

Neuromuscular Regeneration Increases in Response to Agrin and Exercise post-Volumetric Muscle Loss Injury

Eszter Mihaly¹, Neha Chellu¹, Shama R. Iyer², Eileen Su¹, Dallas E. Altamirano¹, Warren L. Grayson¹

¹Johns Hopkins University, Baltimore, MD, ²Marymount University, Arlington, VA

emihaly1@jh.edu; Disclosures: None

INTRODUCTION: Skeletal muscle is well-adapted to regeneration, as muscle satellite cells differentiate upon injury and replace damaged tissue. However, volumetric muscle loss (VML) is a critical sized defect in which the healing mechanism is overwhelmed, and loss of normal structure and function persists. Transplantation of tissue engineered muscle scaffolds have been evaluated as a potential treatment. In past work, we characterized a population of human primary myogenic cells (hPMCs) on an electrospun fibrin scaffold, and demonstrated that the cells are able to align, fuse, and myogenically mature on the scaffold. We successfully enabled muscle regeneration using these cell seeded-scaffolds upon transplantation into a mouse model of VML injury. However, regeneration of the connection between nerves and muscle is also critical to achieving functional recovery, as long term denervation causes muscle atrophy. A promising avenue is the use of rehabilitative exercise to promote nerve regeneration, via the induction of BDNF and other nerve growth factor secretion from muscle. We previously identified a gender-appropriate exercise regimen that improved AChR clustering and neural ingrowth into the injured muscle, but was not sufficient to increase force output or muscle weight. In this study, we attempt to further promote neuromuscular regeneration by the use of agrin-treated scaffolds. Agrin is a proteoglycan endogenously secreted by motor nerve terminals to induce AChR clustering and the formation of a functional neuromuscular junction. Various groups have suggested that the agrin-induced AChR clustering mechanism may be influenced by mechanical strain, but this has not been well-evaluated. In this study, we evaluated the synergistic effect of rehabilitative exercise and agrin-treated cell-seeded scaffolds on neuromuscular regeneration.

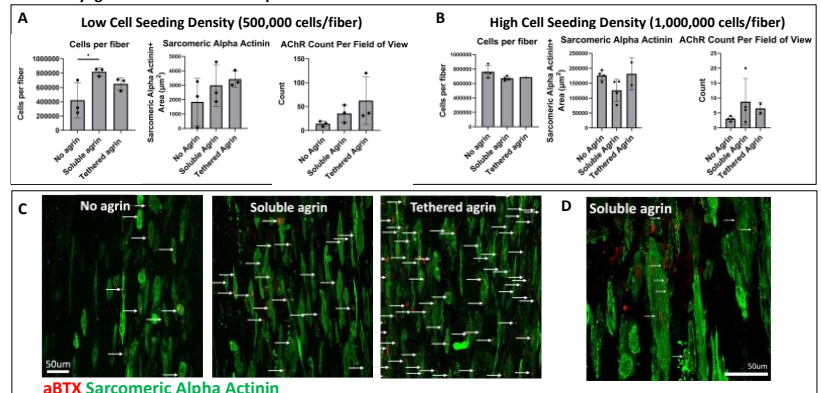
METHODS: Tethered Agrin: electrospun scaffolds were cross-linked with agrin at 100ug/mL using EDC/SNHS chemistry. Soluble Agrin: cell-seeded fibers received agrin in media (300ng/mL) for 2 days. Fibers seeded with hPMCs at 500,000 or 1,000,000 cells/fiber. VML defects were created by 50% resection of the tibialis anterior in male NSG mice, followed by transplantation of 'No Agrin' or 'Tethered Agrin' muscle scaffolds (approved by ACUC protocol). Treadmill regime: 1 hour daily @ 10m/min for 7 weeks. Muscles were cryosectioned, and muscle sections & cell-seeded scaffolds underwent IHC staining. Quantification in FIJI. N≥6 for all groups of mice. Statistical significance determined by t-test, one-way or two-way ANOVA.

