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Efficient generation of endothelial cells from human pluripotent stem cells and characterization of their functional properties

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Abstract: Although endothelial cells (ECs) have been derived from human pluripotent stem cells (hPSCs), large-scale generation of hPSC-ECs remains challenging and their functions are not well characterized. Here we report a simple and efficient three-stage method that allows generation of approximately 98 and 9500 ECs on day 16 and day 34, respectively, from each human embryonic stem cell (hESC) input. The functional properties of hESC-ECs derived in the presence and absence of a TGFβ-inhibitory molecule SB431542 were characterized and compared with those of human umbilical vein endothelial cells (HUVECs). Confluent monolayers formed by SB431542 $^{+}$ hESC-ECs, SB431542^{-}hESC-ECs, and HUVECs showed similar permeability to 10,000 Da dextran, but these cells exhibited striking differences in forming tube-like structures in 3D fibrin gels. The SB431542 $^{+}$ hESC-ECs were most potent in forming tube-like structures regardless of whether VEGF and bFGF were present in the medium; less potent SB431542^{-}hESC-ECs and HUVECs responded differently to VEGF and bFGF, which significantly enhanced the ability of HUVECs to form tube-like structures but had little impact on SB431542^{-}hESC-ECs. This study offers an efficient approach to large-scale hPSC-EC production and suggests that the phenotypes and functions of hPSC-ECs derived under different conditions need to be thoroughly examined before their use in technology development. © 2015 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 104A: 678–687, 2016.

Key Words: endothelial cells, human embryonic stem cells, fibrin gel, tube formation, vascularization

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INTRODUCTION

Endothelial cells (ECs) have many applications in the biomedical field, such as enhancing the patency of engineered vascular grafts, promoting neovascularization in ischemic tissues, vascularizing tissue engineered constructs, and creating *in vitro* models for studying vascular development and screening anti-cancer drugs.[1–5] However, limited availability of human ECs has hindered the progress of endothelial-cell-related technologies. The recent advances in stem cell technology offer a unique opportunity to address this issue. In particular, ECs have been successfully derived from human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs).[6,7] Since hPSCs are able to proliferate extensively, unlimited ECs may be obtained if endothelial differentiation from hPSCs and expansion of hPSC-derived ECs (hPSC-ECs including hESC-ECs and hiPSC-ECs) are efficient.

Various methods have been developed to generate ECs from hPSCs.[8] The first hESC-ECs were isolated from embryoid bodies (EBs), in which hESCs spontaneously developed into the cell types in three germ layers in the presence of serum.[9] Since then, a variety of EB-based protocols have been reported, in which different growth factors and small molecules are used at specific development stages to stimulate EC specification.[10–14] In addition to EB-based methods, co-culture of hPSCs on OP9 (murine bone marrow stromal cells) or other feeder cells is another commonly used method to derive hPSC-ECs.[1,11,15–17] This method provides slightly higher differentiation efficiency than the original EB differentiation protocol, but the contamination from animal derived feeders is a concern for clinical use of these cell products. Endothelial differentiation of hPSCs on surfaces coated with Matrigel or gelatin or inside hydrogels has also been successfully conducted, resulting in improved differentiation efficiencies as compared to the original EB differentiation method.[18–22] In all these differentiation methods, hPSC-derived ECs were sorted with one or multiple endothelial markers such as CD31, CD34, CD144 (VE-cadherin), and KDR.

Extensive studies on endothelial differentiation of hPSCs have provided important insights into the mechanisms of endothelial development, but lack of efficient methods to generate sufficient numbers of hPSC-ECs remains a hurdle to using these cells in translational studies and technology development. In most protocols, differentiation efficiencies are

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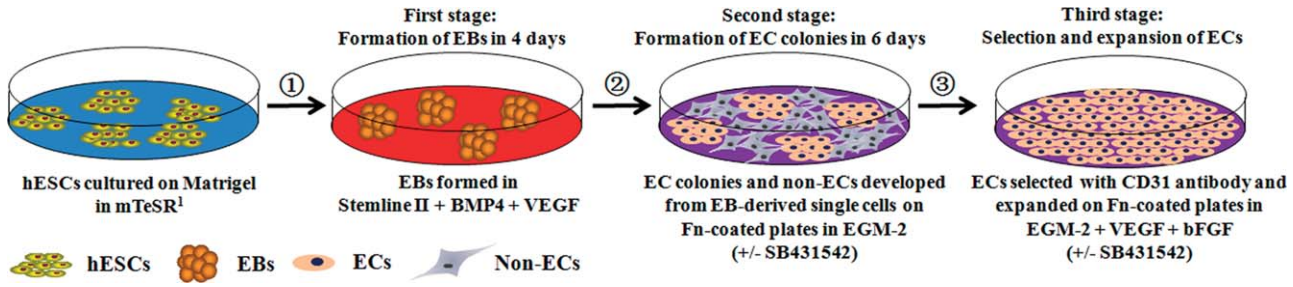

SCHEME 1. The three-stage approach to generate a large amount of ECs from hESCs.

low and the resulting hPSC-ECs have very limited expansion capacity. The recent study by James and colleagues has made significant contribution to addressing this issue.[10] They showed that exposure of differentiating EBs to a TGFβ-inhibitory molecule SB431542 after day 7 resulted in a 10-fold increase in the hESC-EC yield at day 14 and a net 36-fold expansion of hESC-ECs at day 20, producing 7.4 CD31 $^{+}$ VE-cadherin^{+} endothelial cells from every one hESC input over 20 days. This efficiency to generate hPSC-ECs is higher than what reported previously, and the discovery has brought hPSC-ECs closer to translational studies and technology development. Since this study was reported, other laboratories have started to use SB431542 when deriving ECs from hPSCs.[18,23] However, the functional properties of the ECs derived in the presence and absence of SB431542 have not been compared; more efficient differentiation protocols yielding larger amounts of hPSCs-ECs in shorter time periods are desirable.

