Highly Efficient Differentiation of Endothelial Cells from Pluripotent Stem Cells Requires the MAPK and the PI3K Pathways

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Significance Statement
Vascular disease affects millions of people. Endothelial cells can potentially be used to revascularize ischemic areas and to engineer artificial blood vessels and tissues. We not only developed one of the most efficient protocols to derive ECs from pluripotent stem cells, but we have also delineated a detailed mechanistic description of the differentiation process. We revealed that all three MAPK and the PI3K pathways are responsible for induction of EC fate. Furthermore, inhibition of the ERK pathway markedly promoted the differentiation of vascular progenitors to ECs, most likely through regulation of the ETS family transcription factors, ERG and FLI1. We revealed novel roles of the p38 and JNK MAPK pathways on EC differentiation. Furthermore, inhibition of the ERK pathway markedly promoted the differentiation of smooth muscle cells. Finally, we demonstrate that pluripotent stem cell-derived ECs are capable of forming patent blood vessels that were connected to the host vasculature in the ischemic limbs of immune deficient mice. Thus, we demonstrate that ECs can be efficiently derived from hiPSCs and hESCs, and have great potential for vascular therapy as well as for mechanistic studies of EC differentiation. Stem Cells 2017;35:909–919

Abstract
Pluripotent stem cells are a promising source of endothelial cells (ECs) for the treatment of vascular diseases. We have developed a robust protocol to differentiate human induced pluripotent stem cells (hiPSCs) and embryonic stem cells (hESCs) into ECs with high purities (94%-97% CD31\(^{+}\) and 78%-83% VE-cadherin\(^{+}\)) in 8 days without cell sorting. Passaging of these cells yielded a nearly pure population of ECs (99% of CD31\(^{+}\) and 96.8% VE-cadherin\(^{+}\)). These ECs also expressed other endothelial markers vWF, Tie2, NOS3, and exhibited functions of ECs such as uptake of Dil-acetylated low-density lipoprotein and formation of tubes in vitro or vessels in vivo on matrigel. We found that FGF2, VEGF, and BMP4 synergistically induced early vascular progenitors (VPs) from hiPSC-derived mesodermal cells. The MAPK and PI3K pathways are crucial not only for the initial commitment to vascular lineages but also for the differentiation of vascular progenitors to ECs, most likely through regulation of the ETS family transcription factors, ERG and FLI1. We revealed novel roles of the p38 and JNK MAPK pathways on EC differentiation. Furthermore, inhibition of the ERK pathway markedly promoted the differentiation of smooth muscle cells. Finally, we demonstrate that pluripotent stem cell-derived ECs are capable of forming patent blood vessels that were connected to the host vasculature in the ischemic limbs of immune deficient mice. Thus, we demonstrate that ECs can be efficiently derived from hiPSCs and hESCs, and have great potential for vascular therapy as well as for mechanistic studies of EC differentiation.

Introduction
Vascular disease affects millions of people. The severe form of vascular disease leads to limb amputation and life-threatening strokes. Under physiological conditions, blood vessels can be generated by vasculogenesis and angiogenesis. Vasculogenesis refers to the formation of new blood vessels from progenitors, while angiogenesis refers to the formation of new blood vessels from existing blood vessels via migration and proliferation of existing vascular cells [1]. However, these processes are impaired in individuals who have suffered strokes, diabetes or Alzheimer’s [2]. Endothelial cells (ECs), which line the lumen of all blood vessels, play a critical role in regulation of vascular permeability, angiogenesis and tissue regeneration. Human pluripotent stem cells, for example, human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), are capable of self-renewal and differentiation into any cell type in the human body, and thus are attractive resources to generate ECs. Many groups have shown that ECs can be derived...
from hiPSCs and hESCs (reviewed in [3, 4]). These ECs can potentially be used to revascularize ischemic areas and to engineer artificial blood vessels and tissues. The in vitro EC differentiation from pluripotent stem cells also provides a new opportunity to study molecular mechanisms that control endothelial fate. The knowledge gained from mechanistic studies may aid in understanding the pathogenesis of vascular disease as well as developing interventions for vascular disease. However, a highly efficient and cost-effective method to derive ECs from pluripotent stem cells must be established before clinical application can be developed. Furthermore, the mechanisms that govern endothelial fate from pluripotent stem cells need to be further explored.

