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Construction of Continuous Capillary Networks Stabilized by Pericyte-like Perivascular Cells

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Construction of small and continuous capillary networks is a fundamental challenge for the development of three-dimensional (3D) tissue engineering. In particular, to construct mature and stable capillary networks, it is important to consider interactions between endothelial cells and pericytes. This study aimed to construct stable capillary networks covered by pericyte-like perivascular cells, which maintain the lumen of small diameter similar to that of capillary structures *in vivo*. Vascular sprouting, capillary extension, and stabilization were investigated using a 3D angiogenesis model containing human umbilical vein endothelial cells (HUVECs) and mesenchymal stem cells (MSCs) in a microfluidic device. A series of HUVEC:MSC ratios was tested; the ratio was found to be an important factor in the construction of capillary structures. We found that stable capillary networks that were covered by MSC-derived perivascular cells can be constructed at 1:1 HUVEC:MSC ratio. The constructed capillary networks had continuous lumens with <10-μm diameter, which were maintained for at least 21 days. This angiogenic process and basement membrane formation were regulated by HUVEC–MSC interactions.

Keywords: 3D angiogenesis model, microfluidic device, pericytes, mesenchymal stem cells

Impact Statement

Construction of capillary networks is a fundamental challenge for the development of three-dimensional (3D) tissue engineering. However, it is not well understood how to construct stable capillary networks that maintain a luminal size similar to that of capillary structures *in vivo* (i.e., <10 μm diameter). In this study, we demonstrated the construction of stable capillary networks covered by pericyte-like perivascular cells using an *in vitro* 3D angiogenesis model by optimizing interactions between endothelial cells and perivascular cells. Our 3D angiogenesis model can be combined with 3D culture of epithelial cells in the context of vascularization of 3D tissue-engineered constructs.

Introduction

The construction of small and continuous capillary networks is a fundamental challenge in three-dimensional (3D) tissue engineering.[1] In addition, the construction of vital organs such as the liver, pancreas, and kidney by the use of 3D tissue engineering remains a big challenge. Cells of these organs, when cultured in a 3D configuration, require oxygen and nutrients to be delivered and require waste products to be removed through peripheral capillary networks within the thick tissue rather than through diffusion from the tissue surface. Based on the diffusion of oxygen and nutrients, the thickness of tissue-engineered constructs is limited to 100–200 μm in the absence of capillary networks.[2,3] Therefore, it is essential to construct capillary networks for the development of 3D tissues/organs *in vitro*.

The construction of capillary networks has been investigated using two types of *in vitro* models: a 3D angiogenesis model involving vascular formation by sprouting from preexisting blood vessels, and a 3D vasculogenesis model

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involving *de novo* vascular formation mimicking the developmental process.[4–6] Most of these studies were performed using endothelial cells (ECs) alone and focused on early stages of vascular formation, such as sprout formation and capillary extension. These studies were important in elucidating the basic principles of early-stage vascular formation. However, the capillary structures in these studies were formed by ECs alone, which will eventually regress or fuse with neighboring capillaries and result in severely dilated tubular structures.[7,8] Therefore, in addition to initiation and growth of vascular formation, maturation and stabilization of capillary structures become more important in recent vascular tissue engineering studies.

To construct mature and stable capillary networks, it is important to consider interactions between ECs and perivascular cells, such as pericytes and stem cell-derived mural cells.[9] The Davis group has been reporting pioneering work on EC-pericyte interactions both *in vivo* and *in vitro*.[10] On the other hand, in terms of stem cell-derived mural cells, Koike *et al.*[11] reported a pioneering study that showed the importance of perivascular cells, which were derived from mesenchymal precursor cells, on the stabilization of capillary networks *in vivo*. They demonstrated that co-implantation of human umbilical vein ECs (HUVECs) and mesenchymal precursor cells resulted in the formation of long-lasting capillary structures with pericyte-like perivascular cells in mice, whereas implantation of HUVECs alone failed to stimulate the formation of such stable capillary structures. Furthermore, Au *et al.*[12] demonstrated that co-implantation of HUVECs and bone marrow-derived mesenchymal stem cells (MSCs) facilitated the formation of long-lasting functional vasculature, which was covered with MSC-derived perivascular cells. These findings suggest that stable capillary networks can be constructed *in vitro* by the coculture of HUVECs and MSCs.

Recent advances in the development of microfluidic culture platforms have allowed us to investigate cell behavior with the precise control of culture microenvironments, including biochemical and biomechanical factors.[13–15] Microfluidic devices have many advantages compared to conventional culture devices. For example, the cellular distribution can be controlled spatially and temporally in microfluidic devices; this control is useful to investigate interactions between two cell types such as ECs and perivascular cells in the process of capillary formation. In addition, cells can be cultured under flow conditions[16]; this control is important for the replication of *in vivo* microenvironments. These advantages led to the development of *in vitro* angiogenesis/vasculogenesis models using microfluidic devices.[17–23]

Recent *in vitro* studies using microfluidic devices have also demonstrated the importance of perivascular cells on the formation of stable microvascular networks. Jeon *et al.*[24] demonstrated that microvascular networks wrapped with MSC-derived perivascular cells were constructed in a 3D vasculogenesis model containing HUVECs and bone marrow-derived human MSCs. Nevertheless, it is not well understood whether the microvascular networks maintained a luminal size similar to that of capillary structures *in vivo* (i.e., <10 μm diameter), and whether these stable capillary structures can be constructed in a 3D angiogenesis model. In addition, the effect of the HUVEC:MSC ratio on capillary formation is not clear.

On the other hand, Kim *et al.*[8] demonstrated the construction of pericyte-covered microvascular networks in a 3D angiogenesis model containing HUVECs, human placental pericytes, and fibroblasts. Fibroblasts secreted many soluble factors supporting capillary formation, which complicates interactions between HUVECs and pericytes. In addition, the resulting capillary structures have lumens with >30-μm diameter, which is much larger compared with capillary structures *in vivo*, although the protective effect of pericytes from vessel dilation was confirmed. Therefore, it is still a challenge to construct long-lasting capillary networks with small and continuous lumens *in vitro*.

The aim of this study was to construct stable capillary networks covered by pericyte-like perivascular cells, which maintain a small luminal size similar to that of capillary structures *in vivo*. Vascular sprouting, capillary extension, and stabilization were investigated using a 3D angiogenesis model containing HUVECs and MSCs in a microfluidic device. A series of HUVEC:MSC ratios was tested for the construction of capillary networks, and the HUVEC:MSC ratio was found to be an important factor in the construction of capillary structures. Furthermore, we demonstrated that HUVECs and MSCs could construct stable capillary networks, which were covered by pericyte-like perivascular cells, with continuous lumens of <10-μm diameter at an optimal HUVEC:MSC ratio.