Here we report a simple three-stage method to generate abundant hESC-ECs with an efficiency higher than what reported by James and colleagues. Functional properties of hESC-ECs derived in the presence and absence of SB43154, including endothelial permeability of confluent monolayers and tube formation abilities on two-dimensional (2D) Matrigel and in three-dimensional (3D) fibrin gels, were examined and compared. These properties were also compared with those of human umbilical vein endothelial cells (HUVECs), which have been widely used in studying angiogenesis and vasculogenesis in fibrin gels and used as a control for hPSC-derived ECs.[24–27] Striking differences in tube formation abilities of these three EC types have been found, suggesting that the phenotypes and functions of ECs derived from hPSCs under different conditions should be thoroughly studied prior to using these cells for translational studies and technology development.

MATERIALS AND METHODS

Culture of hESCs and HUVECs

The H9 hESCs (WiCell Research Institute, Inc.) were maintained in feeder-free conditions. Cells were cultured in mTeSR^{1} medium (StemCell Technologies) on Matrigel (BD Biosciences)-coated cell-culture plates. The medium was changed daily. The hESCs were passaged with 1 mg/mL dispase (StemCell Technologies) at a split ratio of 1:6 every 4 $\sim$ 6 days.

Human umbilical vein endothelial cells (HUVECs, Lonza) were cultured in EGM-2 medium (Lonza) and passaged with 0.05% trypsin-EDTA (Life Technologies) at a split ratio of 1:3 every 4 $\sim$ 6 days. The medium was changed every other day. HUVECs from passage 3 to passage 6 were used in the experiments.

Differentiation of hESCs to endothelial cells

A three-stage method as illustrated in Scheme 1 was developed to produce a large amount of hESC-derived ECs (hESC-ECs).

The first stage was formation and differentiation of EBs. Colonies of undifferentiated hESCs were dissociated to small cell clumps using 1 mg/mL dispase. The EBs were formed by culturing the cell clumps in ultra-low attachment plates (Costar) in serum-free Stemline II medium (Sigma) supplemented with 50 ng/mL bone morphogenetic protein 4 (BMP4, R&D system) and 50 ng/mL vascular endothelial growth factor (VEGF, R&D system). The EBs were cultured for 4 days, with half medium changed after 2 days.

The second stage was formation of EC-colonies. The EBs cultured for 4 days were collected and dissociated with 0.05% trypsin-EDTA for 5 min, followed by passing the suspension of dissociated EBs through a 22G needle three times and then through a 40 μm cell strainer (BD Falcon). The resulting single cells were collected through centrifugation. The cells were re-suspended in EGM-2 medium or EGM-2 medium supplemented with 10 μ*M* SB431542 (Tocris Bioscience), seeded on fibronectin (FN, BD Biosciences)-coated cell-culture plates at a density of 5,000 cells/cm^{2}, and cultured for 6 days. The media were changed every other day. Colonies surrounded by other cells formed after 3 days and gradually grew larger. To characterize whether the colony-forming cells are ECs, the cells cultured on FN-coated surfaces for 6 days were stained for CD31 and VE-cadherin and counterstained with DAPI. Their ability for Low Density Lipoprotein (LDL) uptake was also examined. Cells were imaged with a 5× objective on a Zeiss Axiovert Observer inverted fluorescence microscope, and the numbers of total cells and ECs (positive for CD31, VE-cadherin, and LDL uptake) in each field were manually counted. Cell densities were calculated via dividing cell numbers by the surface area of a field. The percentage of ECs was calculated via dividing the EC number by the total cell number. Data

were represented as mean ± SD ($n = 5$). These ECs formed in this stage were designated as passage-1 cells.

The third stage was separation and expansion of ECs. Cells cultured in the second stage for 6 days were dissociated with 0.05% trypsin-EDTA, and $CD31^+$ cells were magnetically sorted (EasySep™ Do-It-Yourself Selection Kit, StemCell Technologies). The separated $CD31^+$ cells were seeded on FN-coated cell-culture plates at a density of 10,000 cells/cm^2 and cultured in EGM-2 medium supplemented with 50 ng/mL VEGF and 10 ng/mL bFGF with or without 10 μ*M* SB431542. The medium was changed every 2 days. These cells were designated as passage-2 cells. Further cell expansion was performed by seeding cells on FN-coated cell-culture plates at a density of 10,000 cells/cm^2 and cultured in the same medium.

Proliferation of hESC-ECs cultured with or without SB431542

To analyze the effect of SB431542 on proliferation of hESC-ECs, cells were seeded on FN-coated cell-culture plates at a density of 10,000 cells/cm^2, cultured in EGM-2 medium supplemented with 50 ng/mL VEGF and 10 ng/mL bFGF with or without 10 μ*M* SB431542, and examined on day 6 in each passage. Cells from passage-2 to passage-5 were examined. Cell nuclei were stained with DAPI and imaged with a 10× objective, and cell numbers in six randomly chosen fields were manually counted. Cell densities were calculated by dividing cell numbers by the surface area of a field. Data were represented as mean ± SD ($n = 6$).

Immunocytochemical staining

Cells were washed with PBS and fixed with 3.7% formaldehyde. For CD31 staining, samples were blocked with 2% BSA and incubated with mouse anti-human CD31 (1:200, eBioscience) overnight at 4°C, followed by incubation with the Alexa Fluor® 488 goat anti-mouse secondary antibody (1:500, Molecular Probes) for 30 min. For vonWillebrand factor (vWF) and VE-cadherin staining, samples were permeabilized with 0.2% Triton X-100, blocked with 2% BSA, and incubated with mouse anti-human vWF (1:50, Dako) and mouse anti-human VE-cadherin (1:50, BD Bioscience), respectively, overnight at 4°C, followed by incubation with Alexa Fluor® 555 and Alexa Fluor® 488 goat anti-mouse secondary antibodies (1:500, Molecular Probes), respectively, for 30 min. Cell nuclei were counterstained with DAPI. HUVECs were stained as positive controls of ECs.

LDL uptake

Cells were incubated in EGM-2 medium containing 10 μg/mL Dil-acetylated LDL (Molecular Probes) at 37°C for 5 h. After washing with PBS, cells were fixed with 3.7% formaldehyde and examined on a fluorescence microscope. Human umbilical vein endothelial cells (HUVECs) were used as a positive control.

