Published in final edited form as:

Microvasc Res. 2019 March ; 122: 60–70. doi:10.1016/j.mvr.2018.11.007.

Comparison of organ-specific endothelial cells in terms of microvascular formation and endothelial barrier functions

Hiroyuki Uwamori¹, Yuuichi Ono², Tadahiro Yamashita^{1,3}, Ken Arai⁴, and Ryo Sudo^{1,3}

¹School of Integrated Design Engineering, Keio University, Japan

²Sohyaku. Innovative Research Division, Research Unit/Immunology & Inflammation, Mitsubishi Tanabe Pharma Corporation, Japan

³Department of System Design Engineering, Keio University, Japan

⁴Neuroprotection Research Laboratory, Departments of Radiology and Neurology, Massachusetts General Hospital and Harvard Medical School, USA

Abstract

Every organ demonstrates specific vascular characteristics and functions maintained by interactions of endothelial cells (ECs) and parenchymal cells. Particularly, brain ECs play a central role in the formation of a functional blood brain barrier (BBB). Organ-specific ECs have their own morphological features, and organ specificity must be considered when investigating interactions between ECs and other cell types constituting a target organ. Here we constructed angiogenesis-based microvascular networks with perivascular cells in a microfluidic device setting by coculturing ECs and mesenchymal stem cells (MSCs). Furthermore, we analyzed endothelial barrier functions as well as fundamental morphology, an essential step to build an *in vitro* BBB model. In particular, we used both brain microvascular ECs (BMECs) and human umbilical vein ECs (HUVECs) to test if organ specificity of ECs affects the formation processes and endothelial barrier functions of an engineered microvascular network. We found that microvascular formation processes differed by the source of ECs. HUVECs formed more extensive microvascular networks compared to BMECs while no differences were observed between BMECs and HUVECs in terms of both the microvascular diameter and the number of pericytes peripherally associated with the microvasculatures. To compare the endothelial barrier functions of each type of EC, we performed fluorescence dextran perfusion on constructed microvasculatures. The permeability coefficient of BMEC microvasculatures was significantly lower than that of HUVEC microvasculatures. In addition, there were significant differences in terms of tight junction protein expression. These results suggest that the organ source of ECs influences the properties of engineered microvasculature and thus is a factor to be considered in the design of organ-specific cell culture models.

Corresponding author: Ryo Sudo, Ph.D., Departments of System Design Engineering, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama, 223-8522 Japan, Phone: +81-45-566-1745, sudo@sd.keio.ac.jp.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

angiogenesis; microfluidics; blood brain barrier; permeability

INTRODUCTION

Microvascular networks are essential for correct organ functioning. Each organ has its own vascular characteristics and functions that are maintained through interactions between endothelial cells (ECs) and parenchymal cells within vascular niches (Géraud et al., 2014). Angiocrine and paracrine factors secreted by ECs regulate organ homeostasis and function (Rafii et al., 2016), and the fundamental function of microvascular networks is to deliver oxygen and nutrients throughout organs. ECs that coat microvascular walls adhere to each other through junctional proteins such as VE-cadherin, occludin, and ZO-1 (Greene and Campbell, 2016; Abbott et al., 2010). While the transport of macromolecules and undesired substances such as neurotoxins out of the microvasculature is strongly inhibited by endothelial barrier functions, ECs regulate the active transport of nutrients and oxygen into the organ parenchyma by paracellular and transcellular pathways (Greene and Campbell, 2016; Abbott et al., 2010; Barar et al., 2016). In addition to this universal function, ECs display slightly different features depending on the organ in which they reside. Notably, brain ECs cooperate with astrocytes and pericytes to form a characteristic vascular microenvironment called the blood brain barrier (BBB) (Zhao et al., 2015). Brain microvascular networks strictly prohibit the transport of toxic substances to the brain parenchyma and effectively transfer compounds essential for brain homeostasis. On the other hand, ECs of the kidney glomeruli and liver effectively maintain active transport of blood-borne molecules to the parenchyma (Géraud et al., 2014; Ben-Zvi et al., 2014). In addition to these morphological features of organ-specific ECs, ECs from different organs exhibit tissue-specific expression profiles of transcription factors, angiocrine growth factors, adhesion molecules, and chemokines (Nolan et al., 2013). Such differences in both the structural organization and function of organ-specific ECs suggest that organ specificity should be considered when investigating interactions between ECs and other cell types constituting a target organ.

The brain has organ-specific ECs that play important roles in the functioning of the BBB. Many studies have investigated BBB development and functions because the regulation of the BBB is important for both drug delivery and drug screening (Bicker et al., 2014; Banerjee et al., 2016). Transport properties of the brain capillary endothelium and interactions between ECs and perivascular cells, such as pericytes and astrocytes, have also been the focus of previous studies (Sweeney et al., 2018; Keaney and Campbell, 2015; Helm et al., 2016; Banerjee et al., 2016). By culturing ECs with pericytes and/or astrocytes, cell culture systems have been observed to mimic *in vivo* microvascular environments. In some studies, a Transwell cell culture system was used to obtain transendothelial electrical resistance (TEER) measurements that were further analyzed to estimate endothelial barrier functions. These studies established that endothelial barrier function is augmented when ECs are cultured with astrocytes and pericytes. However, the ECs in these studies formed a two-dimensional (2D) monolayer rather than a native *in vivo* three-dimensional (3D) capillary

structure. It has also been reported that ECs isolated from brain capillaries tend to lose their barrier properties (Banerjee et al., 2016). In addition, ECs are covered by pericytes and astrocytic end-feet and the percentage of pericyte coverage around capillaries are the highest in the brain compared to the other organs (Keaney and Campbell, 2015; Thomsen et al., 2017; Zlokovic, 2008; Daneman et al., 2010; Armulik et al., 2010; Trost et al., 2016). These fundamental features of brain capillaries were not included in previous *in vitro* studies, highlighting the disparity between *in vitro* BBB models and native EC conditions *in vivo*.

