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14. ABSTRACT
Extensive damage to skeletal muscle is a clinically challenging problem both soldiers and civilians, alike. Extensive skeletal muscle injuries due to trauma, congenital defects, or diseases result in functional impairment with severe physical deformity. In addition, in many cases, muscle defects involve nerve damage, which leads to another clinical problem; i.e. denervation, atrophy and permanent functional disability.The standard of care is an autologous muscle flap transfer. However, host muscle tissue availability and donor site morbidity often complicate implementation of this strategy. Tissue-engineered muscles represent an alternative means to repair volumetric muscle defects. Although these tissue-engineered muscles have provided potential solutions to regenerate contractile muscle tissue *in vivo*, the issues related to peripheral nerve regeneration and innervation have not been adequately addressed to date. In this project, we aim to develop an implantable bioprinted engineered muscle prototype with pre-formed neuromuscular junctions (NMJs) and an integrated neurotrophic factor controlled release system. These technologies are intended to accelerate integration with host peripheral nerve to achieve rapid functional muscle restoration. We will develop a 3-dimensnional (3D) bioprinted muscle construct prototype with NMJ pre-formation and neurotrophic factor controlled release to promote rapid nerve integration and innervation of muscle constructs for functional restoration. The muscle construct prototype will be validated both *in vitro* and *in vivo*, in terms of muscle regeneration, peripheral nerve regeneration, innervation, and clinical feasibility for restoring muscle function. We will optimize the prototype and develop manufacturing processes, standard operating procedures (SOPs) and batch records (BRs) to support an FDA Pre-IND application as a step towards clinical deployment.

15. SUBJECT TERMS

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Annual Technical Status Report for

Accelerated Innervation of 3D Bioprinted Muscle Construct with Pre-Fabricated Neuromuscular Junctions and Neurotrophic Factor Release System Research Research

Project No. 2017-614-002

EGS# MT180011

Reporting Period: 10/01/2020 – 09/30/2021

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1. Project Status

a. Accomplishments

i. Milestone completion

1. Task 1.1/D1.1 Determine the effects of neurotrophic factors on skeletal muscle development and acetylcholine receptor (AChR) cluster pre-formation on the bioprinted muscle construct (20% completion during this reporting period, 100% total completion).
    a. Establishing the optimal load of poly-lactic-co-glycolic-acid (PLGA) microspheres into fibrinogen-based bioink and characterizing the viscoelastic properties
    b. Characterizing the morphology of the fibrin-based hydrogels containing PLGA microspheres using scanning electron microscopy

2. Task 1.2/D1.2 Demonstrate neuromuscular junction (NMJ) formation and innervation on the bioprinted skeletal muscle constructs *in vitro* (20% completion during this reporting period, 100% total completion)
    a. Evaluating the biological activity of ciliary neurotrophic factor (CNTF) and glial cell line-derived neurotrophic factor (GDNF) released from PLGA microspheres embedded into a fibrin hydrogel in an *in vitro* assay
    b. Performing an *in vitro* assay to evaluate the effect of neurotrophic factors incorporated into bioprinted muscle constructs on neurite outgrowth and NMJ formation.
    c. Characterizing the effect of CNTF and GDNF on human muscle progenitor cell (hMPC) survival, growth, and differentiation.

3. Task 1.3/D1.3 Evaluate peripheral nerve integration and NMJ formation of the bioprinted skeletal muscle constructs in a rat transposed ectopic nerve model (43% completion during this reporting period, 90% total completion)
    a. Performing implantation surgeries and tissue harvesting in a pilot *in vivo* study to investigate the effects of incorporated neurotrophic factors on peripheral nerve integration into the bioprinted muscle constructs
    b. Performing electrophysiological assessment of the integration of the transposed nerve into the implanted muscle construct
    c. Performing sample processing and histological/immunohistochemical analysis of the harvested tissue samples

4. Task 2.1/D2.1 Create a nerve-muscle injury model in an immunocompromised rat (86% completion during this reporting period, 86% total completion)
    a. Performing implantation surgeries and tissue harvesting

5. Task 2.2/D2.2 Evaluate structural and functional recovery with NMJ formation (15% completion during this reporting period, 15% total completion)

a. Preparing materials and supplies to initiate the *in vivo* studies

ii. Presentations at conferences

1. Aurelia Poerio et al. Sustained release of neurotrophic factors for the innervation of 3D bioprinted skeletal muscle constructs. 5th International Bio-Inspiration N.I.C.E Conference, October 12 – 14, 2020, Nice, France.
2. John Jackson et al. Accelerated Innervation of 3D Bioprinted Muscle Constructs with Pre-Fabricated Neuromuscular Junctions and Neurotrophic Factor Release System, 2021 AUA Annual Meeting, September 10—13, 2021, Las Vegas, NV.
3. Vladimir Mashanov et al. Developing an Approach for Efficient Innervation of Bioengineered Skeletal Muscle Constructs. International Conference on Biofabrication, September 27 – 29, 2021, University of Wollongong, Australia.

b. Reportable Outcomes

i. The optimal load of PLGA microspheres carrying the neurotrophic factors was established (5 mg of microspheres per 1 ml of bioink). The microspheres did not significantly change the viscoelastic properties of the bioink used to manufacture the muscle constructs. The microsphere-laden bioink is thus compatible with bioprinting.

ii. The incorporation of PLGA microspheres does not disrupt the internal porous microstructure of the fibrin hydrogels.

iii. The neurotrophic factors CNTF and GDNF released from the PLGA microspheres into the fibrin hydrogel retain their biological activity, as demonstrated in an *in vitro* assay using chick embryo dorsal root ganglia (DRG) as an experimental model.

iv. An *in vitro* study was initiated to characterize the effect of CNTF and GDNF-encapsulated PLGA microspheres incorporated into bioprinted muscle constructs on neurite outgrowth from chick embryo DRGs. Microscopic analysis showed that CNTF and GDNF released from the bioprinted muscle construct enhance neurite growth from the DRGs. After two days, the quantitative analysis showed that the bioprinted construct containing neurotrophic factors without microspheres showed an increased neurite outgrowth compared to the construct (without microspheres and neurotrophic factors) and the construct containing neurotrophic factors factor-loaded microspheres. We infer that the initial burst release of the neurotrophic factors from the construct without microspheres enhanced the neurite outgrowth compared to the construct containing CNTF and GDNF encapsulated in microspheres at this short-term time point (2 days). Evaluation of the later time points needs to be analyzed to validate the effects of controlled delivery and sustained release of neurotrophic factors using microspheres. The assay has been extended to quantify the neurite outgrowth at later time points (7 days and 14 days).

v. Exposure of hMPCs to CNTF and GDNF at the dosage used in the biofabrication of skeletal muscle constructs does not hinder their proliferation, viability, and differentiation.

vi. In a pilot *in vivo* study of a rat transposed ectopic nerve defect and implantation, all implantation surgeries were completed for the 4-week, 8-week, and 12-week time points. Tissue collections and histological and immunocytochemical analysis are currently underway. Preliminary measurements performed at the 4-week time point show that incorporating CNTF and GDNF into the implanted constructs induced higher neurite sprouting than the control constructs (no neurotrophic factors). At the 4-week time point, the electrophysiological assessment did not show any significant variation in the amplitude of the compound muscle action potential (CMAP) among the experimental groups (i.e., constructs with or without neurotrophic factors). The study was thus extended to later time points (8- and 12-week) with the expectation that a complete functional integration between the sprouting host axons and implanted muscle cells is more likely to develop.

vii. We have established a pre-clinical model of a complex neuromuscular injury using rats. Model creation and implantation surgeries have been completed. The surgical procedures, reliability, and animal health issues have been validated in preliminary tests. Tissue harvesting and electrophysiological data collection are currently in progress. We have started preparing materials and supplies to initiate *in vivo* feasibility studies using the established animal model.

c. Progress Detail

i. Viscoelastic properties of the fibrinogen-based bioink loaded with PLGA microspheres

One of the crucial factors for the functional maturation of the implanted bioengineered muscle construct is its proper innervation. Therefore, it is critical to facilitate the host peripheral nerve growth and migration towards the myotubes within the implanted muscle construct *in vivo*. To enhance the host neural growth and migration, a bioengineered muscle construct capable of sustained delivery of neurotrophic factors is necessary. To this end, we have previously developed and reported a methodology to fabricate PLGA-based microspheres for the controlled and sustained release of neurotrophic factors.

During this reporting period, we tested the viscoelastic properties of the microspheres-loaded bioink and its suitability for bioprinting. Viscosity is one of the most critical characteristics of the bioink that affects its printability and cell viability in the biomanufactured construct. The effect of incorporating the PLGA microspheres on the viscoelastic properties of the acellular bioink is shown in **Fig. 1A–C**. In a shear sweep test **(Fig. 1A)**, the bioink samples loaded with 5 and 10 mg/mL of microspheres showed the same shear-thinning dynamics (i.e., decreasing viscosity under increasing shear rates) as the microsphere-free bioink (i.e., bioink without microspheres).

However, at any given stress value, higher loads of microspheres affect the viscosity characteristics of the bioink-microsphere composites. For example, there is a clear variation in the average viscosity among the three tested formulations (0, 5, and 10 mg/mL) at the shear rate of 0.1 s^{-1} (One-way ANOVA, $F(2,6) = 27.84$, $p = 9.21\times10^{-4}$) **(Fig. 1B)**. The microsphere-laden bioink with the higher microsphere content (10 mg/mL) showed a significantly higher viscosity in comparison with both the microsphere-free bioink ($p = 8\times10^{-4}$) and the microspheres-laden bioink with the lower (5 mg/ml) microsphere content ($p = 5\times10^{-3}$). Notably, the viscosity levels of the microsphere-laden bioink with the lower microsphere content (5 mg/mL) were not statistically different from those of the microsphere-free bioink.

Elasticity is another property that affects the behavior of the encapsulated cells and the interactions between the cells and the extracellular matrix. A standard metric used to characterize the elastic behavior is the storage modulus *(G^0)*, a measure of the elastic or recoverable energy in the sample. One-way ANOVA detected a significant variability in the storage modulus among different formulations of the hydrogels ($F(2,6) = 12.45$, $p = 7\times10^{-3}$). The hydrogels containing the higher microsphere load (10 mg/mL), but not the lower load (5 mg/mL), had a significantly ($p = 6\times10^{-3}$) higher storage modulus than the microsphere-free hydrogels **(Fig. 3C).** This result suggests that microspheres at high concentrations increase the elasticity of the hydrogel-microsphere composite.

Taken together, these data indicate that the higher microsphere load (10 mg/mL) significantly changes the viscosity of the bioink and the elasticity of the resulting cross-linked fibrin hydrogel. On the other hand, the properties of the microsphere-laden formulations with the lower load (5 mg/mL) remain similar to those of the microsphere-free bioink/hydrogel. We previously demonstrated that the bioprinted muscle constructs containing 5 mg/mL of microspheres support the viability and proper development of muscle progenitor cells. Therefore, we used this lower microsphere load (5 mg/mL) in all subsequent experiments.