Endothelial permeability of hESC-ECs

Endothelial permeability of hESC-ECs was examined using trans-well plates. The trans-well membrane (6.5 mm Transwell® with 0.4 μm Pore Polyester Membrane Insert, Costar) was coated with diluted Matrigel (1:100 in PBS) and incubated at 37°C for 1 h before cell seeding. The hESC-ECs derived with SB431542 ($SB431542^+$ hESC-ECs) and without SB431542 ($SB431542^-$hESC-ECs) and HUVECs were seeded on the membrane in the inserts at a density of 500,000 cells/cm^2 and cultured in EGM-2 medium supplemented with 50 ng/mL VEGF and 10 ng/mL bFGF for 2 days. To examine endothelial permeability, 0.1 mL of 1 mg/mL FITC-Dextran solution (MW 10,000, Invitrogen) prepared in EGM-2 medium was added in the upper compartments and 0.6 mL of EGM-2 medium was added to the lower compartments, followed by incubation in a tissue culture incubator for 2 h. The medium from each lower compartment (50 μL) was collected and transferred to 96-well clear bottom plates (Lonza) for fluorescence measurements on a multi-mode microplate reader (Synergy HT, Bio-Tek; λ_{EX} 485 nm; λ_{EM} 525 nm). Fluorescence intensity of each sample was normalized to that of the FITC-Dextran solution added in the upper compartments. Data were represented as mean ± SD ($n = 3$).

To ensure that cells seeded at 5,00,000 cells/cm^2 would form confluent monolayers having cell–cell contacts in 2 days, cells were seeded and cultured in 96-well plates in the same conditions as those in transwells and stained for CD31 and VE-cadherin.

Tube formation on 2D matrigel

Matrigel was added to 48-well plates and allowed to solidify at 37° C for 1 h. The hESC-ECs suspended in EGM-2 medium were seeded on the top of Matrigel and incubated in a tissue culture incubator. Formation of tube-like structures was examined after 24 h. HUVECs were used as a positive control.

Tube formation in 3D fibrin gels

The $SB431542^+$ hESC-ECs, $SB431542^-$hESC-ECs, and HUVECs were dissociated with 0.25% trypsin-EDTA, encapsulated in fibrin gels (2.5 mg/mL fibrinogen and 0.4 U/mL thrombin) at a density of 1 million cells/mL, and cultured in EGM-2 medium or EGM-2 medium supplemented with 50 ng/mL VEGF and 10 ng/mL bFGF. The media were changed every 2 days. Tube formation was examined after 2 and 4 days of culture on a microscope.

Statistical analysis

Statistical analysis was performed using a one-way analysis of variance (ANOVA) with Tukey test for pariwise comparisons. A value of $p < 0.05$ was considered to be a statistically significant difference.

RESULTS

Differentiation of hESCs to ECs

In the first stage of the differentiation method illustrated in Scheme 1, hESCs cultured on Matrigel [Fig. 1(a)] were able to form EBs when cultured on non-adherent surfaces in serum-free Stemline II medium supplemented with BMP4 and VEGF for 4 days [Fig. 1(b)]. The medium was chosen

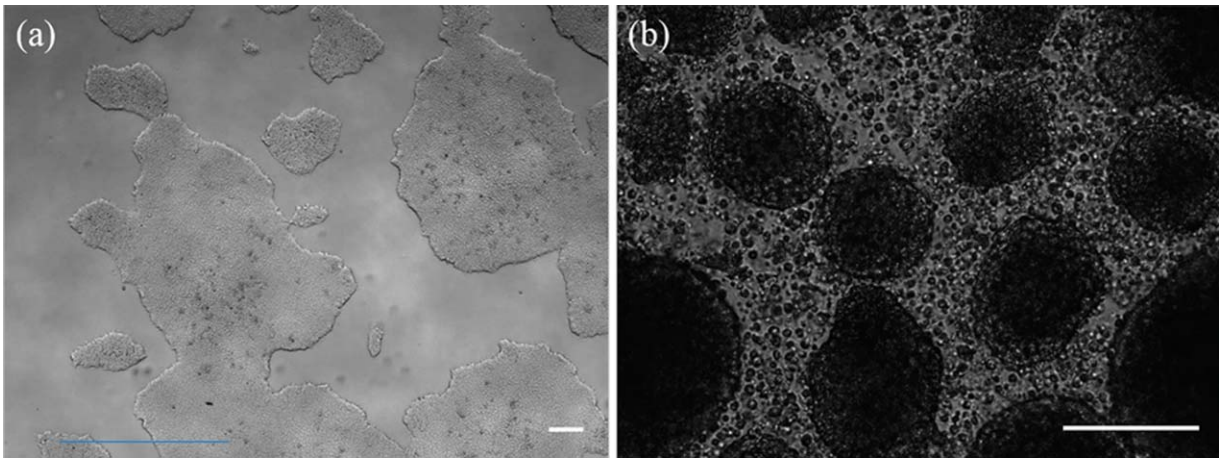

FIGURE 1. Undifferentiated hESCs maintained on Matrigel-coated cell-culture plates (a) and EBs formed in serum-free Stemline II medium after 4 days of culture (b). Scale bars: 200 μm.

because it would stimulate hemangioblast development from hESCs.[13,14]

In the second stage, some single cells dissociated from the EBs were able to form colony-like structures when cultured on FN-coated surfaces in EGM-2 medium with or without SB431542. Two types of cell morphologies were observed in these cultures: the cells in the colony-like structures were smaller and contacting each other; the cells surrounding the colony-like structures were larger and randomly spread [Fig. 2(a,e)]. The cells in the colony-like structures were positively stained for CD31 and VE-cadherin and capable of LDL uptake, suggesting that these cells were committed ECs; while the cells surrounding the colony-like structures were not ECs [Fig. 2(b–d) and (f–h)]. The percentages of $CD31^+$ cells and VE-$cadherin^+$ cells in Figure 2(f, g) were higher than those in Figure 2(b,c). This suggests that production of ECs was enhanced in the presence of SB431542, a TGFβ-inhibitory molecule that stimulates endothelial differentiation from ESCs and proliferation of ESC-derived ECs.[10,28] Comparison of the cultures at the end of stage 2 (day 6 of stage 2 or day 10 of the whole procedure) revealed that addition of SB431542 resulted in a 2.7-fold increase in the absolute number of committed ECs [Fig. 2(i)]. The percentage of ECs in each culture increased from 35.8% in the absence of SB431542 to 56.1% in the presence of SB431542 [Fig. 2(j)]. Since EB-derived cells were seeded at 5,000 cells/cm^2, the data in Figure 2(i) suggest that the yields of passage-1 hESC-ECs at the end of stage 2 were 13.6 and 5.0 ECs from every one hESC input for SB431542 $^+$ hESC-ECs and SB431542$^-$hESC-ECs, respectively.

