Establishment of an *in vitro* vascular anastomosis model in a microfluidic device

Masafumi WATANABE* and Ryo SUDO*.

* School of Integrated Design Engineering, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223-8522, Japan
** Department of System Design Engineering, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223-8522, Japan

E-mail: sudo@sd.keio.ac.jp

Received: 3 December 2018; Revised: 27 February 2019; Accepted: 24 March 2019

Abstract
Formation of vascular anastomoses is critical for the development of transplantable tissue-engineered grafts, because rapid blood perfusion is required for the maintenance of implanted tissue grafts. However, the process of vascular anastomosis remains unclear due to difficulties in observing vascular anastomosis after transplantation. Although several groups have developed *in vitro* models of vascular anastomosis, there is a lack of a suitable *in vitro* anastomosis model that includes perivascular cells. Therefore, we aimed to establish an *in vitro* vascular anastomosis model containing perivascular cells by a combination of human umbilical vein endothelial cell (HUVEC) monoculture and HUVEC-mesenchymal stem cell (MSC) coculture in a microfluidic device. We found that vascular formation was inhibited when HUVECs were seeded on both sides of gel scaffolds, but HUVECs formed vascular networks when they were seeded on one side only. Next, we tested a series of HUVEC:MSC ratios to induce vascular anastomoses. The results demonstrated that addition of MSCs induced vascular anastomosis. In particular, the number of vascular anastomoses was significantly increased at a HUVEC:MSC ratio of 2:8. The process of vascular anastomosis was further investigated by live-cell imaging of green fluorescent protein-expressing HUVECs, which revealed that vascular anastomoses with continuous lumens were constructed during days 8–10. Computational simulation of VEGF concentrations suggested that local VEGF gradients play important roles in vascular formation while the addition of MSCs was critical for anastomosis. This anastomosis model will provide insights for both the development of tissue-engineered grafts and for the construction of large tissues by assembling multiple tissue-engineered constructs.

**Keywords**: Vascular anastomosis, *In vitro* model, Microfluidic device, Human umbilical vein endothelial cell, Mesenchymal stem cell, Chemical gradient

1. Introduction

The demand for organ transplantation has rapidly increased over the past few decades due to a large number of patients with chronic organ failure. However, organ shortage is a critical problem in medical transplantation, including liver and kidney (Caplan, 2016; Hong et al., 2014). To overcome this problem, it is necessary to construct transplantable organ/tissue grafts *in vitro*. Over the last decade, researchers have attempted to construct transplantable bioengineered tissue grafts *in vitro* (Kasuya et al., 2012; Palakkan et al., 2013; Stevens et al., 2017). Despite significant efforts, these studies remain in the early stages of research due to poor graft survival after transplantation (Caralt et al., 2015; Damania et al., 2014).
To improve graft survival, rapid blood perfusion is required for maintenance of implanted tissue grafts after transplantation. For this reason, formation of vascular anastomoses is necessary for transplantation. Previous studies have demonstrated two approaches to construct vascular anastomosis. One is surgical anastomosis between implanted vessels of tissue-engineered grafts and host vessels of recipients (Breuer et al., 2008). The other is anastomosis through self-organization between implanted endothelial cell (EC) networks and host vessels (Sekine et al., 2013; Takebe et al., 2014). In particular, the latter is important for implantation because microvessel-scale anastomosis between implanted vessels and host vessels depends on self-organization of ECs (Bogorad et al., 2015; Huang et al., 2015). For example, a previous study reported transplantation of bioengineered tissue grafts, and succeeded in blood perfusion between engineered vessels and host vessels (Cheng et al., 2011). However, the processes of anastomosis formation between these vessels remained unclear due to difficulties in observation after transplantation.

Several studies have demonstrated \textit{in vitro} vascular anastomosis models using microfluidic devices, which have advantages for investigating processes involved in vascular anastomosis. For example, previous studies have successfully demonstrated the formation of vascular anastomosis in a gel scaffold via angiogenesis and vasculogenesis in human umbilical vein endothelial cell (HUVEC) monoculture (Song et al., 2012; Wang et al., 2014; Yeon et al., 2012). These vascular networks failed to maintain their small luminal size and structures in long-term culture due to the lack of perivascular cells (e.g., mural cells and pericytes). \textit{In vivo} vascular anastomoses are formed between engineered and host vessels, which include both ECs and perivascular cells. Therefore, it is necessary to establish an \textit{in vitro} vascular anastomosis model, including perivascular cells, to further understand the processes of \textit{in vivo} vascular anastomosis formation.

To mimic \textit{in vivo} vascular anastomosis formation, it is important to construct vessels covered with perivascular cells in a microfluidic device. We previously demonstrated coculture of HUVECs and mesenchymal stem cells (MSCs) in a microfluidic device and successfully constructed vascular networks covered with pericyte-like perivascular cells which expressed α smooth muscle actin (αSMA) (Yamamoto et al., 2013). In addition, our recent study demonstrated that vessels constructed in the HUVEC-MSC coculture model were in good agreement with \textit{in vivo} vessels in terms of morphology and function (Yamamoto et al., 2019). Thus, we hypothesized that HUVEC-MSC coculture might be useful for establishment of an \textit{in vitro} vascular anastomosis model by mimicking the \textit{in vivo} vessels covered with perivascular cells.

Here, we investigated how to form vascular anastomoses between vessels with perivascular cells and nascent vessels using a microfluidic device. First, we performed HUVEC monoculture, resulting in ECs that failed to form vascular anastomoses. We then performed HUVEC-MSC coculture to induce angiogenesis, which led to the formation of vascular anastomosis by day 10. This vascular anastomosis was constructed between nascent vessels and stable vessels, which were covered with MSC-derived perivascular cells. Furthermore, the processes of anastomosis formation were monitored using green fluorescent protein-expressing HUVECs (GFP-HUVECs). Finally, the microarchitecture of vascular anastomoses was observed to confirm continuous lumen structures.

