ENGINEERING A THREE-DIMENSIONAL TISSUE MODEL WITH A PERFUSABLE VASCULATURE IN A MICROFLUIDIC DEVICE

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ABSTRACT

In this study, we developed a microfluidic platform for a three-dimensional tissue model with a perfusable capillary network, which will allow, for the first time, a perfusion-culture in a tissue model with a high cell density. Our group previously reported that a spheroid of lung fibroblasts induced angiogenic sprouts from microchannels [1]. In this study, we successfully connected angiogenic sprouts to the vessel-like hollow structure in a spheroid and perfused the formed vascular network through microfluidic channels to the spheroid. This model opens up new techniques for tissue-culture for long-term.

INTRODUCTION

Recent bioengineering techniques have realized the construction of tissue models to screen the drug candidates or to study the morphogenesis during development [2-4]. Despite the significant progress in the field, current limitation is to culture large tissues for a long time. In a three-dimensional tissue, cells need to be located within 150-200 μm from a blood vessel because of the limited supply of oxygen and nutrient [5]. To improve functions of engineered tissues, the development of interconnected three-dimensional vascular networks within tissues plays a crucial role.

A multicellular aggregate, spheroid, is one of the appropriate models to mimic tissue morphology *in vivo*. To construct vessel-like structures in a spheroid, co-culturing target cells with endothelial cells is reported as effective methods [6, 7]. Although these techniques improved the culturable size of spheroids, the diameter of spheroids decreased in time-dependent manner [6]. This implies that the passive diffusion through the network from outside of a spheroid is insufficient to maintain the biological function in a spheroid. To realize the active flow in a vascular network, the connection with a perfusion system is in demand.

Recent techniques for construction of a perfusable vascular networks were roughly categorized into two groups: (1) templating and (2) self-organizing methods [8]. Templating methods are based on casting an extracellular matrices (ECM) material around a removable template and harvesting endothelial cells within the resulting empty channel [9, 10]. Although these approaches have ready-to-use features after the cell culture, the pattern of the vascular network cannot dynamically fit the environment when cultured with a tissue. This prevents the vascular network from its remodeling, which is essential for tissue development or morphogenesis. On the other hand, in self-organizing methods, spontaneous vascularization of endothelial cells is induced by the gradient of growth factors or co-cultures of supporting cells [11, 12]. Vascular networks in self-organizing methods have a similar morphology and permeability to that *in vivo*, and their patterns can be flexibly remodeled by environmental factors. Therefore, vascular networks developed through self-organizing methods better mimic *in vivo* physiological functions than those through templating methods.

By combining self-organizing vascularization method [12] and spheroid culture system, we have studied a new platform for a tissue culture that can induce the formation of a perfusable vascular network in a spheroid. In our previous study, we made a monoculture spheroid comprised of human lung fibroblasts (hLFs) which secrete angiogenic factors [1]. Although the monoculture spheroid successfully induced angiogenic sprouts toward the spheroid, they did not connect to the vessel-like structure inside the spheroid, lacking in the perfusability.

In this study, we cultivated two types of pre-vascularized spheroids, a co-culture (hLFs and Human umbilical vein endothelial cells (HUVECs)) and a tri-culture spheroid (hLFs, HUVECs and breast cancer cell line, MCF-7) in the microfluidic device to efficiently induce the anastomosis between the spheroid and the microchannels. We showed that the vessel-like hollow structure in a spheroid could anastomose with angiogenic sprouts from microchannels. The constructed vascular networks were perfusable and could be applied for assays of a drug delivery into a spheroid. This model provides new platform for a long-term tissue culture.

EXPERIMENTAL METHODS

Cell culture

HUVECs and hLFs were obtained from Lonza (Basel, Switzerland). Green fluorescent protein (GFP) expressing HUVECs and red fluorescent protein (RFP) expressing HUVECs were purchased from Anigio-Proteomie (Boston, MA). MCF-7 was obtained from RIKEN Cell Bank (Ibaraki, Japan). HUVECs, GFP-HUVECs, and RFP-HUVECs were cultured in EGM-2 (Lonza), hLFs were cultured in FGM-2 (Lonza). MCF-7 were cultured in DMEM containing 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA), 50 U/mL penicillin, and 50 μg/mL streptomycin (Thermo Fisher Scientific).