In the third stage, $CD31^+$ cells in the colony-like structures were magnetically sorted and further expanded on FN-coated surfaces in EGM-2 medium supplemented with VEGF and bFGF. These $CD31^+$ cells were seeded at a density of 10,000 cells/cm^2 and cultured to confluence, followed by immunostaining and evaluation of LDL uptake. Expansion of passage-1 cells for 6 days yielded passage-2 hESC-ECs positive for CD31 and VE-cadherin staining and LDL uptake. But expression of vWF in the passage-2 cells was low, suggesting that they were not mature ECs. Expression of vWF was upregulated in passage-3 cells, with [mt]95% of cells stained positive for vWF. The mature phenotypes and functional characteristics of ECs were preserved during further expansion: [mt]95% of passage-5 cells were stained positive for CD31, VE-cadherin, and vWF [(Fig. 3(b–d) and (h-j)]; and they were capable of LDL uptake [Fig. 3(e,k)] and forming tube-like structures on 2D Matrigel [Fig. 3(f,l)].

Some differences between SB431542 $^+$ hESC-ECs and SB431542$^-$hESC-ECs were noticed. When a culture reached confluence, SB431542 $^+$ hESC-ECs were locally aligned in parallel clusters; while SB431542$^-$hESC-ECs did not have such morphological organization [Fig. 3(a–e) and (g–k)]. It was more difficult and took longer time to dissociate SB431542 $^+$ hESC-ECs during subculture, suggesting that these cells might have stronger cell-cell adhesion at confluence. The SB431542 $^+$ hESC-ECs were able to form tube-like structures on Matrigel when seeded at 80,000 cells/cm^2 [Fig. 3(l)]; while the SB431542$^-$hESC-ECs were not able to form tube-like structures at this seeding density, but were able to form tube-like structures when seeded at 120,000 cells/cm^2 [Fig. 3(f)]. These results suggest that SB431542 might affect the cellular properties of hESC-ECs.

The effect of SB431542 on proliferation of hESC-ECs

Proliferation rates of SB431542 $^+$ hESC-ECs and SB431542$^-$hESC-ECs were examined for passage-2 to passage-5 cells [Fig. 4]. In each passage, cells were seeded at 10,000 cells/cm^2 and the cell density on day 6 was characterized. The effect of SB431542 on proliferation of hESC-ECs is obvious: in each passage the proliferation rate of SB431542 $^+$ hESC-ECs was ca. 2– to 3–fold of that of SB431542$^-$hESC-ECs. After 4 times of subculture, cell expansion (the ratio of the total amount of passage-5 cells to the seeded passage-1 cells) was ca. 11-fold for SB431542$^-$hESC-ECs and ca. 695-fold for SB431542 $^+$ hESC-ECs. For both SB431542 $^+$ hESC-ECs and SB431542$^-$hESC-ECs, their proliferation rates gradually decreased as the cell passage number increased. These results, together with the yield of passage-1