2. Materials and Methods
2.1 Fabrication of microfluidic devices
The fabrication process of microfluidic devices used in this study was described previously (Shin et al., 2012). Briefly, master molds of microfluidic devices were produced by UV-photolithography techniques, and the molds were copied to polydimethylsiloxane (PDMS; Sylgard 184, Dow Corning, Midland, MI, USA). The PDMS devices were bonded to cover-glasses after exposure to air plasma, which resulted in formation of microchannels. The microchannels were coated with 1 mg/ml poly-D-lysine solution (Sigma-Aldrich, St. Louis, MO, USA) and rinsed twice with sterile distilled water. The coated microfluidic devices were dried for more than 24 h until use.

A rat-tail type I collagen solution was adjusted to 4 mg/ml and pH 7.4 (Corning, NY, USA) with 0.5 N sodium hydroxide (Wako, Osaka, Japan). Similarly, fibrinogen (Sigma-Aldrich) was dissolved in phosphate-buffered saline...
(PBS) to 4 mg/ml. The fibrinogen and collagen solutions were then mixed at a 9:1 ratio. Finally, the fibrinogen-collagen solution was mixed with 10 unit/ml thrombin solution (Sigma-Aldrich) at a 10:1 ratio and injected into the gel channel of microfluidic devices (750 μm width and 150 μm height) (Fig. 1A–B). Microfluidic devices were then placed in a humidified 5% CO₂ incubator at 37°C for 30 min to promote gelation. Finally, lateral microchannels were filled with cell culture medium and the devices were kept in the incubator until use.

2.2 Cell preparation

HUVECs, GFP-HUVECs, and MSCs were cultured to form vascular anastomosis. HUVECs (Lonza, Basel, Switzerland) and GFP-HUVECs (Angio-Proteomie, Boston, MA, USA) were obtained commercially and cultured in endothelial growth medium (EGM-2; Lonza) in a 5% CO₂ incubator at 37°C. HUVECs and GFP-HUVECs were expanded in collagen-coated cell culture dishes and cells at passages 4–7 were used for experiments.

Isolation of MSCs from human bone marrow was previously described using the LNGFR (CD271) and THY-1 (CD90) surface markers (Mabuchi et al., 2013). The isolated MSCs were expanded in MSC growth medium: Dulbecco’s modified Eagle’s medium with low glucose (Invitrogen, Carlsbad, CA, USA) supplemented with 20% fetal bovine serum (FBS), 20 ng/ml basic fibroblast growth factor (bFGF; PeproTech, Rocky Hill, NJ, USA), and 1% antibiotic-antimycotic (Gibco, Waltham, MA, USA). Cells were then cultured in a humidified 5% CO₂ incubator at 37°C. All experiments were performed with cells at passages 5–7.

2.3 Cell Seeding for the formation of vascular anastomosis

HUVECs were dissociated from cell culture dishes using trypsin-EDTA (Thermo Fisher Scientific, Waltham, MA, USA) and suspended in EGM-2 at a concentration of 1 × 10⁶ cells/ml. MSCs were similarly detached using trypsin-EDTA and suspended in DMEM (Gibco) supplemented with 20% FBS (MSC medium) at a concentration of 1 × 10⁶ cells/ml. First, 10 μl cell suspension of HUVECs were seeded in a microchannel through an outlet of the microchannel (Fig. 1C). Microfluidic devices were then tilted to promote cell attachment to the surface of the gel scaffold. Similarly, the other microchannel was filled with 10 μl cell suspension of HUVECs and MSCs in total at ratios of 2:8, 5:5, and 8:2. Subsequently, microfluidic devices were incubated for 30 min in a humidified 5% CO₂ incubator at 37°C to allow cell attachment. Finally, reservoirs of microchannels were replenished with a 1:1 mixture of EGM-2 and MSC medium supplemented with 10 ng/ml vascular endothelial growth factor (VEGF; R&D Systems, Minneapolis, MN, USA) and 15 ng/ml bFGF. The culture medium was replaced daily, and cells were cultured until day 10. During this culture period, HUVECs extended vascular networks from both microchannels and finally formed vascular anastomosis in the gel scaffold (Fig. 1D–E).
Fig. 1 Schematic illustrations of an *in vitro* vascular anastomosis model in a microfluidic device. (A–B) A schematic illustration of a microfluidic device. (C) Human umbilical vein endothelial cells (HUVECs, red) and mesenchymal stem cells (MSCs, green) were seeded into microchannels and attached to the surfaces of the gel scaffold. (D) MSCs migrated into the gel and induced the formation of vascular sprouts. (E) Finally, microvessels extended from both sides of the gel contacted each other and formed vascular anastomosis (asterisks).

### 2.4 Immunofluorescent staining and imaging of cultured cells

A rabbit anti-CD146 antibody and a mouse anti-αSMA antibody (Sigma-Aldrich) were used as primary antibodies labeling HUVECs and pericyte-like perivascular cells, respectively (Yamamoto et al., 2019). Alexa Fluor 594-conjugated anti-rabbit IgG (Invitrogen) and Alexa Fluor 488-conjugated anti-mouse IgG (Invitrogen) were used as secondary antibodies.

Samples were fixed in 4% paraformaldehyde for 15 min at 25°C and permeabilized with 0.1% Triton X-100 for 5 min. After rinsing with PBS, samples were treated with BlockAce (DS Pharma Biomedical, Osaka, Japan) for 1 h to inhibit non-specific staining. Samples were then incubated overnight with primary antibodies, followed by incubation with secondary antibodies. Thereafter, samples were incubated with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) for staining cell nuclei.