To bridge this gap, it is imperative that a successful 3D brain microvascular network be constructed. An *in vitro* BBB model requires two key features: the construction of functional capillary-level microvascular networks and the coverage of pericytes and astrocytic end-feet around the vasculatures. The construction of capillary-level microvasculatures is particularly challenging, especially in terms of capillary diameter: indeed, most previously reported microvasculatures have been between 20 and 100 μm (Kim et al., 2015; Kim et al., 2013; Nguyen et al., 2013), much larger than that of real capillaries. Toward the establishment of an *in vitro* BBB model, we first focused on constructing capillary-level microvascular networks with pericyte coverage. Previous studies that have constructed *in vitro* microvascular networks have used microfluidic cell culture platforms in which cells are cultured in 3D gel scaffolds mimicking an *in vivo* extracellular matrix (ECM) (Kim et al., 2015; Kim et al., 2013). We created an angiogenesis model using ECs and human mesenchymal stem cells (MSCs) (Yamamoto et al., in press). These studies have used human umbilical vein endothelial cells (HUVECs).

In this study, we developed a microfluidic cell culture platform by coculturing human brain microvascular endothelial cells (BMECs) and MSCs, an essential milestone in the construction of an *in vitro* BBB model. To compare the characteristics of organ-specific ECs, HUVECs were also cultured with MSCs as opposed to BMECs. MSCs were used in the coculture because of their ability to differentiate into pericytes both *in vivo* (Koike et al., 2004) and *in vitro* (Yamamoto et al., 2013; Yamamoto et al., in press). We assessed whether organ-specific ECs affect vascular formation processes and whether microvascular networks constructed using such ECs exhibit their own characteristics in terms of endothelial barrier functions and fundamental morphology.

MATERIALS AND METHODS

Cell culture

BMECs and HUVECs were obtained from Cell Systems (Kirkland, WA, USA) and Lonza (Basel, Switzerland), respectively. Both BMECs and HUVECs were cultured in EGM-2 (Lonza) and expanded in fibronectin-coated culture dishes for no more than seven passages.

The isolation of MSCs has previously been described (Yamamoto et al., 2013; Yamamoto et al., in press). Briefly, MSCs were isolated from human bone marrow using LNGFR (CD271) and Thy-1 (CD90) surface markers. First, bone marrow mononuclear cells (Poietics™; Lonza, Walkersville, MD, USA) were suspended at 1 to 5×10^7 cells/mL in ice-cold Hank's balanced salt solution supplemented with 2% fetal bovine serum (FBS), 10 mM HEPES, and 1% penicillin/streptomycin. Then the cells were stained for 30 min on ice with a monoclonal

antibody. LNGFR-PE (Miltenyi Biotec, Bergisch Gladbach, Germany) and Thy-1-APC (BD Pharmingen, CA, USA) were used as antibodies. To eliminate dead cells from flow cytometric analyses, propidium iodide (2 µg/mL) was used. Flow cytometric analyses and sorting were performed on a triple-laser MoFlo (Beckman Coulter, CA, USA) or FACS Vantage SE (Becton Dickinson, Heidelberg, Germany).

MSCs were expanded in the MSC growth medium: Dulbecco's modified Eagle's medium with low levels of glucose (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 20% FBS, and 1% antibiotic-antimycotic (Gibco, Gaithersburg, MD, USA). MSCs were used to promote vascular formation and stabilize vascular structures (Yamamoto et al., 2013; Yamamoto et al., in press). MSCs were used at passages 6–8.

Cells were cultured in a humidified 5% CO₂ incubator at 37°C. A 1:1 mixture of EGM-2 and the MSC medium was used for the coculture of ECs and MSCs in a microfluidic device.

Preparation of microfluidic devices

The construction process of the microfluidic device used in this study has previously been described (Shin et al., 2012). Briefly, the microfluidic device was made of polydimethylsiloxane (PDMS; Silgard 184, Dow Corning, Midland, MI, USA) and was produced using soft lithography with SU-8 patterned wafers. After peeling cured PDMS from an SU-8 mold, it was cut into pieces and punched to make media inlets and gel inlets. To create microchannels, each device was plasma-bonded with a cover glass. After the microchannels were coated with 1 mg/mL poly D-lysine solution (Sigma-Aldrich, St. Louis, MO, USA), each device was rinsed twice with sterile deionized water and dried.

This microfluidic device has three parallel microchannels: the central channel filled with hydrogel and two microchannels filled with culture medium (Fig. 1). The central channel has two gel inlets of 1.2 mm diameter (Fig. 1A, arrowheads), and the two microfluidic channels have two media inlets of 4.0 mm in diameter (Fig. 1A, arrows). The widths of these channels were 750 µm and 500 µm, respectively (Fig. 1B). The height of each channel was 135 µm.

Hydrogel filling and cell seeding in microfluidic devices

Prepolymerized fibrinogen solution (4 mg/mL) was dissolved in PBS and injected into the central channel through the two gel inlets immediately after thrombin (10 units/mL, Sigma-Aldrich) was added to the solution to promote gelation. After injection of the pre-gel solution, devices were placed in a humidified 5% CO₂ incubator at 37°C for 30 min to complete the polymerization of the gel solution in the central channel. Then the microfluidic channels were filled with culture medium to prevent the gel from drying out. The microfluidic devices were kept in these conditions until use.

After dissociation from culture dishes using TrypLE (Gibco), BMECs, HUVECs, and MSCs were suspended in their own culture medium at a concentration of 1×10^6 cells/mL. After aspiration of the media from reservoirs of the microfluidic device, 1×10^4 cells of BMECs or HUVECs were seeded into the prepared device using a microchannel. When seeding the cells, devices were tilted by 90° to allow them to adhere to the gel surface. Then the devices

were incubated for 30 min in a humidified 5% CO₂ incubator at 37°C to complete the cell attachment on the gel surface. After seeding ECs into a microchannel, MSCs were seeded into the same microchannel. Culture medium was exchanged and phase-contrast images were captured on a daily basis to monitor the process of cell migration and microvascular formation inside the 3D gel region.