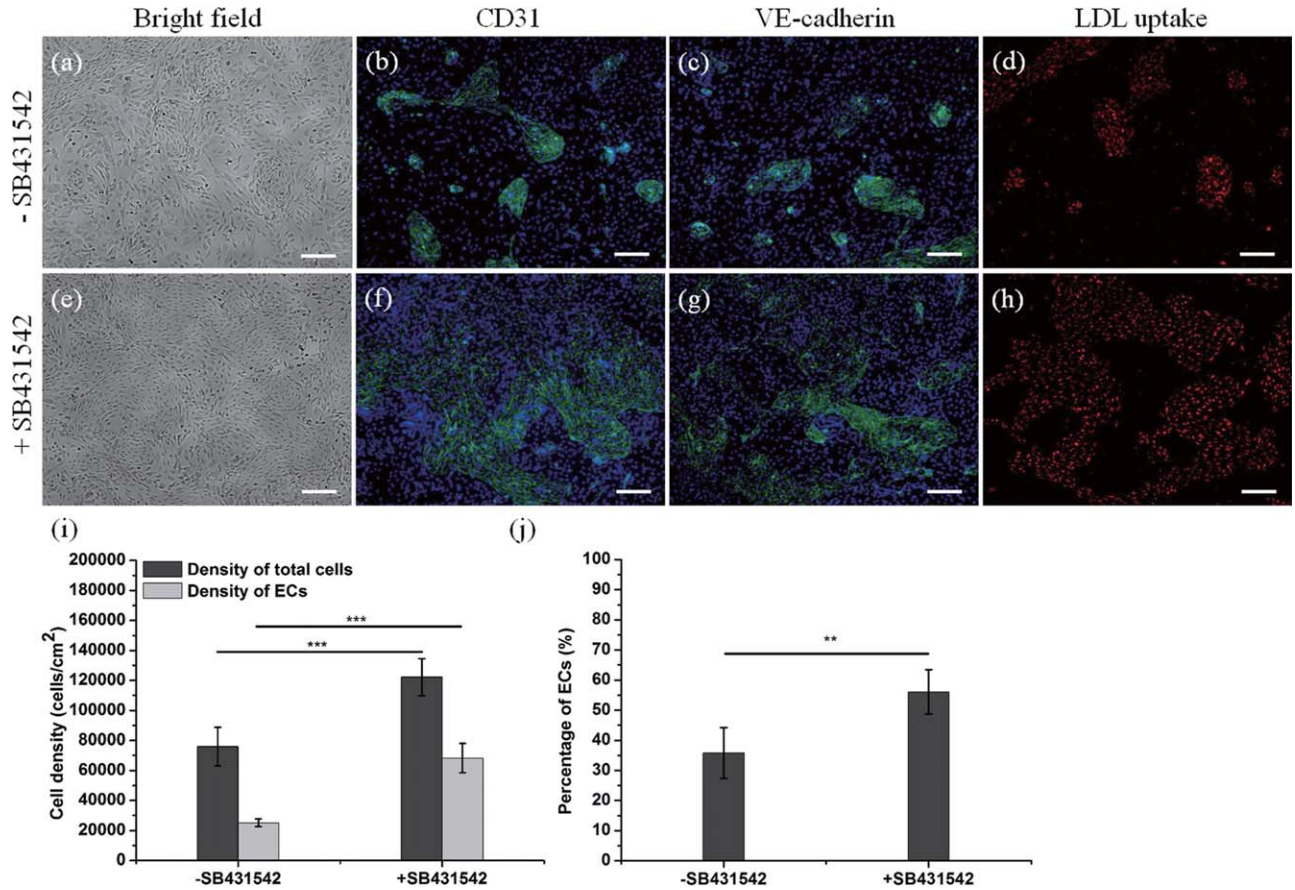

FIGURE 2. Generation of ECs by culturing EB-derived single cells on FN-coated surfaces for 6 days. (a, e) The EB-derived single cells formed colony-like structures surrounded by cells having a different morphological organization. (b, c, f, g) The cells in the colony-like structures were positively stained for CD31 and VE-cadherin, showing phenotypic characteristics of ECs. Green: CD31 and VE-cadherin; blue: DAPI. (d, h) The cells in the colony-like structures were capable of LDL uptake, showing the functional characteristic of ECs. (i) The densities of total cells and ECs after 6 days of culture in the presence or absence of SB431542. (j) The percentage of ECs after 6 days of culture. Cells were cultured with or without SB431542. ** $p < 0.01$ and *** $p < 0.001$. Scale bars: 200 μm.

hESC-ECs, suggested that large numbers of hESC-ECs could be produced through this 3-stage method: ca. 98 passage-2 SB431542 $^{+}$ hESC-ECs were generated in 16 days and ca. 9500 passage-5 SB431542 $^{+}$ hESC-ECs were generated in 34 days from every one hESC input. We conducted the cell differentiation, isolation, and proliferation procedures multiple times, and the proliferation behavior was reproducible.

Endothelial permeability of hESC-ECs

Since the barrier function of ECs is critical in physiological conditions, endothelial permeability of confluent monolayers of hESC-ECs was examined using a trans-well system that allowed material transport across endothelium to be mimicked. It was shown that HUVECs, SB431542 $^{+}$ hESC-ECs, and SB431542^{-}hESC-ECs seeded at 500,000 cells/cm^2 and cultured in EGM-2 medium containing VEGF and bFGF all formed confluent monolayers having cell-cell contacts in 2 days [Fig. 5(a–f)]. Immunofluorescent staining revealed that CD31 and VE-cadherin, two intercellular junction markers, were both present at cell-cell interfaces. Permeability of the EC monolayers formed in transwells under the same seeding and culture conditions was characterized by examining transport of FITC-Dextran (MW 10000) across the monolayers. When FITC-Dextran was added in the upper compartment of a transwell, the fluorescence of the medium in its lower compartment revealed the permeability of the EC monolayer: the more permeable an EC monolayer was, the higher fluorescence intensity would be expected. The results in Figure 5(g) showed that monolayers formed from both SB431542 $^{+}$ hESC-ECs and SB431542^{-}hESC-ECs had similar permeability as HUVEC monolayers under the conditions of the experiments.

Tube formation of hESC-ECs in 3D fibrin gels

The ability of hESC-ECs to form tube-like structures in 3D fibrin gels was examined, and striking differences were observed among SB431542 $^{+}$ hESC-ECs, SB431542^{-}hESC-ECs, and HUVECs (Fig. 6). The SB431542 $^{+}$ hESC-ECs could organize and assemble into tube-like structures in fibrin gels more rapidly than the SB431542^{-}hESC-ECs and HUVECs. After 2 days of culture, elongated tubes were obviously observed in fibrin gels encapsulating SB431542 $^{+}$