Three-dimensional (3D) fluorescence images were obtained using a confocal laser-scanning microscope (LSM700; Carl Zeiss, Oberkochen, Germany). The 2D projected images were generated from 3D fluorescence images using maximum intensity Z-projection in ImageJ (National Institutes of Health, Bethesda, MD, USA).

### 2.5 Quantitative analysis of vascular networks

Geometrical characteristics of vascular networks (e.g., vascular distance, total length, and number of anastomoses) were analyzed using 2D phase-contrast images or projected images of 3D confocal data generated by ImageJ. Maximum vascular distance was defined as the distance between the gel surface and the vascular tip. The entire gel region was divided into 6 regions as shown in Fig. 2C and maximum distance of vascular networks was measured in each region.
This distance was measured using a straight-line tool in ImageJ. Total length of vascular networks was measured by multiple lines that were drawn along the long axis of the vessel using the straight-line tool. Vascular anastomosis was defined by a continuous lumen structure between microvessels, which was confirmed by cross-sectional images of vascular networks. The number of vascular anastomoses was counted manually.

2.6 Diffusion simulation of VEGF in HUVEC-MSC coculture

The concentration of VEGF in the hydrogel scaffold was calculated by Fick’s second law using a finite element simulation software, COMSOL Multiphysics 5.2a (COMSOL Inc., Burlington, MA, USA). To create 3D models of microfluidic devices, gel channels, medium channels, and medium ports were modeled in COMSOL. VEGF distributions were calculated based on a previous study (Jeong et al., 2011), using a diffusion coefficient of VEGF in the medium of $2.5 \times 10^{-9}$ m$^2$/s, and that in the gel scaffold of $6.6 \times 10^{-11}$ m$^2$/s (Helm et al., 2005; Sudo et al., 2009), respectively. The consumption rate of VEGF in HUVECs in microchannels was assumed to be 20 ng/day/10$^6$ cells (Zandstra et al., 1997), and VEGF secretion from MSCs in microchannels was assumed to be 24 ng/day/10$^6$ cells (Beckermann et al., 2008). Colormaps of VEGF distribution at 24 h were obtained.

2.7 Live-cell imaging for monitoring the process of in vitro vascular anastomosis formation

To monitor the formation of vascular anastomosis, GFP-HUVECs were cultured with MSCs in microfluidic devices. GFP-HUVECs were seeded in a microchannel, while both GFP-HUVECs and MSCs were seeded in the other channel. The cell culture medium was replenished daily, and fluorescence images were taken every other day until day 10 to monitor the process of anastomosis formation using a fluorescence microscope (Axio Observer Z1; Carl Zeiss).

2.8 Observation of the detailed structure of in vitro vascular anastomoses

Z-stack fluorescence images were acquired with 40× objective lens (EC Plan-Neofluar, NA 1.30; Carl Zeiss) to examine the detailed structure of vascular anastomosis between HUVECs and GFP-HUVECs using a confocal laser-scanning microscope (LSM700). Projected and cross-sectional images were generated from the z-stack fluorescence images using maximum intensity Z-projection and orthogonal views in ImageJ, respectively. To demonstrate vascular anastomosis, 3D reconstruction images were generated from the z-stack fluorescence images by Imaris 7.7.0 software (Bitplane, Badenerstrasse, Zurich, Switzerland).

2.9 Statistical analysis

Experiments were repeated at least twice to confirm reproducibility of results. Data are presented as means ± SEM. Tukey’s multiple comparison test was used to test for differences, which were considered statistically significant at $p < 0.05$. Statistical analyses were performed using SPSS (IBM, Armonk, NY, USA).

3. Results

3.1 Vascular formation was inhibited in HUVEC-HUVEC monoculture

First, we cultured HUVECs in one of two microchannels to confirm the ability of vascular formation. In this “HUVEC-alone” condition, HUVECs attached to one side of the gel scaffold and formed vascular sprouts. These vascular sprouts gradually extended across the gel scaffold and finally developed into vascular networks. The constructed vessels reached to the other microchannel across the gel scaffold of 750 μm width by day 10 (Fig. 2A). Next, we cultured HUVECs in both microchannels to assess whether HUVECs form vascular anastomosis in vitro. In this “HUVEC-HUVEC” condition, HUVECs attached to both sides of the gel scaffold. In contrast to the HUVEC-alone condition, vascular sprouts were hardly observed, at least by day 10 (Fig. 2B). Although some sprouts were formed, they were not stable and eventually regressed (Fig. 2B, arrowheads). Quantitative analysis was performed focusing on maximum
vascular distance (Fig. 2C), which demonstrated that the elongation of the constructed vessels was significantly inhibited in the HUVEC-HUVEC condition, compared to the HUVEC-alone condition (Fig. 2D). Specifically, maximum vascular distance was 430 ± 46.6 µm in the HUVEC-alone condition, while this figure was 99.9 ± 20.8 µm in the HUVEC-HUVEC condition on day 10.