Immunofluorescence staining of cultured cells

For the imaging of microvasculatures constructed on days 3 and 7, samples were fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100 for 5 min. After permeabilization, samples were treated with BlockAce (Dainippon Pharmaceutical, Japan) for 1 h to block nonspecific staining. Then the samples were incubated overnight with primary antibodies, sheep anti-PECAM-1 antibody (1:100 dilution; R&D Systems) for detection of BMECs and HUVECs, and mouse anti- α -SMA antibody (1:200 dilution; Sigma-Aldrich), mouse anti-neural/glial antigen 2 (NG2) antibody conjugated phycoerythrin (1:50 dilution; R&D systems) and rabbit anti-platelet-derived growth factor receptor beta (PDGFR β) antibody (1:100 dilution, Clone Y92; abcam) for detection of pericytes differentiated from MSCs. Rabbit anti-VE-cadherin antibody (1:100 dilution; CST Technologies), rabbit anti-occludin antibody (1:100 dilution; Invitrogen), and mouse anti-ZO-1 antibody (1:50 dilution; Invitrogen) were used to detect junctional proteins. In addition, rat anti-type IV collagen antibody (1:50 dilution; Shigei Medical Research Institute) and rabbit anti-laminin antibody (1:200 dilution; LSL) were used to detect basement membrane proteins. The following day, samples were incubated with secondary antibodies (1:100 dilution): Alexa Fluor 488-conjugate anti-mouse IgG (Invitrogen), Alexa Fluor 555-conjugate anti-sheep IgG (Invitrogen), or Alexa Fluor 647-conjugate anti-rabbit IgG (Invitrogen) and Alexa Fluor 405-conjugate anti-rabbit IgG (Invitrogen) at room temperature for 2 h. Finally, the samples were incubated with 4, 6-diamidino-2-phenylindole (DAPI; Invitrogen) to stain cell nuclei. The samples were rinsed twice with PBS between each step. Z-stack fluorescent images were obtained using a confocal laser-scanning microscope (LSM700, Carl Zeiss, Germany). The 2D projected images were constructed from Z-stack fluorescent images and analyzed using the ImageJ software program (National Institutes of Health, Bethesda, MD, USA).

Quantification and statistical analysis of microvascular network formation

To quantify the characteristics of the constructed microvascular networks, cells were fixed with 4% paraformaldehyde and stained with antibodies as described above. After Z-projection images were obtained using ImageJ (NIH), sprout length and microvascular length were manually measured using Image J. After 2D projection images were generated with z-stack images of microvasculatures, sprout length and microvascular length in the 2D projection images were traced using the freehand line tool. The length of the traced lines was measured to quantify sprout length and microvascular length. Sprout number and branch points were quantified by manually counting the sprout number and branch points in each image. Pericyte numbers were quantified by counting α -SMA-expressing cell nuclei on the outer surfaces of the microvascular structures. To quantify the outer microvascular diameter, manual measurements using the orthogonal view (X-Z plane) of immunofluorescence images were captured. The diameter was measured at 1/3 and 2/3 of the width of the central

channel along the Y-axis (Fig. 3D). Quantitative analyses of junctional proteins such as VE-cadherin, ZO-1, and occludin were performed using ImageJ. After thresholding each image, image calculator, a plugin of ImageJ, was used to calculate fluorescence intensity of junctional proteins in ECs constituting microvasculatures. Experiments were repeated at least three times to confirm the reproducibility of results. Data are presented as means $\pm$ SEMs. A Student's *t*-test was used to test for differences between the two groups, which were considered statistically significant at $P < 0.05$.

Dextran perfusion and measurement of microvascular permeability coefficient

To analyze microvascular permeability, fluorescein isothiocyanate-conjugated dextran (FITC-dextran, 2000 kDa) or Texas Red-conjugated dextran (Texas Red-dextran, 70 kDa) were introduced into the constructed microvascular networks. Following aspirated removal of culture media from the microfluidic channels, 10 μ L of 50 μ g/mL FITC-dextran or 50 μ g/mL Texas Red-dextran was added to microchannels containing seeded ECs and MSCs. Fluorescence images were obtained every 10 s using the 20 $\times$ objective lens of a confocal laser-scanning microscope (LSM700).

Permeability coefficients of BMEC/HUVEC microvasculatures were calculated using the following equation assuming that microvascular structure is circular (Kim et al., 2013; Bichsel et al., 2015):

$$P = \frac{r}{2} \times \frac{dI/dt}{I_0}$$

where r is vessel radius, I_0 is the initial intravascular fluorescence intensity and was not changed during the dextran perfusion experiment, and dI/dt is the change in fluorescence intensity per unit time in the perivascular region within 3 μ m from the capillary wall.

RESULTS

Initial vascular sprout formation in BMEC/HUVEC-MSC cocultures

First, we focused on an early stage of microvascular formation recapitulated by BMEC-MSC coculture and HUVEC-MSC coculture. Both ECs and MSCs were seeded in the same microchannel and attached to the surface of the gel region (Fig. 2A, Day 0). These cells then actively migrated into the gel region from the start of coculture regardless of EC origin (Fig. 2A, Day 2, arrowheads). Corresponding phase-contrast images demonstrated that these cells migrated across the gel region within 3 days of seeding (Fig. 2A, Day 3).

Immunofluorescence images showed that vascular sprouts stained with PECAM-1 were formed by day 3 (Fig. 2B, arrowheads). Quantitative analyses of the immunofluorescence images revealed that both sprout number and microvascular length were significantly greater in HUVEC-MSC cocultures than in BMEC-MSC cocultures (Fig. 2B–D). These results suggest that vascular formation in the early stage is different depending on EC origin.

Microvascular network formation in BMEC/HUVEC-MSC cocultures

Next, we performed EC-MSC cocultures until day 7 to observe the extension and the maturation of constructed microvasculatures. According to phase-contrast microscopy, the number of migrating cells into the gel region was increased on day 7 compared to that on day 3 (Fig. 3A versus Fig. 2A). Microvascular networks were constructed by day 7 regardless of EC origin, which was observed by immunostaining microvascular structures with PECAM-1, α -SMA and DAPI (Fig. 3B). Enlarged images showed that some perivascular cells expressing α -SMA, which were derived from MSCs, wrapped around microvascular configurations (Fig. 3C).