RESULTS: A primary human myogenic cell source (hPMC) was successfully induced into aligned, multi-nucleated myotubes on electrospun fibrin scaffolds. Two different cell seeding concentrations were evaluated: 500,000 cells/fiber and 1,000,000 cells/fiber. In the lower cell seeding group, the addition of agrin significantly increased the proliferation of myogenic cells, and agrin-treated fibers trended towards increased myogenic maturity (Fig 1A). In the higher cell-seeding group, the fiber was presumably saturated with cells and the agrin treatment produced no additional benefit in cell proliferation or myogenic maturity (Fig 1B). In both cell-seeding groups, the addition of agrin trended towards increased numbers of AChR clusters. In the subsequent study, No Agrin and Tethered Agrin fibers seeded with hPMCs were implanted into a mouse model of VML injury (Fig 2A). These animals were further separated into groups with and without rehabilitative exercise. The implantation of Tethered Agrin fibers significantly increased the weight of the injured muscle, as compared to an untreated control (Fig 2B). Functional testing revealed a trend towards increasing force output with the Agrin group (although not significant, Fig 2C-D). The addition of exercise to the No Agrin group trended towards increasing force output, but produced no change with the Tethered Agrin group.

DISCUSSION: In previous work, we developed a successful model of exercise-induced regeneration by shortening the recovery period and lengthening the exercise period. hPMC-seeded scaffolds implanted into the defect not only survive for a period of 8+ weeks, but induce almost complete regeneration (90% of contralateral) of the muscle volume, with a strong trends towards functional improvement over an untreated defect. In this study, we evaluated the synergistic effect of agrin treatment and exercise. *In vitro*, Agrin-treated fibers seeded with hPMCs trended towards increased myogenic proliferation, maturity, and AChR clustering. Upon implantation, Agrin-treated groups showed better outcomes in terms of the injured muscle weight and force output. The addition of exercise to the No Agrin group trended towards increased force output; however, the addition of rehabilitative exercise did not further enhance neuromuscular regeneration in the Agrin group. Considering that the average weight of the injured muscle in the Agrin groups was close to 100% of the contralateral muscle weight, it is likely that these treatment methods have maximized neuromuscular recovery in this injury model (highly regenerative young mice with acute VML injuries). To better evaluate the combinatorial effects of rehabilitative exercise and agrin treatment, a model of chronic injury with young and aged mice will be utilized in the future.

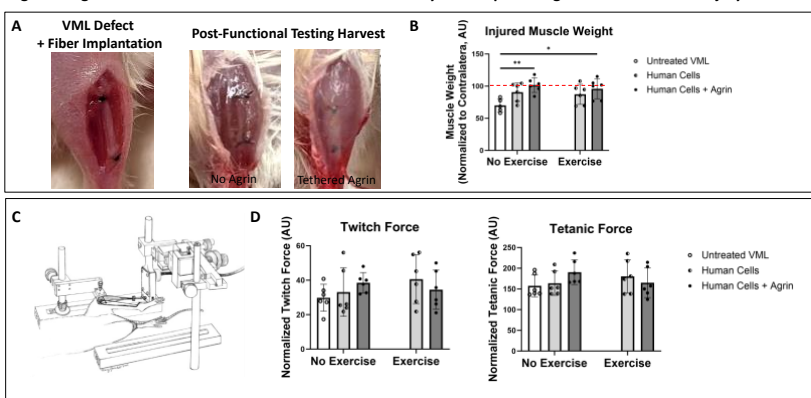
CLINICAL RELEVANCE: VML contributes to 92% of the muscle injuries in military members who have retired for medical reasons. The projected cost of VML injury over the patient's lifetime exceeds \$300,000. VML injuries contribute to significant long-term disability of service men and women.

Figure 1. Agrin Treatment Promotes Myogenic Cell Differentiation, Myogenic Maturity, and AChR Clustering on Primary Human Myogenic Cells on 3D Electrospun Fibers.



A, B. Cell proliferation on electrospun fibers (as evaluated by Quant-iT PicoGreen assay), and expression of Sarcomeric Alpha Actinin and AChR with low seeding density (A, 500,000 cells/fiber) and high seeding density (B, 1,000,000 cells/fiber). C. Representative images of Sarcomeric Alpha Actinin and Alpha-Bungarotoxin staining. D. Close-up image of staining.

Figure 2. Agrin-Tethered Scaffolds Seeded With Human Primary Cells Improve Regeneration After VML Injury.



A. Surgical creation of VML defect and fiber implantation; images of muscle during post-functional testing harvest. B. Weight of harvested muscle. C. Schematic of functional testing – tibialis anterior tendon is isolated and secured to a force sensor, and the peroneal nerve is electrically stimulated. D. Resulting Twitch and Tetanic force readings.