We made a co-culture spheroid (hLFs and HUVECs) and a tri-culture spheroid (hLFs, HUVECs and MCF-7) in a 96-well plate (Sumitomo Bakelite, Tokyo, Japan). For a co-culture spheroid, hLFs and HUVECs were mixed at a ratio of 4:1 (2.0×10^4 cells: 5.0×10^3 cells) in 200 μL EGM-2 and cultured for 4 days. For a tri-culture spheroid, hLFs, HUVECs and MCF-7 were mixed at a ratio of 3:1:1 (1.5×10^4 cells: 5.0×10^3 cells: 5.0×10^3 cells) in same volume of EGM-2 (200 μL) and cultured for 2 days.

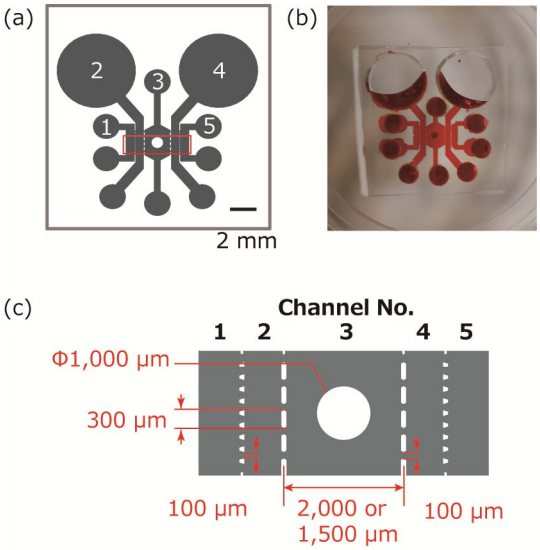

Figure 1: Overview of the microfluidic device. (a) The design of a microfluidic device, comprised of five microchannels. Each number indicates channel number. (b) The microfluidic device filled with red ink. (c) High magnification view of the red rectangle area in (a). Each channel was separated by microposts. The well in the channel 3 was used for culture of a spheroid.

Fabrication of the microfluidic device

The fabrication method of our microfluidic device was described previously [1]. Briefly, the microfluidic device was made of polydimethylsiloxane (PDMS) comprised of five microchannels (Fig. 1).

It was fabricated through conventional soft lithography and replica molding. A 100-μm thick master with a positive relief of negative photoresist SU-8 (MicroChem, Westborough, MA) was made by photolithography on a silicon wafer. The master was treated with trichloro(1H,1H,2H-perfluorooctyl)silane (Sigma, St Louis, MO). A 10:1 (w/w) mixture of PDMS pre-polymer and curing agent (Dow Corning Toray, Tokyo, Japan) was poured on the master, yielding negative replica-mold structure. Using biopsy punch (Sterile Dermal Biopsy Punch, 1, 2, and 6 mm diameters, Kai Industries, Tokyo, Japan), inlets, outlets, and a spheroid culture well were punched out. The diameters were 6 mm for the inlets of channels 2 and 4, 2 mm for the inlets and outlets of the others, and 1 mm for the spheroid well. After removing dust, the PDMS slab and glass cover slip (24 mm × 24 mm, Matsunami Glass, Osaka, Japan) were treated with oxygen plasma for 40 s for irreversible bonding; this was completed by curing at 80°C for 12 h.

Cell seeding in the microfluidic device

A spheroid was collected from a 96-well plate and embedded in a fibrin–collagen gel (2.5 mg/mL fibrinogen, Sigma, St Louis, MO), 0.15 U/mL aprotinin (Sigma), 0.2 mg/mL collagen (Corning, Corning, NY), and 0.5 U/mL thrombin (Sigma) in Dulbecco's phosphate-buffered saline (PBS, Roman, Tokyo, Japan). A spheroid was injected to a well in channel 3 (See Fig. 1(c)) and set at the bottom of the well. After polymerization of the fibrin–collagen at 37°C for 15 min, EGM-2 was added into channels 1, 2, 4, and 5.