Materials and Methods

Preparation of microfluidic devices

The fabrication process followed for the microfluidic device used in this study was described previously.[15] Briefly, the microfluidic device was fabricated from polydimethylsiloxane (PDMS; Sylgard 184; Dow Corning, Midland, MI) and was produced by soft lithography using SU-8 patterned wafers. The PDMS device, copied using an SU-8 mold, was plasma bonded with a coverglass to form the microchannels (Fig. 1A). Once the microchannels were coated with 1 mg/mL poly-D-lysine solution (Sigma-Aldrich, St. Louis, MO), the device was rinsed twice with sterile deionized water and dried. A rat tail collagen type I solution (3 mg/mL, pH 7.4; BD Biosciences, San Jose, CA) was injected into the gel region through the two collagen gel inlets (Fig. 1A) and placed in the humidified 5% CO_2 incubator at 37°C for 30 min to facilitate polymerization. The microfluidic channels were then filled with culture medium and the devices were kept in the incubator until use. The microfluidic device had two parallel microchannels separated by collagen gel (Fig. 1B). Widths of the microchannel and collagen gel region were 500 and 600 μm, respectively, while the heights of both were 180 μm (Fig. 1B).

Cell isolation and culture

MSCs were isolated from human bone marrow using the LNGFR (CD271) and THY-1 (CD90) surface markers.[25] First, bone marrow mononuclear cells (Poietics™; Lonza, Walkersville, MD) were suspended at $1–5 \times 10^7$ cells/mL in ice-cold Hank's balanced salt solution supplemented with 2% fetal bovine serum (FBS), 10 mM HEPES, and 1% penicillin/streptomycin. Cells were then stained for 30 min on ice with a monoclonal antibody. The antibodies used

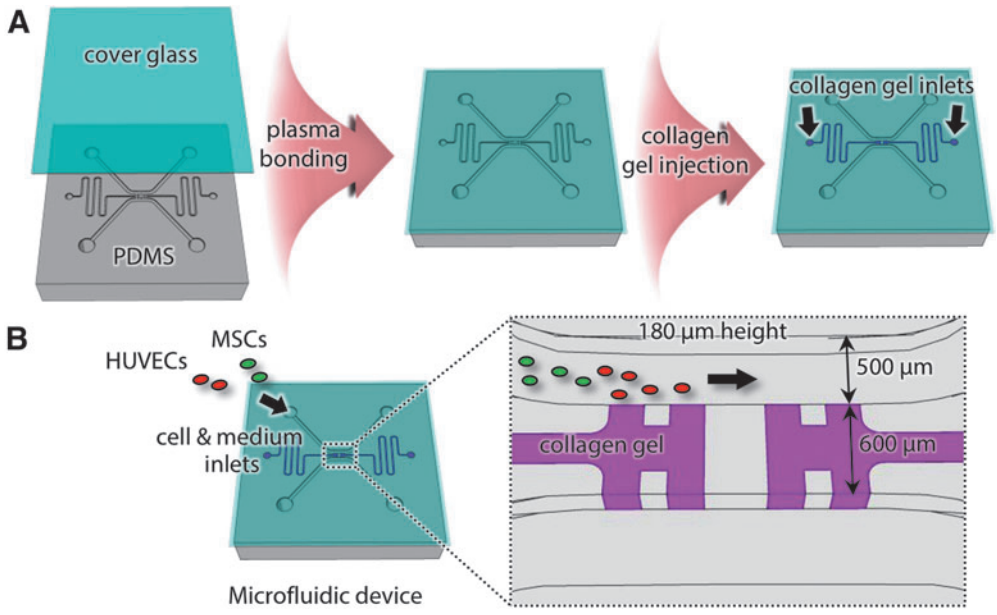

FIG. 1. Schematic illustration of a microfluidic device. **(A)** A PDMS device copied using an SU-8 mold was plasma bonded with a cover glass to form microfluidic channels. Collagen solution was then injected from the two inlets into the gel region and was placed in an incubator for gelation. **(B)** Two parallel microchannels were separated by the collagen gel. HUVECs and MSCs were seeded into the same channel. HUVECs, human umbilical vein endothelial cells; MSCs, mesenchymal stem cells; PDMS, polydimethylsiloxane.

were LNGFR-PE (Miltenyi Biotec, Bergisch Gladbach, Germany) and THY-1-APC (BD Pharmingen). Propidium iodide (2 μg/mL) was used to eliminate dead cells from the flow cytometric analysis. The flow cytometric analysis and sorting were performed on a triple-laser MoFlo (Beckman Coulter) or FACSVantage SE (Becton Dickinson, Heidelberg, Germany).

The isolated MSCs were expanded in the MSC growth medium: Dulbecco's modified Eagle medium with low glucose (Invitrogen) supplemented with 20% FBS, 20 ng/mL basic fibroblast growth factor (bFGF; Pepro Tech, Rocky Hill, NJ), and 1% penicillin/streptomycin. Cells were then cultured in a humidified 5% CO_2 incubator at 37°C. All experiments were performed with the cells at passages 6–8.

We used HUVECs to construct capillary structures because they have been widely used for the study of angiogenesis both *in vitro* and *in vivo*. HUVECs (Lonza) and green fluorescent protein-expressing HUVECs (GFP-HUVEC; Angio-Proteomie) were commercially obtained and cultured in the endothelial growth medium (EGM-2; Lonza). HUVECs and GFP-HUVECs were then expanded in collagen-coated culture dishes for no more than six passages.

HUVEC-MSC coculture in a microfluidic device

First, HUVECs were seeded by injecting a 10-μL cell suspension (1×10^6 cells/mL) in a microchannel. The device was tilted and incubated for 30 min in a humidified 5% CO_2 incubator at 37°C to allow cells to attach on the surface of collagen gel. MSCs were subsequently seeded in the same channel by injecting a 10-μL cell suspension ($0.1–1 \times 10^6$ cells/mL) for a series of HUVEC:MSC ratios—1:1, 2:1, 5:1, and 10:1. HUVECs alone were also cultured as controls for the experiments. These ratios were selected based on previous studies on EC-MSC coculture in two dimensional (2D) and 3D conditions.[7,26,27] In addition, in concordance with a previous study, the number of pericytes is comparable to or less compared with ECs *in vivo*.[28]

Cells were cultured for 21 days in a 1:1 mixture of EGM-2 and MSC growth medium supplemented with 10 ng/mL bFGF and 10 ng/mL vascular endothelial growth factor (VEGF; R&D Systems, Minneapolis, MN). The culture medium was replaced every day. Phase-contrast images were taken every day to monitor cell migration into the collagen gel. Immunofluorescence staining of the cells or live-cell imaging was performed as described below to investigate 3D angiogenesis.

Live-cell imaging

For live-cell imaging, GFP-HUVECs were used to distinguish HUVECs exhibiting green fluorescence, while MSCs were labeled with DiI to distinguish MSCs exhibiting red fluorescence. Briefly, MSCs were cultured in a 60-mm dish and incubated for 1 h with the Vybrant CM-DiI cell-labeling solution (1:200 dilution; Invitrogen) to stain cell membranes. Cells were then rinsed with warm phosphate-buffered saline (PBS), incubated in MSC growth medium for >1 h, and used for subsequent experiments. GFP-HUVEC and DiI-MSC morphogenesis were observed daily until day 21 using a confocal laser-scanning microscope (LSM700; Carl Zeiss, Hallbergmoos, Germany).