We aimed to optimize the differentiation of pluripotent stem cells into ECs that will have the capacity to treat vascular disease. In addition, we intended to elucidate the molecular mechanisms that instruct endothelial fate. Herein, we report a highly efficient protocol for the differentiation of ECs from hESCs and hiPSCs that yields a nearly pure population of ECs without cell sorting. We further reveal important roles of the MAPK and PI3K signaling pathways in the determination of endothelial lineages. Finally, we show that ECs derived from pluripotent stem cells are capable of forming blood vessels in ischemic mouse limbs. These findings significantly advance approaches for deriving ECs from pluripotent stem cells, as well as our understanding of endothelial fate determination.

**Materials and Methods**

**Cell Cultures for hiPSCs and hESCs**

Human iPSCs line, IMR-90-4iPSC and human ESC line, H9, were purchased from WiCell Research Institute (Madison, WI). Both hiPSCs and hESCs were passaged every 6-7 days by manually picking the colonies with 1-ml pipet tips and seeded onto mouse embryonic fibroblasts (Global Stem, Gaithersburg, MD) in hESC culture media as manufacturer’s instructions.

**Differentiation of ECs from hiPSCs and hESCs**

Differentiation of ECs from hiPSCs and hESCs was progressively induced in three stages. Briefly, hiPSC or hESCs cells were manually passaged as small clusters onto hESC-qualified Matrigel (Corning, Corning, NY)-coated culture plates in MEF conditioned hESC medium with an additional 10 ng/ml of FGF2. Matrigel coating was performed according to the manufacturer’s suggestion (Corning). After 1 day, culture medium was switched to StemDiff APEL medium (STEMCELL Technologies, Cambridge, MA) with 6 µM of CHIR99021 (Tocris Bioscience, Avonmouth, UK) for 2 days. Next, the cells were cultured in StemDiff APEL medium (STEMCELL Technologies) supplemented with 25 ng/ml bone morphogenetic protein (BMP4) (R&D Systems, Minneapolis, MN), 10 ng/ml FGF2 (R&D Systems), and 50 ng/ml vascular endothelial growth factor (VEGF) (R&D Systems) for 2 days. In some experiments, individual or combinations of BMP4 (25 ng/ml), FGF2 (10 ng/ml), and VEGF (50 ng/ml) were added at this stage. Cells were lifted with Accutase (Innovative Life Technologies, San Diego, CA) at day 4 and seeded onto p100 culture dishes at 5 × 10^4–1 × 10^5 cells per cm2 in EC Growth Medium MV2 (ECGM-MV2, PromoCell) with an additional 50 ng/ml VEGF. The medium was changed every 2 days for 4-6 days to generate ECs. In some experiments, these ECs were passaged every 3-5 days in the last stage medium. Human umbilical vein endothelial cells (HUVECs) were cultured in the EC Growth Medium MV2 (PromoCell).

The following inhibitors were used in some culture conditions indicated in the text. U0126 (EMD4Biosciences, Darmstadt, Germany) and GCD-0994 (Selleckchem, Houston, TX) are inhibitors for the ERK MAPK pathway. SB202190 (EMD4Biosciences) and BIRB 796 (Selleckchem) are inhibitors for the p38 MAPK pathway. JNK Inhibitor II (EMD4Biosciences) and JNK-IN-8 are inhibitors for the JNK MAPK pathway.

**Quantitative RT-PCR (qRT-PCR)**

RNA isolation was performed according to the manufacturer’s instructions using QiAshredder and RNeasy Mini Kit (Qiagen, Germantown, MD). The RNA concentration was measured using NanoDrop 2000 spectrophotometer (Thermo Scientific, Carlsvad, CA) and 500 ng RNA from each sample was converted to cDNA using the High-Density cDNA Conversion Kit (Applied Biosystems) according to the manufacturer’s protocol. The cDNA (500 ng) from each sample was used for quantitative PCR in the presence of specific primers and SYBR Green PCR Master Mix (Applied Biosystems) to quantitate the gene expression of interest. Thermal cycling was conducted in the ABI-7300-Real Time-PCR System (Applied Biosystems). Primers for tested genes are listed in Supporting Information Table 1. Ct value was first normalized to the internal GAPDH level and presented as fold increase compared to the average of the control group.

