# Injectable Agrin/IGF-1-loaded Nanoparticle Hydrogel with Syngeneic Myoblasts for Functional Recovery Following Chronic Denervation

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**INTRODUCTION:** In humans, axonal regeneration in damaged nerves occurs at approximately 1 mm/day, often leading to chronic loss of innervation to target muscles during this period. Chronic denervation of muscles results in significant loss of muscle mass which precludes significant functional recovery. Previous work has demonstrated that agrin and insulin-like growth factor 1 (IGF-1) promote the formation of neuromuscular junctions, improved vascularity, and myoblast proliferation in a rat chronic denervation model[1]. Despite increases in innervation and vascularity of muscles treated with agrin and IGF-1, significant muscle atrophy remains an issue following a chronic injury, which has not been addressed in previous studies. Direct injection of agrin, IGF-1, or myoblasts has resulted in strong immune responses and inconsistent effects of agrin and IGF-1. Nanofibril hydrogel composites (NHC) made of polycaprolactone fibrils and hyaluronic acid have been demonstrated to improve long-term viability of mesenchymal stem cells (MSCs) [2] and provide predictable agrin and IGF-1 release[1] while limiting immune rejection of cells and promoting angiogenesis. Here we hypothesize that a combination of agrin, IGF-1, and syngeneic myoblasts delivered in NHC will promote engraftment of myoblasts, alleviate functional deficits due to chronic denervation and increase neural infiltration.

**METHODS:** All animal procedures were carried out as approved by institutional ACUC. *Myoblast Isolation and Viability:* Myoblast were isolated from GFP$^+$ Lewis rats (F344-Tg(UBC-EGFP)F455Rrrc) and non-GFP$^+$ Lewis rats using either a serial pre-plating protocol as described by Yoshioka et al (2020)[3] (Protocol 1) or by expansion from explants as described by Shahini et al (2018)[4] (Protocol 2). Cells were used at passages 2 to 4 for myoblast injection. NHC/Agrin/IGF-1 gel was manufactured as described previously [2] with agrin and IGF-1 at 12.5 µg/mL and 250 µg/mL, respectively. Live/Dead assay was carried out on 100µL of NHC/Agrin/IGF-1 with 2.5x10$^6$ myoblasts, as per the manufacturer's instructions to determine the viability of cells following injection from a 22-guage needle. *Composite Hydrogel Injection*: 9-10-week-old Lewis rats received median nerve transection with a 12-week delay of ulnar to median nerve transfer to create an anterior compartment forepaw chronic denervation model. Animals ($n=6$ per group) received 100 µL of PBS injections, myoblasts in NHC/Agrin/IGF-1 or myoblasts in PBS starting at 33 weeks post denervation, or no chronic denervation (transection with immediate transfer at timepoint as treatment groups). Treatment was given in 3 doses, 2- and 4- weeks apart. Following surgeries, animals received weekly forepaw stimulated grip strength testing (SGST) and histological assessment to follow 6-weeks following the last injection (**Fig. 1**). To decouple the histological effects of NHC, agrin, IGF-1, and myoblast, animals ($n=2$) were injected with GFP$^+$ myoblasts with PBS, NHC, NHC and agrin, NHC and IGF-1 or NHC/agrin/IGF-1 in the denervated forepaw. Animals were sacrificed 6 weeks following their last injection for histological assessment of injected cell engraftment, motor end plate regeneration, implanted and host muscle myofiber cross-sectional area, and angiogenesis. *Statistics:* Forearm grip strengths were compared among groups each week with statistical assessment using analysis of variance (ANOVA) with Tukey post hoc assessment for differences between individual groups.

**RESULTS***:* Primary myoblast showed high viability 1 hour post extrusion from a 22G hypodermic needle. The onset of a plateau of functional recovery occurred in all groups at 16 weeks with the most remarkable improvement in SGST seen in the positive control group. Myoblasts treatment was given at 33 weeks post-injury. It showed a trend towards improvement in the functional recovery at 2 weeks (**Fig. 2A**), with statistically significant ($p<0.05$) improvement starting at 3- and 4-weeks post-injection 1 in NHC/myoblast/nanoparticles and PBS/myoblast groups, respectively (**Fig. 2B**). Histological assessment of myofiber cross-sectional area (CSA), motor endplate regeneration, angiogenesis, immune response is currently in progress.

