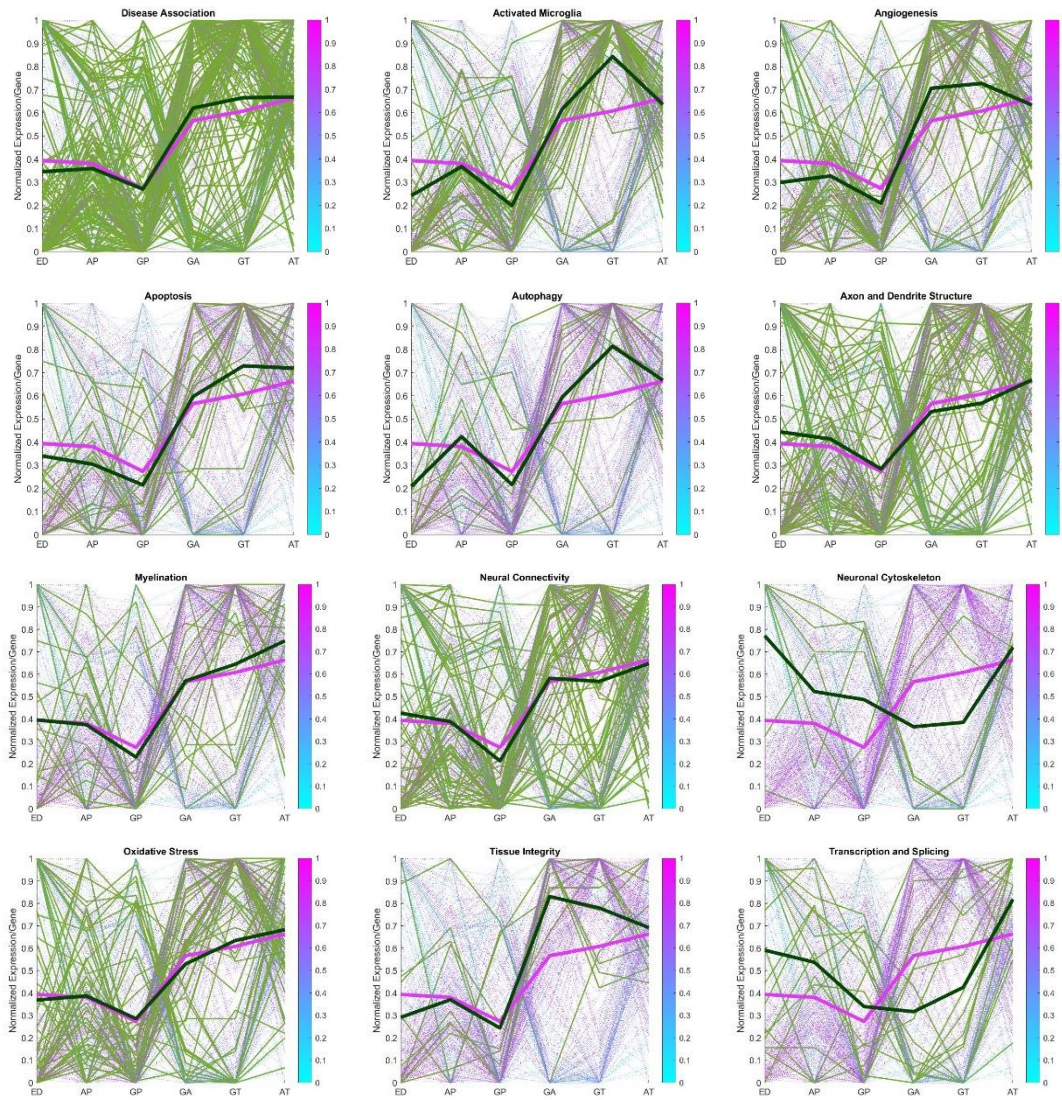


Figure 21. Pie chart category counts represent how associated gene groups changed with treatment.

Of note, trophic signaling genes were far higher in peroneal and tibial nerve treatments for decellularized muscle grafts than were for autografts. We then investigated further into the which genes are of most interest based on their categorical association and their significance level compared to all treatments.