To evaluate vascular formation by BMECs and HUVECs, we compared characteristics of micro vasculatures formed by these ECs. Specifically, quantitative analyses were performed for microvascular length, branch points, outer diameter, and pericyte number. As expected from the results of vascular formation in the early stages as shown in Figure 2, HUVECs formed more complex microvascular networks compared to BMECs. For example, the length of constructed microvascular networks was greater in HUVEC-MSC cocultures than in BMEC-MSC cocultures (Fig. 3E). Branch points were also greater in HUVEC-MSC cocultures (Fig. 3F), but microvascular outer diameter did not differ between cell types (Fig. 3G). Finally, pericyte numbers were comparable between cocultures (Fig. 3H).

Basement membrane formation and pericyte coverage around microvasculatures

Next, we investigated the formation of basement membranes and the coverage of pericytes around microvasculatures, an important stage in vascular maturation. Immunofluorescence staining revealed that basement membrane proteins such as laminin and type IV collagen were expressed along with microvasculatures regardless of EC origin (Fig. 4A, 4B). Laminin was localized in the basal domain of the microvascular configurations (arrowheads, Fig. 4A), suggesting that ECs constituting microvasculatures recognized apical-basal polarity. Type IV collagen was localized in the outer space of microvasculatures. Some type IV collagen was co-expressed with α -SMA (arrows, Fig. 4A), suggesting potential cooperation between ECs and MSCs in type IV collagen production. In terms of pericyte coverage, we performed immunofluorescence staining of microvasculatures for pericyte markers, NG2 and PDGFR β , in addition to α -SMA. These markers were also expressed in perivascular cells in both BMEC-MSC and HUVEC-MSC cocultures (Fig. 4C, 4D). Cross-sectional images showed that these pericytes wrapped PECAM-1-positive microvasculatures. These results indicate that the constructed microvasculatures became mature and stabilized within 7 days of EC-MSC coculture.

Permeability assay of microvascular walls using fluorescence dextran solution

Next, we tested endothelial barrier function of 7-day-old microvasculatures constructed from either BMECs or HUVECs because the BBB is a specific feature of neurological tissue. Fluorescence dextran solution was perfused into microvascular networks, and time-lapse imaging was performed to investigate the time course of the dextran distribution. First, we succeeded in introducing fluorescence dextran solution into constructed capillary-level microvasculatures with diameter < 10 μ m (Fig. 5A–C). Cross-sectional images of microvasculatures clearly demonstrated that the luminal space was filled with the dextran

solution without leakage (Fig. 5B). Next, we analyzed the diffusion of the dextran solution from the intravascular region to the extravascular region. Quantitative analyses of the fluorescence intensity showed that the intensity of the dextran solution significantly decreased across the capillary wall (Fig. 5D). To analyze endothelial barrier function, we quantified the time transition of fluorescence intensity of dextran in the extravascular region within 3 μm from the capillary wall. The extravascular fluorescence intensity increased gradually with increasing time in both BMEC and HUVEC microvasculatures (Fig. 5E). The permeability coefficients of 70 kDa dextran solution across capillary walls were $0.910 \pm 0.405 \times 10^{-7}$ cm/s for BMEC microvasculatures and $2.743 \pm 0.550 \times 10^{-7}$ cm/s for HUVEC microvasculatures, respectively (Fig. 5F). These results indicate that the endothelial barrier function of BMEC microvasculatures was significantly more robust than that of HUVEC microvasculatures.

Junctional protein expression in microvascular networks

We hypothesized that the different permeabilities of the different microvasculatures were related to differences in endothelial junctional properties, in particular the increased tightness of BMEC microvascular walls. Therefore, we performed immunofluorescence staining of endothelial junctional proteins such as VE-cadherin, ZO-1, and occludin to investigate the junctional properties of 7-day-old microvasculatures. VE-cadherin was expressed in intercellular regions and the cytoplasm of both BMECs and HUVECs (Fig. 6A, VE-cadherin). ZO-1 expression was localized in intercellular regions and the cytoplasm of BMEC microvasculatures while that in HUVEC microvasculatures was found only in cytoplasmic regions (Fig. 6A, ZO-1). Occludin was expressed in the cytoplasm of both BMECs and HUVECs contributing towards microvasculature structures (Fig. 6A, Occludin).

Quantitative analyses of VE-cadherin expression showed no significant differences between BMEC and HUVEC microvasculatures (Fig. 6B). Conversely, expression levels of ZO-1 and occluding were significantly higher in BMEC microvasculatures (Fig. 6C, 6D). These results demonstrate that the endothelial junctional properties differed by EC origin.

Inflammatory response of BMEC microvasculature induced by thrombin

Finally, we tested the inflammatory response of microvasculatures focusing on BMECs because it is important for the study of BBB barrier functions in pathological states. BMEC microvasculatures were incubated with culture medium supplemented with 1 unit/mL thrombin to induce an inflammatory response. Before thrombin stimulation, the fluorescence dextran solution introduced in a microchannel passed through the microvascular networks across the gel region (Fig. 7A, 7C). The dextran solution came out of open ends of microvasculatures toward the other microchannel. The fluorescence dextran solution was detected in the other microchannel, the intensity of which increased with time (Fig. 7A, asterisks). However, the BMEC microvasculatures became leaky following thrombin stimulation (Fig. 7B). Although the solution was detected in the other microchannel, the increase in intensity was attenuated (Fig. 7B, asterisks). Moreover, it was detected throughout extravascular regions of the gel scaffold (Fig. 7B), suggesting that it passed across the walls of microvasculatures. To quantify these observations, fluorescence intensity of the dextran-perfused microvasculatures was measured along the line across

microvasculatures (Fig. 7C, enlarged image). The time-series of fluorescence intensity profiles clearly showed that fluorescence intensity corresponding to extravascular regions increased with increasing time after thrombin stimulation (Fig. 7C–E, open circles).