Immunofluorescence staining of cultured cells

Cells were fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100 for 5 min. After rinsing with PBS, cells were treated with BlockAce (Dainippon Pharmaceutical, Osaka, Japan)

for 1 h to inhibit nonspecific staining. Cells were then incubated with primary antibodies, a mouse anti-α smooth muscle actin (αSMA) antibody (1:200 dilution, Clone 1A4; Sigma-Aldrich) for pericytes[29] or a rabbit anti-CD146 antibody[7] or a sheep anti-CD31 antibody (1:100 dilution; R&D Systems) for HUVECs, followed by incubation with secondary antibodies. These included the Alexa Fluor 488-conjugated anti-mouse IgG (1:200 dilution; Invitrogen) or Alexa Fluor 594-conjugated anti-rabbit/sheep IgG (1:200 dilution; Invitrogen), respectively. Thereafter, some samples were incubated with Alexa Fluor 594-conjugated phalloidin (1:50 dilution; Invitrogen) for actin and 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) for staining nuclei. To confirm the MSC-derived perivascular cells showing pericyte characteristics, cells were also incubated with anti-neural/glial antigen 2 (NG2) antibody conjugated phycoerythrin (1:100 dilution; R&D Systems).[30] Z-stack fluorescent images were taken using a confocal laser-scanning microscope (LSM700). The projected images were generated with the z-stack fluorescent images using ImageJ (National Institutes of Health, Bethesda, MD).

Quantitative analysis of capillary structures

Quantitative analysis was performed to evaluate constructed capillary structures. We measured lumen diameter using immunofluorescence images taken by a confocal microscope. The z-stack images were imported in ImageJ to examine cross-sectional images. The existence of capillary lumens was carefully confirmed using the cross-sectional images parallel to *xy*, *yz*, and *zx* planes. Capillary lumen diameter was measured at 50 μm intervals using cross-sectional images, which were perpendicular to the axis of capillary structures.

Capillary length was also measured using ImageJ. First, 2D projected images were generated with z-stack images of capillary structures. Next, the capillary structures in the 2D projected images were traced using the freehand line tool. Finally, the length of the traced lines was measured. The number of branching points was manually counted using the 2D projected images of the capillary structures.

The number of HUVECs and pericytes was quantified by counting the number of nuclei stained with DAPI in confocal images, which were randomly selected. HUVECs and pericytes were distinguished by immunofluorescence staining of these cells, which were positive for CD31 and αSMA, respectively.

Quantitative analysis of capillary stabilization

The correlation coefficient was calculated based on the corresponding live-cell fluorescent images to analyze capillary stabilization. The corresponding images of GFP-HUVECs in the HUVEC-MSC coculture were taken every day until day 21. The correlation coefficient between the images taken on day x and day $x+1$ was calculated using MATLAB based on the following definition:

$$\text{Correlation coefficient} = \frac{\sum_{l=0}^{N-1}\sum_{k=0}^{N-1}\left(f[k][l]-\bar{f}\right)\left(t[k][l]-\bar{t}\right)}{\sqrt{\sum_{l=0}^{N-1}\sum_{k=0}^{N-1}\left(f[k][l]-\bar{f}\right)^2}\sqrt{\sum_{l=0}^{N-1}\sum_{k=0}^{N-1}\left(t[k][l]-t\right)^2}}$$

where f and t are the $N\times N$ pixel images taken on day x and day $x+1$, respectively. $f[k][l]$ indicates pixel intensity, while $\bar{f}$ and $\bar{t}$ indicate average intensities. The value of the correlation coefficient ranges between 0 and 1, and a correlation coefficient value of 1 indicates that the images taken on day $x+1$ is same as that taken on day x. As the value approaches 1, it indicates that the constructed capillary structures are stable.

Fluorescent dextran imaging

Fluorescent dextran imaging was performed to confirm that luminal spaces of capillary structures were continuous from an endothelial monolayer on a microchannel. Capillary structures, which were formed on day 5 at a GFP-HUVEC:MSC ratio of 1:1, were fixed with 4% paraformaldehyde for 15 min at room temperature. After rinsing with PBS, cell nuclei were stained with DAPI. Thereafter, the culture medium in a microchannel was replaced with the medium supplemented with 15 μg/mL rhodamine B isothiocyanate-dextran (2000 kDa; Sigma-Aldrich). Fluorescent images were taken in 5 min using a confocal laser-scanning microscope.

Statistical analyses

Experiments were repeated at least twice to confirm repeatability of the results. Data are presented as mean ± standard error of the mean. A Student's *t*-test was used to test for differences, which were considered statistically significant at $p<0.05$.

Results

Effect of the HUVEC:MSC ratio on angiogenic processes

We performed the HUVEC-MSC coculture with a series of HUVEC:MSC ratios, 1:1, 2:1, 5:1, and 10:1 to find an optimal ratio for constructing stable capillary structures. First, GFP-HUVECs were seeded in a microchannel and incubated to allow cells to attach on collagen gel for 30 min. DiI-labeled MSCs were subsequently seeded in the same channel (Fig. 2A). Because it was difficult to distinguish between HUVECs and MSCs by phase-contrast microscopy, GFP-HUVECs and DiI-labeled MSCs were used for monitoring live-cell behaviors by fluorescent microscopy.

At a HUVEC:MSC ratio of 1:1, only MSCs migrated into the collagen gel on day 1 (arrows, Fig. 2B). HUVECs subsequently migrated into the collagen gel to form vascular sprouts on day 4 and co-localized with MSCs migrating into the gel. These vascular sprouts extended and developed into branching capillary networks on day 7. At a HUVEC:MSC ratio of 2:1, both MSCs (arrows, Fig. 2B) and HUVECs (arrowheads, Fig. 2B) migrated into the collagen gel on day 1. Some HUVECs formed vascular sprouts and partially developed into capillary-like structures on day 7. On the other hand, at HUVEC:MSC ratios of 5:1 and 10:1, HUVECs dominantly migrated into the collagen gel to form