To investigate this inhibitory vascular formation in the HUVEC-HUVEC condition, we performed computational simulation, focusing on VEGF concentration, because establishment of a VEGF gradient is an important factor to regulate vascular formation. In the initial condition, there was no VEGF gradient because both microchannels were filled with culture medium containing 10 ng/ml VEGF (data not shown). However, HUVECs consumed VEGF, resulting in changes in VEGF concentration. In the present study, we performed HUVEC monoculture in static culture. Thus, concentrations of VEGF varied depending on the VEGF consumption rate of cells in channels due to the absence of fresh medium perfusion. The simulation results indicated that a VEGF gradient was formed by 24 h in the HUVEC-alone condition (Fig. 3A), while in the HUVEC-HUVEC condition, a flat distribution was obtained at 24 h (Fig. 3B). These results indicated that VEGF distribution varied depending on initial cell distribution.
Fig. 2 The processes of vascular formation in HUVEC monoculture. (A) A schematic illustration of the “HUVEC-alone” condition. HUVECs were seeded in a microchannel and attached to one side of the gel scaffold. HUVECs formed vascular sprouts on day 1, which developed into networks extending into the entire gel by day 10. Arrowheads indicate tips of constructed vessels. (B) A schematic illustration of the “HUVEC-HUVEC” condition. HUVECs were seeded in both microchannels and attached to both sides of the gel scaffold. Although HUVECs formed some vascular sprouts, these were not stable and eventually regressed (arrowheads). (C) A schematic diagram for the measurement of maximum vascular distance. (D) Quantitative analysis of maximum vascular distance. Scale bars: 200 μm (A), (B). *p < 0.05 (N = 3, n = 11).
Fig. 3 Computational simulation of VEGF gradients in HUVEC monoculture. (A) The overall and enlarged views of colormap show the distribution of vascular endothelial growth factor (VEGF) in the “HUVEC-alone” condition at 24 h of culture. The concentration gradient of VEGF was maintained across the gel scaffold. (B) The overall and enlarged views of colormap show the distribution of VEGF in the “HUVEC-HUVEC” condition at 24 h. No VEGF gradient was observed in the gel scaffold.

3.2 HUVEC-MSC coculture promoted formation of in vitro vascular anastomosis

Recently, we reported that MSCs promoted the formation of microvascular networks in a microfluidic device (Yamamoto et al., 2019). Thus, HUVEC-MSC coculture was performed to support vascular anastomosis in a microfluidic device. Specifically, we added MSCs to the HUVEC-HUVEC culture condition to induce angiogenesis. Moreover, a series of HUVEC:MSC ratios were tested in HUVEC-MSC coculture (Fig. 4A). Immunofluorescence images showed that vascular formation was dependent on HUVEC:MSC ratios. At a HUVEC:MSC ratio of 2:8, vascular networks were extended from both microchannels and they formed vascular anastomoses by day 10 (Fig. 4B, asterisks). Furthermore, enlarged images showed that these vascular networks were covered with perivascular cells, which were positive for a pericyte marker, αSMA (Fig. 4B(i), (ii)). In contrast, vascular anastomoses were less frequently observed at HUVEC:MSC ratios of 5:5 and 8:2 (Fig. 4C, D).

Quantitative analysis showed that both maximum vascular distance and total length of vascular networks were significantly greater at the HUVEC:MSC ratio of 2:8, compared to the other ratios (Fig. 4E, F). Moreover, the number of vascular anastomoses was significantly increased at the HUVEC:MSC ratio of 2:8, which was >3-times greater than the other ratios (Fig. 4G).

We also performed computational simulation of VEGF concentrations in the HUVEC-MSC coculture at the HUVEC:MSC ratios of 2:8, 5:5, and 8:2 in static culture. Similar to the HUVEC monoculture, there was no VEGF gradient in the initial condition because both microchannels were filled with culture medium containing 10 ng/ml VEGF (data not shown). However, HUVECs consumed VEGF while MSCs produced VEGF, resulting in changes in local VEGF concentration near cells, depending on HUVEC:MSC ratios. The simulation results showed that VEGF gradients were formed by 24 h under all conditions (Fig. 5A–C). Notably, the steepest VEGF gradient was detected at the HUVEC:MSC ratio of 2:8. This coculture condition was used for anastomosis formation in the following experiments.
Fig. 4 Comparison of *in vitro* vascular anastomosis models in HUVEC-MSC coculture at different HUVEC:MSC ratios. (A) Schematic illustrations of cell culture conditions. HUVECs were seeded in microchannels, while both HUVECs and MSCs were seeded in the other channels at different HUVEC:MSC ratios (2:8, 5:5, 8:2). (B–D) Formation of vascular anastomosis in microfluidic devices. The fluorescence image represents the formation of vascular networks (CD146, red) covered with perivascular cells (αSMA, green) on day 10. Cell nuclei were stained with DAPI. Arrowheads indicate vascular anastomosis. Enlarged images (i) and (ii) correspond to the boxes (i) and (ii) in B, respectively. (E–G) Quantitative analyses of maximum vascular distance (E), total length of vascular networks (F), and the number of vascular anastomoses (G) on day 10. Scale bars, 200 μm (B–D) and 50 μm (i, ii). *p < 0.05 (N = 3, n = 10).
Fig. 5 Computational simulation of VEGF gradients in HUVEC-MSC coculture at different HUVEC:MSC ratios. (A–C) The overall and enlarged views of colormaps and graphs show the distribution of VEGF at 24 h in HUVEC-MSC coculture at HUVEC:MSC ratios of 2:8 (A), 5:5 (B), and 8:2 (C).

3.3 Live-cell imaging of GFP-HUVECs in the process of vascular anastomosis formation

To further investigate the process of vascular anastomosis formation, live-cell imaging of GFP-HUVECs was performed until day 10 in the HUVEC-MSC coculture at the HUVEC:MSC ratio of 2:8. In this experiment, GFP-HUVECs were seeded in a microchannel, while both GFP-HUVECs and MSCs were seeded in the other microchannel (Fig. 6A). First, vascular sprouts were formed on day 2, which extended from the side of HUVECs alone (Fig. 6B, arrowheads). These vascular sprouts gradually extended across the gel scaffold, while vascular sprouts also extended from the side of HUVECs and MSCs by day 4 (Fig. 6C, arrowheads). These vascular sprouts extended from both microchannels and continued to extend. Vascular interconnections between the tips of these vessels were formed during days 6–8 (Fig. 6D, E, asterisks). Finally, vascular anastomoses were established in the microfluidic device by day 10 (Fig. 6F, asterisks).
Fig. 6 Live-cell imaging of GFP-HUVECs in the process of vascular anastomosis formation. (A) GFP-HUVECs were seeded in microchannels, while both GFP-HUVECs and MSCs were seeded in the other channels at a HUVEC:MSC ratio of 2:8. (B–D) GFP-HUVECs formed vascular sprouts on day 4 from both sides of the microchannel and then extended vascular networks on day 6. Arrowheads indicate the tips of vascular networks. (E–F) Microvessels extended from different microchannels contacted each other and formed vascular anastomosis (asterisks) after day 8. Scale bar, 200 μm.