DISCUSSION

We demonstrated an *in vitro* angiogenesis model using BMECs/HUVECs and MSCs. Both microvasculatures had continuous lumens with average diameters close to 10 μm . In addition, the microvasculatures were sparsely covered by pericytes and expressed basement membrane proteins such as laminin and type IV collagen. These results indicate that the engineered microvasculatures were stable. Furthermore, we succeeded in introducing fluorescence dextran solution into the microvascular networks and analyzing their endothelial barrier functions. The calculated permeability coefficients of BMEC and HUVEC microvasculatures were $0.910 \pm 0.405 \times 10^{-7}$ cm/s and $2.743 \pm 0.550 \times 10^{-7}$ cm/s, respectively. These permeability coefficients are close to that of *in vivo* microvessels (Yuan et al., 2009; Shi et al., 2014). Permeability coefficients of *in vitro* microvasculatures perfused with 70 kDa dextran were also measured, which ranged from 4.5×10^{-7} to 2×10^{-6} cm/s (Lee et al., 2014; Sobrino et al., 2016; Bichsel et al., 2015; Lee et al., 2014). Taken together, these data indicate that the microvasculatures constructed in this study had robust barrier functions. In this study, microvasculatures were covered by α -SMA-expressing pericytes, suggesting that endothelial barrier function was supported by these pericytes and through interactions between ECs and pericytes.

We have shown here that microvascular networks can indeed be constructed in BMEC-MSC as well as HUVEC-MSC coculture systems. Notably, the microvasculatures of different cell origin had different features. For example, both sprout formation and microvascular length were greater in HUVEC-MSCs than in BMEC-MSCs on day 3, the initiation stage of vascular formation. Previous studies have reported that MSCs produce angiogenic factors such as VEGF and bFGF (Nassiri and Rahbarghazi, 2014; Bronckaers et al., 2014; Volarevic et al., 2011). Our results suggest that HUVECs were more sensitive to angiogenic factors than BMECs, or that HUVECs have greater potential than BMECs in terms of vascular formation, which might be due to their organ-specific characteristics. BMEC and HUVEC microvasculatures were also compared on day 7, the maturation stage of vascular formation. Similar to the initiation stage of vascular formation, HUVECs constructed more extensive networks in terms of both microvascular length and branch points. However, there were no significant differences between BMECs and HUVECs in terms of vessel diameter and the number of pericytes covering microvasculatures. A previous study reported that brain capillaries have higher pericyte coverage than any other capillaries (Daneman et al., 2010). Further investigation is needed to clarify whether reported differences in pericyte coverage are observed in our EC-MSC cocultures 3D models.

Pericytes stabilize capillary structures through the Ang1-Tie2 signaling pathway, resulting in inhibition of angiogenic processes (Ribatti et al., 2011; Armulik et al., 2005). The construction of microvascular networks was realized in two possible steps: EC sprout formation and elongation, and the stabilization of capillary networks by the coverage of pericytes differentiated from MSCs. In addition, the expression of basement membrane

proteins such as laminin and type IV collagen were observed in both BMEC and HUVEC microvasculatures, suggesting that engineered microvasculatures are both stable and mature within 7 days of culture.

We performed fluorescence dextran perfusion into constructed microvascular networks to compare endothelial barrier functions. Instant leakage was not observed. Furthermore, the permeability coefficient was significantly lower in HUVEC microvasculatures. This indicates a more robust barrier function in BMEC microvasculatures. Tight junction proteins such as ZO-1 and occludin are key components that regulate endothelial paracellular barrier functions (Greene and Campbell, 2016; Reinhold and Rittner, 2017). VE-cadherin is also a key regulator of endothelial barrier functions (Gavard, 2014; Koch and Claesson-Welsh, 2012; Dejana, 1996). Therefore, the difference in barrier function observed in this study might be attributable to the junctional properties of each constructed microvasculature. To verify this, we performed immunofluorescence staining for VE-cadherin, ZO-1, and occludin. The expressions of ZO-1 and occludin were significantly higher in BMEC microvasculatures than in HUVEC microvasculatures, while VE-cadherin expression was confirmed in both microvasculatures at the same level. These data indicate that the lower permeability coefficients of BMEC microvasculatures are attributable in part to the higher expression of tight junction proteins. VE-cadherin and ZO-1 were localized in intercellular regions while occludin was not. Further investigation on occludin localization and its effect on endothelial barrier functions is required.

Finally, we demonstrated the potential of our BMEC microvasculatures. A previous study presented an *in vitro* vascular model using HUVECs with bone marrow stromal cells to mimic barrier function and analyze the role of mural cells in vascular inflammation (Alimperti et al., 2017). Inflammatory factors such as lipopolysaccharides, thrombin, and tumor necrosis factor α have been used to induce an inflammatory response in vasculatures *in vitro*. Vasculatures treated with thrombin have shown the greatest increase in permeability among these inflammatory factors, and thrombin disrupts tight junctions (Hassanpour et al., 2017) and VE cadherin-dependent cell–cell contact (Konstantoulaki et al., 2003). Therefore, we selected thrombin as an inflammatory factor and found that BMEC microvasculatures showed leaky characteristics after treatment. This suggests that our *in vitro* BMEC-MSCC coculture model recapitulated an endothelial response to the proinflammatory stimulus, which may potentially be of use in the study of BBB pathologies.

CONCLUSION

We performed BMEC-MSCC and HUVEC-MSCC cocultures to establish an *in vitro* brain angiogenesis model and compare the effects of EC origin on the formation of microvasculatures. The formation process differed depending on EC origin. Moreover, BMEC and HUVEC microvascular networks exhibited different characteristics in terms of endothelial barrier functions as well as fundamental morphology. In addition, we also demonstrated the inflammatory response of microvasculatures constructed with BMECs. Our results suggest that the organ source of ECs has to be considered when creating an organ-specific cell culture model because the organ-specificity can affect interactions between microvasculatures and parenchymal cells in the model.

ACKNOWLEDGEMENTS

We appreciate Dr. Yumi Matsuzaki at Shimane University and Dr. Yo Mabuchi at Tokyo Medical and Dental University for providing MSCs. This work was partially supported by Japan Society for Promotion of Science (16H03173, 18K19937) and NIH (R01 NS065089).