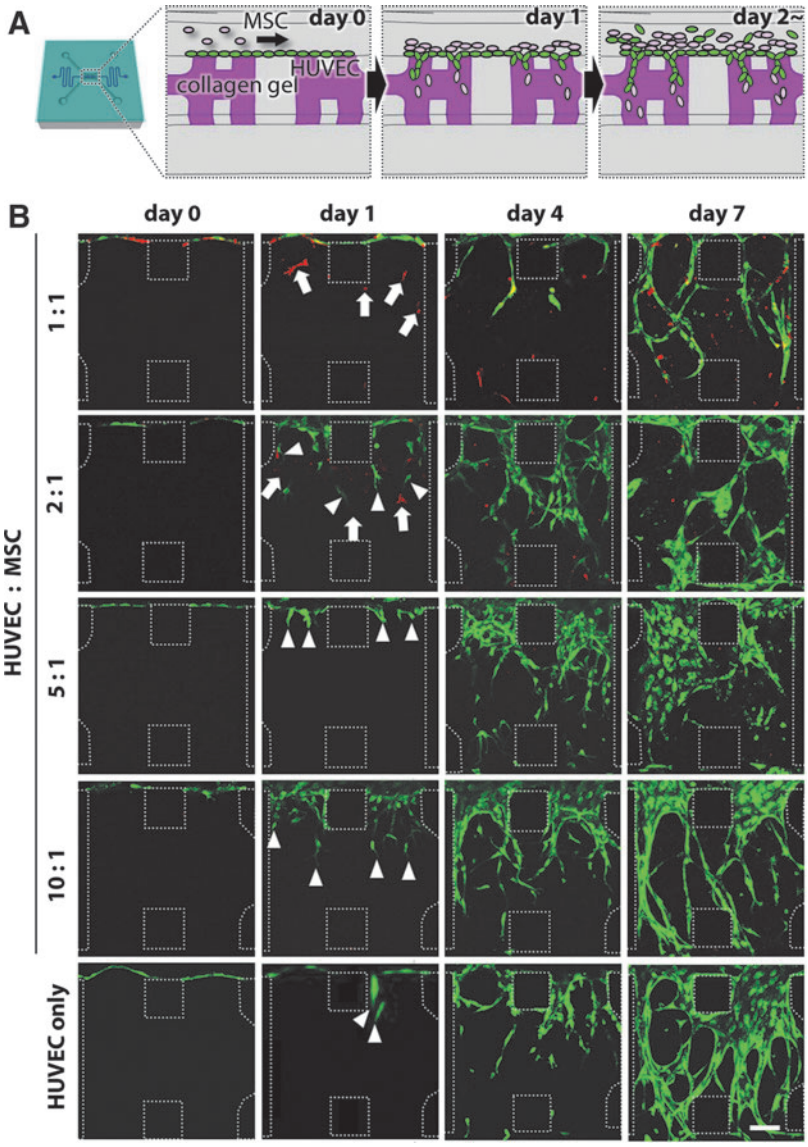

FIG. 2. Process of capillary formation for a series of HUVEC:MSC ratios. **(A)** Schematic illustration of the HUVEC-MSC coculture. After HUVECs attached to the surface of collagen gel, MSCs were added to the same channel. Both cell types migrated into the collagen gel and formed capillary structures. **(B)** Representative fluorescence images were obtained by monitoring HUVECs (*green*, GFP) and MSCs (*red*, DiI) in coculture at a series of HUVEC:MSC ratios. As a control, only HUVECs were also cultured. *Arrowheads* and *arrows* indicate sprouting HUVECs and migrating MSCs, respectively. Scale bar, 100 μm. GFP, green fluorescent protein.

vascular sprouts on day 1 (arrowheads), while few MSCs were detected in the gel region. These vascular sprouts extended and fused with neighboring capillaries, resulting in the formation of severely dilated tubular structures on day 7, which is similar to the result obtained in control experiments (Fig. 2B, HUVEC only).

To analyze the effect of the HUVEC:MSC ratio on angiogenic activities, we quantified sprout numbers from days 1–3 and capillary length on day 7. At a HUVEC:MSC ratio of 1:1, few HUVECs migrated into the collagen gel and formed vascular sprouts on day 1, while more vascular sprouts were observed with a decrease in MSCs (Fig. 2B). This tendency was confirmed by quantitative analysis of sprout number. The sprout number at a HUVEC:MSC ratio of 1:1 was significantly lower than that observed at the other ratios on day 1 (Fig. 3A). Although the sprout number increased from days 1 to 3, the same tendency was observed at least until day 3. In contrast, quantitative analysis of capillary length revealed that the capillary length on day 7 at a HUVEC:MSC ratio of 1:1 was significantly longer than that observed at the other ratios on day 7 (Fig. 3B). These results indicate that capillary structures were well constructed at a HUVEC:MSC ratio of 1:1. Therefore, we performed the following experiments at a HUVEC:MSC ratio of 1:1.

3D structure of constructed capillary networks at a HUVEC:MSC ratio of 1:1

The 3D structure of constructed capillary networks was further investigated at a HUVEC:MSC ratio of 1:1. In addition, we performed immunofluorescence staining for αSMA, a pericyte marker,[29] to confirm that MSCs differentiated into perivascular cells. Confocal microscope images revealed that some MSCs attaching to HUVECs expressed αSMA, whereas no MSCs expressed αSMA in the initial culture condition. MSC-derived perivascular cells, which were positive for αSMA, localized along HUVEC capillary structures (Fig. 4A). Cross-sectional images clearly showed that these capillary structures had lumens. However, the lumens were not continuous on day 7. Furthermore, a 3D projection image showed that αSMA-positive perivascular cells covered the outer surface of HUVEC capillary structures (Fig. 4B).

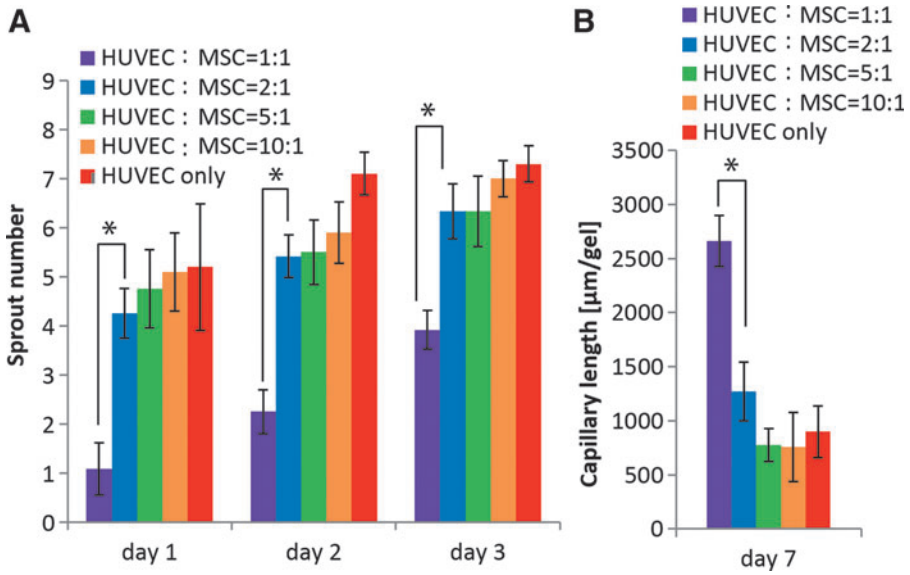

FIG. 3. Quantitative analysis of microvascular formation in the HUVEC-MSC coculture for a series of HUVEC:MSC ratios. Only HUVECs were cultured as a control. **(A)** Number of sprouts formed on days 1, 2, and 3. Sprout number at a 1:1 HUVEC:MSC ratio was significantly less than that at other ratios. **(B)** Total length of capillary networks, whose outer diameter was <20 μm. Capillary length at a 1:1 HUVEC:MSC ratio was significantly more than that at other ratios. Data are shown as the mean ± SEM. $*p < 0.05$. SEM, standard error of the mean.