3.4 Investigation of the detailed structure of in vitro vascular anastomosis

To investigate the detailed structure of vascular anastomosis, vessels were three-dimensionally observed by confocal microscopy. In this experiment, GFP-HUVECs were seeded in one of the two microchannels to distinguish vessels extended from each microchannel, while both HUVECs and MSCs were seeded in the other channel at a HUVEC:MSC ratio of 2:8 (Fig. 7A). Vascular structures were fixed on day 10 and stained for CD146, an endothelial marker. Immunofluorescence imaging showed that the upper part of the vessel was positive for both CD146 and GFP (Fig. 7B, i–ii), while the lower part of the vessel was positive for CD146 (Fig. 7B, iii). This represents vascular anastomosis because the upper part of the vessel was extended from the microchannel of GFP-HUVECs alone while the lower part of the vessel was extended from the microchannel of both HUVECs and MSCs. Corresponding 3D reconstruction images indicated that vascular anastomosis with continuous lumens was constructed (Fig. 7C–E).
Fig. 7 Detailed structure of vascular anastomosis between HUVECs and GFP-HUVECs. (A) A schematic illustration of vascular anastomosis between HUVECs and GFP-HUVECs. GFP-HUVECs were seeded in microchannels, while both HUVECs and MSCs were seeded in the other channels at a HUVEC:MSC ratio of 2:8. (B) Projected confocal image showing vascular anastomosis labeled with CD146 (HUVECs) and GFP (GFP-HUVECs). Cross-sectional images correspond to the lines i–iii. (C) 3D reconstruction image of vascular anastomosis. (D, E) 3D reconstructed images show that both GFP-HUVECs and HUVECs formed continuous lumens. Scale bars, 20 μm (B) and 10 μm (C).

3.5 Pro-angiogenic effects of VEGF gradient in HUVEC-HUVEC monoculture

Finally, we performed HUVEC-HUVEC monoculture with VEGF gradient to investigate whether VEGF gradient can induce the formation of vascular anastomosis in this model. To generate a VEGF gradient across the gel scaffold, one channel was filled with culture medium containing 50 ng/ml VEGF, while the other channel was filled with culture medium containing 10 ng/ml VEGF. In this condition, HUVECs formed vascular sprouts, while few vascular sprouts were observed without VEGF gradient (Fig. 8A, B). Quantitative analysis revealed that the VEGF gradient significantly promoted vascular formation in the HUVEC-HUVEC condition. The maximum vascular distance in the presence of VEGF gradient was approximately 2.5-times greater than that without VEGF gradient (Fig. 8C). However, vascular anastomoses were not formed even in the presence of VEGF gradient.
Fig. 8 HUVEC-HUVEC monoculture with VEGF gradient. (A) A schematic illustration and phase-contrast images of the “HUVEC-HUVEC” condition. An arrowhead indicates a vascular sprout. (B) A schematic illustration and phase-contrast images of the “HUVEC-HUVEC + VEGF gradient” condition. Arrowheads indicate vascular sprouts. (C) Quantitative analysis of maximum vascular distance. Scale bars: 50 μm (A), (B). *p < 0.05 (N = 2, n = 8).

4. Discussion

4.1 Inhibition of vascular sprouting in “HUVEC-HUVEC” monoculture

In the present experiments, HUVEC monoculture was performed to form vascular anastomosis in a microfluidic device in vitro. Contrary to our expectations, vascular formation was significantly inhibited when HUVECs were seeded in both microchannels (“HUVEC-HUVEC” condition). This can be explained by VEGF gradient profiles in “HUVEC-alone” and “HUVEC-HUVEC” conditions. Simulation results indicated that a VEGF gradient across the gel scaffold was maintained in the “HUVEC-alone” condition, whereas it is not maintained in the “HUVEC-HUVEC” condition because HUVECs consumed VEGF supplemented in the medium in both microchannels. We also verified the effect of VEGF gradient in the HUVEC-HUVEC condition, which resulted in enhanced vascular formation. These results suggest that VEGF gradient is one of the most important factors for regulating angiogenesis.

It is well-known that chemical gradients (e.g., VEGF and S1P) facilitate EC migration and vascular sprouting (van Duinen et al., 2018; Liu et al., 2007). Previous studies have reported that VEGF regulates the formation and orientation
of vascular sprouts, which are parallel to the VEGF gradient (Shamloo et al., 2012; Wu et al., 2014). Furthermore, VEGF is also known to regulate EC morphogenesis via induction and activation of specific matrix metalloproteases (MMPs), which play important roles in EC migration (Funahashi et al., 2011). However, vascular anastomoses were not formed even in the HUVEC-HUVEC condition in the presence of VEGF gradient. Although VEGF gradient enhanced angiogenesis, it was not sufficient for the formation of vascular anastomoses in this model. Therefore, HUVEC monoculture was not suitable for the formation of vascular anastomosis.