Abbreviations:

2D	two dimensional
3D	three dimensional
BBB	blood brain barrier
bFGF	basic fibroblast growth factor
BMEC	brain microvascular endothelial cell
EC	endothelial cell
MSC	mesenchymal stem cell
PBS	phosphate buffered saline
PDMS	poly-dimethylsiloxane
VEGF	vascular endothelial growth factor

REFERENCE

- Abbott NJ, Patabendige AAK, Dolman DEM, Yusof SR, Begley DJ, 2010 Structure and function of the blood-brain barrier. *Neurobiol. Dis* 37, 13–25. <https://doi.org/10.1016/j.nbd.2009.07.030> [PubMed: 19664713]
- Alimperi S, Mirabella T, Bajaj V, Polacheck W, Pirone DM, Duffield J, Eyckmans J, Assoian RK, Chen CS, 2017 Three-dimensional biomimetic vascular model reveals a RhoA, Rac1, and N-cadherin balance in mural cell–endothelial cell-regulated barrier function. *Proc. Natl. Acad. Sci* 114, 201618333 <https://doi.org/10.1073/pnas.1618333114>
- Armulik A, Abramsson A, Betsholtz C, 2005 Endothelial/pericyte interactions. *Circ. Res* 97, 512–523. <https://doi.org/10.1161/01.RES.0000182903.16652.d7> [PubMed: 16166562]
- Armulik A, Genové G, Mäe M, Nisancioglu MH, Wallgard E, Niaudet C, He L, Norlin J, Lindblom P, Strittmatter K, Johansson BR, Betsholtz C, 2010 Pericytes regulate the blood-brain barrier. *Nature* 468, 557–561. <https://doi.org/10.1038/nature09522> [PubMed: 20944627]
- Banerjee J, Shi Y, Azevedo HS, 2016 In vitro blood–brain barrier models for drug research: state-of-the-art and new perspectives on reconstituting these models on artificial basement membrane platforms. *Drug Discov. Today* 21, 1367–1386. <https://doi.org/10.1016/j.drudis.2016.05.020> [PubMed: 27283274]
- Barar J, Rafi MA, Pourseif MM, Omidi Y, 2016 Blood-brain barrier transport machineries and targeted therapy of brain diseases. *BioImpacts* 6, 225–248. <https://doi.org/10.15171/bi.2016.30> [PubMed: 28265539]
- Ben-Zvi A, Lacoste B, Kur E, Andreone BJ, Mayshar Y, Yan H, Gu C, 2014 Mfsd2a is critical for the formation and function of the blood-brain barrier. *Nature* 509, 507–511. <https://doi.org/10.1038/nature13324> [PubMed: 24828040]
- Bichsel CA, Hall SRR, Schmid RA, Guenat OT, Geiser T, 2015 Primary Human Lung Pericytes Support and Stabilize In Vitro Perfusable Microvessels. *Tissue Eng Part A* 21, 2166–2176. <https://doi.org/10.1089/ten.tea.2014.0545>

- Bicker J, Alves G, Fortuna A, Falcão A, 2014 Blood-brain barrier models and their relevance for a successful development of CNS drug delivery systems: A review. *Eur. J. Pharm. Biopharm* 87, 409–432. <https://doi.org/10.1016/j.ejpb.2014.03.012> [PubMed: 24686194]
- Bronckaers A, Hilkens P, Martens W, Gervois P, Ratajczak J, Struys T, Lambrechts I, 2014 Mesenchymal stem/stromal cells as a pharmacological and therapeutic approach to accelerate angiogenesis. *Pharmacol. Ther* 143, 181–196. <https://doi.org/10.1016/j.pharmthera.2014.02.013> [PubMed: 24594234]
- Daneman R, Zhou L, Kebede AA, Barres BA, 2010 Pericytes are required for blood-brain barrier integrity during embryogenesis. *Nature* 468, 562–566. <https://doi.org/10.1038/nature09513> [PubMed: 20944625]
- Dejana E, 1996 Endothelial adherens junctions: implications in the control of vascular permeability and angiogenesis. *J. Clin. Invest* 98, 1949–1953. <https://doi.org/10.1172/JCI118997> [PubMed: 8903311]
- Gavard J, 2014 Endothelial permeability and VE cadherin: A wacky comradeship. *Cell Adhes. Migr* 8, 158–164. <https://doi.org/10.4161/cam.29026>
- Géraud C, Koch PS, Goerdts S, 2014 Vaskuläre Nischen: Endothelzellen als multifunktionale gewebe- und standortspezifische Teamplayer im gesunden und erkrankten Organismus. *JDDG - J. Ger. Soc. Dermatology* 12, 685–689. <https://doi.org/10.1111/ddg.12402>
- Greene C, Campbell M, 2016 Tight junction modulation of the blood brain barrier: CNS delivery of small molecules. *Tissue Barriers* 4, e1138017 <https://doi.org/10.1080/21688370.2015.1138017> [PubMed: 27141420]
- Hassanpour S, Langlotz CP, States U, 2017 HHS Public Access 29–39 <https://doi.org/10.1016/j.artmed.2015.09.007> Information
- Helm MW Van Der, Meer AD Van Der, Eijkel JCT, Van A, Berg D, Segerink LI, Helm MW Van Der, Meer AD Van Der, Eijkel JCT, Berg A Van Den, Segerink LI, 2016 Microfluidic organ-on-chip technology for blood-brain barrier research. *Tissue Barriers* 8370, 1–13. <https://doi.org/10.1080/21688370.2016.1142493>
- Keaney J, Campbell M, 2015 The dynamic blood-brain barrier. *FEBS J* 282, 4067–4079. <https://doi.org/10.1111/febs.13412> [PubMed: 26277326]
- Kim J, Chung M, Kim S, Jo DH, Kim JH, Jeon NL, 2015 Engineering of a biomimetic pericyte-covered 3D microvascular network. *PLoS One* 10, 1–15. <https://doi.org/10.1371/journal.pone.0133880>
- Kim S, Lee H, Chung M, Jeon NL, 2013 Engineering of functional, perfusable 3D microvascular networks on a chip. *Lab Chip* 13, 1489–500. <https://doi.org/10.1039/c3lc41320a> [PubMed: 23440068]
- Koch S, Claesson-Welsh L, 2012 Signal transduction by vascular endothelial growth factor receptors. *Cold Spring Harb. Perspect. Med* 2, 1–21. <https://doi.org/10.1101/cshperspect.a006502>
- Koike N, Fukumura D, Gralla O, Au P, Schechner JS, Jain RK, 2004 Tissue engineering: creation of long-lasting blood vessels. *Nature* 428, 138–139. <https://doi.org/10.1038/428138a> [PubMed: 15014486]
- Konstantoulaki M, Kouklis P, Malik AB, 2003 Protein kinase C modifications of VE-cadherin, p120, and β -catenin contribute to endothelial barrier dysregulation induced by thrombin. *Am. Physiol. - Lung Cell. Mol. Physiol* 285, L434–L442. <https://doi.org/10.1152/ajplung.00075.2003>
- Lee H, Kim S, Chung M, Kim JH, Jeon NL, 2014 A bioengineered array of 3D microvessels for vascular permeability assay. *Microvasc. Res* 91, 90–98. <https://doi.org/10.1016/j.mvr.2013.12.001> [PubMed: 24333621]
- Nassiri SM, Rahbarghazi R, 2014 Interactions of Mesenchymal Stem Cells with Endothelial Cells. *Stem Cells Dev* 23, 319–332. <https://doi.org/10.1089/scd.2013.0419> [PubMed: 24171705]
- Nguyen D-HT, Stapleton SC, Yang MT, Cha SS, Choi CK, Galie P. a, Chen CS, 2013 Biomimetic model to reconstitute angiogenic sprouting morphogenesis in vitro. *Proc. Natl. Acad. Sci. U. S. A* 110, 6712–7. <https://doi.org/10.1073/pnas.1221526110> [PubMed: 23569284]
- Nolan DJ, Ginsberg M, Israely E, Palikuqi B, Poulos MG, James D, Ding B. Sen, Schachterle W, Liu Y, Rosenwaks Z, Butler JM, Xiang J, Rafii A, Shido K, Rabbany SY, Elemento O, Rafii S, 2013 Molecular Signatures of Tissue-Specific Microvascular Endothelial Cell Heterogeneity in Organ