After 24 h incubation to remove bubbles from a microchannels, suspension of HUVECs were prepared at 5 × 10^6 cells/mL in the EGM-2 medium and introduced to channels 2 and 4. By tilting the microfluidic device at 90 degrees and incubating for 30 min, HUVECs were adhered on the fibrin surface of channel 3 (Fig. 2, top). Other two channels were used for medium reservoirs (channels 1 and 5). The device was tilted 90° and incubated at 37°C in 5% CO_2 for 30 min to allow HUVECs to adhere to the fibrin gel surface. This process was repeated for channel 4, and HUVECs adhered onto the other side of the fibrin gel. Angiogenesis was induced by cultivation for 8-21 days after introduction of a spheroid (Fig. 2, bottom).

Fluorescence staining

For live cell imaging, MCF-7 were labeled with CellTracker™ Green CMFDA (CellTracker green, Thermo Fisher Scientific, Waltham, MA) in a 100-mm dish.

For nuclei and lectin-FITC staining of HUVECs, cells were fixed with 4% paraformaldehyde (PFA, Wako, Osaka, Japan) for 6–12 h and incubated with 10 μg/mL Hoechst 33342 (Thermo Fisher Scientific) and 5 μg/mL lectin-FITC for 12–24 h. The cells were subsequently rinsed with PBS three times to remove extracellular dyes and stored at 4°C until observation.

Imaging

Bright-field and fluorescent images were obtained using an inverted fluorescence microscope (IX71, Olympus, Tokyo, Japan) equipped with a charge-coupled device camera (ORCA-R2 Digital CCD, Hamamatsu Photonics, Shizuoka, Japan). Cross-sectional images were acquired at 1-μm intervals in the vertical direction with a confocal microscope (FluoView FV1000 confocal, Olympus). Images were stored as sequential files in TIFF format and analyzed with ImageJ software (National Institutes of Health, Bethesda, MD).

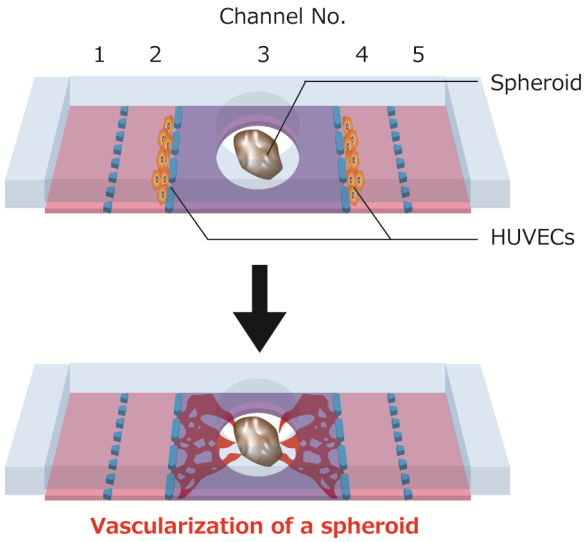

Figure 2: Schematic overview of vascularization of a spheroid. After seeding a spheroid (channel 3) and HUVECs (channels 2 and 4), the device was cultured for 8-21 days to induce angiogenic sprouts toward a spheroid.

RESULTS AND DISCUSSION

Vessel-like structure in a spheroid help anastomosis.

In the previous study, we confirmed that it was difficult to make a perfusable vascular network in a LF monoculture spheroid because angiogenic sprouts from microchannels 2 and 4 could not be anastomosed in a spheroid. In this study, we firstly investigated whether pre-formed a vessel-like structure in a spheroid would help anastomosis between sprouts from channels 2 and 4. Figure 3 shows the fluorescent micrographs of co-culture and tri-culture spheroid after 2 or 4 days in a 96-well plate. HUVECs in a spheroid formed vessel-like structures which uniformly distributed in a spheroid. The histological analysis confirmed no necrosis in the spheroid core.