### 4.2 Establishment of an in vitro vascular anastomosis model in HUVEC-MSC coculture

As we found that HUVEC monoculture failed to form vascular anastomosis even in the presence of VEGF gradient, HUVEC-MSC coculture was conducted to induce vascular sprouting and anastomosis formation. The important findings of the present study are as follows: 1) the amount of MSCs added to the HUVEC monoculture significantly affected vascular formation, 2) greater numbers of vascular anastomoses were formed at a HUVEC:MSC ratio of 2:8, and 3) the addition of MSCs was critical for the formation of vascular anastomosis in our model. Two factors can help to explain these findings.

First, vascular formation was varied by different VEGF gradient profiles due to the different amount of MSCs added to the HUVEC-MSC coculture. As we confirmed by computational diffusion analysis, the steepest VEGF gradient was maintained at 24 h at a HUVEC:MSC ratio of 2:8. This is consistent with the greatest number of vascular anastomoses at a HUVEC:MSC ratio of 2:8. However, this cannot explain vascular extension from the side of HUVECs and MSCs.

Second, vascular formation was promoted by other secreted or juxtacrine factors of MSCs (Nassiri et al., 2014). There are many evidences that MSCs promote vascular formation (Jeon et al., 2014; McFadden et al., 2013), which may induce the formation of vascular networks in a microfluidic device. Our recent study also demonstrated that HUVEC-MSC coculture promoted EC sprouting and migration along MSC networks in the gel scaffold (Yamamoto et al., 2019). These findings suggested that multiple factors including VEGF gradient were involved in the formation of vascular networks and anastomoses in this model. In particular, HUVECs might extend vascular networks in response to a local gradient of chemical factors secreted by the MSCs distributed in the gel scaffold. This is consistent with our results that microvessels were extended from both microchannels, and there was subsequent formation of vascular contact between microvessels. In addition, other groups reported that ECs at the tips of adjacent sprouts would align ECM between them by a traction-mediated effect, which can also facilitate vascular anastomosis formation (Vernon and Sage, 1995).

The addition of MSCs is also critical to mimic in vivo vascular anastomosis. Vascular anastomosis in this study was constructed between microvessels formed by HUVECs alone, and by those formed in HUVEC-MSC culture. Our results demonstrated the construction of microvessels covered with MSC-derivived perivascular cells, which was similar to in vivo microvessels because in vivo vascular anastomosis is regulated by not only EC but also perivascular cells (Hirschi and D’amore, 1996; Ribatti et al., 2011). Therefore, this culture model can recapitulate in vivo vascular anastomosis between engineered and host vessels.

### 4.3 Comparison of in vitro and in vivo anastomosis formation

As we succeeded in establishing an in vitro model of vascular anastomosis, the processes and detailed structures of anastomoses were investigated by live-cell imaging of GFP-HUVECs. Previous studies have reported the processes of vascular anastomosis formation during embryonic development using animal models (e.g., zebrafish). These studies described that a multistep process is involved in vascular anastomosis, including EC migration, vascular contact, and luminal formation (Herwig et al., 2011; Lenard et al., 2013). In particular, VE-cadherin protein was shown to play critical roles in the early steps of vascular anastomosis, such as recognition of tip cells, contact formation, and lumen formation.

We also confirmed that vascular anastomosis was formed through EC migration and vascular contact, finally resulting in the formation of continuous lumens. In addition, other groups reported that in vivo vascular anastomosis formation
between arterial and venous vessels in zebrafish is regulated by a chemokine receptor, cxcr4a, and its ligand, cxcl12b (Bussmann et al., 2011). Our anastomosis model may provide detailed insight to further elucidate the mechanism of in vivo anastomosis formation. Additional investigation will be needed to evaluate vascular functions after anastomosis generation, such as barrier functions and antithrombotic properties.

5 Conclusion

In the present study, we successfully established an in vitro vascular anastomosis model in a microfluidic device. First, we revealed that vascular formation was inhibited in “HUVEC-HUVEC” cell culture conditions. Next, we tested HUVEC-MSC coculture to induce vascular anastomosis. The results demonstrated that the addition of MSCs induced the formation of vascular anastomoses. Computational simulation suggested that local VEGF gradients play an important role in vascular formation. However, since HUVECs failed to form vascular anastomoses in HUVEC-HUVEC conditions even in the presence of VEGF gradient, we found that the addition of MSCs was critical for vascular anastomoses in our culture model. This anastomosis model will provide useful information for the development of tissue-engineered grafts in terms of vascular anastomosis formation between engineered and host vessels to overcome the problem of poor graft survival after transplantation. Moreover, this model can provide insights to construct larger tissue by assembling multiple tissue-engineered constructs by forming vascular anastomosis between these tissues.

Acknowledgement

We are grateful to Dr. Yo Mabuchi (Tokyo Medical and Dental University) and Yumi Matsuzaki (Shimane University) for generously providing mesenchymal stem cells (MSCs). We thank Dr. Yamato Kikkawa (Tokyo University of Pharmacy and Life Science) for generously providing anti-CD146 antibody. This work was supported, in part, by Japan Society for Promotion of Science (16H03173, 18K19937).

References

Beckermann, B.M., Kallifatidis, G., Groth, A., Frommhold, D., Apel, A., Mattern, J., Salnikov, A.V., Moldenhauer, G., Wagner, W., Diehlmann, A., Saffrich, R., Schubert, M., Ho, A.D., Giese, N., Büchler, M.W., Friess, H., Büchler, P., and Herr, I., VEGF expression by mesenchymal stem cells contributes to angiogenesis in pancreatic carcinoma, British Journal of Cancer, Vol. 99, No. 4 (2008), pp. 622–631

Bogorad, M.I., DeStefano, J., Karlsson, J., Wong, A.D., Gerecht, S., and Searson, P.C., Review: In vitro microvessel models, Lab on a Chip, Vol. 15, No. 22 (2015), pp. 4242–4255.