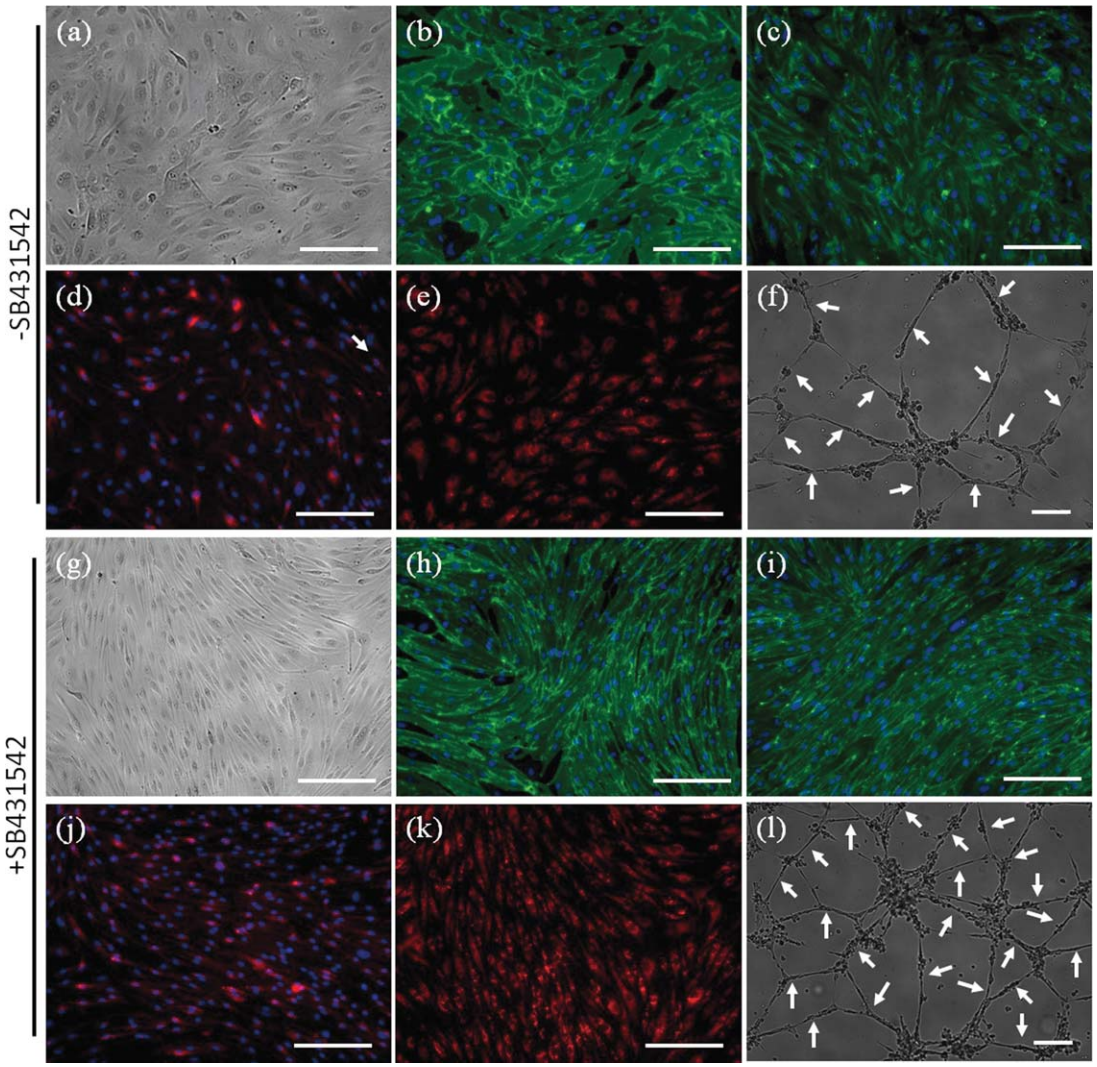

FIGURE 3. Characterization of passage-5 hESC-ECs. (a, g) Bright field images. (b, h) CD31 staining. (c, i) VE-cadherin staining. (d, j) vWF staining. (e, k) LDL uptake. (f, l) Tube formation on 2D Matrigel. Tube-like structures are indicated by white arrows. Cells were seeded at a density of 10,000 cells/cm^2 and cultured to confluence with SB431542 (g-l) or without SB431542 (a-f) prior to immunostaining and evaluation of LDL uptake. Scale bars: 200 μm.

hESC-ECs [Fig. 6(c,f)]; whereas only a few short tubes appeared in fibrin gels encapsulating HUVECs or SB431542$^-$hESC-ECs [Fig. 6(a,b,d,e)]. After 4 days of culture, longer and more tube structures had developed in gels encapsulating HUVECs [Fig. 6(g,j)], whereas gels encapsulating SB431542$^-$hESC-ECs still only contained a few short tubes [Fig. 6(h,k)]. In gels encapsulating SB431542$^+$ hESC-ECs, the tubes became longer and formed denser networks after 4 days of culture [Fig. 6(i,l)]. The effects of VEGF and bFGF on tube formation were also different for SB431542$^+$ hESC-ECs, SB431542$^-$hESC-ECs, and HUVECs: VEGF and bFGF enhanced tube formation of HUVECs [Fig. 6(g,j)], whereas they did not affect tube formation of SB431542$^+$ hESC-ECs and SB431542$^-$hESC-ECs [Fig. 6(b,c,e,f,h,I,k,l)]. Taken together, SB431542$^+$ hESC-ECs had excellent ability to form tube-like structures in fibrin gels. The ability of tube formation of these three cell types was different

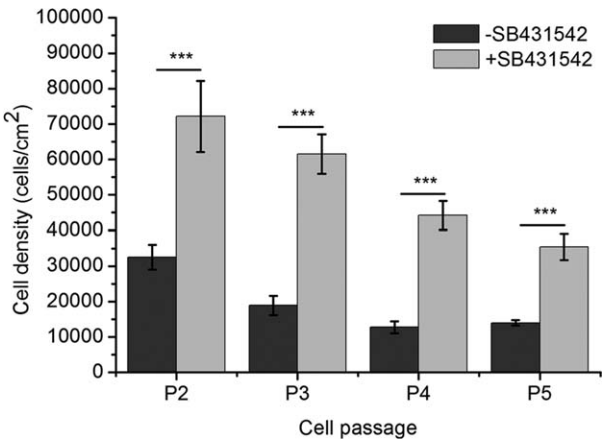

FIGURE 4. The proliferation profiles of hESC-ECs (from passage-2 to passage-5) cultured with and without SB431542. *** $p < 0.001$.