- Maintenance and Regeneration. *Dev. Cell* 26, 204–219. <https://doi.org/10.1016/j.devcel.2013.06.017> [PubMed: 23871589]
- Rafii S, Butler JM, Ding B, Sen, 2016 Angiocrine functions of organ-specific endothelial cells. *Nature* 529, 316–325. <https://doi.org/10.1038/nature17040> [PubMed: 26791722]
- Reinhold A, Rittner H, 2017 Barrier function in the peripheral and central nervous system—a review. *Pflügers Arch. - Eur. J. Physiol* 469, 123–134. <https://doi.org/10.1007/s00424-016-1920-8> [PubMed: 27957611]
- Ribatti D, Nico B, Crivellato E, 2011 The role of pericytes in angiogenesis. *Int. J. Dev. Biol* 55, 261–268. <https://doi.org/10.1387/ijdb.103167dr> [PubMed: 21710434]
- Shi L, Zeng M, Sun Y, Fu BM, 2014 Quantification of Blood-Brain Barrier Solute Permeability and Brain Transport by Multiphoton Microscopy. *J. Biomech. Eng* 136, 31005 <https://doi.org/10.1115/1.4025892>
- Shin Y, Han S, Jeon JS, Yamamoto K, Zervantonakis IK, Sudo R, Kamm RD, Chung S, 2012 Microfluidic assay for simultaneous culture of multiple cell types on surfaces or within hydrogels. *Nat. Protoc* 7, 1247–59. <https://doi.org/10.1038/nprot.2012.051> [PubMed: 22678430]
- Sobrinho A, Phan DTT, Datta R, Wang X, Hachey SJ, Romero-López M, Gratton E, Lee AP, George SC, Hughes CCW, 2016 3D microtumors in vitro supported by perfused vascular networks. *Sci. Rep* 6, 1–11. <https://doi.org/10.1038/srep31589> [PubMed: 28442746]
- Sweeney MD, Sagare AP, Zlokovic BV, 2018 Blood-brain barrier breakdown in Alzheimer disease and other neurodegenerative disorders. *Nat. Rev. Neurol* 14, 133–150. <https://doi.org/10.1038/nrneurol.2017.188> [PubMed: 29377008]
- Thomsen MS, Routhe LJ, Moos T, 2017 The vascular basement membrane in the healthy and pathological brain. *J. Cereb. Blood Flow Metab* 37, 3300–3317. <https://doi.org/10.1177/0271678X17722436> [PubMed: 28753105]
- Trost A, Lange S, Schroedl F, Bruckner D, Motloch KA, Bogner B, Kaser-Eichberger A, Strohmaier C, Runge C, Aigner L, Rivera FJ, Reitsamer HA, 2016 Brain and Retinal Pericytes: Origin, Function and Role. *Front. Cell. Neurosci* 10, 1–13. <https://doi.org/10.3389/fncel.2016.00020>
- Volarevic V, Arsenijevic N, Lukic ML, Stojkovic M, 2011 Concise review: Mesenchymal stem cell treatment of the complications of diabetes mellitus. *Stem Cells* 29, 5–10. <https://doi.org/10.1002/stem.556> [PubMed: 21280154]
- Yamamoto K, Tanimura K, Mabuchi Y, Matsuzaki Y, Chung S, Kamm RD, Ikeda M, Tanishita K, Sudo R, 2013 The stabilization effect of mesenchymal stem cells on the formation of microvascular networks in a microfluidic device. *J. Biomech. Sci. Eng* 8, 114–128. <https://doi.org/10.1299/jbse.8.114>
- Yamamoto K, Tanimura K, Watanabe M, Sano H, Uwamori H, Mabuchi Y, Matsuzaki Y, Chung S, Kamm R, Tanishita K, Sudo R, 2018 Construction of continuous capillary networks stabilized by pericyte-like perivascular cells. *Tissue Eng Part A* 0, ten.TEA.2018.0186. <https://doi.org/10.1089/ten.TEA.2018.0186>
- Yuan W, Lv Y, Zeng M, Fu BM, 2009 Non-invasive measurement of solute permeability in cerebral microvessels of the rat. *Microvasc. Res* 77, 166–173. <https://doi.org/10.1016/j.mvr.2008.08.004> [PubMed: 18838082]
- Zhao Z, Nelson AR, Betsholtz C, Zlokovic BV, 2015 Establishment and Dysfunction of the Blood-Brain Barrier. *Cell* 163, 1064–1078. <https://doi.org/10.1016/j.cell.2015.10.067> [PubMed: 26590417]
- Zlokovic BV, 2008 The Blood-Brain Barrier in Health and Chronic Neurodegenerative Disorders. *Neuron* 57, 178–201. <https://doi.org/10.1016/j.neuron.2008.01.003> [PubMed: 18215617]

Fig. 1. Schematic illustration of an EC-MSc coculture in a microfluidic device.