Brennan, M.P., Dardik, A., Hibino, N., Roh, J.D., Nelson, G.N., Papademitris, X., Shinoka, T., and Breuer, C.K., Tissue-engineered vascular grafts demonstrate evidence of growth and development when implanted in a juvenile animal model, Annals of Surgery, Vol. 248, No. 3 (2008), pp. 370–376.

Bussmann, J., Wolfe, S.A., and Siekmann, A.F., Arterial-venous network formation during brain vascularization involves hemodynamic regulation of chemokine signaling, Development, Vol. 138, No. 9 (2011), pp. 1717–1726.

Caplan, A.L., Finding a solution to the organ shortage, Canadian Medical Association Journal, Vol. 188, No. 16 (2016), pp. 1182–1183.

Caralt, M., Uzarski, J.S., Iacob, S., Obergfell, K.P., Berg, N., Bijonowski, B.M., Kiefer, K.M., Ward, H.H., Wandinger-Ness, A., Miller, W.M., Zhang, Z.J., Abecassis, M.M., and Wertheim, J.A., Optimization and critical evaluation of decellularization strategies to develop renal extracellular matrix scaffolds as biological templates for organ engineering and transplantation, American Journal of Transplantation, Vol. 15, No. 1 (2015), pp. 64–75.

Cheng, G., Liao, S., Kit Wong, H., Lacorre, D.A., di Tomaso, E., Au, P., Fukushima, D., Jain, R.K., and Munn, L.L.,
Engineered blood vessel networks connect to host vasculature via wrapping-and-tapping anastomosis, Blood, Vol. 118, No. 17 (2011), pp. 4740–4749.

Damania, A., Jain, E., and Kumar, A., Advancements in in vitro hepatic models: Application for drug screening and therapeutics, Hepatology International, Vol. 8, No. 1 (2014), pp. 23–38.

van Duinen, V., Zhu, D., Ramakers, C., van Zonneveld, A.J., Vulto, P., and Hankemeier, T., Perfused 3D angiogenic sprouting in a high-throughput in vitro platform, Angiogenesis, Vol. 22, No. 1 (2019), pp. 157–165.

Funahashi, Y., Shawber, C.J., Sharma, A., Kanamaru, E., Choi, Y.K., and Kitajewski, J., Notch modulates VEGF action in endothelial cells by inducing Matrix Metalloprotease activity, Vascular Cell, Vol. 3, No. 1 (2011), pp. 1–12.

Helm, C.L., Fleury, M.E., Zisch, A.H., Boschetti, F., and Swartz, M.A., Synergy between interstitial flow and VEGF directs capillary morphogenesis in vitro through a gradient amplification mechanism, Proceedings of the National Academy of Sciences of the United States of America, Vol. 102, No. 44 (2005), pp. 15779–15784.

Herwig, L., Blum, Y., Krudewig, A., Ellertsdottr, E., Lenard, A., Belting, H.G., and Affolter, M., Distinct cellular mechanisms of blood vessel fusion in the zebrafish embryo, Current Biology, Vol. 21, No. 22 (2011), pp. 1942–1948.

Hirschi, K.K., and D’Amore, P.A., Pericytes in the microvasculature, Cardiovascular Research, Vol. 32, No. 4 (1996), pp. 687–698.

Hong, J.C., Venick, R., Yersiz, H., Kositamongkol, P., Kaldas, F.M., Petrowsky, H., Farmer, D.G., Agopian, V., McDiarmid, S.V., Hiatt, J.R., and Busuttill, R.W., Liver transplantation in children using organ donation after circulatory death: A case-control outcomes analysis of a 20-year experience in a single center, JAMA Surgery, Vol. 149, No. 1 (2014), pp. 77–82.

Huang, Y., Tong, D., Zhu, S., Wu, L., Mao, Q., Ibrahim, Z., Lee, W.P., Brandacher, G., and Kang, J.U., Evaluation of microvascular anastomosis using real-time, ultra-high-resolution, fourier domain doppler optical coherence tomography, Plastic and Reconstructive Surgery, Vol. 135, No. 4 (2015), pp. 711–720.

Jeon, J.S., Bersini, S., Whisler, J.A., Chen, M.B., Dubini, G., Charest, J.L., Moretti, M., and Kamm, R.D., Generation of 3D functional microvascular networks with human mesenchymal stem cells in microfluidic systems, Integrative Biology, Vol. 6, No. 6 (2014), pp. 555–563.

Jeong, G.S., Han, S., Shin, Y., Kwon, G.H., Kamm, R.D., Lee, S.H., and Chung, S., Sprouting angiogenesis under a chemical gradient regulated by interactions with an endothelial monolayer in a microfluidic platform, Analytical Chemistry, Vol. 83, No. 22 (2011), pp. 8454–8459.

Kasuya, J., Sudo, R., Tamogami, R., Masuda, G., Mitaka, T., Ikeda, M., and Tanishita, K., Reconstruction of 3D stacked hepatocyte tissues using degradable, microporous poly(d,l-lactide-co-glycolide) membranes, Biomaterials, Vol. 33, No. 9 (2012), pp. 2693–2700.

Lenard, A., Ellertsdottr, E., Herwig, L., Krudewig, A., Sauteur, L., Belting, H.G., and Affolter, M., In vivo analysis reveals a highly stereotypic morphogenetic pathway of vascular anastomosis, Developmental Cell, Vol. 25, No. 5 (2013), pp. 492–506.

Liu, L., Ratner, B.D., Sage, E.H., and Jiang, S., Endothelial cell migration on surface-density gradients of fibronectin, VEGF, or both proteins, Langmuir, Vol. 23, No. 22 (2007), pp. 11168–11173.