Confocal microscope images of capillary structures demonstrated that fluorescent dextran added to a microchannel remained within the luminal space, suggesting that the luminal space was continuous from an endothelial monolayer on a microchannel (Supplementary Fig. S1 and Supplementary Video S1; Supplementary Data are available online at www.liebertpub.com/tea).

Stability of capillary networks at a HUVEC:MSC ratio of 1:1

Capillary networks are covered by pericytes and are stabilized *in vivo*. To investigate the stabilization effect of perivascular cells, we monitored the growth of capillary networks constructed in the HUVEC-MSC coculture at a HUVEC:MSC ratio of 1:1. Corresponding GFP-HUVECs and DiI-labeled MSCs were monitored daily by fluorescent microscopy until day 21. First, MSCs migrated into the collagen gel on day 1 (arrows, Fig. 5A, day 1). Second, HUVECs migrated toward the MSCs migrating into the collagen gel from days 2 to 3 (arrowheads, Fig. 5A, days 2 and 3). Third, some MSCs co-localized with HUVECs, forming vascular sprouts from days 4 to 5 (Fig. 5A, days 4 and 5). These vascular sprouts gradually extended and finally developed into branching networks from days 6 to 7 (Fig. 5A, days 6 and 7). The growth of capillary networks was monitored until day 21, revealing that the outline of capillary networks was maintained after day 7, although they continued to grow slowly (Fig. 5A, days 7, 14, 21). In contrast, HUVECs continuously degraded the collagen gel and capillary structures were fused with neighboring ones, resulting in the formation of severely dilated tubular

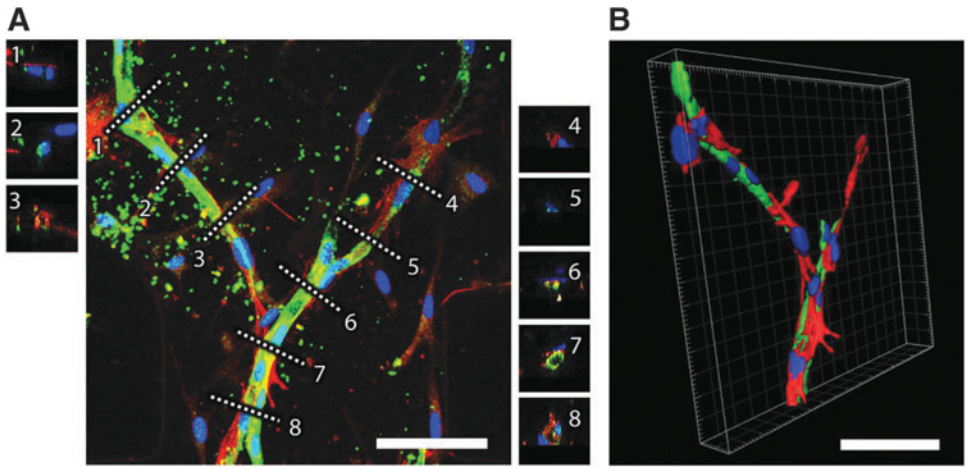

FIG. 4. Confocal microscopy images of capillary structures on day 7 in the HUVEC-MSC coculture at a 1:1 HUVEC:MSC ratio. HUVECs, pericytes, and nuclei are shown in *green* (CD31), *red* (αSMA), and *blue* (DAPI), respectively. **(A)** A 2D projection image reconstructed using z-stack images. Cross-sectional images correspond to lines 1–8. Lumens can be seen in lines 3 and 6–8; parts of lines 1, 2, 4, and 5 showed no lumens, suggesting that some sections of the capillaries formed continuous lumens, whose inner diameter was <10 μm. **(B)** A 3D projection image indicating that HUVECs formed capillary structures covered by MSC-derived pericyte-like perivascular cells. Scale bars, 100 μm. 2D, two dimensional; 3D, three dimensional; αSMA, α smooth muscle actin; DAPI, 4′,6-diamidino-2-phenylindole.