**DISCUSSION:** Implanted myoblast cell populations have been shown to result in histological improvement in muscle regeneration in injury models, but this has not been demonstrated with injectable hydrogels for chronic denervation-induced skeletal muscle atrophy. It was demonstrated that primary syngeneic myoblasts resulted in significant functional improvement in middle-aged rats with chronic denervation of the forepaw. NHC/agrin/IGF-1 combined with myoblast provided significant improvement in forearm grip strength at 6 weeks post-first injection. Nanoparticles used here allow controlled release of agrin and IGF-1 over 30 days, while NHC allows at least 50% local retention of MSCs at 14 days post injection compared 50% retention occurring at 5 days when cells are delivered in PBS[2]. It is assumed that drug release and cell retention dynamics seen with MSCs hold true for injected myoblast and would explain the more subtle functional improvement seen from PBS injections compared to NHC-mediated injections. It is expected that histological analysis will show the greatest difference in motor end plate regeneration in NHC-based treatment. Additionally, based on results from other hydrogel delivery studies, it is anticipated that mean myofiber CSA in host tissue will also increase and lead to partial or complete resolution of skeletal muscle atrophy. The additional trophic benefits of agrin and IGF-1 could potentially reduce the effective dose of myoblast needed to elicit a clinically relevant benefit compared to previous studies using autologous myoblast injections.

**CLINICAL RELEVANCE:** This work shows promise in functional recovery in chronic denervated muscles by building on the known benefits of agrin and IGF-1 in promoting myoblast proliferation and neuronal regeneration while compensating for muscle atrophy.

**REFERENCES: 1**. Hanwright, P. J. *et al. Biomaterials* **280**, 121244 (2022). **2.** Yao, Z. C. *et al. Small* **18**, (2022). **3.** Yoshioka, K. *et al. Front Cell Dev Biol* **8**, (2020). **4.** Shahini, A. *et al. Stem Cell Res* **30**, 122–129 (2018)

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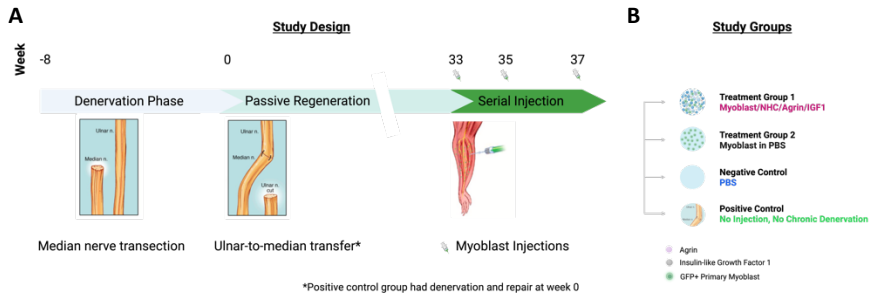


**Figure 1. Schematic of Study Design.** **A**. Animals received ulnar-to-median transfer immediately (positive control) or 8 weeks following median nerve transection. **B**. Grip strength testing was done to determine the functional plateau, after which treatment commences with PBS or myoblast in NHC with nanoparticles or PBS.

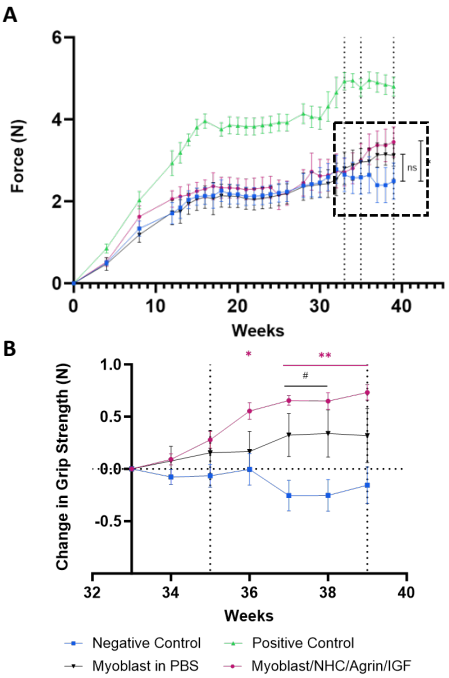


**Figure 2. Syngeneic myoblasts cause significant improvement in SGST.** **A**. Significant difference between forepaw grip strength was noted at 6 weeks following first injection in NHC group. **B.** Relative to negative control, improvement was noted as early as 3 weeks post injection. NHC group: $p<0.05$, $^{**}p<0.001$; PBS groups # $p<0.05$. Dotted lines: injections