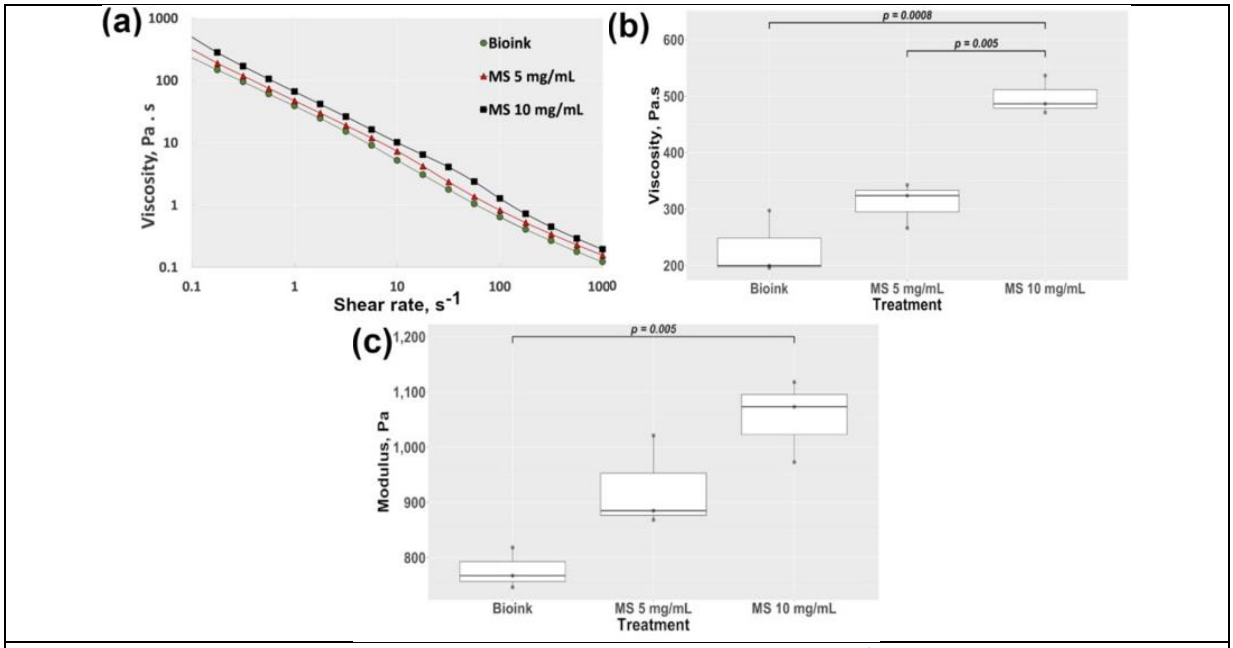

Figure 1. Viscoelastic properties of the microsphere-free and microsphere-laden bioink/hydrogels. (a) Shear viscosity of the microsphere-free bioink (circles) and bioink loaded with 5 mg/mL (triangles) or 10 mg/mL (squares) of microspheres. (b) Average viscosity of the bioink formulations at a share rate of 0.1 s^{-1}. (c) The storage modulus of the cross-linked hydrogel samples. *Bioink* – microsphere-free bioink; *MS 5 mg/mL* – bioink containing 5 mg/mL of microspheres; *MS 10 mg/mL* – bioink containing 10 mg/mL of microspheres.

ii. Microarchitecture of the fibrin-based bioink loaded with PLGA microspheres

To determine how microspheres interact with the cross-linked fibrin hydrogel at the structural level, we compared microscopic characteristics of both the microsphere-free and microsphere-laden fibrin hydrogels. The microsphere-laden hydrogels preserve the highly porous structure characteristic of fibrin hydrogels (compare **Fig. 2A and B**), which provides an interconnected network essential for cell nutrition, proliferation, and migration. Furthermore, the microspheres embedded in the hydrogel appear to be firmly attached to the fibrin network, supporting our strategy to use them as a neurotrophic factor delivery system.

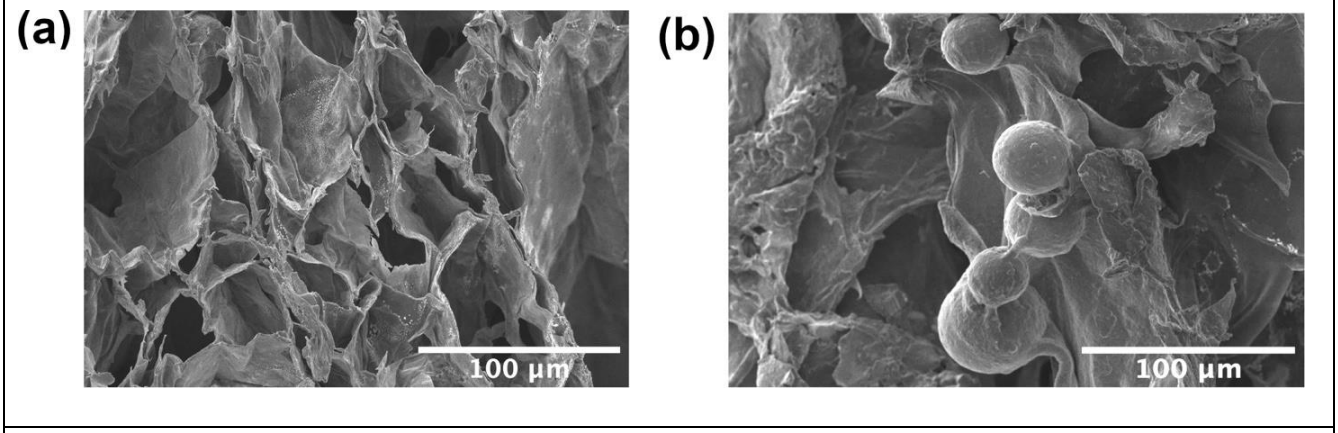

Figure 2. Microarchitecture of the microsphere-free and microsphere-laden bioink/hydrogels. Scanning electron microscopy (SEM). **(a)** Cross-linked microsphere-free hydrogel. **(b)** Microsphere-laden (10 mg/mL) hydrogel.

iii. Neurotrophic factors released from microspheres embedded in a fibrin hydrogel retain their biological activity

We previously demonstrated in a gradient assay that the combination of two neurotrophic factors - CNTF and GDNF - facilitates the growth and migration of nerves to the source of the chemical gradient. In this reporting period, we investigated whether these neurotrophic factors would retain their biological activity during their sustained release from the PLGA microspheres.

We designed the following *in vitro* neurite growth assay involving embryonic chick DRGs as a neural tissue source cultured on acellular microsphere-free and microsphere-loaded hydrogels with or without the CNTF/GDNF combination (Fig. 3). To prepare the hydrogels, 40 µm cell strainers were placed into wells of a 6 well plate and filled with cross-linked acellular fibrin bioink (hydrogel). The DRGs were extracted from stage 33 (~E8) chick embryos and then positioned on top of the hydrogel.

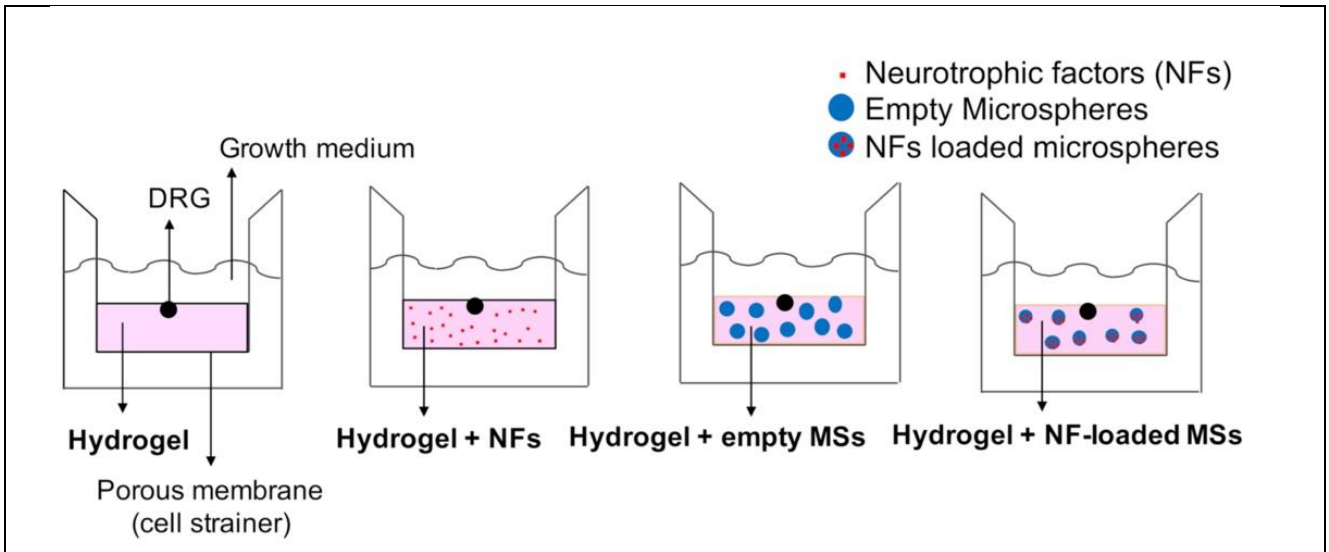

Figure 3. Design of the DRG neurite growth assay: diagram of the experimental setup. Four preparations were involved: the control hydrogel *(Hydrogel)*, the hydrogel with freely dissolved neurotrophic factors *(Hydrogel + NFs)*, the hydrogel containing suspended empty microspheres *(Hydrogel + empty MSs)*, and the hydrogel containing suspended microspheres loaded with the neurotrophic factors *(Hydrogel + NF-loaded MSs)*. The hydrogels were cast into inserts manufactured from 40 µm cell strainers, which were then placed into wells of a 6-well plate containing the culture medium

The assay involved four different hydrogel formulations:

(a) Hydrogel only (no neurotrophic factors (NFs) and no microspheres (MSs)) (negative control),
(b) Hydrogel + NFs (NFs mixed directly into the hydrogel without MSs) (positive control),
(c) Hydrogel + empty MSs (MSs (5 mg/mL) without NFs),
(d) Hydrogel + NF-loaded MSs (5 mg/mL of CNTF/GDNF-loaded MSs).

The amount of the neurotrophic factors in groups (b) and (d) was the same and determined based on the encapsulation efficiency of 75%. The plates were incubated at 37˚C for 2, 7, and 14 days, with the medium being changed every other day. The samples were then fixed in 10% neutral buffered formalin and immunostained with an anti-neurofilament heavy polypeptide antibody to visualize the neurites growing from the DRGs **(Fig. 4)**.

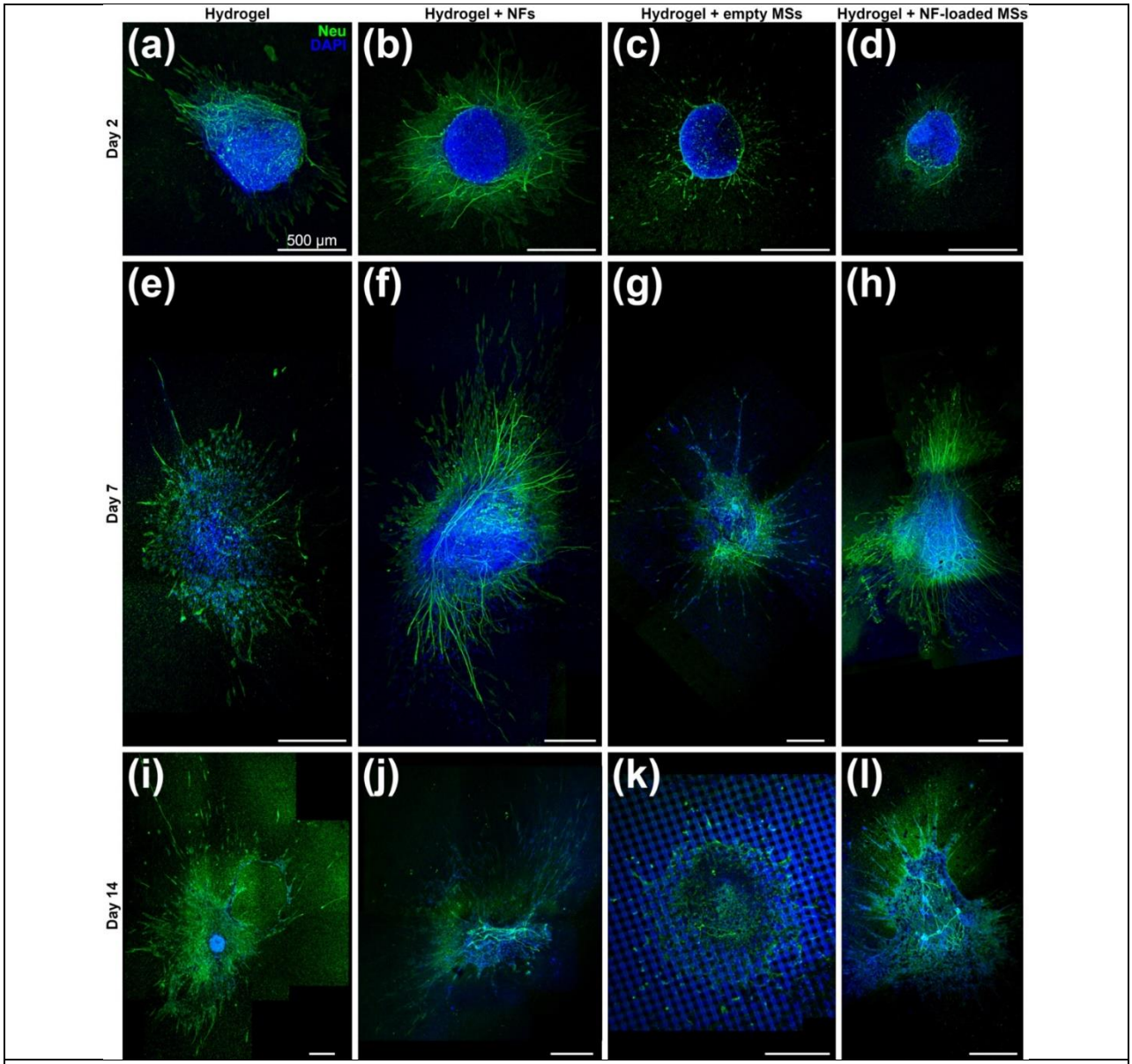

Figure 4. Representative micrographs of DRGs in the *in vitro* neurite outgrowth assay. Four treatment groups were involved: microsphere-free hydrogel with no NFs *(Hydrogel)* (a, e, i); microsphere-free hydrogel with CNTF and GDNF directly mixed into the bioink *(Hydrogel + NFs)* (b, f, j); hydrogel with empty microspheres *(Hydrogel + empty MSs*); and hydrogel containing CNTF/GDNF-loaded microspheres *(Hydrogel + NF-loaded MSs)* (d, h, l). The DRGs were fixed at three time points: day 2 (a – d), day 7 (e – h), and day 14 (i – l). The DRGs were stained with antibodies recognizing neurofilaments *(NF, green)*. Nuclei were stain with DAPI *(blue)*. All images are maximum intensity Z-projections of confocal stacks.

