Journal of Bioscience and Bioengineering
VOL. 119 No. 2, 212–216, 2015

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Microfluidic devices for construction of contractile skeletal muscle microtissues

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Received 9 April 2014; accepted 7 July 2014
Available online 30 July 2014

Cell-culture microchips mimicking tissue/organ-specific functions are required as alternatives to animal testing for drug discovery and disease models. Although three-dimensional (3D) cell culture microfluidic devices can create more biologically relevant cellular microenvironments and higher throughput analysis platforms of cell behavior than conventional techniques, devices for skeletal muscle cells have not been developed. In the present study, we aimed to develop microfluidic devices for 3D cultures of skeletal muscle cells. Skeletal muscle cells mixed with a collagen type-I solution was introduced into the microchannel for cells (MC-C) and was gelated. Then, the medium was introduced into the microchannel for medium (MC-M). During this process, connecting microchannels (Con-MCs) prevented leakage of the collagen solution mixed with cells from MC-C to MC-M and supplied the nutrients from the medium in MC-M to the cells in MC-C. Skeletal muscle microtissues cultured in the microchannel for a week consisted of myotubes were confirmed by histological analysis and immunofluorescence staining. The skeletal muscle microtissues in the microchannel contracted in response to externally applied electrical stimulation (1 and 50 Hz). These results indicate that the functional skeletal muscle microtissues were constructed in the microchannel. Thus, the microfluidic device for culturing 3D skeletal muscle microtissues presented in this study has a potential to be used for drug discovery and toxicological tests.

[**Key words:** BioMEMS; Skeletal-muscle-on-a-chip; Myoblasts; Myotubes; Differentiation; Active tension]

Development of *in vitro* human-tissue or organ models that are biologically relevant is required for drug discovery and disease modeling. Organs-on-chips, or cell-culture microchips that mimic specific organ functions, are promising technologies (1,2); the chips mimicking liver (3), lung (4), heart (5), blood–brain barrier (6), gut (7), and kidney (8) processes have been developed.

Skeletal muscle has unique features, such as huge multinucleated cells, myotubes, and highly ordered sarcomere structures and the cells. It functions as an actuator for physical movement of the body and assumes various physiological functions, including glucose homeostasis and body temperature maintenance. Defects of these functions cause various diseases, such as diabetes, adiposity, and age-related or disuse muscle atrophy. Many research groups, including our group, have developed various technologies for establishing skeletal-muscle-on-a-chip. Alignment of myoblasts and myotubes has been guided by microfeature techniques (9,10) and hydrogel macrochannels (11). Single myotubes have been micropatterned using elastic stencil masks (12). Contractile behavior of micropatterned myotubes has been individually controlled by localized electrical stimulation using microelectrode arrays (13). The contractile force generated by myotubes has been measured by Si-MEMS cantilever devices (14,15), UV-cross-linked collagen films (16–19), polydimethylsiloxane (PDMS) thin films (20), PDMS microposts (21,22), and so on (23). Furthermore, the fabrication of three-dimensional (3D) muscle constructs with neuromuscular junctions have been reported (24).

Microfluidic cell culture systems have been employed to create more biologically relevant cellular microenvironments and higher throughput analysis platforms of cell behavior than conventional techniques (25,26). However, the technologies described above have been used in combination with conventional cell culture techniques, or batch cultures using dishes or wells. To date, several groups have reported the development of two-dimensional (2D) cultures of skeletal muscle cells using microfluidic systems. Futai et al. developed a palm-sized microfluidic recirculation system in which a Braille display module was used for fluid control and showed that C2C12 myoblasts could proliferate and differentiate into myotubes in the microchannel (27). Tourovskaia et al. developed a microfluidic perfusion system in which micropatterned myoblasts were differentiated into myotubes in the microchannel and demonstrated a membrane receptor labeling assay to a region smaller than a myotube (28). Because 3D skeletal muscle cell cultures are proposed to provide a more physiologically relevant *in*

