Anisotropic Network Formation of Neurons and Endothelial Cells in Engineered Muscle Tissue Construct

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Statement of Purpose: Complex structural organization in the human body is a key factor to produce the appropriate tissue functionality. In mature skeletal muscle, the muscle fibers are highly oriented and the bundle structure is essential to produce its mechanical functions. We have developed a micropatterned thermoresponsive cell culture substrate to prepare an anisotropic cell sheet composed of aligned skeletal muscle myoblasts. Based on cell sheet-based tissue engineering, 3D muscle tissue with aligned orientation was producible by layering multiple anisotropic cell sheets [1]. In native skeletal muscle tissue, neurons and vasculatures are also well organized, both structurally and functionally. The aim of this study is to create an oriented neural and vascular network through a simple cell sheet layering process.

Methods: The original procedures for the preparation of micropatterned thermoresponsive surfaces have been reported previously [2]. Briefly, thermoresponsive polymer poly(*N*-isopropylacrylamide) (PIPAAm) was grafted on glass substrates, and then hydrophilic polymer poly(*N*-acryloylmorpholine) (PAcMo) was further polymerized spatio-selectively through photolithographic process, resulting in the stripe patterns composed of PAcMo-*b*-PIPAAm block polymer brush and PIPAAm brush regions (50 μm / 50 μm stripes).

Human skeletal muscle myoblasts were seeded onto the patterned thermoresponsive surface. After reached confluence, the cells were harvested as a single cell sheet by lowering culture temperature to 20 °C. To layer multiple cell sheets, this myoblast sheet was manipulated using a gelatin gel-coated plunger. After attaching to the cell sheet, the gelatin gel was transferred onto another cell sheet. When the two myoblast sheets were layered, human induced pluripotent stem (iPS) cell-derived neurons and/or human umbilical vein endothelial cells (HUVECs) were sandwiched between the two cell sheets to encapsulate these cells within the cell sheet construct.

Results: Myoblasts were aligned on the surface and the thermally induced detachment of the aligned myoblasts resulted in a single continuous cell sheet. Using gelatin gel-coated plunger, the anisotropic myoblast sheet was transferred onto the second anisotropic myoblast sheet while maintaining the designed orientation. Figure 1a shows a microscopic image of neurofilament-positive cells spreading within the two-layered myoblast sheets. Neurite outgrowth was guided by the anisotropy (myoblast orientation) of the cell sheets, and they finally formed an oriented network. Figure 1b shows that endothelial cells also formed vascular-like branching networks after being sandwiched between two myoblast sheets. In the absence of the top cell layer, HUVECs simply adhered as single cells onto the myoblast sheet. On the other hand, the presence of the top cell layer triggered cellular signaling in the endothelial cells and then formed a branching structure. This change in the morphogenic response induced by cell sheet layering was found previously by our group. Importantly in this study, the tubular structure showed anisotropy originated from the orientation of the myoblast sheets. This indicated that the cell sheet layering allowed endothelial cells to form a branching network by recognizing the anisotropy of the 3D microenvironment.

Finally, a muscle tissue model was constructed using three cell types by mixing neurons and endothelial cells then seeding them onto an anisotropic myoblast sheet. They formed both neurite outgrowths and a vascular-like branching network uniformly throughout the whole area within the cell sheet construct. That is to say, this 3D microenvironment allowed all three types of cells to self-organize native-like microstructures within the tissue construct. While this biomimetic microstructure is obviously important to understand biological actions found in native tissue, we need to focus on the functionality of the tissue construct. In future work the structurally organized tissue construct shown here is expected to be effectively functionalized within our newly developed culture system [3].

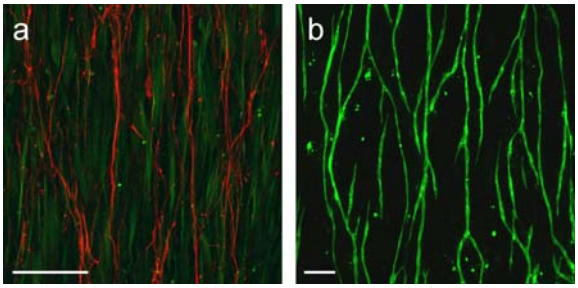

Figure 1. Formation of anisotropic cellular networks in multilayered myoblast sheets.
Neurons (a) or HUVECs (b) were sandwiched between two anisotropic myoblast sheets. At Day 5, confocal microscopic images were taken. Neurofilament (red) and desmin (green) were stained fluorescently in Figure 1a. HUVECs were stained with fluorescein-labeled ulex europaeus agglutinin I in Figure 1b. Scale bar: 100 μm.

Conclusions: We have shown a novel technique to create a muscle tissue construct through a cell sheet layering process. Uniquely, the structure of the networks made of neurons and endothelial cells can be controlled by using anisotropic myoblast sheets. The structural design in this muscle tissue construct and sequential functionalization in future work could lead to truly biomimetic tissue generation, and development of in-vitro physiological tissue models.

References: [1] Takahashi H. et al. *Biomaterials*. 2013; 34: 7372-7380. [2] Takahashi H. et al. *Biomacromolecules*. 2011; 12: 1414-1418. [3] Sakaguchi K. et al. *Sci. Rep.* 2013; 3: 1316.