Next, we injected a spheroid into a microfluidic device and evaluated whether angiogenesis could be induced from channels 2 and 4. HUVECs attached to the fibrin gel (Fig. 4(a) and (b)) formed angiogenic sprouts and reached to the spheroid after 2 days in device-culture. Figure 4(c) indicated the confocal micrographs of the center of the device at 14 days. Angiogenic sprouts from channels 2 and 4 connected spheroid and microchannels. These blood vessels had continuous lumens, whose diameters are 50-200 µm (Fig. 4(d)). In addition, the histological and immunohistological analysis showed that the vascular network was uniformly distributed in the spheroid. We also confirmed that tri-culture spheroid could also induce a similar pattern of a vascular network.

Collectively, we showed that a spheroid with pre-formed vessel like structures induced a continuous vascular network, which could connect the spheroid with microchannels. The spheroid had two features: 1) It could induce angiogenic sprouts from microchannels by the angiogenic factors from hLFs in the spheroid. 2) It could anastomose the vessel-like structures in the spheroid and the angiogenic sprouts from microchannels. Next, we tested whether these vascular networks could be applied to perfusion culture and drug delivery.

Constructed vascular networks were perfusable.

To evaluate the perfusability of our vascular network, FITC-dextran (70 kDa) was introduced from channel 2 (Fig. 5). FITC-dextran from channels 2 flowed through the constructed vascular network and the spheroid without any leakage. The permeability coefficient of the reconstructed vascular network was $0.56 \pm 0.39 \times 10^{-6}$ cm/s for 70 kDa FITC-dextran. These results indicated that our vascular network was perfusable and close to that *in vivo*.

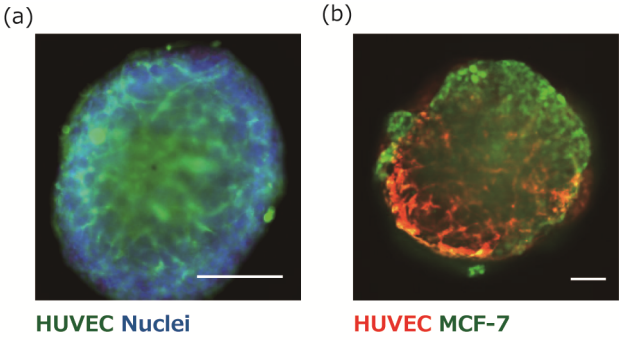

Figure 3: Fluorescent micrographs of (a) a co-culture and (b) a tri-culture spheroid. In (a), HUVECs were labeled by FITC-conjugated lectin from Ulex europaeus and all cellular nuclei were labeled by Hoechst 33342. In (b), HUVECs were labeled by RFP and MCF-7 were labeled by CellTracker Green. Scale bar = 200 µm.