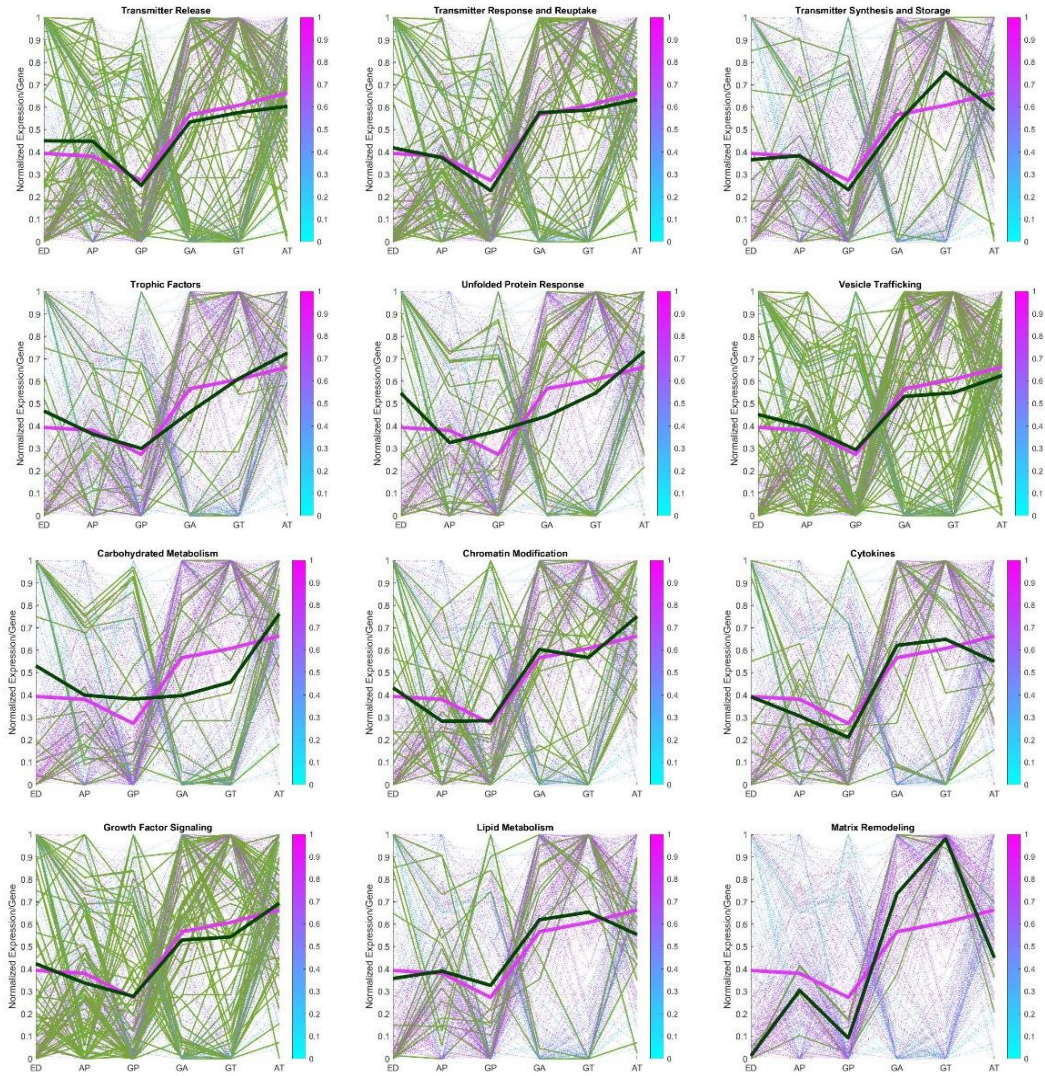


Figure 22. Parallel Coordinate Plots-MODE coloring to demonstrate changes in expression patterns amongst the groups.

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.