The neurite outgrowth was quantified using a range of growth parameters **(Fig. 5)**, including:

(1) the total neurite outgrowth,
(2) average neurite length,
(3) longest neurite length,
(4) number of neurites per ganglion.

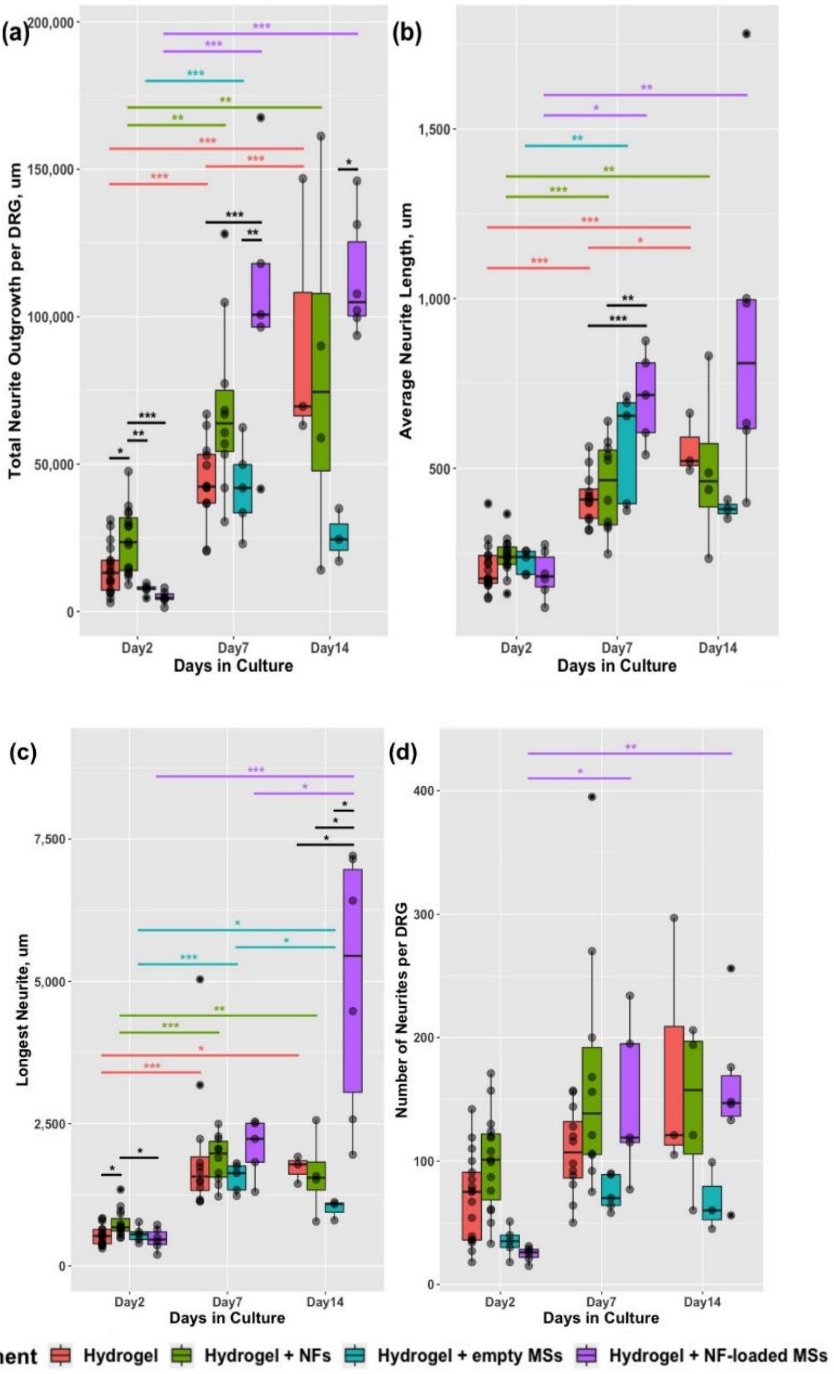

Figure 5. Quantitative effect of the CNTF/GDNF either directly dissolved in the hydrogel *(Hydrogel + NFs)* or encapsulated in PLGA microspheres *(Hydrogel + NF-loaded MSs)* on the DRG neurite outgrowth. The two control cohorts involved the microsphere-free hydrogel with no NFs *(Hydrogel)* and the hydrogel loaded with empty microspheres *(Hydrogel + Empty MSs)*. (a) Total neurite outgrowth per ganglion. (b) Average neurite length. (c) Length of the longest neurite in DRGs. (d) The number of neurites per ganglion. The significance level annotations are shown in black for the treatment cohorts at each time point and in the respective color for each cohort across the time points.

The statistical analysis detected a significant effect of the treatment on the above growth metrics. On day 2, the DRGs in group b (Hydrogel + NFs) significantly surpassed the other three groups in the total neurite outgrowth **(Figs. 4A - D, 5A)**. In addition, the longest neurites in this group were significantly longer than in group a (Hydrogel only) and group d (Hydrogel + NF-loaded MSs) **(Fig. 5C)**. Notably, no difference between group a (Hydrogel only) and group d (Hydrogel + NF-loaded MSs) was observed in terms of neurite outgrowth at this early time point **(Figs. 4D, 5)**.

On day 7, the DRGs in group d (Hydrogel + NF-loaded MSs) started to surpass the other three groups in the growth metrics. The total neurite outgrowth in this group was significantly more extensive than in group a (Hydrogel only) and group c (Hydrogel + empty MSs). The total outgrowth in the Hydrogel + NF-loaded MSs group (group d) also showed a trend (p = 0.078) to be higher than in group b (Hydrogel + NFs) **(Figs. 4E – H, 5A)**. The average neurite length in this group also significantly increased compared to that in groups a (Hydrogel only) and b (Hydrogel + NFs) **(Fig. 5B)**.

On day 14 **(Fig. 4I – L)**, the total outgrowth in the Hydrogel + NF-loaded MSs group (group d) was significantly higher than in the Hydrogel + empty MSs group (group c) **(Fig. 5A)**. This group also surpassed the other three groups in the maximum neurite length **(Fig. 5C)**.

In addition to the treatment effect on the growth metrics at individual time points, the two-factor ANOVA test also showed a significant variation in temporal growth dynamics among the treatment groups. Although all four cohorts experienced an increase in the total neurite outgrowth and average neurite length between day 2 and day 7 **(Fig. 5A, B)**, the temporal growth pattern of the DRGs in the Hydrogel + NF-loaded MSs (group d) was distinct from that of the other groups. Only this group showed a significant increase in neurite number over time **(Fig. 5D)** and also an increase in the maximum neurite length over the later phase of the experiment (between days 7 and 14) **(Fig. 5C)**.

Taken together, these data suggest that the NFs directly dissolved in the hydrogel (group b, Hydrogel + NFs) accelerate the early neurite outgrowth. In contrast, NFs delivered in the PLGA microspheres (group d, Hydrogel + NF-loaded MSs) facilitate neurite growth over a longer time scale and significantly increase the maximum neurite length and the number of neural processes per ganglion. Thus, our results demonstrate the utility of PLGA microspheres as a delivery vehicle for neurotrophic factors, as they are capable of efficiently encapsulating bioactive compounds and releasing them in a sustained manner over an extended period without loss of biological activity.

iv. Effect of neurotrophic factors embedded in bioprinted muscle constructs on neurite growth

After optimizing and demonstrating that the microsphere-based neurotrophic factor delivery system facilitates neurite growth when incorporated into acellular hydrogels, we then proceeded to confirm the suitability of this system for incorporation into cellularized bioprinted skeletal muscle constructs. Here, we employ a neurite growth assay, in which chick embryonic DRG explants are cultured on top of biomanufactured muscle constructs containing hMPC (30 million cells per mL of fibrin hydrogel). Before planting the DRGs, pre-formation of AChR clusters was induced in differentiated muscle cells with the agrin treatment.

Our design involves three experimental groups:

(a) Control - constructs containing no neurotrophic factors and no microspheres

(b) NFs - microsphere-free constructs with CNTF and GDNF (0.5 µg/mL each) directly mixed into the hydrogel;

(c) MSs - constructs containing CNTF/GDNF-loaded PLGA microspheres, with the overall load of neurotrophic factors matching that in group b.

The bioprinted muscle constructs and DRGs were co-cultured for 2 days, fixed, and immunostained with anti-neurofilament and anti-AChR antibodies to detect neurites and post-synaptic AChR clusters on muscle cells (Fig. 6).