Mabuchi, Y., Morikawa, S., Harada, S., Niibe, K., Suzuki, S., Renault-Mihara, F., Houlihan, D.D., Akazawa, C., Okano, H., and Matsuzaki, Y., LNGFR+THY-1+VCAM-1hi cells reveal functionally distinct subpopulations in mesenchymal stem cells, Stem Cell Reports, Vol. 1, No. 2 (2013), pp. 152–165.

McFadden, T.M., Duffy, G.P., Allen, A.B., Stevens, H.Y., Schwarzmair, S.M., Plesnila, N., Murphy, J.M., Barry, F.P., Guldberg, R.E., and O’Brien, F.J., The delayed addition of human mesenchymal stem cells to pre-formed endothelial cell networks results in functional vascularization of a collagen-glycosaminoglycan scaffold in vivo, Acta Biomaterialia, Vol. 9, No. 12 (2013), pp. 9303–9316.
Nassiri, S.M., and Rahbarghazi, R., Interactions of mesenchymal stem cells with endothelial cells, *Stem Cells and Development*, Vol. 23, No. 4 (2013), pp. 319–332.

Palakkam, A.A., Hay, D.C., Anil Kumar, P.R., Kumary, T.V., and Ross, J.A., Liver tissue engineering and cell sources: issues and challenges, *Liver International*, Vol. 33, No. 5 (2013), pp. 666–676.

Ribatti, D., Nico, B., and Crivellato, E., The role of pericytes in angiogenesis, *International Journal of Developmental Biology*, Vol. 55, No. 3 (2011), pp. 261–268.

Sekine, H., Shimizu, T., Sakaguchi, K., Dobashi, I., Wada, M., Yamato, M., Kobayashi, E., Umezu, M., and Okano, T., In vitro fabrication of functional three-dimensional tissues with perfusable blood vessels, *Nature Communications*, Vol. 4, (2013), pp. 1310–1399.

Shamloo, A., Xu, H., and Heilshorn, S., Mechanisms of Vascular Endothelial Growth Factor-Induced Pathfinding by Endothelial Sprouts in Biomaterials, *Tissue Engineering Part A*, Vol. 18, No. 3–4 (2012), pp. 320–330.

Shin, Y., Han, S., Jeon, J.S., Yamamoto, K., Zervantonakis, I.K., Sudo, R., Kamm, R.D., and Chung, S., Microfluidic assay for simultaneous culture of multiple cell types on surfaces or within hydrogels, *Nature Protocols*, Vol. 7, No. 7 (2012), pp. 1247–1259.

Song, J.W., Bazou, D., and Munn, L.L., Anastomosis of endothelial sprouts forms new vessels in a tissue analogue of angiogenesis, *Integrative Biology*, Vol. 4, No. 8 (2012), pp. 857–862.

Stevens, K.R., Scull, M.A., Ramanan, V., Fortin, C.L., Chaturvedi, R.R., Knouse, K.A., Xiao, J.W., Fung, C., Mirabella, T., Chen, A.X., McCue, M.G., Yang, M.T., Fleming, H.E., Chung, K., de Jong, Y.P., Chen, C.S., Rice, C.M., and Bhatia, S.N., In situ expansion of engineered human liver tissue in a mouse model of chronic liver disease, *Science Translational Medicine*, Vol. 9, No. 399 (2017), pp. 1–10.

Sudo, R., Chung, S., Zervantonakis, I.K., Vickerman, V., Toshimitsu, Y., Griffith, L.G., and Kamm, R.D., Transport-mediated angiogenesis in 3D epithelial coculture, *FASEB Journal*, Vol. 23, No. 7 (2009), pp. 2155–2164.

Vernon, R.B., and Sage, E.H., Between molecules and morphology. Extracellular matrix and creation of vascular form, *The American Journal of Pathology*, Vol. 147, No. 4 (1995), pp. 873–83.

Wang, X., Phan, D.T., Sobrino, A., George, S.C., Hughes, C.C., and Lee, A.P., Engineering anastomosis between living capillary networks and endothelial cell-lined microfluidic channels, *Lab on a Chip*, Vol. 16, No. 2 (2014), pp. 282–290.

Wu, Y., Al-Ameen, M.A., and Ghosh, G., Integrated Effects of Matrix Mechanics and Vascular Endothelial Growth Factor (VEGF) on Capillary Sprouting, *Annals of Biomedical Engineering*, Vol. 42, No. 5 (2014), pp. 1024–1036.

Yamamoto, K., Tanimura, K., Mabuchi, Y., Matsuzaki, Y., Chung, S., Kamm, R.D., Ikeda, M., Tanishita, K., and Sudo, R., The stabilization effect of mesenchymal stem cells on the formation of microvascular networks in a microfluidic device, *Journal of Biomechanical Science and Engineering*, Vol. 8, No. 2 (2013), pp. 114–128.

Yamamoto, K., Tanimura, K., Watanabe, M., Sano, H., Uwamori, H., Mabuchi, Y., Matsuzaki, Y., Chung, S., Kamm, R.D., Tanishita, K., and Sudo, R., Construction of continuous capillary networks stabilized by pericyte-like perivascular cells, *Tissue Engineering Part A*, Vol. 25, No. 5–6 (2019), pp. 499–510.

Yeoh, J.H., Ryu, H.R., Chung, M., Hu, Q.P., and Jeon, N.L., In vitro formation and characterization of a perfusable three-dimensional tubular capillary network in microfluidic devices, *Lab on a Chip*, Vol. 12, No. 16 (2012), pp. 2815–2822.

Zandstra, P.W., Conneally, E., Petzer, A.L., Piret, J.M., and Eaves, C.J., Cytokine manipulation of primitive human hematopoietic cell self-renewal, *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 94, No. 9 (1997), pp. 4698–703.