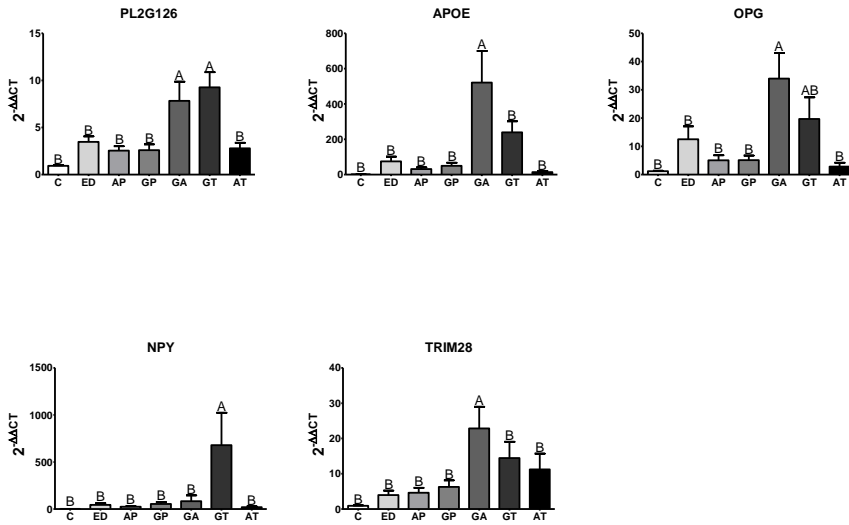


Figure 24. Isolated mRNA from muscle lysates was assessed using real time qPCR to detect fold change in gene expression. Primers tested were Pl2g126, Apoe, Spp1 (OPG), Npy, and Trim28.

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.

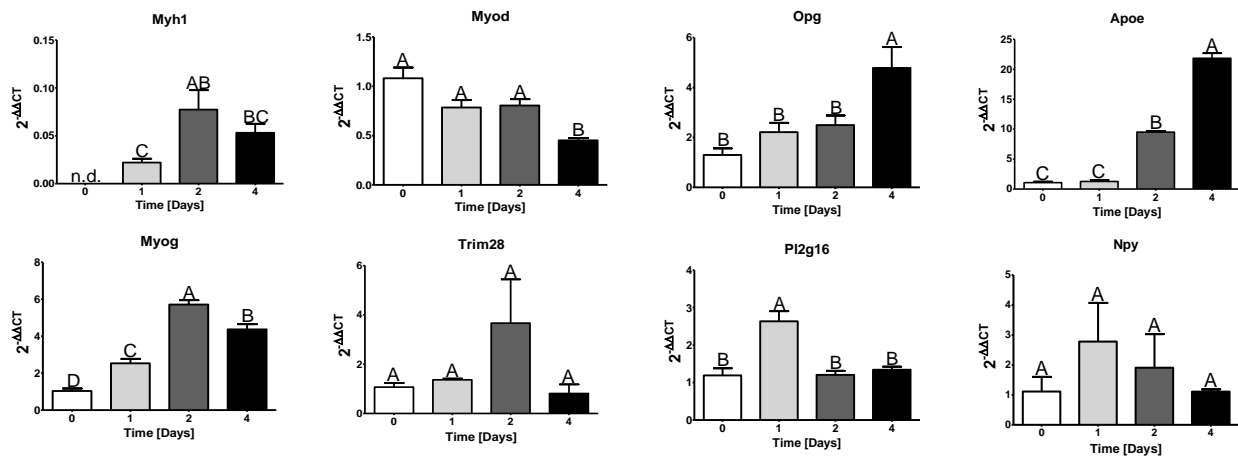


Figure 25. Myogenic differentiation in C2C12 mouse myoblasts over a time course of 0, 1, 2, and 4 days. Myogenic factors confirm successful differentiation of the cells, and Spp1 (Opg)

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

Specific Aim 3: Examine the effect of muscle graft neurotization on functional muscle recovery.

Major Task 5: Muscle Function tests following neurotization

Subtask 1: Muscle function to be measured using electrical stimulation of the sciatic nerve.

Muscle force was recorded throughout the duration of this experiment at 2, 4, and 8 weeks by stimulating the posterior crural muscles.

Subtask 2: Assess data from muscle function tests.