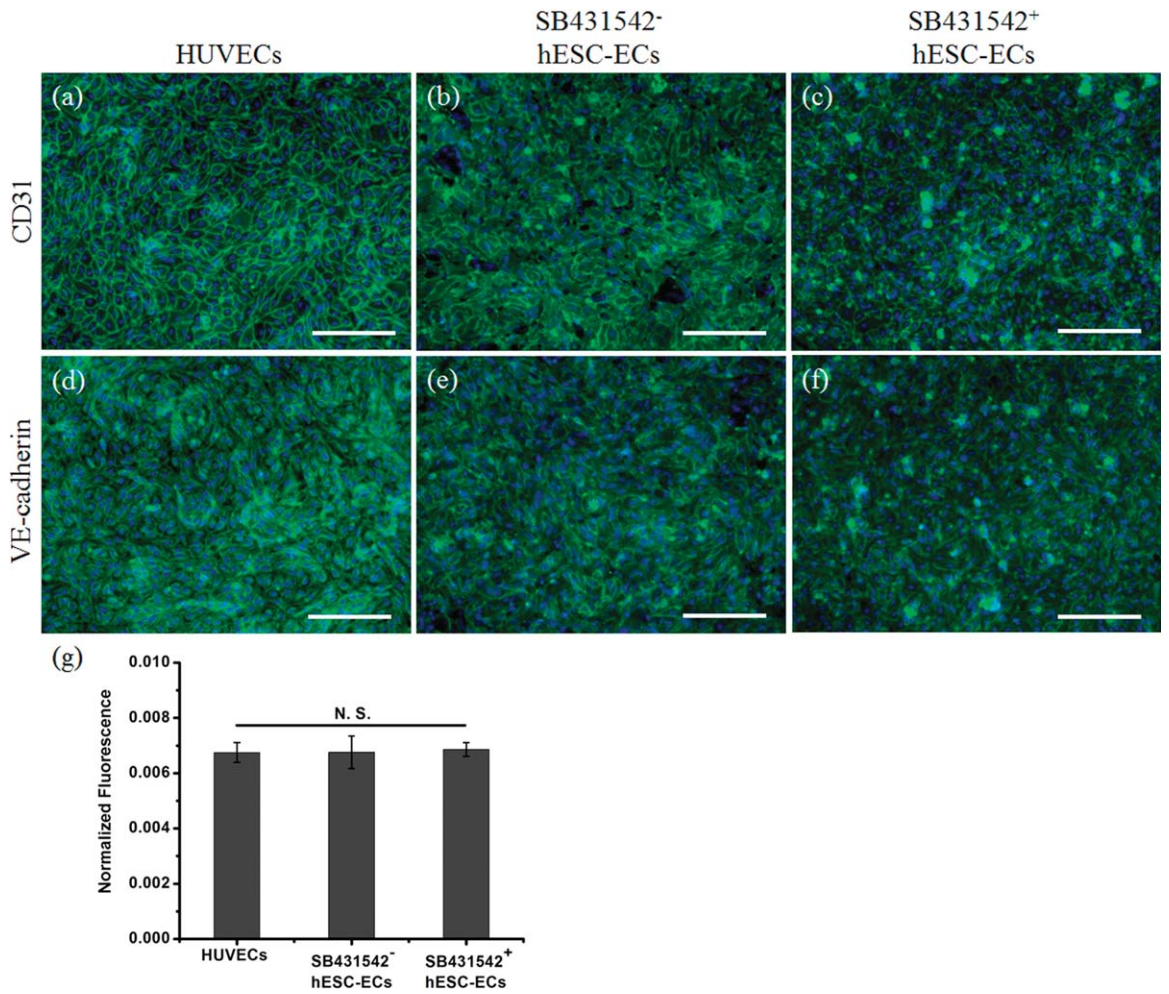

FIGURE 5. Characterization of endothelial permeability of hESC-ECs. (a-f) Cells formed confluent monolayers having cell-cell contacts. (g) The permeability of monolayers of HUVECs, SB431542 $^{+}$ hESC-ECs, and SB431542^{-}hESC-ECs was similar as revealed from transport of FITC-Dextran across the monolayers. N. S. No significant difference. Scale bars: 200 μm.

and ranked as SB431542 $^{+}$ hESC-ECs, HUVECs, and SB431542^{-}hESC-ECs (from higher to lower).

DISCUSSION

Although ECs were successfully derived from hESCs [mt]10 years ago and various differentiation methods and protocols have been reported since then[8,9], large-scale generation of hPSC-ECs remains challenging and it is a hurdle to using these cells in translational studies and technology development. Here we report a simple and efficient three-stage method that allows quick production of a large amount of hESC-ECs. In the first stage, EBs were cultured in serum-free Stemline II medium supplemented with BMP-4 and VEGF for 4 days to promote mesodermal and hemangioblast differentiation. In the second stage, single cells dissociated from EBs were cultured on FN-coated surfaces in EGM-2 medium for 6 days to drive endothelial differentiation. Interestingly, ECs positive for CD31 and VE-cadherin were all located in colony-like structures surrounded by non-ECs. Since single cells were plated at the beginning of this stage, ECs in each colony-like structure were most likely derived from the same parent cell, suggesting that cells underwent extensive proliferation while differentiating toward ECs in this stage. In the third stage, CD31^{+} cells were sorted to obtain immature passage-1 ECs, which were positive for VE-cadherin and capable of LDL uptake, but expressed low levels of vWF. Expansion of passage-1 cells in the presence of VEGF and bFGF resulted in mature ECs at passage-3, and all EC markers were maintained during further expansion to passage-5.

This method offers higher production efficiency of hESC-ECs than previously reported approaches. Lu et al. cultured EBs of hESCs in Stemline II medium supplemented with BMP-4 and VEGF for 3.5 days, followed by culture of the single cells dissociated from the EBs in expensive blast-colony growth medium.[13,14] On day 9.5, about 0.35-0.52 hemangioblasts (which had the potential to develop into ECs under appropriate conditions) were produced from each hESC input. In our method, single cells dissociated