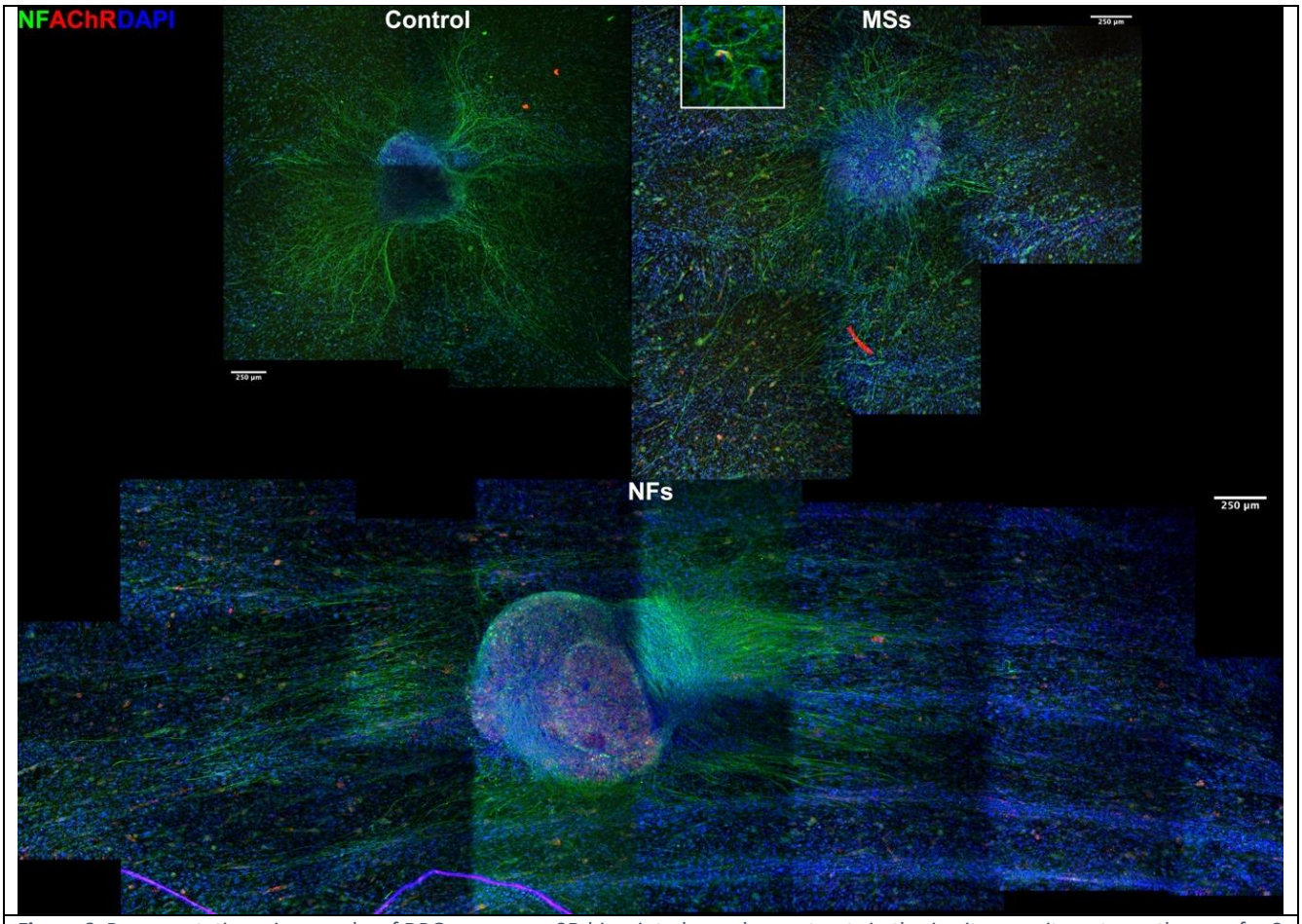

Figure 6. Representative micrographs of DRGs grown on 3D bioprinted muscle constructs in the *in vitro* neurite outgrowth assay for 2 days. Three experimental groups were involved: *Control* constructs containing no neurotrophic factors and no microspheres; constructs containing PLGA microspheres *(MSs)* loaded with CNTF and GDNF; and microsphere-free constructs with CNTF and GDNF *(NFs)* directly incorporated into the fibrin hydrogel. The samples were immunostained with anti-neurofilament *(NF, green)* and anti-AChR *(red)* antibodies. The nuclei were stained with DAPI *(blue)*. All images are maximum intensity Z-projections of confocal stacks.

The following metrics were quantified to assess the neurite outgrowth using the immunofluorescent staining images (Fig. 7):

- Number of neurites per ganglion,
- Total neurite outgrowth
- Average neurite length
- Longest neurite per ganglion

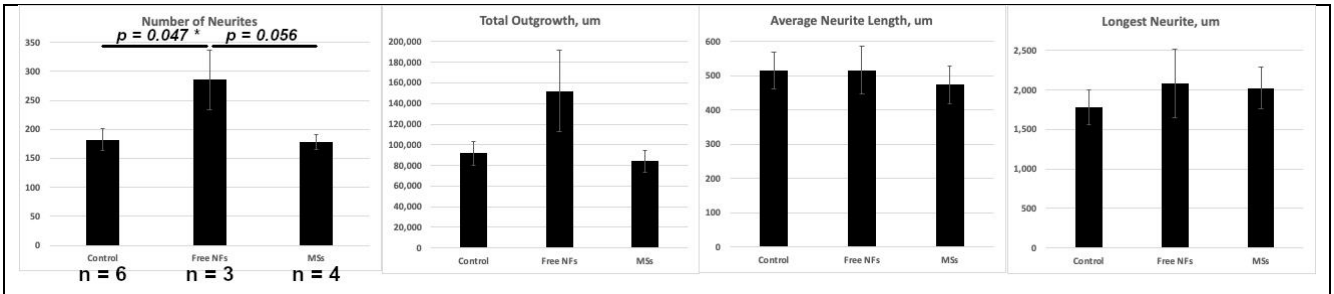

Figure 7. Neurite outgrowth in DRGs culture on 3D bioprinted muscle constructs in the *in vitro* neurite outgrowth assay for 2 days. Three experimental groups were involved: control constructs containing no neurotrophic factors and no microspheres *(Control)*; microsphere-free constructs with CNTF and GDNF directly incorporated into the fibrin hydrogel *(Free NFs)*, and constructs containing PLGA microspheres loaded with the matching amount CNTF and GDNF *(MSs)*.

The quantitative analysis showed that at this early time point, the DRGs grown on the microsphere-free constructs with CNTF and GDNF directly mixed into hydrogel formed a significantly higher number of neurites and had a trend to show a more extensive total outgrowth than the explants in the other two cohorts (control and constructs with neurotrophic factor-laden microspheres). These data are in agreement with our previously reported experiments that involved cell-free hydrogels (see above). Initially, the microsphere-free hydrogels containing freely dissolved neurotrophic factors induced active neurite sprouting at early time points. However, the hydrogels containing the microsphere-based neurotrophic factor delivery system outperformed the microsphere-free hydrogels over more extended periods. We are currently extending this neurite outgrowth assay to later time points, including 7 days and 14 days.

v. Effect of CNTF and GDNF on viability, proliferation, and differentiation of hMPCs

As described above, we have previously established that two neurotrophic factors – CNTF and GDNF – when applied in a 1:1 combination induced directional neurite growth towards the source of the cytokine gradient *in vitro*. We then developed a protocol to fabricate bioprinted muscle constructs containing these neurotrophic factors for subsequent *in vivo* studies in animal models. Even though the data accumulated so far suggest that this combination of neurotrophic factors would facilitate the nerve growth into the constructs, the effect of these signaling molecules on the muscle progenitors has remained unknown. To this end, we initiated a study to test the effect of CNTF and GDNF on hMPCs.

The experimental parameters, including time in culture, base medium composition, and concentration of CNTF/GDNF, matched those established earlier to fabricate the muscle constructs. Four experimental cohorts were involved:

- Control (no neurotrophic factors)
- CNTF (0.5 µg/mL)
- GDNF (0.5 µg/mL)
- CNTF+GDNF (0.5 µg/mL each)

hMPCs (Passage 5) were seeded in 48-well plates, 6 wells per treatment cohort, in the Growth Medium (DMEM/High Glucose with 20% fetal bovine serum, 2% chick embryo extract, 1% penicillin/streptomycin) and incubated overnight. After 24 hours, the medium was replaced with the Differentiation Medium (DMEM/high glucose with 2% horse serum, 1% insulin/transferrin/sodium selenite, 250 nM dexamethasone, 1% penicillin/streptomycin, and 150 ng/mL agrin) with or without the neurotrophic factors, as described above.

To assess hMPCs growth/viability, an alamarBlue assay was performed at days 0, 3, 5, and 8 (Fig. 8). The main conclusion from this experiment is that over 8 days of culture (the time period corresponding to the pre-implantation incubation of the bioprinted constructs), the neurotrophic factors, administered either individually or in combination, did not hinder the growth/viability of the hMPCs, as there was either no statistical difference between the neurotrophic factor-treated and control cohorts, or a slight increase in growth/viability, as in the case of the GDNF-only cohort.

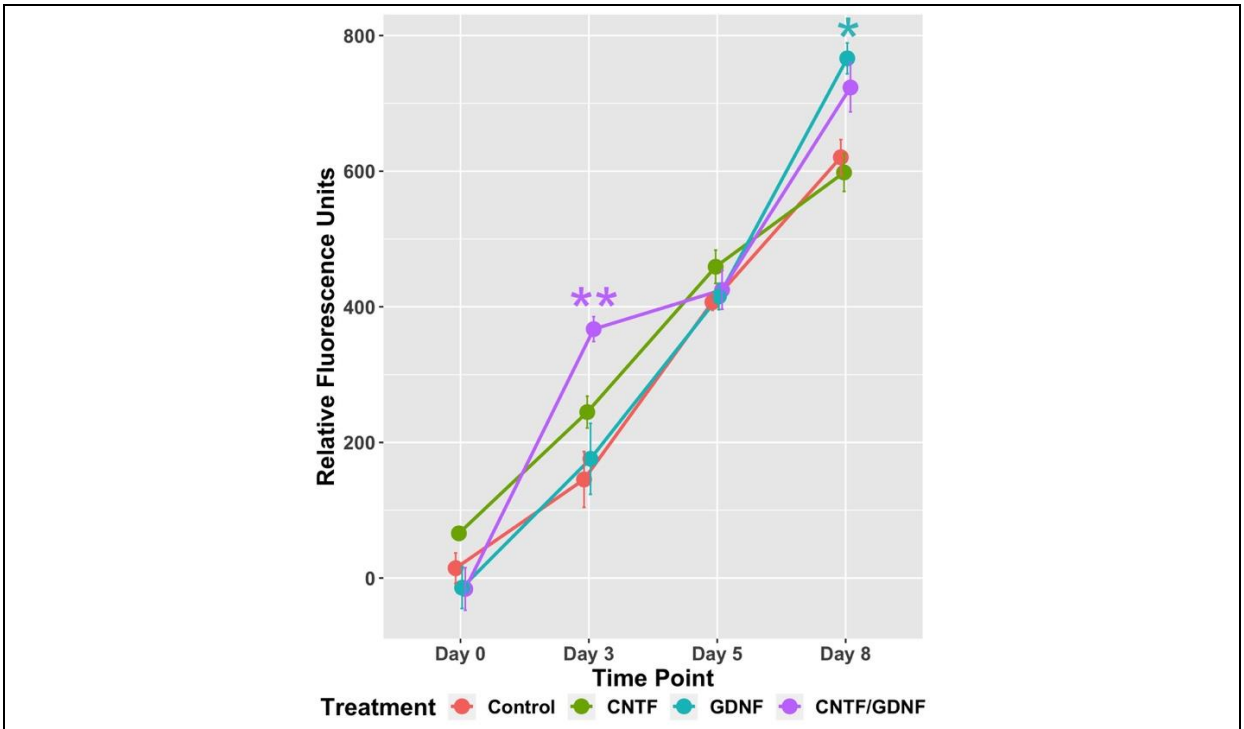

Figure 8. AlamarBlue cell growth/viability assay. hMPCs were incubated in 2D *in vitro* culture in either the base Differentiation Medium (Control) or in the same medium containing CNTF and/or GDNF at 0.5 µg/mL. * $p < 0.05$; ** $p < 0.01$

We next assessed the effect of CNTF and GDNF on hMPC differentiation and ability to pre-from AChR clusters. The cells were seeded and treated as above, and then fixed on day 5 and 8 and processed for immunocytochemistry with an anti-myosin heavy chain (MHC) and anti-AChR antibodies (Fig. 9).

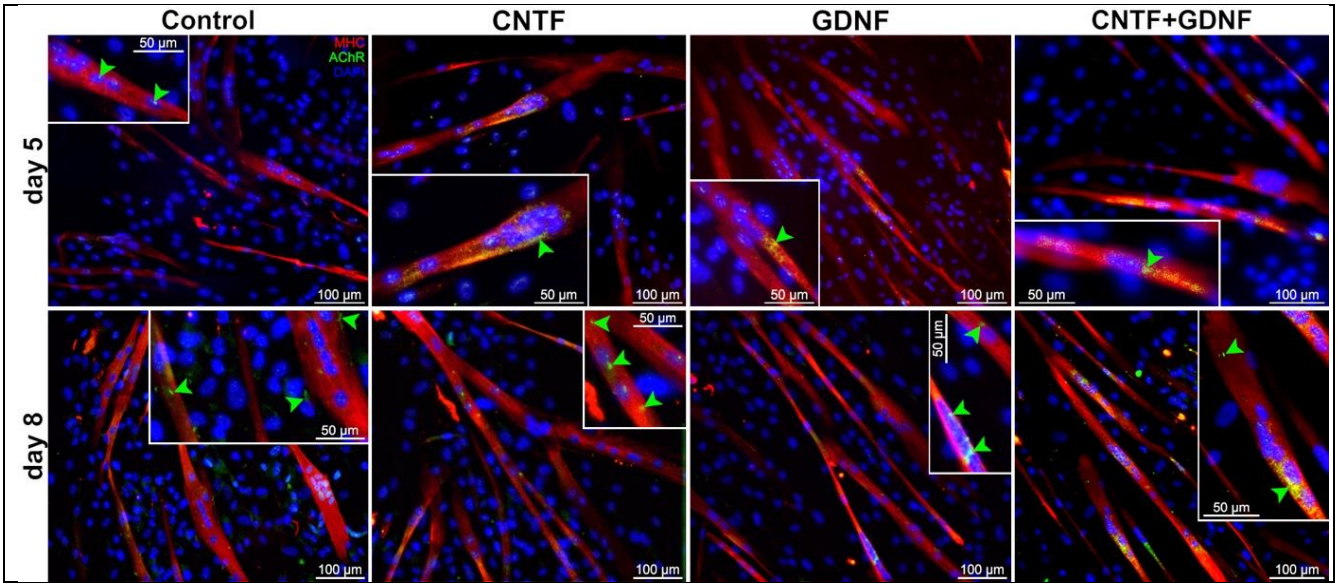

Figure 9. Representative images of hMPCs incubated in 2D *in vitro* culture in either the base Differentiation Medium (Control) or the same medium containing CNTF and/or GDNF at 0.5 µg/mL. The cells were fixed on day 5 and day8 and immunostained with antibodies against myosin heavy chain (MHC, red) and acetylcholine receptors (AChR, green). *Arrowheads* indicate forming AChR clusters.