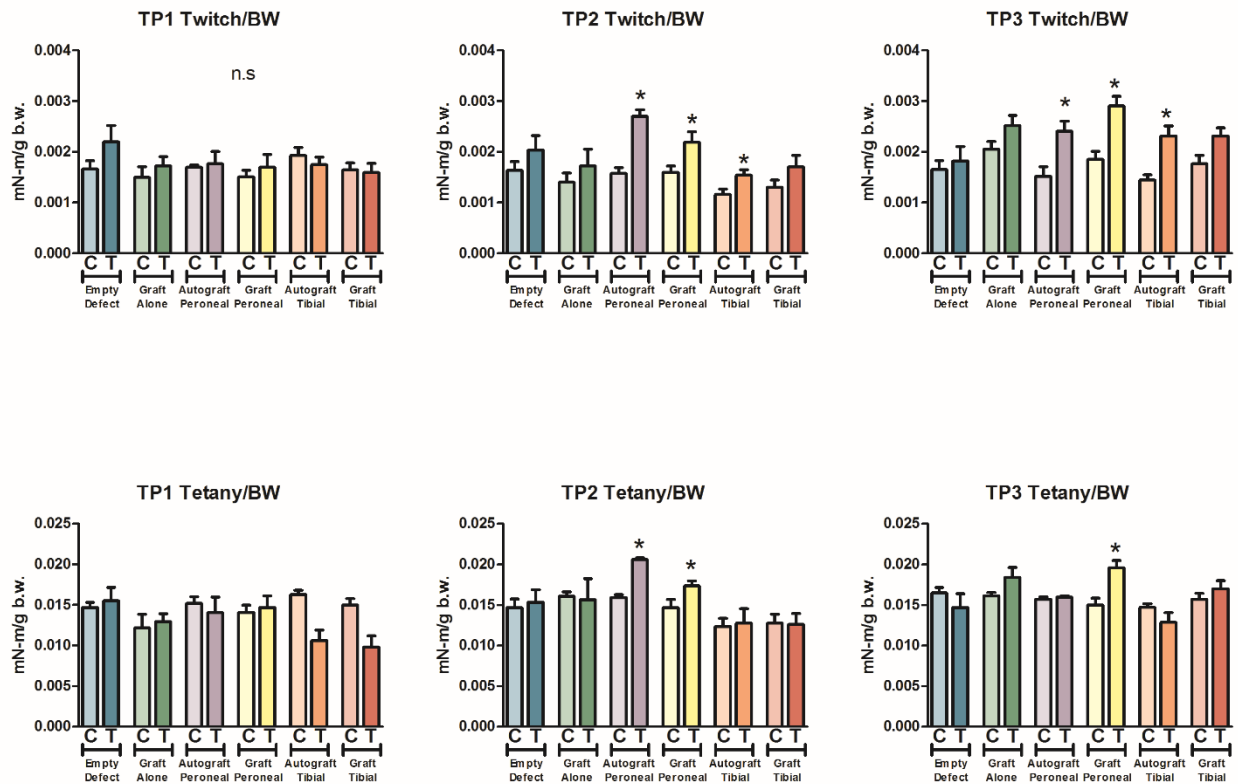
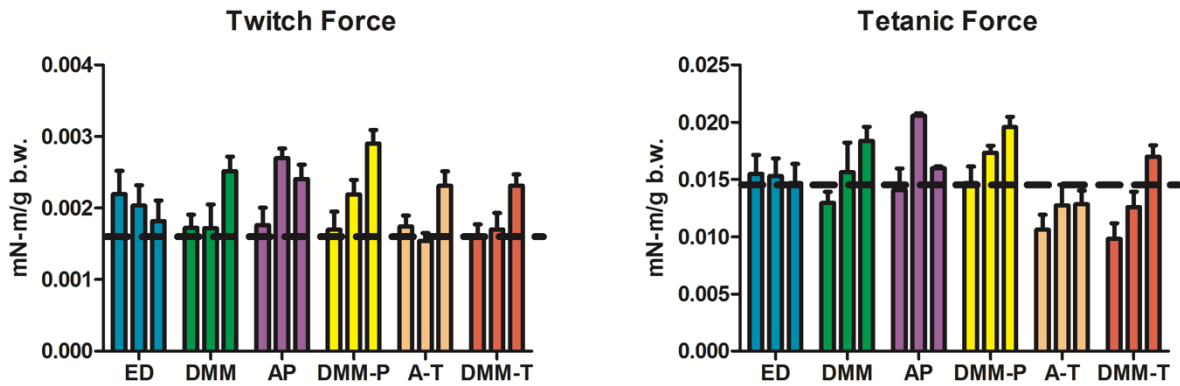


Figure 26. In vivo specific muscle force generated over 8 weeks compared to contralateral (uninjured) leg. * indicates a significant difference from control legs (p<0.05).