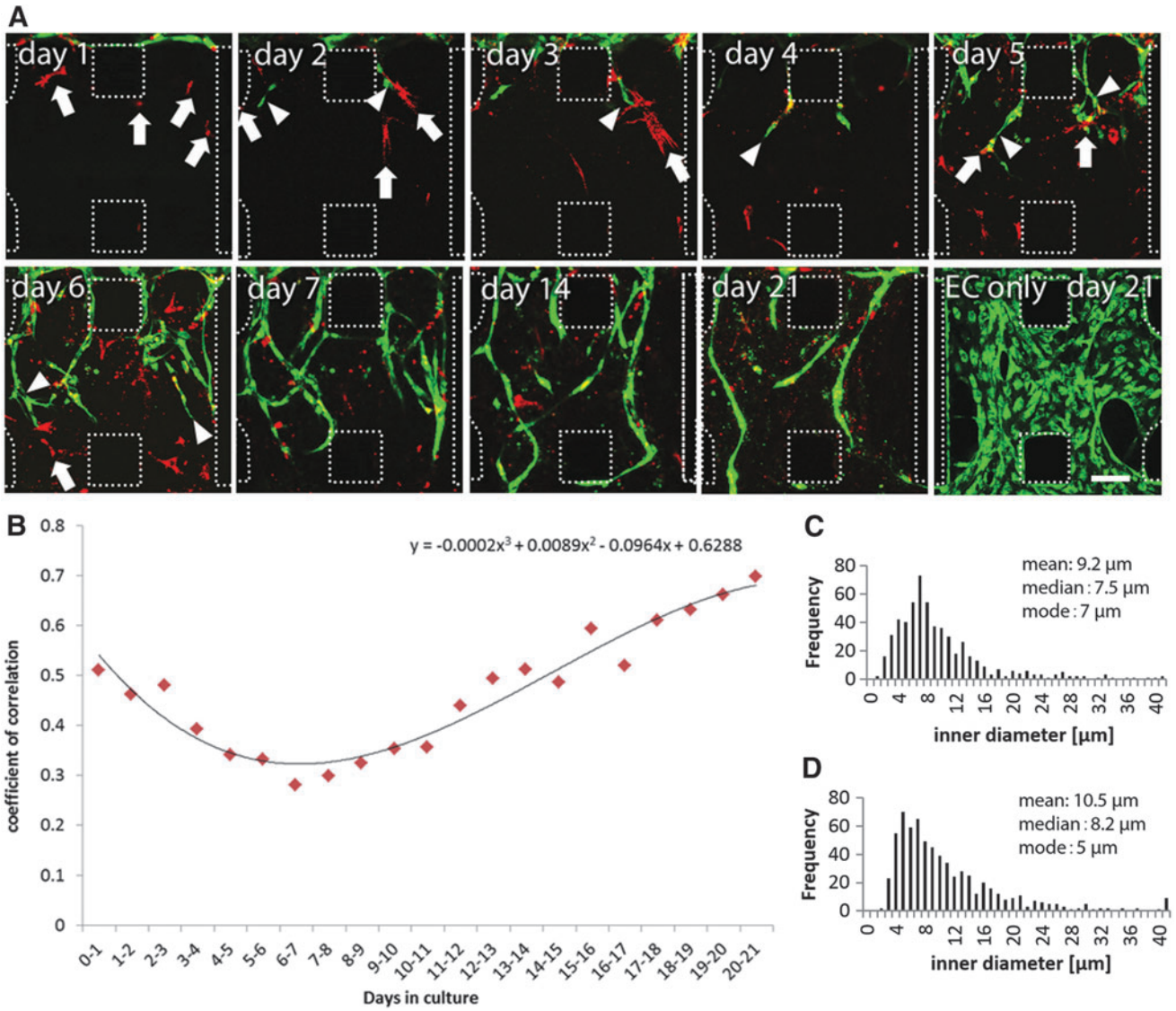

FIG. 5. Live-cell images for monitoring and characterizing the process of capillary formation in detail. **(A)** Capillary formation was monitored using confocal microscopy until day 21. HUVECs and MSCs are shown in *green* (GFP) and *red* (DiI), respectively. *Arrows* and *arrowheads* indicate penetrating MSCs and HUVECs, respectively. As a control, only HUVECs were also cultured. Scale bar, 100 µm. **(B)** Correlation coefficient was calculated to evaluate dynamical changes during capillary formation. **(C, D)** Histograms of the inner diameter of constructed capillaries on days 14 and 21, respectively.

structures or endothelial monolayers in the HUVEC monoculture (Fig. 5A, EC only, day 21).

We then calculated the correlation coefficient between fluorescence images of capillary networks on a couple of sequential days among 21-day culture to quantitatively analyze day-by-day variation in capillary network formation (Fig. 5B). The correlation coefficient value decreased until day 7, which corresponds to capillary formation in early stages of the culture. However, this value increased after day 7, which indicates that the day-by-day variation reduces after day 7, suggesting structural stabilization of capillary networks.

To investigate the stability of capillary networks further, the inner diameter of constructed capillary structures was measured on days 14 and 21. The mean, median, and mode of the inner diameter on day 14 were 9.2, 7.5, and 7.0 µm, respectively, while those on day 21 were 10.5, 8.2, and 5.0 µm, respectively (Fig. 5C, D). The modes of the inner diameter of constructed capillary structures were maintained within 10 µm at least until day 21, whereas capillary structures tended to fuse with each other and form larger structures in the HUVEC monoculture.

Although we found that capillary structures were stabilized from days 7 to 21, we measured the capillary length and number of branching points to analyze the growth of capillary networks quantitatively. The results revealed that capillary length gradually increased from days 7 to 21 (Supplementary Fig. S2A), while the number of branching points significantly increased from days 7 to 21 (Supplementary Fig. S2B).

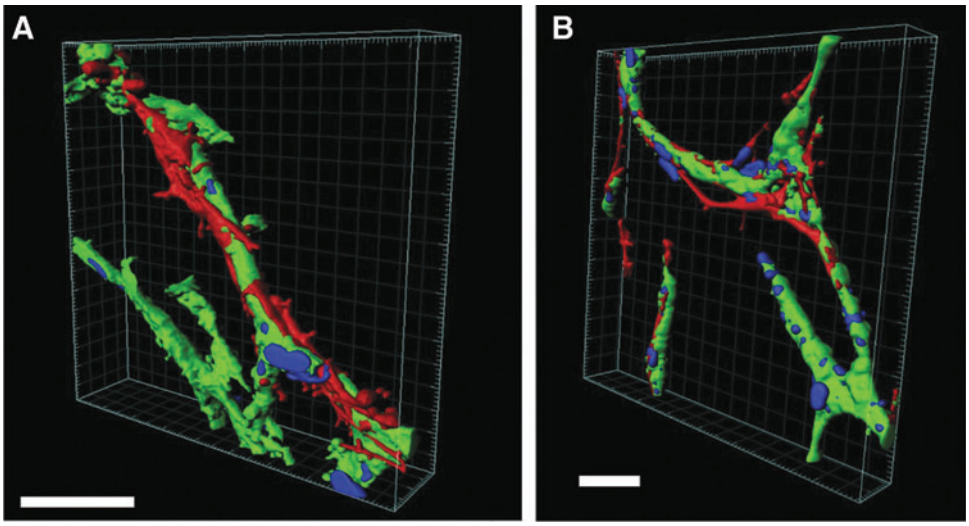

FIG. 6. 3D projection images of capillary structures on days 14 **(A)** and 21 **(B)** in the HUVEC-MSC coculture at a 1:1 HUVEC:MSC ratio. HUVECs, pericytes, and nuclei are shown in *green* (CD146), *red* (αSMA), and *blue* (DAPI), respectively. Scale bars, 50 μm.

To further confirm that capillary structures covered by perivascular cells are maintained in long-term culture, we performed immunofluorescence staining for HUVECs (green, CD146), perivascular cells (red, αSMA), and nuclei (DAPI) on days 14 and 21. Similar to the capillary structures observed on day 7, HUVECs maintained capillary structures covered by perivascular cells on days 14 and 21 (Fig. 6A, B). In contrast to the partially formed lumens in capillary structures on day 7, capillary structures showed continuous lumens on days 14 and 21, the diameter of which was <10 μm (Supplementary Fig. S3). Cross-sectional images also revealed that these capillary structures were covered by MSC-derived perivascular cells (Supplementary Fig. S3). MSC-derived perivascular cells showing pericyte characteristics were also confirmed by staining with anti-NG2 antibody. Confocal images revealed that NG2-positive perivascular cells distributed around the capillaries on day 14 (Supplementary Fig. S4A). High magnification images showed that these perivascular cells attached to the outer surface of capillaries (Supplementary Fig. S4B).

To investigate the growth of pericytes in long-term culture, we counted the number of αSMA-positive perivascular cells (pericytes) covering capillary structures on days 7, 14, and 21, which were found to be 6, 13, and 19 cells/gel region, respectively (Fig. 7A). This increasing number of pericytes resulted in the variation of the pericyte:HUVEC ratio of constructed vascular structures. The pericyte:HUVEC ratios on days 7, 14, and 21 were 1:7.6, 1:5.6, and 1:5.0, respectively (Fig. 7B).