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2014.07.003

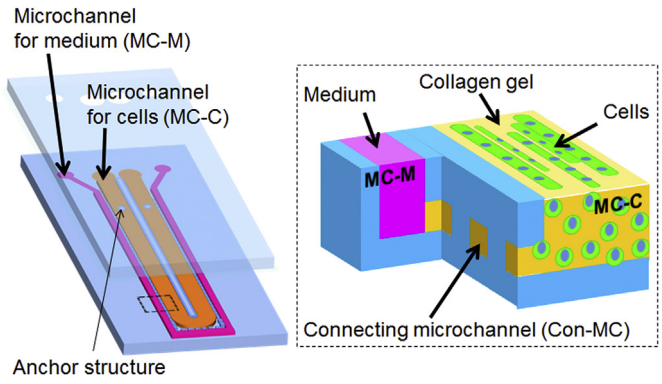

FIG. 1. Schematic drawing of developed microfluidic device.

vitro skeletal muscle model than 2D culture models (29,30), further development of microfluidic cell culture systems for 3D skeletal muscle cell cultures is warranted. Recently, Anene-Nzelu et al. developed a microfluidic cell culture system that used a micro-grooved substrate and showed that the substrate could align 3D cellular constructs cultured in the microchannels (31). However, there have been relatively few attempts to develop 3D skeletal muscle cell cultures in microchannels of microfluidic systems and, to the best of our knowledge, no reports are available on development of microfluidic devices that can be used to construct 3D skeletal muscle tissues in microchannels that contract in response to stimulation.

Therefore, in the present study, we aimed to develop microfluidic device to construct 3D contractile skeletal muscle tissues in microchannels (Fig. 1). We cultured skeletal muscle cells mixed with hydrogel in microchannels of the developed device and confirmed that the resulting skeletal muscle microtissues in the microchannels contracted in response to externally applied electrical stimulation.

MATERIALS AND METHODS

Device fabrication process The device design and fabrication process flow used are shown in Figs. S1 and 2. SU-8 3050 resist (SU-8; MicroChem Corp., Newton, MA, USA) was spin-coated at 3000 rpm for 30 s on a silicon wafer, patterned by photolithography using mask no. 1 and developed using SU-8 developer (MicroChem Corp.). Then, the SU-8 was spin-coated at 1000 rpm for 30 s on the wafer, patterned by photolithography using mask no. 2, and developed. Pre-cured PDMS mixture (Sylgard 184, Dow Corning, Midland, MI, USA) was cured on the patterned wafer at 75°C for 10 min, and the PDMS slab with micropatterns was removed from the wafer. The inlets and outlets of the microchannels were made by biopsies (φ1.5 mm; Kai Industry, Gifu, Japan). The pattered PDMS slab was irreversibly bonded with a PDMS surface formed on a glass surface, as described previously (32).

Medium diffusion assay Type-I collagen (3.0 mg/ml, Cellmatrix Type A, Nitta Gelatin Inc., Osaka, Japan), 5× Dulbecco's modified Eagles medium (DMEM), and reconstitution buffer were mixed together in a ratio of 7:2:1, according to the manufacturers' instruction. This solution was introduced into the microchannel for cells (MC-C) using a micropipette and was then gelated for 10 min in a 37°C, 5% CO_2 incubator. Then, the pipette tip filled with the DMEM (Invitrogen, Carlsbad, CA, USA) containing 2% horse serum and 1 mg/ml FITC-Albumin (Sigma) was attached to a hole of the microchannel for medium (MC-M), and the empty pipette tip was attached to the other hole of the MC-M. Diffusion of FITC-Albumin within the collagen gel was monitored using confocal laser scanning microscopy.

Cell culture Murine skeletal muscle cell line C2C12 cells were used. The C2C12 cells were maintained in growth medium (GM) consisting of DMEM (Invitrogen) containing 10% fetal bovine serum, 100 U/ml potassium penicillin G, and 100 μg/ml streptomycin sulfate. The C2C12 cells were differentiated in medium (DM) consisting of DMEM (Invitrogen) containing 2% horse serum, 100 U/ml potassium penicillin G, and 100 μg/ml streptomycin sulfate. The medium change was performed every day.