Muscle twitch and tetanic force were measured for all treatment legs and their contralateral control legs. Data demonstrated that both autograft and DMM treated legs with the addition of a peroneal swing over produced far better force than DMM grafts alone, or graft treated legs with a tibial nerve neurotization. More, DMM treated with a peroneal nerve produced higher force levels for both twitch

and tetanic force measurements compared to control. Finally, it appeared that twitch force improved due to neurotization in the same manner as DMM graft alone with a sudden increase in force at week



8. This suggests tibial nerves did not adequately contribute to muscle fiber activation. Additional measurements were also determined such as $\frac{1}{2}$ relaxation time, max rate of contraction, time to $\frac{1}{2}$ max contraction, and force-time integration. Data are not shown however to preserve space, but those data will be reported in the final manuscript.

Figure 27. Representation of measured twitch and tetanic force over 8 weeks as compared to control leg. Dashed bar represents the average control leg. Note that empty defect legs while they did not display a significant reduction in force production, force did not change over time.

Subtask 3: Examine immunohistochemical stains for proteins that regulate calcium flux.

Based on our findings from the first immunohistochemical stained muscle sections we believed this examination of RyR and SERCA staining to be redundant information. In addition, if muscle was contracting at all in the injury site then we could assume that calcium was being regulated.

Subtask 4: Determine protein levels for RyR and SERCA using Western blot.

We used RT-qPCR to assess levels of RyR and SERCA in samples from neurotized and non-neurotized animals.

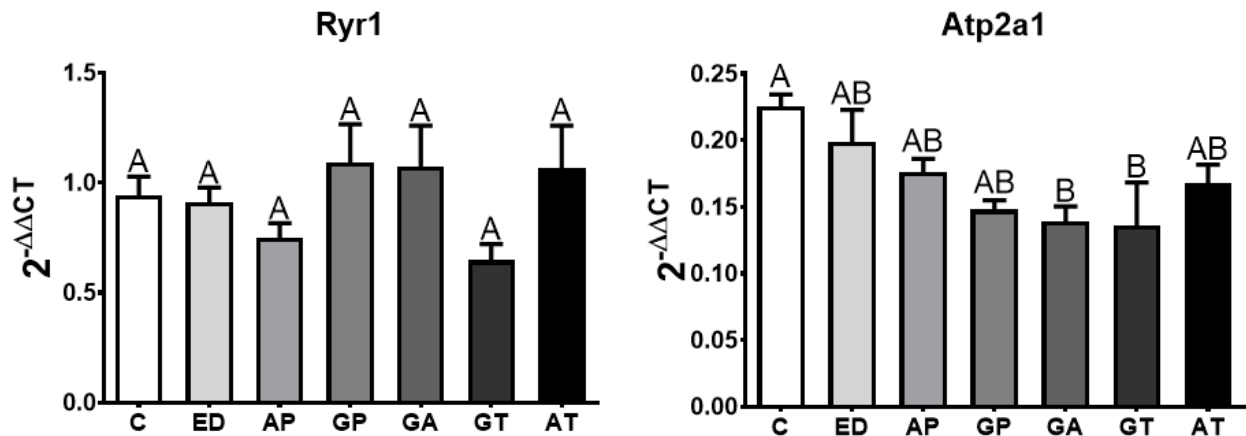


Figure 28. No change in RyR expression levels and SERCA expression decreased more in DMM treated VML sites compared to autograft treated sites. Neurotization did not appear to affect SERCA expression.

Milestone #3: Manuscript on neurotization of muscle grafts and its effect on muscle function. This milestone is in process with a manuscript being prepared for publication in Nature Bioengineering.

Conclusions: Collectively these data indicate that DMM and autograft implants integrate into the injury site very well and are able to support some new muscle fiber growth; however, other data also indicate these graft areas are denervated. NCAM staining indicated that DMM and autograft implants are denervated while Nanostring showed that an upregulation of muscle markers for regeneration. Furthermore, neurotization studies were successful for both tibial and peroneal nerve surgeries. Peroneal nerve surgeries were less time consuming and implanted a nerve directly from a “live” nerve. In contrast, tibial nerve surgeries were more time consuming since a harvested nerve had to be sutured to the tibial nerve. In addition, increases in force production were delayed in tibial nerve animals since it takes longer for axons to connect from the tibial host nerve to the recipient nerve. Nanostring analysis demonstrated that tibial nerve treated VML sites showed the best results in terms of muscle regeneration markers, inflammation markers, and neural regeneration markers. This was confirmed in histological assays where we qualitatively observed improved tissue appearance. Quantitative measurements are ongoing and will be completed by the end of September. Overall, our results demonstrated that neurotization treatments for VML show promising results, and that tibial nerve treatments were the most optimal by comparison. Yet, it remains unclear what exactly causes the improvements. Moreover, future research should investigate other methods to encourage axon growth in addition to vascular growth into the VML treated site. Our findings could potentially shift the paradigm of VML treatment strategies, where prevalent denervation needs to be treated appropriately or fibrosis and fatty tissue infiltrate will continue to develop within zones of injury/repair.