Basement membrane formation around capillary structures covered by perivascular cells

Basement membranes are formed between capillary structures and pericytes *in vivo*, and are important for the maintenance of capillary structures. Therefore, we investigated the formation of basement membranes around capillary structures constructed at a HUVEC:MSC ratio of 1:1. Specifically, immunofluorescence staining was performed for basement membrane proteins, such as laminin and collagen type IV. Both laminin and collagen type IV proteins were detected along the capillary structures constructed in the HUVEC-MSC coculture on day 21 (Fig. 8A, B, coculture). Cross-sectional images of the capillary structures showed that capillary

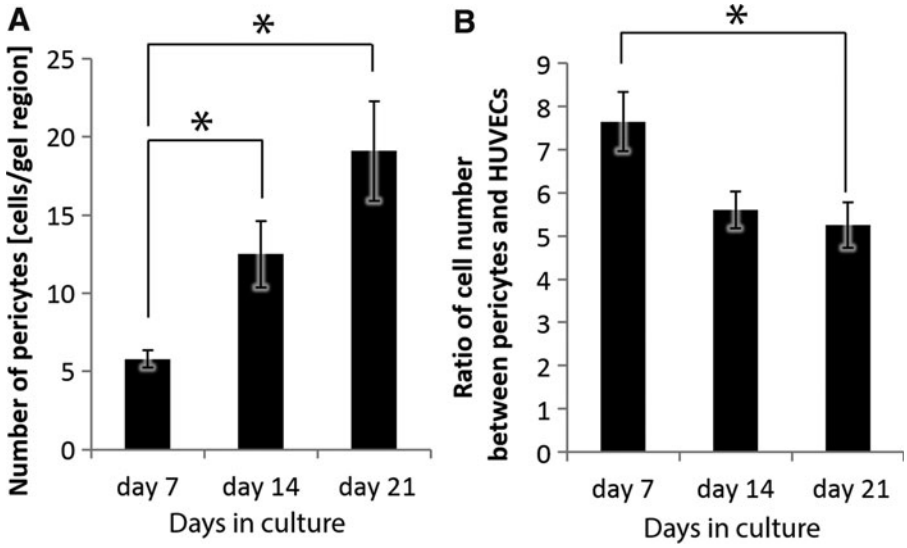

FIG. 7. Growth of pericytes in the HUVEC-MSC coculture. **(A)** Number of pericytes covering the capillary structures was measured per gel region, and was 6, 13, and 19 cells on days 7, 14, and 21, respectively. **(B)** Ratio of the number of pericytes to that of HUVECs was measured. The pericyte:HUVEC ratio was 1:7.6, 1:5.6, and 1:5.0 on days 7, 14, and 21, respectively. Data are shown as the mean ± SEM. $*p < 0.05$.

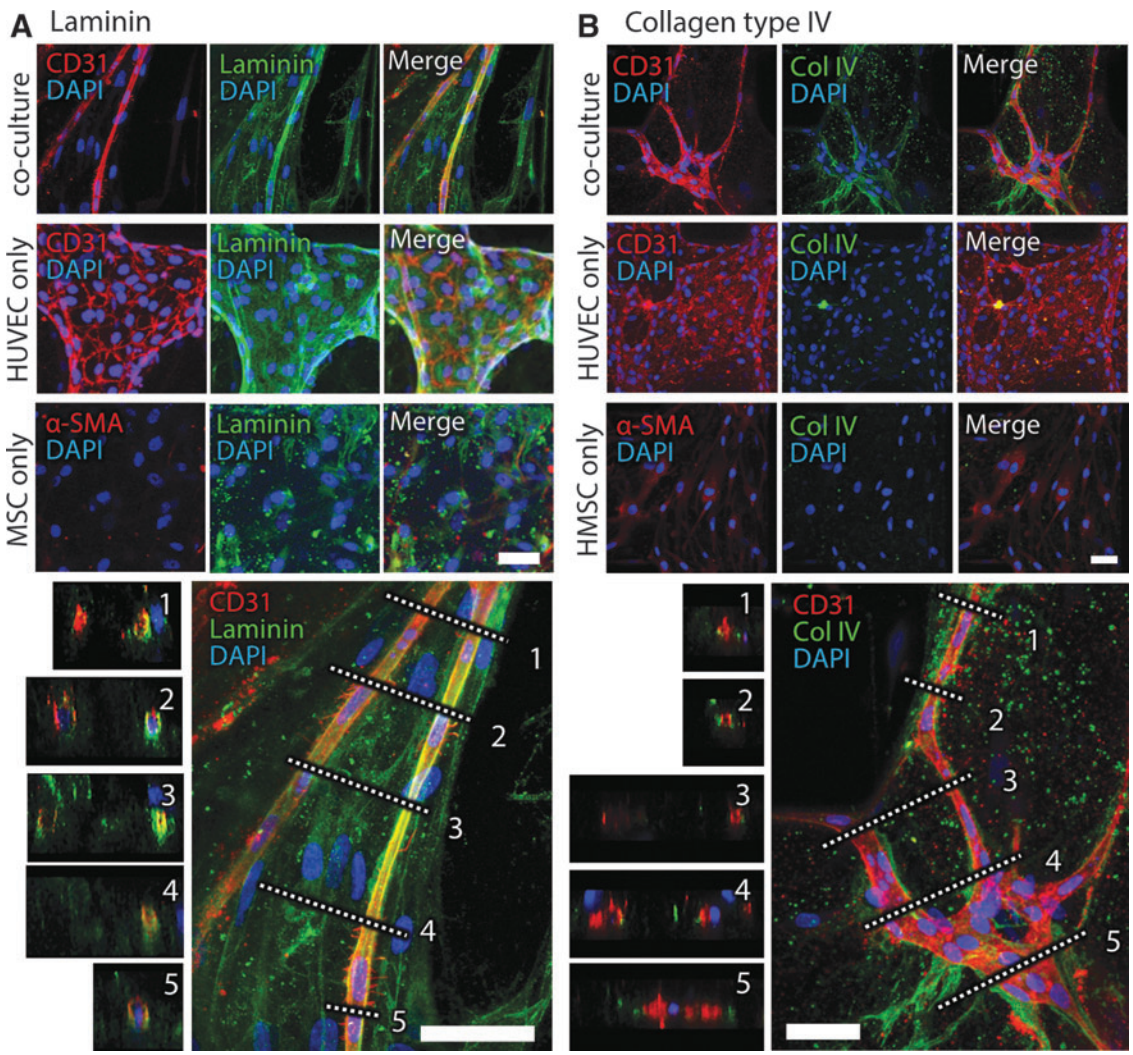

FIG. 8. Immunofluorescence images of the basement membrane proteins around the capillary structures on day 21 in the HUVEC-MSC coculture at a 1:1 HUVEC:MSC ratio. **(A, B)** Immunofluorescence staining was performed to detect HUVECs (CD31, *red*), laminin or collagen type IV (*green*), and nuclei (*blue*), respectively. Cross-sectional images correspond to lines 1–5. Scale bars, 50 μm.

structures had continuous lumens and that these proteins deposited around the capillary structures. Laminin was detected in both HUVEC and MSC monocultures (Fig. 8A, HUVEC only and MSC only). In contrast, collagen type IV was not detected in HUVEC or MSC monocultures (Fig. 8B, HUVEC only and MSC only).