Construction of microtissues in microchannels Type-I collagen (3.0 mg/ml, Nitta Gelatin Inc.), 5× DMEM, and reconstitution buffer were mixed together in a ratio of 7:2:1, according to the manufacturers' instruction. Then, C2C12 cells were suspended in the solution at 1.0×10^7 cells/ml and placed in ice water until use. The solution containing cells was introduced into the MC-C using a micropipette and was then gelated for 10 min in a 37°C, 5% CO_2 incubator. Then, to drive the medium flow, the pipette tip filled with DM was attached to an MC-M hole, and the empty pipette tip was attached to the other MC-M hole. The tips were removed and new tips with and without DM were set in the same manner daily.

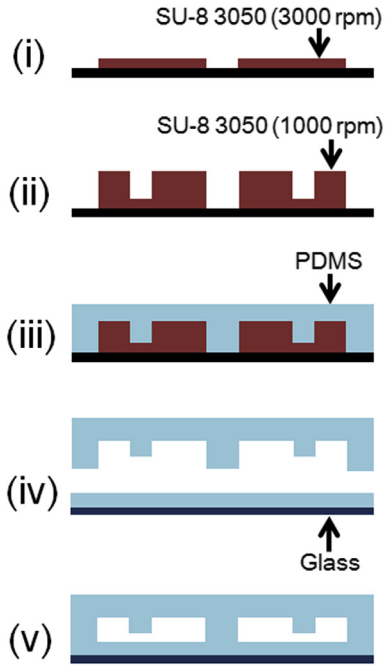

FIG. 2. Device fabrication. SU-8 photoresist was spin-coated on the Si wafer before it was exposed to UV light through a photomask. The exposed resist was then developed using SU-8 developer (i, ii). Polydimethylsiloxane (PDMS) was poured onto the wafer and cured (iii). The PDMS slab was then peeled from the wafer and bonded to the PDMS thin layer that was spin-coated on the glass (iv, v).

Hematoxylin and eosin staining Microtissues were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde phosphate buffer solution for 10 min. The microtissues were stained with eosin, taken from the device, and embedded in paraffin blocks. Five-micrometer thick paraffin sections were stained with hematoxylin.

Fluorescence microscopy Constructed microtissues were not taken out from the device during the procedure. The microtissues were washed with PBS and fixed in 4% paraformaldehyde phosphate buffer solution for 20 min, skinned in PBS containing 0.2% Triton-X 100 for 6 min, and washed with PBS for 10 min. Then, they were blocked in PBS containing 1% bovine serum albumin (PBS-BSA) for 60 min, washed with PBS for 10 min and immersed in PBS-BSA containing a monoclonal anti α-actinin (sarcomeric) antibody (EA-53, Sigma, Saint Louis, MO, USA; 1:200) for 120 min. After washing with PBS for 10 min, the microtissues were immersed in PBS-BSA containing secondary antibody conjugated with Alexa Fluor 488 (Invitrogen; 1:1000) for 60 min and rinsed with PBS for 10 min. Observations were performed using a fluorescence microscope (BX-51; Olympus, Tokyo, Japan).

Electrical stimulation The microtissues cultured in the device were stimulated electrically. The device was placed on a hot plate to maintain the medium temperature at 37°C. Two pipette tips filled with DM were attached to the inlet and outlet of the MC-M and gold electrodes (φ0.15 mm gold wire; The Nilaco Corporation, Tokyo, Japan) were inserted into these tips to be immersed in the DM. Electrical pulses (±20 V, 10 ms of pulse width, rectangular wave form) designed by the LabView software (National Instruments, Austin, TX, USA) were generated with a PC (33). The contraction of microtissues were observed using a microscope (CKX-31; Olympus) equipped with video camera (iVIS HF M52; Canon, Tokyo, Japan). The displacement of region of interest in the contracting microtissues was measured every 100 ms using Image J software.

RESULTS AND DISCUSSION

Device design and fabrication A schematic illustration of the device is shown in Fig. 1. The device has MC-M and MC-C. These hairpin-like microchannels are connected together via microchannels (Con-MCs). The cell cultures were processed using the microchannel device as follows: (i) collagen solution mixed