Engineering Pre-Vascularized Skeletal Muscle with Physiologically-Relevant Cellular Organization for Treatment of Volumetric Muscle Loss

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Statement of Purpose: Traumatic musculoskeletal injury often results in impaired endogenous tissue regeneration and revascularization capacity. A successful therapeutic intervention must restore vasculature, muscle function, and physiological anatomical structure. Towards this goal, we bioengineered parallel-aligned skeletal muscle constructs that mimic the physiological orientation of native vasculature and muscle tissue, to examine their therapeutic potential in a model of volumetric muscle loss (VML). We **hypothesized** that coculture of vascular endothelial cells (ECs) with skeletal muscle myoblasts on highly ordered three-dimensional scaffolds will produce parallel-aligned multi-nucleated myotubes interspersed with aligned capillary-like structures that augment muscle regeneration *in vivo*.

Methods: To generate ordered three-dimensional scaffolds, a shear-based extrusion process was used to generate parallel-aligned nanofibrillar collagen scaffolds [1]. In brief, the procedure involved extruding high concentration rat tail monomeric collagen I in acetic acid from a syringe tip directly into neutral pH buffer to induce instantaneous fibrillogenesis along the direction of extrusion. To generate randomly oriented fibrillary scaffolds, the extrusion speed was reduced by >10-fold to induce random orientation in fibrillogenesis. Mouse myoblasts (C2C12) and human microvascular endothelial cells (HMEC-1) were cocultured on the scaffolds for up to 9 days. In some experiments, the myoblasts were fluorescently labeled with green fluorescence protein (GFP) and luciferase, whereas ECs were labeled with mRuby fluorescence protein. The cell-seeded scaffolds were paced at 1Hz to examine uniformity of contraction and contractile force. In addition, immunofluorescence staining was performed to quantify myoblast alignment, myotube formation, and EC organization using antibodies targeting myosin heavy chain (myotubes) and CD31 (ECs), respectively. For *in vivo* studies, scaffolds were implanted into mice with 20% volumetric muscle loss in the anterior tibealis (TA) muscle. Implanted scaffolds consisted of: 1) acellular aligned scaffolds: 2) aligned scaffold with myoblasts; 3) aligned scaffolds with myoblasts and ECs; 4) randomly oriented scaffolds with myoblasts; and 5) randomly oriented scaffolds with myoblasts and ECs (n>5). After 3 weeks, the scaffolds were harvested for histological analysis of muscle and vascular regeneration.

Results: We generated collagen scaffolds composed of either non-aligned or parallel-aligned nanofibrils. *In vitro*, myoblasts and ECs on aligned scaffolds were within 10 degrees from the direction of the nanofibrils, whereas on randomly oriented scaffolds the cells were randomly distributed (Fig. 1). Myotube lengths and nuclei incorporation were 2 times greater on aligned scaffolds than on non-aligned scaffolds. The presence of ECs significantly increased myotube length and nuclei incorporation on aligned scaffolds. Upon electrical stimulation at 1 Hz, the aligned muscle constructs demonstrated coordinated contraction properties compared to non-aligned constructs. Next, fluorescently and bioluminescently labeled mouse myoblasts and ECs were co-cultured on the scaffolds and were transplanted into a VML mouse model for 21 days. *In vivo*, the region of tissue injury treated with constructs composed of aligned myoblasts and ECs demonstrated significant re-

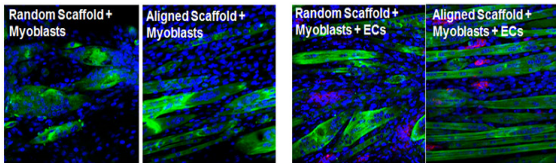

Fig. 1. Myoblasts and ECs become highly aligned and longer on the nanofibrillar scaffolds

vascularization and blood perfusion, based on the percentage of CD31+ blood vessels that co-stained with systemically delivered lectin. Furthermore, bioluminescence imaging of myoblast survival demonstrated increased proliferation and sustained survival of myoblast when co-cultured with ECs on aligned nanofibrillar scaffolds. Histological analysis of myosin heavy chain with co-localization of GFP for transplanted myoblasts demonstrated significantly greater numbers of myofibers, suggesting improved muscle regeneration in the myoblast+EC on aligned scaffold treatment group, compared with non-aligned and cell-free constructs.

Conclusions: This work demonstrates that pre-vascularized engineered muscle using aligned nanofibrillar scaffolds mimic the spatial organization of native muscle and has important translational potential as a muscle graft to enhance muscle regeneration in a diseased injury model.

References: 1. Nakayama KH, Hong G, Lee JC *et al*. Aligned-braided nanofibrillar scaffold with endothelial cells enhances arteriogenesis. *ACS Nano* 9(7), 6900-6908 (2015).