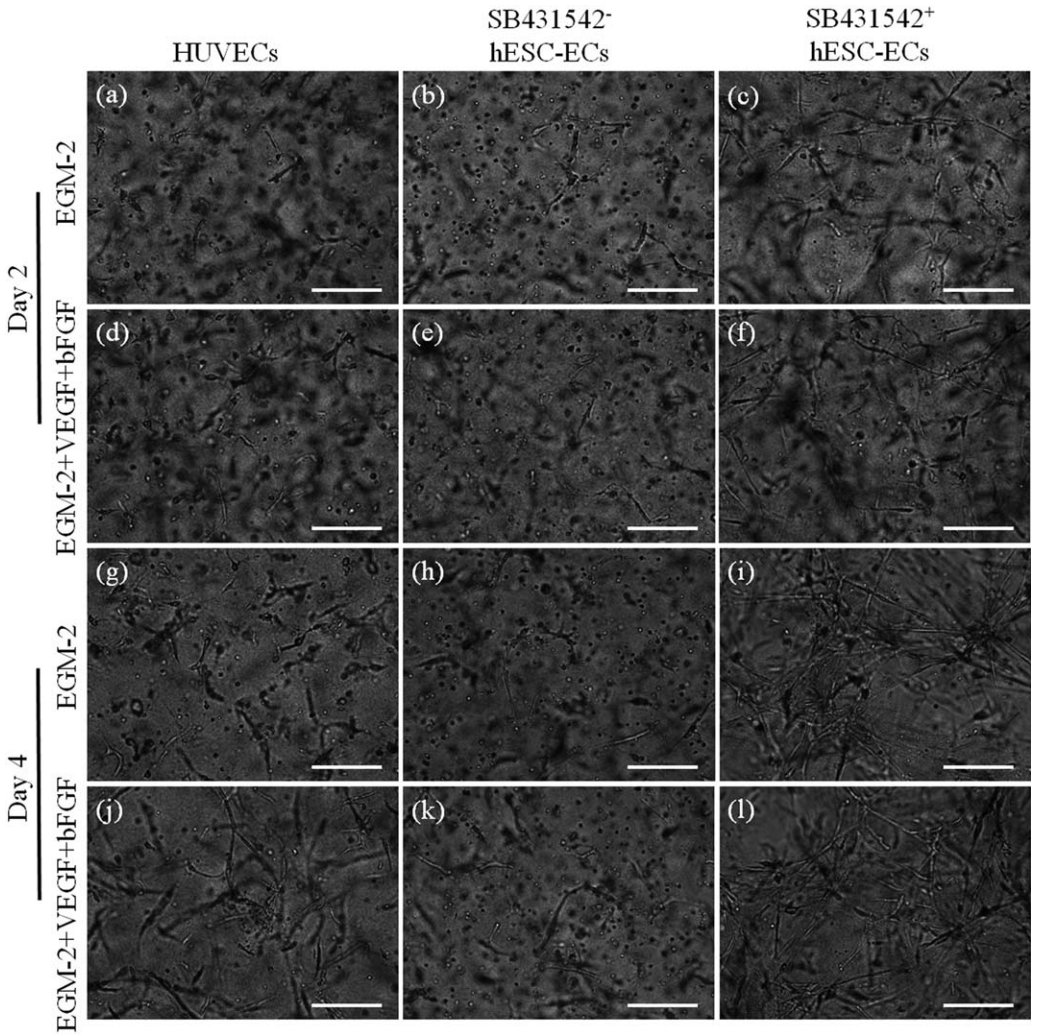

FIGURE 6. Tube formation of HUVECs (a, d, g, h), SB431542$^-$hESC-ECs (b, e, h, k) and SB431542 $^+$ hESC-ECs (c, f, i, l) after 2 and 4 days of culture in 3D fibrin gels. Cells were cultured in EGM-2 medium (a-c and g-i) or EGM-2 medium supplemented with VEGF and bFGF (d-f and j-l). Scale bars: 200 μm.

from the EBs were cultured in much less expensive EGM-2 medium. On day 10, colony-like structures containing CD31 $^+$ VE-cadherin$^+$ cells formed and the yield of ECs (passage-1) was 5 ECs from each hESC input in the absente of SB431542 and 13.6 ECs from each hESC input in the presence of SB431542.

The passage-1 hESC-ECs isolated with an anti-CD31 antibody could be further expanded on FN-coated surfaces in EGM-2 medium supplemented with VEGF and bFGF. We observed that addition of SB431542 significantly increased the proliferation rate of hESC-ECs, consistent with the finding of James and colleagues. They reported that 7.4 ECs were generated from each hESC input in 20 days when SB431542 was added on day 7.[10] In our method, ca. 98 ECs were generated from each hESC input in 16 days when SB431542 was added in the second and third stages, suggesting higher efficiency of this method. It allowed production of ca. 9500 passage-5 SB431542 $^+$ hESC-ECs from each hESC input on day 34—such efficient production may well address the previously existing challenge to obtain sufficient numbers of human ECs for translational studies and technology development.

Since James and colleagues reported that SB431542 could enhance expansion of hESC-ECs, this strategy has been used to derive ECs from hPSCs in several laboratories.[18,23] However, it has remained unclear whether hPSC-ECs derived in the presence and absence of SB431542 have the same functional properties. We examined barrier function of confluent monolayers formed by SB431542 $^+$ hESC-ECs, SB431542$^-$hESC-ECs, and HUVECs, respectively, by using a transwell assay. It was shown that these monolayers had similar permeability to dextran having molecular weight of 10000. During subculture, we observed that enzymatic dissociation of confluent SB431542 $^+$ hESC-ECs took longer time than that of confluent SB431542$^-$hESC-ECs and HUVECs, suggesting that the components and the levels of cell adhesion proteins expressed on these cells were different. One explanation for similar endothelial permeability of

these three cell types despite the difference in their cell adhesion proteins is that these proteins contribute to permeability regulation differently. Striking differences among SB431542 $^{+}$ hESC-ECs, SB431542^{-}hESC-ECs, and HUVECs were observed in their ability to form tube-like structures in 3D fibrin gels. The SB431542 $^{+}$ hESC-ECs showed most potent ability to form tube-like structures in 3D fibrin gels regardless of whether VEGF and bFGF were present in the medium. Between the less potent SB431542^{-} hESC-ECs and HUVECs, their responses to VEGF and bFGF were different: these two growth factors significantly enhanced the ability of HUVECs to form tube-like structures; while their impact on SB431542^{-}hESC-ECs was little.

The finding that SB431542 $^{+}$ hESC-ECs and SB431542^{-}hESC-ECs have functional differences calls for thorough investigation of the phenotypes and functions of ECs derived from hPSCs in the presence of SB431542 prior to using these cells for translational studies and technology development. In particular, the investigation of the proteins mediating cell adhesion, cell migration, and matrix remodeling and the proteins related to EC functions, such as endothelial nitric oxide synthase and prostacyclin, is essential for elucidating the molecular mechanisms underlying functional differences in future studies. Investigation of the effects of other activin receptor-like kinase inhibitors on endothelial differentiation of hPSCs will also provide mechanistic understanding of the differentiation. In addition, the markers of subtypes of ECs (arterial, venous, and lymphatic) need to be evaluated as it has been shown that hPSC-derived ECs can be biased toward arterial ECs over venous ECs under certain *in vitro* differentiation conditions [29]; and different EC subtypes function differently. Comparison with controls other than HUVECs, such as human arterial endothelial cells (HAECs) and human microvascular endothelila cells (HMVEC), will be necessary and will provide insightful information.

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