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.

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?

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.

Information resulting from this project was not explicitly disseminated to the public in outreach programs. However, we did take advantage of VCU outreach programs that involve high school students within the City of Richmond and Henrico County areas. In these programs we discussed the process of neurotization with students from a very broad perspective along with its application to bioengineering problems.

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

This is the final reporting period. We are actively applying for additional funding from PRORP and we are expanding on this research.

- 4. IMPACT:** *Describe 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).

The impact of this project 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. IHC staining has also confirmed that neurotization treatment encourages more mature muscle fibers to develop with evidence of innervation.

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.

Neurotization is a surgical technique that has been used in the area of peripheral nerve surgery, but its use is far less than nerve transfers. This is because it requires a donor site which may not be an available option. One of the areas that we are working on is to determine other bioengineering strategies that would allow for the use of an acellular nerve graft that is connected to an existing nerve via an end-to-side nerve transfer. This would donate axons to the acellular graft and allow for a “new” peripheral nerve to regenerate. As part of this technology development, we are testing a new electrical stimulation device that could accelerate axon growth and reduce the time needed to grow a new nerve.

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.*

We have perfected our decellularized muscle matrix (DMM) and applied for a provisional patent during the course of this grant period. In addition, a company (Sarcogenics, LLC) was developed recently using to begin testing DMM and optimizing it for use in muscle injuries. It should be noted though that no expenses for DMM optimization were used from the money provided in this contract. DMM optimization expenses came from the Junior Investigator Grant from MTF Biologics.

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.

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.

Significant changes in use or care of human subjects

No human subjects are used in this study.

No significant changes.

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).*

Olson LC, Redden JT, Gilliam L, Nguyen T, Vossen JA, Cohen DJ, Schwartz Z, **McClure MJ**. Human adipose-derived stromal cells delivered on decellularized muscle improve muscle regeneration and regulate RAGE and P38 MAPK Bioengineering. 2022. *In press.*

Olson LC, Redden JT, Schwartz Z, Cohen DJ, ***McClure MJ**. Advanced Glycation End-Products in Skeletal Muscle Aging. *Bioengineering*. 2021. Acknowledged federal support.

***McClure MJ**, Olson LC, Cohen DJ, Ramey AN, Zhang S, Boyan BD, Schwartz Z. Foxn1^{RNU} Rats Demonstrate an Improved Ability to Regenerate Muscle in a Volumetric Muscle Injury Compared to Sprague Dawley Rats. 2021. *Bioengineering*. Acknowledged federal support.

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).*

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. Redden JT, Deng J, Cohen DJ, Olson LC, Boyan BD, Schwartz Z, **McClure MJ**. Muscle-Nerve Interaction Differentially Alters Extracellular Matrix Remodeling between Two Models of Paralysis. Society for Biomaterials. April 27-30, 2022. Baltimore, MD.
2. Redden JT, Cohen DJ, Olson LC, Krebs L, Bendale G, Isaacs JE, Schwartz Z, **McClure MJ**. Neurotization of Decellularized Muscle Matrix Improves Functional Recovery and Promotes Unique mRNA Profiles in a Volumetric Muscle Loss Model. Society for Biomaterials. Virtual. April 20-23, 2021.
3. Olson LC, Cohen DJ, Vossen JA, Huang Y, Imming EA, Boyan BD, Schwartz Z, **McClure MJ**. Stem cells delivered on decellularized muscle to support muscle regeneration. International Association of Dental Research. March 18-21, 2020. Washington, DC.
4. Olson LC, Cohen DJ, Boyan BD, Schwartz Z, **McClure MJ**. The Immunodeficient State of Athymic Rats Enhances the Ability of Decellularized Muscle Matrix to Support Regeneration in a Volumetric Muscle Injury. Military Health System Research Symposium. Orlando, FL. August, 2019

- **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”.

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: 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.

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.*

- 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.*