A: A microfluidic device and an enlarged image of its central channel filled with fibrin gel (gel region). The central channel is sandwiched between two microchannels filled with cell culture media (microfluidic channels). Fibrin gel pre-polymer solution was injected into the central channel from two gel inlets (arrowheads) immediately after thrombin was introduced to the pre-polymer solution. Culture medium was added using the inlets of the microfluidic channels (arrows) following gel polymerization. B: Schematic illustration of cell seeding and microvascular formation. A cell suspension of ECs and MSCs was added to one microfluidic channel and cells were attached to the side wall of the fibrin gel (left). ECs migrated into the fibrin gel to form microvascular structures (red) covered with pericytes differentiated from MSCs (green) (right).

Fig. 2. An early stage of EC-MSc coculture.

A: Phase-contrast images of BMEC-MSc (left) and HUVEC-MSc (right) cocultures on days 0–3. Arrowheads indicate migrating cells at the front edge. B: Representative immunofluorescence images of ECs and MSCs on day 3. Arrowheads indicate vascular sprouts stained with PECAM-1 (green). Cell nuclei were stained with DAPI (blue). C: Quantitative analyses of the number of EC sprouts. D: Quantitative analyses of microvascular length. Data are shown as the mean $\pm$ SEM (N=3, n = 9). *p<0.05.

Fig. 3. A late stage of EC-MSC coculture.

A: Representative phase-contrast images of ECs and MSCs on day 7 (left: BMEC-MSC, right: HUVEC-MSC). B: Corresponding immunofluorescence images of ECs (PECAM-1, red) and perivascular cells (α -SMA, green) on day 7. Cell nuclei were stained with DAPI (blue). C: Enlarged images of constructed microvasculatures, corresponding to boxes 1 and 2 in panel B, respectively. D: Orthogonal views of constructed microvasculatures showing microvascular diameters. E-H: Quantitative analyses of microvascular length (E), branch

point (F), microvascular outer diameter (G), and pericyte number (H) in both BMEC-MSC and HUVEC-MSC cocultures. Data are shown as the mean $\pm$ SEM (N=3, n = 9). *p<0.05.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 4. Immunofluorescence staining for basement membrane proteins and pericytes. Representative immunofluorescence images of constructed microvasculatures (PECAM-1), pericytes (α -SMA, NG2 and PDGFR β), basement membrane proteins (laminin and type IV collagen), and cell nuclei (DAPI) on day 7 in BMEC-MSC coculture (A, C) and HUVEC-MSC coculture (B, D). Arrowheads indicate laminin localization around microvasculatures. Arrows indicate the colocalization of α -SMA and type IV collagen. Perivascular cells, which were positive for NG2 and PDGFR β , were observed around microvasculatures (C,

D). Cross-sectional images corresponding to the dotted lines in C and D showed the coverage of pericytes around microvasculatures.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 5. Fluorescence dextran perfusion into constructed microvasculatures.

A: Immunofluorescence image of microvascular networks filled with FITC-dextran solution (2000 kDa). Dextran solution was introduced into microvascular networks on day 7. B: An enlarged image corresponding to the box in panel A and cross-sectional images of a dextran-perfused capillary (diameter < 10 μm). C: Immunofluorescence image of a dextran-perfused capillary on day 7. Fluorescence dextran solution (Texas red; 70 kDa) was introduced into microvascular networks. D: Representative fluorescence intensity distribution in intravascular and extravascular regions quantified along the axis of the white line in panel C. E: Representative time transition of fluorescence dextran intensity in extravascular regions. F: Quantitative analyses of permeability coefficients for perfused microvasculatures in BMEC-MSC and HUVEC-MSC cocultures. Data are shown as the mean $\pm$ SEM (N=3, n 3). *p<0.05.

Fig. 6. Junctional properties of constructed microvasculatures in EC-MSC cocultures.
A: Immunofluorescence images of constructed microvascular networks stained with VE-cadherin, ZO-1, occludin, PECAM-1 and DAPI. VE-cadherin, ZO-1, and occludin were expressed in the constructed microvasculatures. Arrowheads indicate intercellular expressions of junctional proteins. Dotted lines in the enlarged image of HUVEC-MSC ZO-1 represent the microvascular configuration. B, C, D: Quantitative analyses of VE-cadherin (B), ZO-1 (C), and occludin (D) expressions in the microvasculatures. Data are shown as the mean $\pm$ SEM (N=3, n = 12). *p<0.05.

Fig. 7. Inflammatory response of BMEC microvasculatures.

A: Time-lapse images of microvasculatures perfused with the dextran solution on day 5. Images were obtained every 30 s for 330 s using a confocal microscope. Asterisks represent FITC-dextran solution detected in the other microchannel, which passed through the microvasculatures. B: Time-lapse images of the thrombin-treated microvasculatures perfused with dextran solution on day 5. Asterisks represent the dextran solution detected in the other microchannel. The fluorescence intensity in extravascular spaces within the gel region was significantly increased after thrombin stimulation. C: A merged image of phase-contrast and fluorescence images showing microvasculatures perfused with FITC-dextran solution (2000 kDa) on day 5. Dotted lines in an enlarged image represented outlines of microvasculatures. D,E: Quantitative analysis of fluorescence intensity before (D) and after (E) thrombin stimulation along the line in the enlarged image in C. Fluorescence intensity profiles at three

time points ($t = 0$ s, 150 s, 330 s) were shown. Open and closed circles represent extravascular and intravascular regions, respectively.