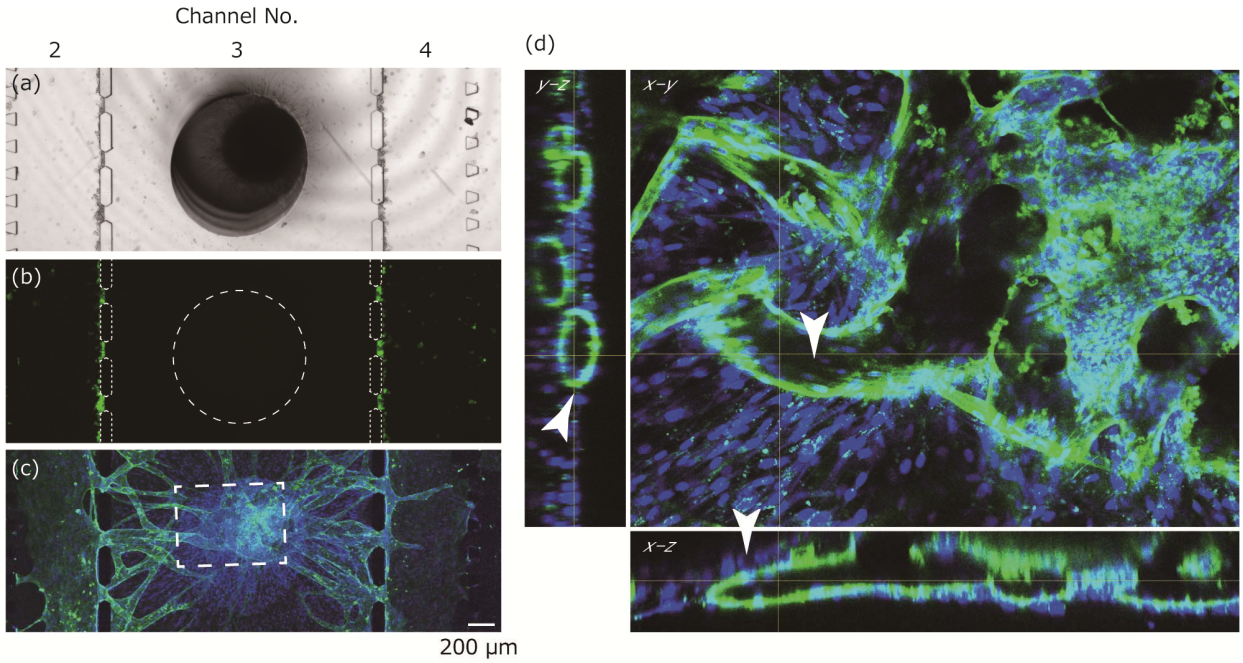

Figure 4: Vascular network formation induced by co-culture spheroid in the microfluidic device. Nuclei and HUVECs were fluorescently labeled by CellTracker green on day 0 and by Hoechst 33342 and lectin-FITC on day 14. Bright-field micrograph of the device on day 0 (a) and fluorescent micrographs on day 0 (b) and day 14 (c). (d) Confocal micrographs of the rectangle area in (c). Arrows indicate continuous vascular lumens.

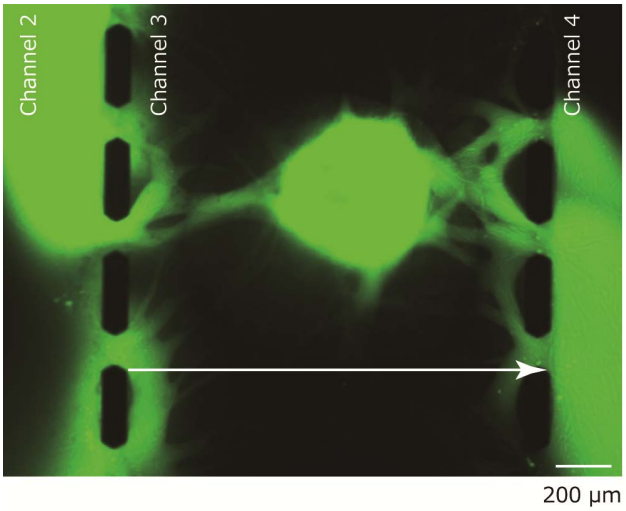

Figure 5; Perfusion of FITC-dextran (70 kDa) in vascular networks constructed in a spheroid. The arrow indicates the flow direction of FITC-dextran.

We investigated a drug administration to the spheroid using a constructed vascular network. The cellular membrane permeable reagents (Calcein Red Orange) could be delivered to the inner cells of the spheroid significantly faster than the diffusion from the microchannels. These results indicate that the supply of nutrients and oxygens can be achieved with the engineered perfusable vascular network, which is analogous to physiological functions of vascular networks *in vivo*.

CONCLUSION

In this study, we developed a method to create a three-dimensional cellular spheroid with a perfusable vascular network in a microfluidic device. We found that pre-formed vessel-like structures in a spheroid were effective to anastomose the spheroid and angiogenic sprouts from microchannels so that a perfusable vascular network was constructed in a spheroid. We also showed that the engineered vascular network could administer the biological substances to the interior of spheroid. Our model provided a new platform a long-term tissue culture system.

ACKNOWLEDGEMENTS

This work was supported by JST CREST, JSPS KAKENHI (25600060, 16K16386), AMED-PRIME, Mizuho Foundation for the Promotion of Sciences. Microfabrication was supported by Kyoto University Nano Technology Hub. Y.N. is supported by Tateisi Science and Technology Foundation for his travel.

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