To assess the muscle progenitor cell differentiation, we quantified three different metrics (Fig. 10):

- the percent of nuclei in MHC-positive cells (relative to the total number of nuclei)
- the relative area occupied by MHC-positive cells
- the area of MHC-positive cells

Statistical analysis (one-way ANOVA or Kruskal-Wallis tests, as appropriate) revealed that despite a significant increase in differentiation metrics over time, there were no statistical differences among the treatment cohorts at either of the two time points. These data, therefore, indicate that the neurotrophic factors CNTF and GDNF, chosen to facilitate the innervation of biomanufactured muscle constructs, did not affect the differentiation of the hMPCs.

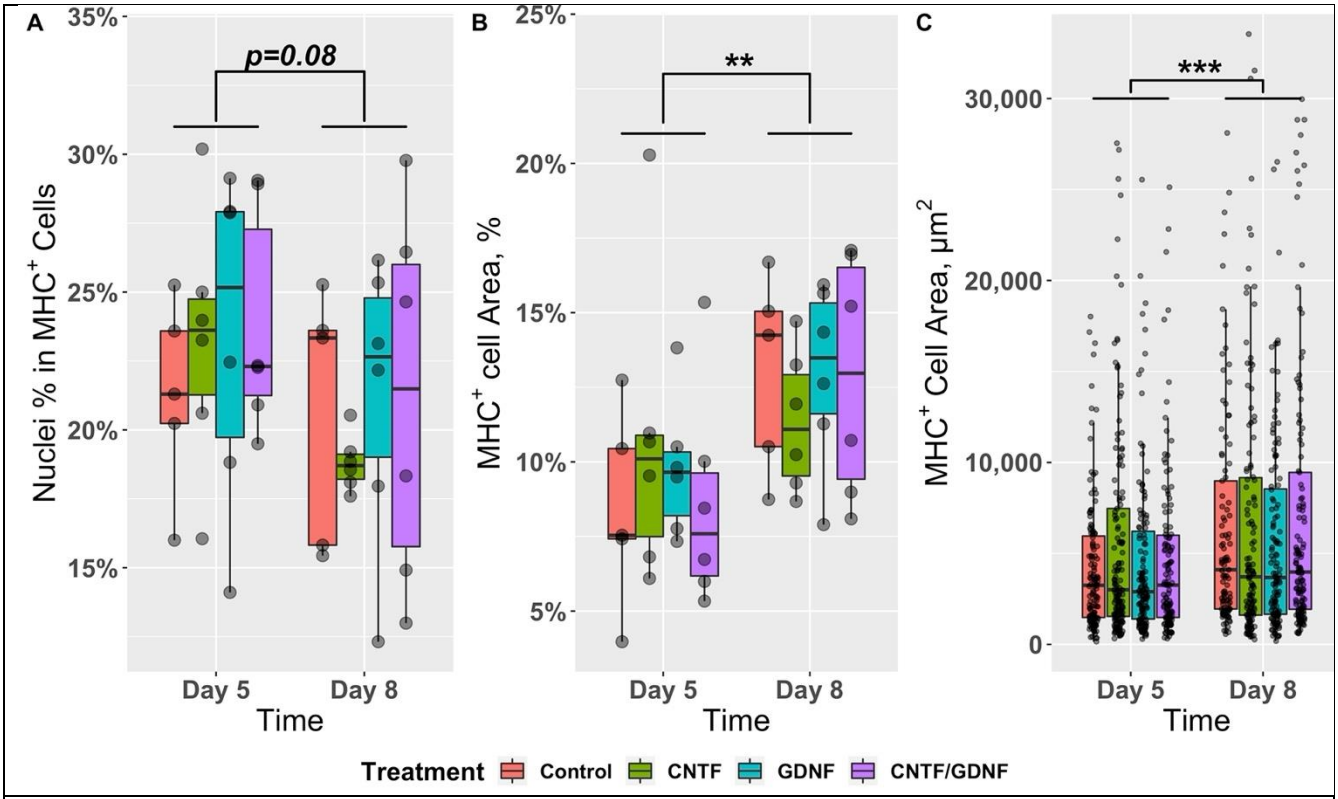

Figure 10. hMPC differentiation metrics in response to treatment with CNTF and/or GDNF at 0.5 µg/mL for 5 or 8 days. (A) Percentage of nuclei contained in MHC-positive cells; (B) Cumulative area occupied by MHC-positive cells relative to the total area of the field of view; (C) Size (area) of individual MHC-positive cells. ** $p < 0.01$; *** $p < 0.001$

To assess the effect of CNTF and GDNF on the efficiency of AChR cluster formation on the differentiated hMPCs, the following metrics were used (Fig. 11):

- AChR cluster area percentage (calculated as a fraction of the AChR cluster area on MHC-positive cells relative to the total area of MHC-positive cells);
- AChR cluster density (calculated as the number of AChR clusters per µm^2 of the MHC-positive cell area);
- Number of AChR clusters per MHC-positive cell

Statistical analysis showed a significant increase in all three metrics in the treatment cohorts over time but no differences among the treatment groups at individual time points. The data suggest that CNTF and GDNF did not affect the ability of the hMPCs to pre-form AChR clusters in the presence of agrin, which was included in the Differentiation Medium.

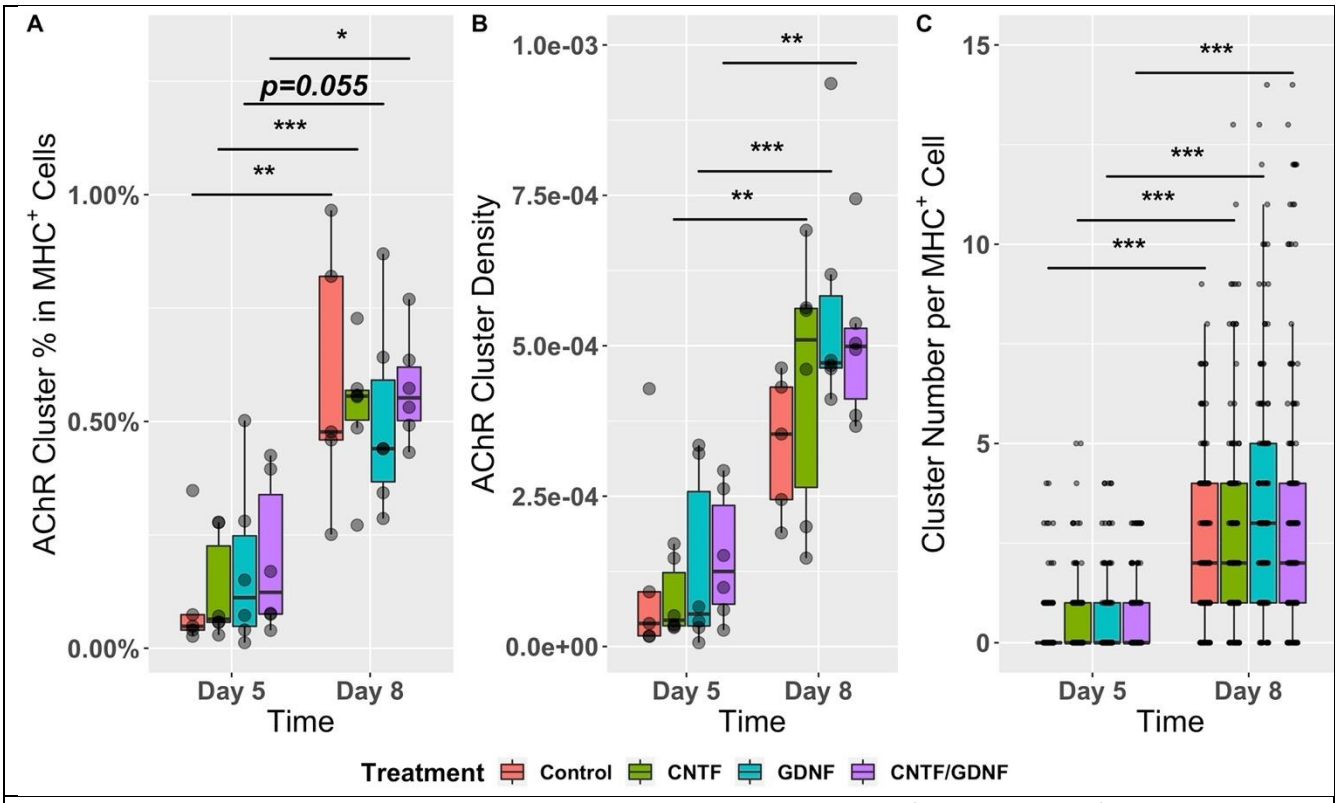

Figure 11. AChR cluster formation metrics metrics in response to treatment with CNTF and/or GDNF at 0.5 µg/mL for 5 or 8 days. (A) AChR cluster percentage area in MHC-positive cells; (B) AChR cluster density MHC-positive cells; (C) Number of AChR clusters in individual MHC-positive cells. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

vi. A pilot *in vivo* study of bioprinted muscle implants with neurotrophic factor-loaded microspheres in an ectopic transposed nerve defect model

We have initiated a pilot *in vivo* study to confirm whether the combination of CNTF and GDNF promotes the host neurite migration and ingrowth into the implanted skeletal muscle constructs in the *in vivo* environment. The bioprinted constructs contain 30 million patient-derived hMPCs per mL of bioink. The pre-formation of AChR clusters on the printed hMPCs was induced by incubating the cross-linked constructs with agrin (150 ng/mL) for 8 days post-fabrication. The study involves three experimental groups:

(a) *Control* - Bioprinted muscle construct without neurotrophic factors

(b) *NFs* - Bioprinted microsphere-free muscle construct with neurotrophic factors (CNTF and GDNF, 0.5 µg/mL) directly dissolved in the bioink

(c) *NF-loaded MSs* - Bioprinted muscle construct with CNTF/GDNF-loaded microspheres (the total amount of the neurotrophic factors matches that in the group (b)

Four animals were used in each treatment group. In all three cohorts, the constructs are wrapped around the distal end of the dissected common peroneal nerve (CPN) and implanted under the fascia of the right gluteus muscle of athymic male rats (bodyweight 250-300 g) **(Fig. 12)**. We are harvesting the tissue samples at three time

points -- 4 weeks, 8 weeks, and 12 weeks post-implantation - and proceed with the histological and immunohistochemical analysis.