Discussion

Construction of 3D capillary networks covered by perivascular cells

The most important achievement of this study is the construction of stable capillary networks covered by MSC-derived perivascular cells in an *in vitro* 3D angiogenesis model created in a microfluidic device. Median diameters of the constructed capillaries on days 14 and 21 were comparable to the size of capillaries *in vivo*, which are <10 μm. Recent studies from other groups also reported that the size of constructed vasculatures became smaller in coculture with perivascular cells compared to that in the culture of ECs alone.[8,24,31] However, the diameter of the vasculatures in these studies was >20 μm, which are much larger than the diameters of capillaries *in vivo*. Since 3D tissue-engineered constructs are vascularized by capillary networks rather than by larger blood vessels, it is important to construct the most peripheral blood vessels in the context of bottom-up approaches to 3D tissue engineering.[32]

A series of HUVEC:MSC ratios was tested in this study, and we found that a 1:1 ratio promoted stable capillary formation with perivascular cells, while other ratios resulted in the formation of severely dilated tubular structures with few perivascular cells. These results suggest that the existence of perivascular cells is important for preventing capillary dilation. An *in vivo* study using retinal vasculature reported that perivascular cells were recruited to the preformed endothelial plexus and stabilized the vasculature.[33] Similarly, MSC-derived perivascular cells stabilized constructed capillaries in this study. Although it is possible that capillary structures are constructed even at lower HUVEC ratios, they were not tested in this study due to the fact that the number of pericytes was comparable to or less compared with ECs *in vivo*.[28]

Interestingly, the capillary length was significantly longer at a 1:1 HUVEC:MSC ratio on day 7 compared to that at other ratios, whereas the sprout number was significantly lower. Two types of HUVEC-MSC interactions could explain this. At a 1:1 HUVEC:MSC ratio, sprout formation was inhibited on day 1 by HUVEC-MSC interactions, while sprout formation was observed on day 1 in lower MSC ratios. This inhibitory effect of MSCs was also observed in our previous study when MSCs and HUVECs were seeded in separate microchannels.[7] However, the characteristics of MSCs appeared to vary after migrating into collagen gel and led to vascular sprout formation with guiding endothelial tip cells. Shorter capillary length at 2:1, 5:1, and 10:1 HUVEC:MSC ratios might be due to the lack of these MSC-derived guiding cells. This guidance is consistent with that seen in previous studies, reporting a pericyte-driven angiogenic process in which sprouting of nascent vessels was guided by migrating pericytes.[34,35]

Maturation and remodeling of constructed 3D capillary networks

Maturation of constructed capillary networks is closely related to the recruitment of perivascular cells. In this study, capillary networks, which were constructed at least on day 7, were covered by MSC-derived perivascular cells. We previously confirmed that MSCs started to differentiate into αSMA-positive perivascular cells within 2 days because of the direct HUVEC-MSC contact, while MSCs not in contact with HUVECs did not express αSMA.[7] In this study, MSCs differentiated into αSMA-positive perivascular cells because both HUVECs and MSCs were seeded into the same channel, resulting in the direct HUVEC-MSC contact, which enhanced differentiation of the MSCs. We also confirmed that the perivascular cells were also positive for NG2, the other pericyte marker. These perivascular cells wrapped capillary structures on day 7, suggesting that the MSCs differentiated into pericyte-like cells. Similarly, Rohringer *et al.*[30] reported that adipose-derived stem cells differentiated into NG2-positive perivascular cells in coculture with ECs in fibrin gels.

Pericytes are associated with microvessels (10–100 μm diameters) or capillaries (<10 μm diameters) *in vivo*. Capillary pericytes align parallel to the longitudinal axis of the vessel, while microvessel pericytes align circumferentially.[9] Perivascular cells in this study aligned parallel to the longitudinal axis of the vessel on days 7, 14, and 21 (Figs. 4 and 6), suggesting that the MSCs differentiated into capillary pericytes. However, only αSMA was used to confirm MSC differentiation into pericytes. Further investigation will clarify the phenotypic transition of MSCs toward pericytes in more detail.

Maturation of capillary networks in terms of lumen formation occurred during days 7–21 in this study. Partially formed lumens of capillary networks on day 7 developed into continuous luminal structures during days 14–21. According to the correlation coefficient analysis, the outline of the capillary networks was not changed markedly after day 7. However, the capillary length and number of branching points still gradually increased during days 7–21. These results suggest that the framework capillaries were constructed by day 7, while maturation and remodeling of the constructed capillaries occurred during days 7–21.

The number of perivascular cells increased with culture time in this study, which also indicates maturation of constructed capillary networks. The pericyte:HUVEC ratio is different depending on tissues or organs *in vivo*. The ratio is reported to be 1:1 for the retina and the central nervous system, 1:10 for the lung, and 1:100 for the striated muscle.[28] The pericyte:HUVEC ratio measured in this study, 1:7.6, 1:5.6, and 1:5.0 on days 7, 14, and 21, respectively, did not fit to that for any of these tissues and organs. Recently, the role of organ-specific capillary ECs has been identified.[36] Using the culture model in this study, MSCs can be cultured with ECs from various organs instead of HUVECs, which might lead to insights into the control of the pericyte:EC ratio.

Stabilization of the constructed 3D capillary networks

In this study, framework capillaries were constructed by day 7 and they were stable for at least 21 days. This stabilization of constructed capillaries can be due to the association with perivascular cells. An increasing number of pericytes covered the capillary structures during days 7–21, which appeared to be associated with the formation of continuous lumens and maintenance of their diameter at <10 μm. It is reported that ECs secrete platelet-derived growth factor B (PDGF-B) and induce pericyte recruitment, which leads to the stabilization of capillary structures.[29] In this study, luminal size and framework capillaries were stabilized in the HUVEC-MSC coculture, while capillary length and number of branching points continued to increase slowly during days 7–21. These results suggest that luminal size and framework capillaries were stabilized by the interaction between MSC-derived pericytes and endothelial stalk cells, while endothelial tip cells were guided by the pericytes.[35]

In addition to the investment of capillaries with pericytes, formation of a basement membrane is important for the stabilization of constructed capillary networks.[29] In this study, we confirmed that the basement membrane proteins, laminin and collagen type IV, deposited around capillary structures covered by MSC-derived pericytes. However, in HUVEC or MSC monocultures, laminin deposition was confirmed, while collagen type IV deposition was not detected. Since dilation of capillary structures was observed in HUVEC monocultures, collagen type IV deposition appears to be more important for the stabilization of capillary structures. It is reported that EC-pericyte interactions induce basement membrane formation, which is a critical step in vessel maturation.[37,38] In particular, tissue inhibitor of metalloproteinase (TIMP)-3 derived from pericytes was important for collagen type IV deposition and the restriction of the capillary diameter.[37]

In conclusion, we demonstrated the construction of stable capillary networks covered by MSC-derived perivascular cells using an *in vitro* 3D angiogenesis model in a microfluidic device. The constructed capillary networks had continuous lumens with <10-μm diameter, which were maintained for at least 21 days. HUVEC-MSC interactions regulated this angiogenic process and basement membrane formation. A series of HUVEC:MSC ratios was tested and

we found that stable capillary networks could be constructed at a 1:1 HUVEC:MSC ratio. This 3D angiogenesis model is useful to further investigate the function of stable capillary structures covered by perivascular cells, and eventually can be combined with 3D culture of epithelial cells in the context of vascularization of 3D tissue-engineered constructs.

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Disclosure Statement

No competing financial interests exist.

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