Harnessing sphingosine-1-phosphate signaling and nanotopographical cues to regulate skeletal muscle maturation and vascularization

Jonathan H. Tsui[1], Kajohnkiart Janebodin[1,2], Nicholas Ieronimakis[1,2], David M. P. Yama[1], Hee Seok Yang[1,3], Rakchanok Chavanachat[1], Aislinn L. Hays[2], Morayma Reyes[2,4], and Deok-Ho Kim[1]

Dept. of Bioengineering, University of Washington, Seattle, WA, USA; [2]Dept. of Pathology, University of Washington, Seattle, WA, USA; [3]Dept. of Nanobiomedical Science Medicine, Dankook University, Cheonan, South Korea; [4]Dept. of Pathology, Albert Einstein College of Medicine, Bronx, New York, USA

Statement of Purpose: The loss of skeletal muscle function and volume due to traumatic injury and myopathies is a significant healthcare problem for which there are currently few interventions. In this study, we developed an approach in which the benefits of biomimetic nanotopography and sustained biomolecular signaling could be harnessed synergistically to induce the formation of structurally organized skeletal muscle tissues that are both mature and vascularized. Biodegradable substrates were nanopatterned to mimic the structure of muscle extracellular matrix (ECM) and were subsequently functionalized with sphingosine-1-phosphate (S1P), a sphingolipid G-protein-coupled receptor ligand known to have potent angiogenic and myogenic effects.

Methods: *Substrate Fabrication and Functionalization with S1P:* Substrates were generated using a capillary force lithography (CFL) technique in which PLGA is nanopatterned with a mold applied with constant pressure and heat. For this study, substrates with 800 x 800 x 600 nm (groove width x ridge width x groove depth) feature sizes were used. Fabricated substrates were functionalized with S1P using 3,4-dihydroxy-L-phenylalanine (DOPA).

Transgenic Mouse Generation: Mice harboring the tamoxifen-inducible knockin/knockout Pax7CreERT2 allele were mated with mice homozygous for the mT/mG flox, homozygous for the S1P1 flox, and heterozygous for the GCamP3 flox to generate each Cre-lox system.

Cell Isolation and Culture: Mice were sacrificed 3 days after cardiotoxin-induced injury. Injured muscles were harvested under sterile conditions and cleaned of any fat and tendons before enzyme digestion. Digested tissue was passed through strainers to remove debris, muscle fibers, and multinucleated cells. Resulting mononuclear cells were then seeded onto each respective substrate at a density of 1,500 cells/cm^2 and cultured for 10 days.

Tissue Characterization: Expression of protein and genetic transcription markers for myogenic and angiogenic development was analyzed using immunohistochemical staining and qRT-PCR. Functional assessment of differentiated myotubes was accomplished with computational analysis of both bright-field and fluorescent live-cell imaging.

Results: Nanopatterned substrates induced a greater degree of structural organization in the form of aligned myotubes (Fig. 1A). These substrates also appeared to enhance the myogenic development of cultured progenitor cells, as a greater number of MHC$^+$ myotubes were observed in the patterned environment. When coupled with S1P signaling, myogenesis and subsequent maturation was further enhanced. The expression of *MyoD*, *MyoG* and *Myh15*, which are expressed in late-stage or terminally differentiated muscle cells, was greater in cells not only on patterned substrates, but also significantly in cells on substrates with 175 µM S1P (Fig. 1B). Experiments were conducted to determine whether the maturation observed as a result of the synergistic signaling from S1P and nanopatterning translated to improvements in myotube function. Myotube contraction displacement and velocity, as well as Ca^{2+} flux, was significantly increased in tissues cultured on nanopatterned substrates with 175 µM S1P.

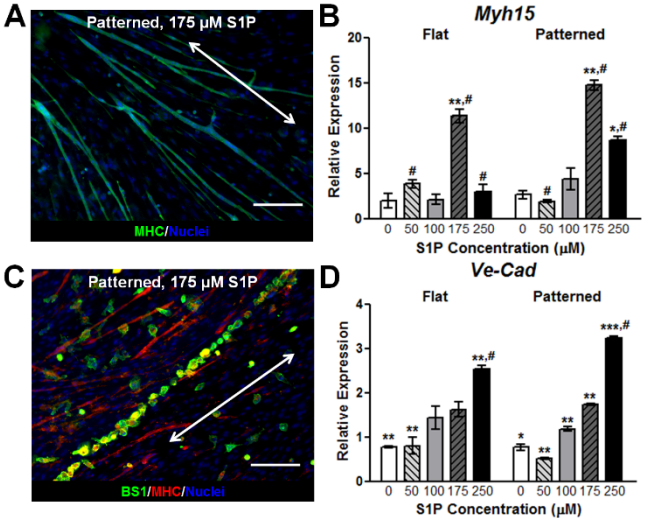

Figure 1. (A) Representative image of aligned myotubes stained for MHC. (B) Expression of *Myh15* as a function of S1P concentration. (C) Representative image of cells stained for BS1. (D) Expression of *Ve-Cad* as a function of S1P concentration.

The potency of S1P as angiogenic factor was observed with increasing concentrations corresponding to a greater number of BS1$^+$ cells (Fig. 1C). The expression of genes related to endothelial adhesion (*CD31*), function (*vWF*), integrity (*Ve-Cad*), and vascular tone regulation (*eNOS*) were also found to increase with S1P concentration (Fig. 1D).

Conclusions: We have demonstrated that by harnessing signaling cues from biomimetic nanotopography and from S1P, it is possible to enhance the maturation and overall function of cultured skeletal muscle progenitors in conjunction with an enhanced neovascularization capability, all without the need for incorporating multiple large proteins or growth factors. In future work, by combining S1P signaling with cell-sheet fabrication techniques that enable the generation of highly-ordered 3D tissues, we aim to generate skeletal muscle tissues that are primed for prolonged myogenic development and vascularization *in vivo*.