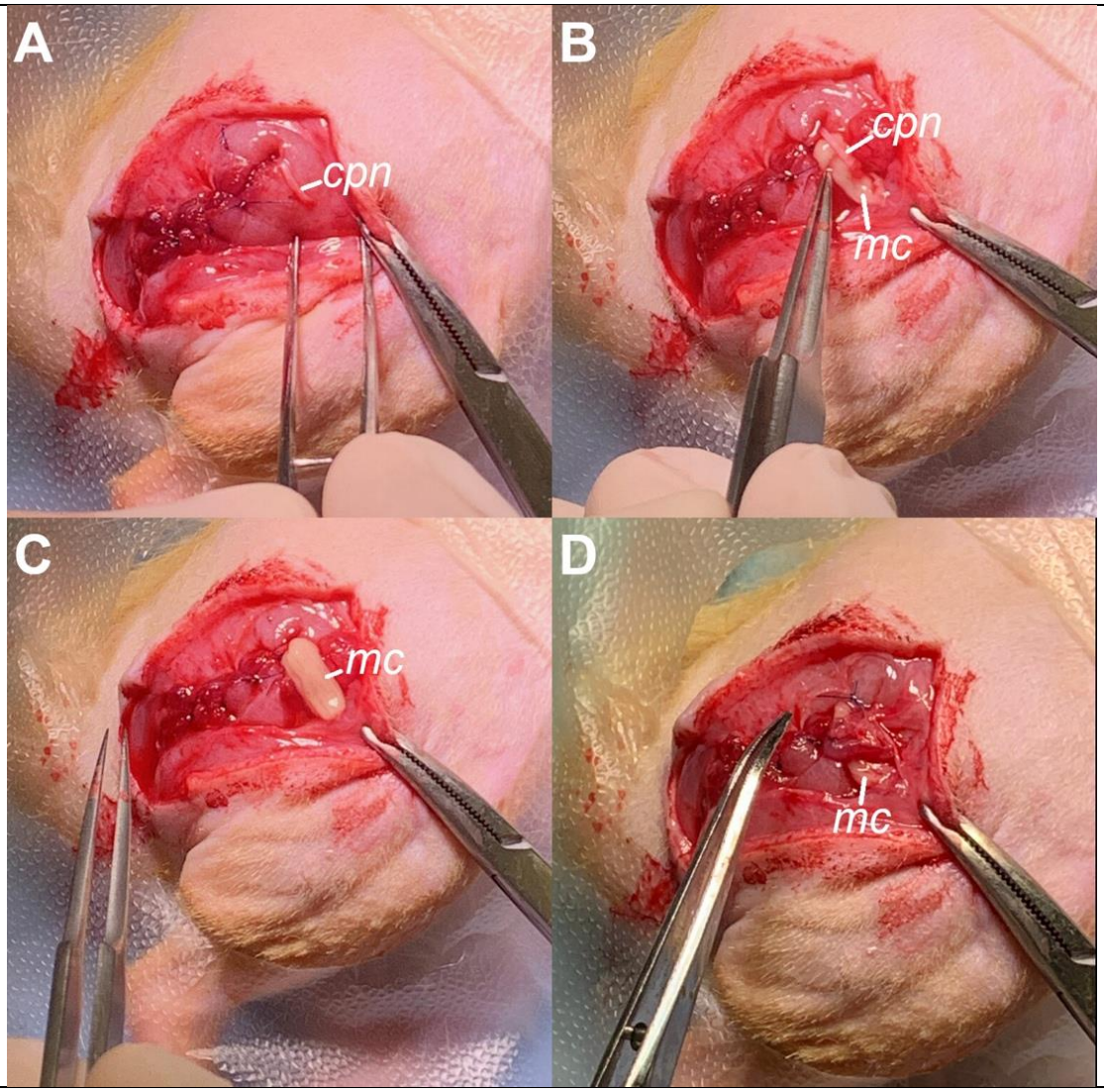

Figure 12. Transposed nerve model, implantation surgery. **(A)** The distal end of the common peroneal nerve *(cpn)* is dissected and placed on the surface of the gluteus muscle. **(B)** The superficial fascia of the gluteus muscle is opened, and one-half of the bioprinted muscle construct *(mc)* is placed on top of the muscle. The distal end of the CPN is placed on top of the construct. **(C)** The distal end of the CPN is overlaid by the second half of the construct so that the nerve is sandwiched between the two layers of the bioprinted muscle construct. **(D)** The implanted construct is covered with the facia, which is sutured in place.

So far, we have been analyzing the tissue samples from the 4 weeks time point **(Fig. 13)**. To quantitatively assess the growth and migration of the host neurites into the implanted muscle constructs, we employed two metrics **(Fig. 14)**:

- the number of neurites per implant area;
- the total length of the sprouting neurites per implant area

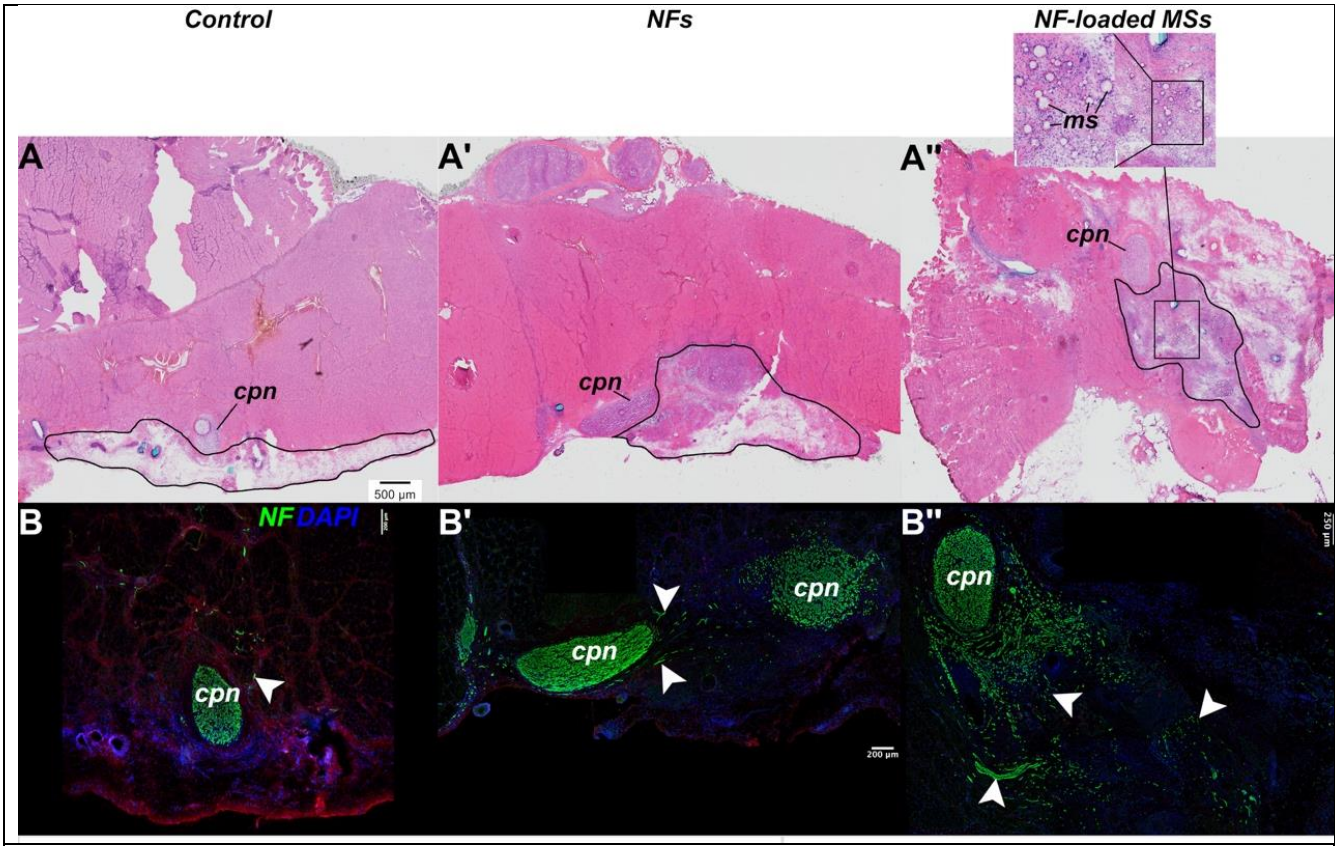

Figure 13. Histological and immunohistochemical analysis of the samples from the transposed nerve study at 4 weeks post-implantation. The control group contains biofabricated muscle implants without any extrinsic neurotrophic factors. The second group *(NFs)* contains CNTF and GDNF freely dissolved in the extracellular matrix of the implant. The third group *(NF-loaded MSs)* contains the matching load of the neurotrophic factors encapsulated in PLGA microspheres suspended throughout the hydrogel of the construct. **(A-A'')** Sections stained with hematoxylin and eosin. The implanted constructs are outlined. **(B-B'')** Sections immunostained with an anti-neurofilament antibody *(green)* to visualize the sprouting neurites *(arrowheads)*. The nuclei are stained with DAPI *(blue)*. *cpn* – transposed common peroneal nerve; *ms* - microspheres.

Even though the statistical analysis has not yet shown any significant differences among the treatment cohorts at 4 weeks time point, both image analysis (Fig. 13B –B'') and quantitative metrics (Fig. 14) demonstrate a trend of the constructs containing the freely dissolved neurotrophic factors (NFs group) and the constructs containing neurotrophic factor-loaded microspheres (NF-loaded MSs group) to induce higher neurite sprouting from the distal end of the transposed nerve in comparison with the control constructs (no neurotrophic factors). Note that only three out of four biological replicates were quantitatively analyzed so far for two out of three experimental groups (Fig. 14). The inclusion of the fourth sample is expected to increase the statistical power of the analysis. The relevant results will be included in the following report.

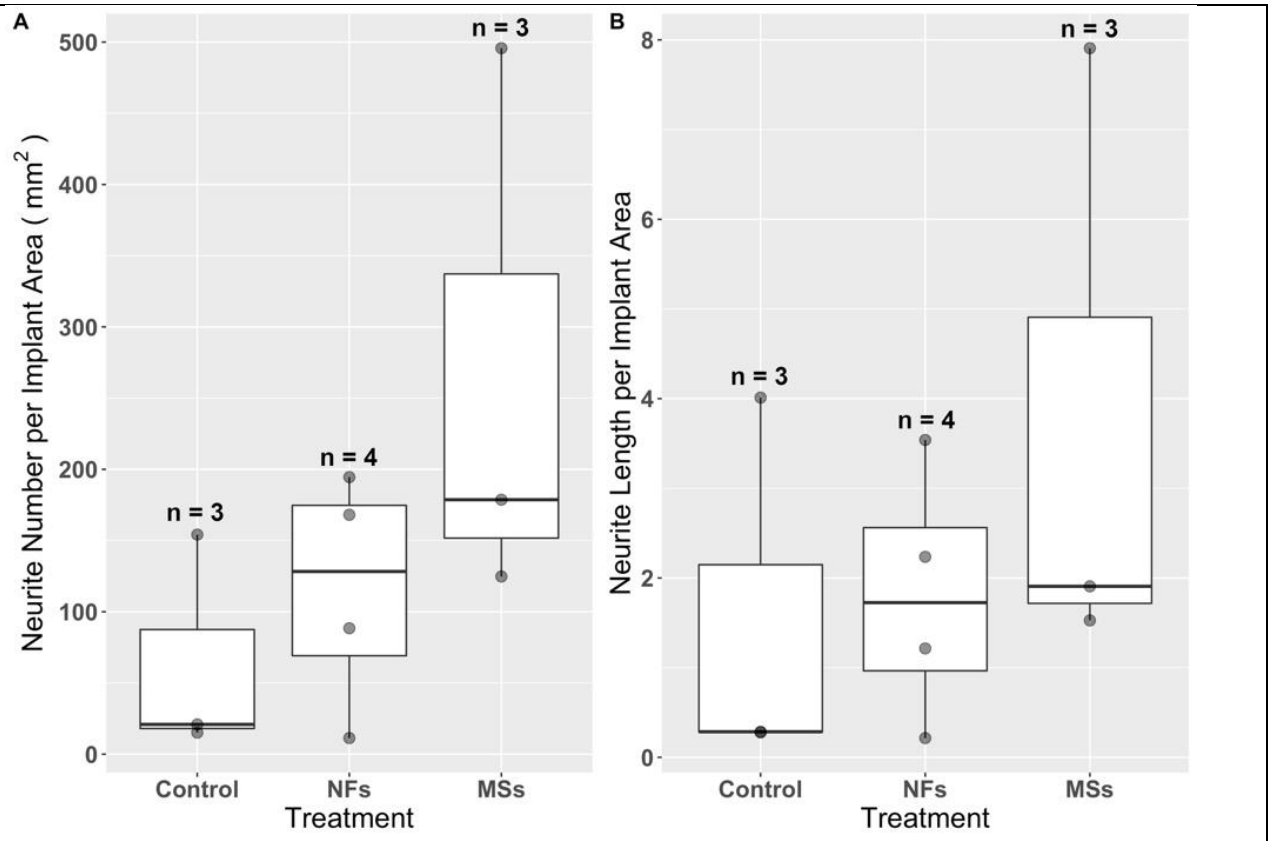

Figure 14. Quantitative metrics of the neurite sprouting from the transposed host common peroneal nerve in the implanted skeletal muscle constructs at 4 weeks post-implantation. (A) Number of neurites per mm^2 of the implant area. (B) Total sprouting neurite length (mm) per mm^2 of the implant area. The *Control* group contains biofabricated muscle implants without any extrinsic neurotrophic factors. The second group *(NFs)* contains CNTF and GDNF freely dissolved in the extracellular matrix of the implant. The third group *(NF-loaded MSs)* contains the matching load of the neurotrophic factors encapsulated in PLGA microspheres suspended throughout the hydrogel of the construct.

In addition to histological and immunohistochemical analysis, we performed the electrophysiological assessment of the integration of the transposed nerve into the implanted muscle construct. Prior to tissue harvesting, the transplanted CPN was stimulated by 2 mA, and the response from the area corresponding to the implant was recorded using the Sierra Wave electromyography (EMG)/nerve conduction velocity (NCV) instrument (Cadwell) (Implant). Similar readings were also recorded from the control contralateral normal leg in each animal (Contralateral Muscle - not innervated with CPN).

The amplitude of the compound muscle action potential (CMAP) recorded from the implanted construct did not show any significant variation across the experimental group and was significantly higher than in the untreated contralateral leg (Fig. 15). These results suggested that even though extensive neurite sprouting did occur in response to the neurotrophic factors incorporated into the muscle implants, proper physiological integration between the host nervous system and the implanted muscle cells has not yet occurred at this early time point (4 weeks). We addressed this issue by extending our transposed nerve paradigm to two later time points – 8 weeks and 12 weeks. All additional implantation surgeries have been completed. The electrophysiological assessments and tissue harvesting are currently in progress.

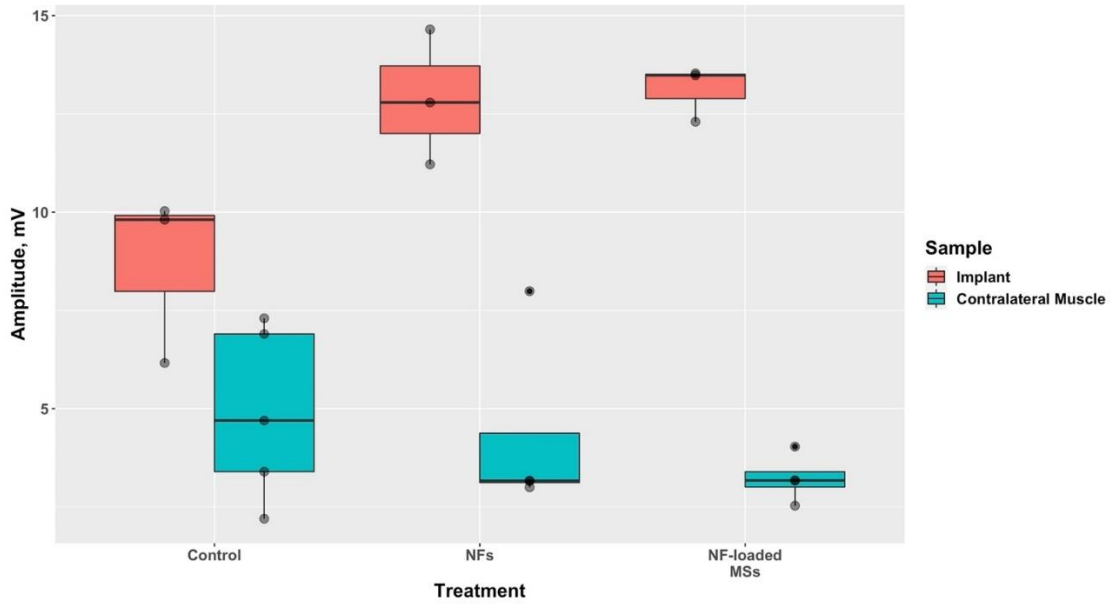

Figure 15. The amplitude of the compound muscle action potential (CMAP) in the transposed nerve model study at 4 weeks post-implantation. The control group contains biofabricated muscle implants without any extrinsic neurotrophic factors. The second group *(NFs)* contains CNTF and GDNF freely dissolved in the extracellular matrix of the implant. The third group *(NF-loaded MSs)* contains the matching load of the neurotrophic factors encapsulated in PLGA microspheres suspended throughout the extracellular matrix of the construct. Four animals were analyzed in each treatment group (n = 4). The data were recorded from the implanted muscle construct *(Implant)* and the untreated contralateral gluteus muscle *(Contralateral Muscle).*

vii. Development of a pre-clinical muscle-nerve injury model in rats

As described above, we have shown that the bioprinted muscle construct containing neurotrophic factor-incorporated PLGA microspheres releases the neurotrophic factors in a controlled and sustained manner. We also demonstrated that the neurotrophic factors released from the PLGA microspheres facilitate neurite sprouting *in vitro*. The ectopic transposed nerve model study above showed that the bioprinted muscle construct with the neurotrophic factor delivery system also promotes the host neurite migration and ingrowth in the *in vivo* settings.

The next step is to determine whether the bioprinted muscle construct with the neurotrophic factor delivery system is beneficial for the recovery of muscle structure and function in complex peripheral nerve-muscle injuries. To this end, we have established a reliable and reproducible muscle-nerve defect model in rats.

The model creation procedure involves removing 30% of the tibialis anterior (TA) muscle in immunocompromised rats. The nerve defect is created by complete transection of the common peroneal nerve and resection of 10 mm of its distal end. Two bioprinted muscle constructs are implanted into the TA muscle defect, and the distal end of the severed nerve is directed to the implants through a silicone tube conduit filled with 0.2% collagen hydrogel containing 100 ng/mL of CNTF and GDNF **(Fig. 16)**. The model creation study involves two experimental cohorts:

a) the *Injury-only* group, in which the neuromuscular injury is created as above, but no implants are grafted
b) the *Neurotrophic Factor-loaded Microsphere* group containing the matching load of the neurotrophic factors encapsulated in PLGA microspheres suspended in the hydrogel.

- The contralateral leg of rats (without defect and implantation) will be used as a normal group.

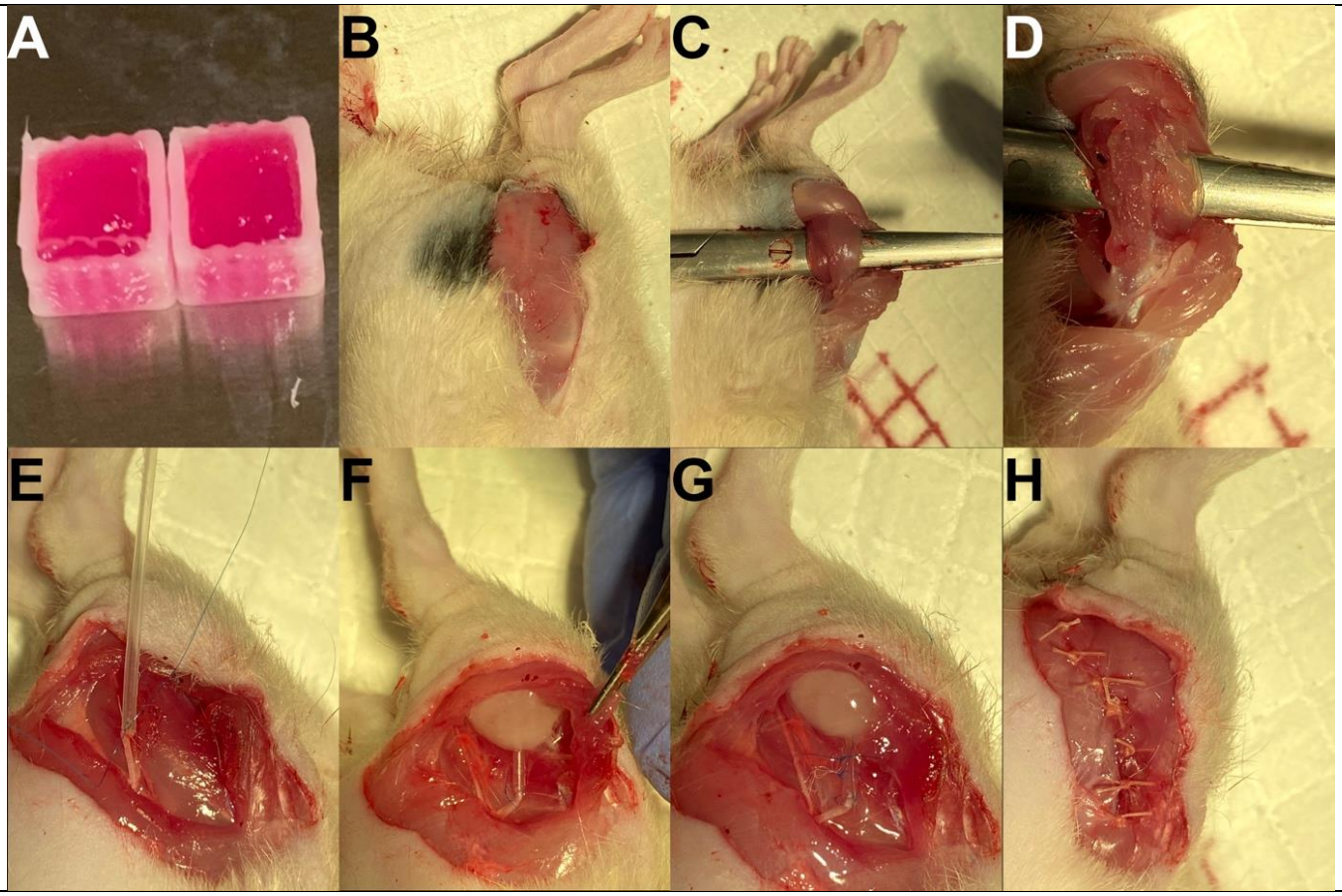

Figure 16. The surgical procedure to create pre-clinical nerve-muscle injury. **(A)** Bioprinted muscle constructs used in the implantation surgery. **(B)** An incision is made in the skin of the left hind limb. **(C)** The TA muscle is exposed, and 30% of its weight is removed **(D)**. **(E)** A 10 mm-long distal segment of the common peroneal nerve is removed, and a conduit (a silicone tube filled with a CNTF/GDNF-containing hydrogel) is attached to the distal end of the nerve stump. **(F)** Two biomanufactured muscle constructs are implanted into the TA muscle defect, and the distal end of the nerve conduit is sandwiched between the implants. **(G)** The conduit is sutured in place to the underlying host muscle tissue to prevent displacement. **(H)** The incision is closed with a suture.

We completed all model creation and implantation surgeries during the reporting period. Preliminary tests of surgical procedures, reliability, and animal health issues have been carried out for 4 weeks follow-up. The TA muscle tissue samples harvested from the animals in the injury-only (no treatment) cohort showed the reduced volume of entire TA muscles and clear signs of atrophy without regeneration of remained muscles (Fig. 17 A). On the other hand, the animals that received muscle implants with neurotrophic factors and had the CPN directed to the implants were much less atrophied and showed more normal tissue structure (Fig. 17B). The neurotrophic factors-encapsulated microspheres were observed in the implanted sites. We have proceeded with functional data collection, tissue harvesting and histological processing, staining, and imaging of the tissue samples.

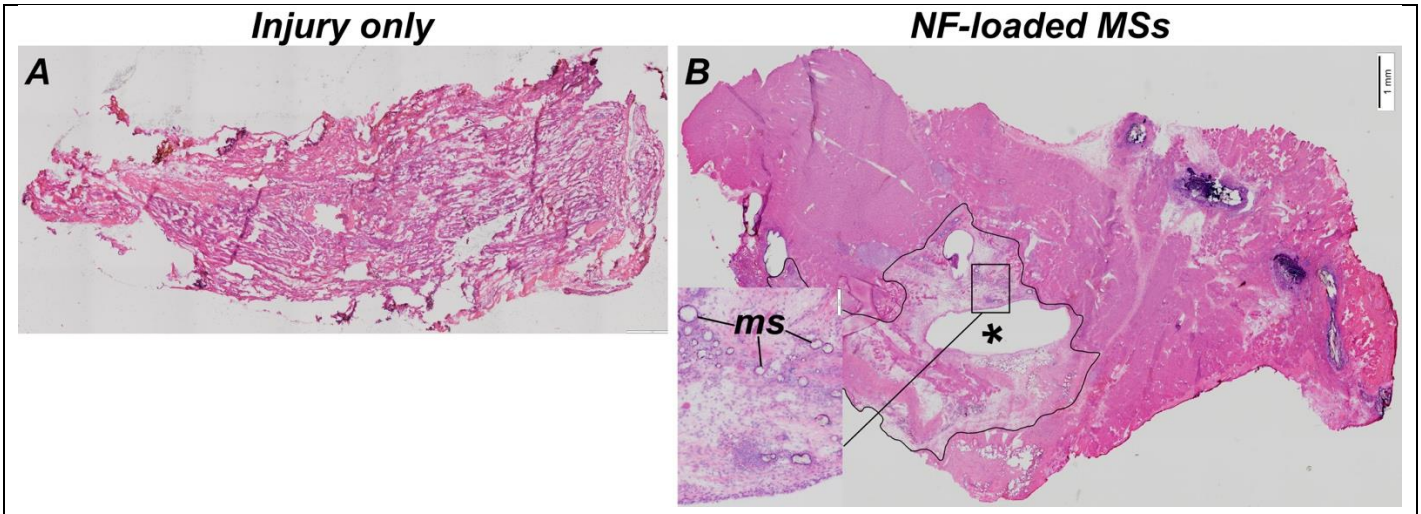

Figure 17. Representative micrograph of the TA muscle from the preclinical muscle-nerve injury *in* vivo study at 4 weeks post-surgery. **(A)** Injury-only group (the animals underwent neuromuscular injury, and no implant was grafted). (B) Neurotrophic Factor-loaded Microsphere group (the inured animals received biofabricated skeletal muscle implants containing CNTF/GDNF-loaded microspheres, and the CPN was directed to the construct using a silicone tube conduit). Cryosection stained with hematoxylin and eosin. The implant area is outlined. The inset shows a higher magnification view of the implant area with still visible microspheres *(ms)*. *Asterisk* indicates the silicone tube nerve conduit.

viii. *In vivo* feasibility study of restoring muscle function with bioprinted skeletal muscle constructs and nerve grafts in a pre-clinical muscle-nerve injury model in rats

After the ongoing muscle-nerve injury model creation study is completed, we will determine the feasibility of using the bioprinted muscle construct to recover muscle structure and function in the muscle-nerve injury model. We have prepared materials and supplies needed for initiating the *in vivo* feasibility study in this reporting period, including cell culture media and supplements, cell culture ware, biomaterials and chemicals, bioprinting supplies.

2. Future Plans

In the following year of the project, we will be focusing on the following tasks:

Task 1. Development and validation of bioprinted skeletal muscle constructs with NMJ formation capacity and neurotrophic factor release system

Task 1.3 Evaluate peripheral nerve integration and NMJ formation of the bioprinted skeletal muscle constructs in a rat transposed ectopic nerve model.

We will complete analyzing the histological and electrophysiological data from our transposed ectopic nerve model.

Task 2. Determine the clinical feasibility of restoring muscle function with bioprinted skeletal muscle constructs and nerve grafts in a pre-clinical animal model of traumatic muscle injury.

Task 2.1 Create a nerve-muscle injury model in an immunocompromised rat.

We will complete tissue harvesting and collecting and processing the functional data.

Task 2.2 Evaluate structural and functional recovery with NMJ formation.

We will perform implantation surgeries and analyze the collected tissue samples with histological and immunohistochemical techniques to evaluate the innervation and functional integration of the implanted muscle constructs.

Task 3. Development and validation of manufacturing processes, standard operating procedures (SOPs) and batch records (BRs) for the prototype product under GLP conditions to support FDA applications and clinical translation.

Task 3.1 Develop and validate BRs and SOPs to support a Pre-IND application.

We will initiate developing the BRs and SOPs.

3. Problems / Issues.

a. Current Problems / Issues

The social distancing guidelines implemented because of the continuing COVID-19 pandemic have been somewhat relaxed. However, we are still experiencing a shortage of experimental materials (cell culture media, plasticware, chemicals) due to delayed production and stockout issues of several vendors.

b. Anticipated Problems / Issues

The continuing shortage of experimental materials and supplies and delayed shipping may cause a delay in implementing experiments, but we are trying to plan experiments appropriately to complete the proposed project works in time.

4. Financial Health

The project is mostly on track to meet the major milestones. The research team is fully assembled. The PIs and all team members continue to process the data and quantitative analysis, perform *in vitro* and *in vivo* experiments and write manuscripts. The research team regularly tracks the progress of the project through weekly all-hands meetings.

5. Personnel Effort

Personnel	Role	Percent Effort
Yoo, James	Principal Investigator	15% - 20%
Jackson, John	Co-Investigator	14% - 15%
Ju, Young Min	Co-Investigator	5% - 20%
Kim, Ickhee	Co-Investigator	20% - 21%
Kim, Ji Hyun	Co-Investigator	30%

Lee, Sang Jin	Co-Investigator	15%
Vaughan, Bill	Co-Investigator	0% - 5%
Cranfill, Jeff	Technician	0% - 10%
Mashanov, Vladimir	Research Associate	100%
Perez, Jovanna	Technician	20% - 40%
Young, Anna Marie	Technician	0% - 10%
Wilson, Jennifer Marie	Lab Technician	10% - 40%

6. Protocol and Activity Status

a. Human Use Regulatory Protocols

TOTAL PROTOCOLS: 1 (one) human use protocol is required to complete this project.

PROTOCOLS:

Protocol: IRB00051342
Title: Discarded skeletal muscle tissue for use in laboratory cell processing and scaffold development
Target required for clinical significance: 30
Target approved for clinical significance: 30
Submitted to and Approved by:

- Submitted to HRPO on 01/16/2019
- Approved by HPRO on 08/14/2019

STATUS: Approved.

b. Use of Human Cadavers for RDT&E, Education or Training

TOTAL ACTIVITIES: No RDT&E, education or training activities involving human cadavers will be performed to complete the Statement of Work (SOW).

c. Animal Use Regulatory Protocols

TOTAL PROTOCOLS: 1 (one) animal use protocols is required to complete this project

PROTOCOLS:

Protocol: A18-153
Title: Accelerated Innervation of Engineered Tissue Construct
Target required for statistical significance: 1344
Target approved for statistical significance: 1344
Submitted to and Approved by:

- IACUC protocol #A18-153 was approved on 12/12/2018.
- Amendment #1 for IACUC study #A18-153 was approved on 12/18/2018.

- Amendment #2 for IACUC study #A18-153 was approved on 12/28/2018 (personnel change request, ACURO approval is not needed).
- ACURO appendix for IACUC protocol A18-153 was submitted to ACURO on 1/15/2019.
- Additional information for IACUC protocol A18-153 was submitted to ACURO on 4/9/2019 per ACURO's request.
- ACURO approved on 4/30/2019
- Amendment #3 for IACUC study #A18-153 was approved on 07/08/2019 (personnel change request, ACURO approval is not needed).
- Amendment #4 for IACUC study #A18-153 was approved on 09/04/2019 (personnel change request, ACURO approval is not needed).
- Amendment #5 for IACUC study #A18-153 was approved on 10/02/2019 (personnel change request, ACURO approval is not needed).
- Amendment #6 for IACUC study #A18-153 was approved on 02/06/2020 (ACURO approved on 2/13/2020)
- Amendment #7 for IACUC study #A18-153 was approved on 03/02/2020 (ACURO approved on 3/10/2020)
- Amendment #8 for IACUC study #A18-153 was approved on 03/18/2020 (personnel change request, ACURO approval is not needed).
- Amendment #9 for IACUC study #A18-153 was approved on 03/24/2020 (Withdrawn from ACURO after the communication with ACURO)
- Amendment #10 for IACUC study #A18-153 was approved on 04/07/2020 (personnel change request, ACURO approval is not needed).
- Amendment #11 for IACUC study #A18-153 was approved on 06/25/2020 (ACURO approved on 07/07/2020)
- Amendment #12 for IACUC study #A18-153 was approved on 11/24/2020 (ACURO approved on 12/2/2020)
- Amendment #13 for IACUC study #A18-153 was approved on 04/21/2021 (personnel change request, ACURO approval is not needed)
- Amendment #14 for IACUC study #A18-153 was approved on 06/02/2021 (personnel change request, ACURO approval is not needed)

STATUS: Approved

Annual Business Status Report for

Accelerated Innervation of 3D Bioprinted Muscle Construct with Pre-Fabricated Neuromuscular Junctions and Neurotrophic Factor Release System Research Research

Project No. 2017-614-002

EGS# MT180011

Reporting Period: 10/01/2020 – 09/30/2021

MTEC Research Project Awardee

James Yoo

John Jackson, Young Min Ju, Ickhee Kim, Ji Hyun Kim, Sang Jin Lee

Research Project Technical POC

James Yoo

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Winston Salem, NC 27107

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Submitted: 11/2/2021

1. CURRENT STAFF

Personnel	*% of Effort on project*
James Yoo: Principal Investigator	15% - 20%
John Jackson: Co-Investigator	14% - 15%
Young Min Ju: Co-Investigator	5% - 20%
Ickhee Kim: Co-Investigator	20% - 21%
Ji Hyun Kim: Co-Investigator	30%
Sang Jin Lee: Co-Investigator	15%
Bill Vaughan: Co-Investigator	0% - 5%
Jeff Cranfill: Technician	0% - 10%
Vladimir Mashanov: Research Associate	100%
Jovanna Perez: Technician	20% - 40%
Anna Marie Young: Technician	0% - 10%
Jennifer Marie Wilson: Lab Technician	10% - 40%

2. CURRENT EXPENDITURES

A. Cost Reimbursable Contracts

Contract Expenditures	Current Annual Expenditures	*Cumulative To Date Expenditures*
Labor (Personnel and Fringe)	$232,757.89	$ 666,691.94
Supplies/Materials	$ 15,904.31	$ 93,819.31
Travel	$ 260.00	$ 10,039.04
Equipment	$	$
Subcontractors and Consultants	$	$
Other Direct Costs	$127,399.18	$ 276,328.81
Indirect Costs	$206,976.76	$ 575,783.52
Total	**$583,298.14**	**$1,622,662.62**

B. Fixed Priced Contracts: n/a

C. Cost Share Contributions: n/a

3. STATUS OF MILESTONES

MTEC Milestone Number	Milestone Description	Due Date	% Completed this Reporting Period	Cumulative % Complete
1	Project Kick Off	10/15/2018		100%

2	Quarterly Report 1 (October - December, Technical and Business Reports)	01/25/2019		100%
3	Quarterly Report 2 (January - March, Technical and Business Reports)	04/25/2019		100%
4	Quarterly Report 3 (April- June) Technical and Business Reports)	07/25/2019		100%
5	Annual Report 1	10/25/2019		100%
6	Quarterly Report 4 (October-December, Technical and Business Reports)	01/25/2020		100%
7	Quarterly Report 5 (January - March, Technical and Business Reports)	04/25/2020		100%
8	Quarterly Report 6 (April-June, Technical and Business Reports)	07/25/2020		100%
9	Annual Report 2	10/25/2020	100%	100%
10	Quarterly Report 7 (October-December, Technical and Business Reports)	01/25/2021	100%	100%
11	Quarterly Report 8 (January - March, Technical and Business Reports)	04/25/2021	100%	100%
12	Task 1.1/D1.1 Determine the effects of neurotrophic factors on skeletal muscle development and acetylcholine receptor (AChR) pre-formation on the bioprinted muscle construct.	06/25/2021	20%	100%
13	Task 1.2/ D1.2 Demonstrate NMJ formation and innervation in bioprinted skeletal muscle constructs, in vitro.	06/25/2021	20%	100%
14	Quarterly Report 9 (April - June, Technical and Business Reports)	07/25/2021	100%	100%
15	Annual Report 3	10/25/2021		
16	Task 1.3/D1.3 Evaluate peripheral nerve integration and NMJ formation of the bioprinted skeletal muscle constructs in a rat transposed ectopic nerve model.	12/25/2021	43%	90%
17	Task 2.1/D2.1 Create a nerve-muscle injury model in rat.	12/25/2021	86%	86%

18	Quarterly Report 10 (October-December, Technical and Business Reports)	01/25/2022		
19	Quarterly Report 11 (January - March, Technical and Business Reports)	04/25/2022		
20	Quarterly Report 12 (April - June, Technical and Business Reports)	07/25/2022		
21	Annual Report 4	10/25/2022		
22	Task 2.2/D2.2 Evaluate structural and functional recovery with NMJ formation.	11/30/2022	15%	15%
23	Task 3.1/D3.1 Develop and validate BRs and SOPs to support a Pre-IND application.	11/30/2022		
24	Final Technological and Business Report	11/30/2022		

4. NONTRADITIONAL DEFENSE CONTRACTOR PARTICIPATION: n/a

5. DEVIATION FROM PROJECT PLAN

None