**Flow Cytometry**

Cells were lifted with Actuase and filtered using a 70 µm nylone mesh (Fisher Scientific, San Jose, CA). Cells were then washed once with phosphate buffered saline (PBS) (HyClone, Logan, Utah) and resuspended in 1:20 dilution of MACS buffer (Miltenyi Biotec, San Diego, CA) for 30 minutes with testing antibodies at 1:100 dilution. Antibodies used were FITC-labeled mouse anti-human CD31, APC-labeled mouse anti-human CD34, and PE-labeled mouse anti-human CD144 (BD Biosciences). Cells were centrifuged, washed once in PBS and analyzed using a Beckman Coulter Cytomics FC 500 (Indianapolis, IN). CXP Analysis software was used to generate data.

**Immunofluorescence Staining**

Cultured cells and tissue sections were fixed using either 4% paraformaldehyde (Affymetrix, Cleveland, Ohio) for 20 minutes or acetone for 5-10 minutes. Immunostaining was performed as previously described [5]. CD31 (Bethyl, Montgomery, TX), α-smooth muscle actin (α-SMA, Sigma-Aldrich, St. Louis, MO) and vWF (Abcam, Cambridge, MA) were used at a 1:200, 1:400, and 1:400 dilutions, respectively. Images were taken with either Nikon Eclipse Ti or Keyence BZ-9000 microscope (BioRevo, Elmwood Park, NJ).

**Dil-ac-LDL Uptake Assay**

Dil fluorescent dye-labeled acetylated low density lipoprotein (Dil-ac-LDL) (Biomedical technologies, Tewksbury, MA) at 10 µg/ml in ECGM MV2 medium was added to hiPSCs, HUVECs, day 10 hiPSC-ECs, or hESC-ECs, and incubated for 4 hours at 37°C. Cells were washed three times with PBS. Images were taken with a Nikon Eclipse Ti.
Tube Formation In Vitro and Vasculogenesis In Vivo

For in vitro tube formation, hiPSC-ECs or hESC-ECs were cultured on a solid layer of growth factor reduced Matrigel (Corning) in a 96-well plate (1.5 × 10^6 cells per well) in EC Growth Medium MV2 (PromoCell). Images were taken at 12-24 hours after plating. To assess vasculogenesis in vivo, 1 × 10^6 hESC-ECs were mixed with a 1/30 diluted Matrigel and injected subcutaneously into the hind limbs of the immune deficient NOD/SCID/L2Rγ−/− (NSG) mice. Two weeks after cell transplantation, the matrigel plug was dissected out and frozen in Optimal Temperature Compound, and was sectioned at 30 μm thickness using a Cryotome FSE (Thermo Scientific). Tissue slides were fixed in 4% paraformaldehyde for 20 minutes and subjected to immunostaining using rabbit anti-human CD31 antibody (Bethyl) as described in the prior section.

Ischemic Mouse Model

NSG mice underwent femoral artery ligation followed by a cut at the femoral artery to induce ischemia in one of their hind limbs. One day after the surgery, either saline or 1 × 10^6 hiPSC-ECs were injected intramuscularly into the ischemic limb. Ten weeks after cell transplantation, these mice (six received saline and six received hiPSC-ECs) were injected with FITC-dextran at 1 mg in 100 ml PBS via tail vein to label all blood flow in the animals, and subsequently sacrificed 10 minutes after the injection. The ischemic leg muscles were removed and frozen in Optimal Temperature Compound (Tissue-Tek, Torrance, CA). Tissues were sectioned at 20 μm thickness using a Cryotome FSE. Tissue slides were fixed in acetone for 5-8 minutes. Human CD31 cells were immunostained using rabbit anti-human CD31 antibody (Bethyl) as described in the prior section.

Statistics

In all qRT-PCR analysis, there were 3-4 samples per group. Data is presented as average ± standard deviation. Analysis of variance (ANOVA) was performed to detect whether a significant difference existed between groups with different treatments, and Tukey’s multiple comparisons test was used for post-analysis. A p value of .05 or less will indicate significant difference between samples in comparison. Probability (p) less than .05 was considered statistically significant.

RESULTS

Inducing Vascular Progenitors from HiPSCs by Activation of the WNT Signaling Followed by the VEGF, BMP, and FGF Signaling Pathways

It has been previously established that activation of the WNT signaling pathway induces mesoderm differentiation from pluripotent stem cells [6, 7]. Since vascular lineage cells are derived from mesoderm during embryogenesis [8], we first induced mesoderm from hiPSCs in 2 days using the GSK3 inhibitor (GSK) CHIR99021 at 6 μM, which activated the WNT signaling pathway. Next, we investigated whether treatment of hiPSC-derived mesoderm with VEGF, BMP4, or FGF2 individually or in combinations for 2 days is beneficial in the induction of vascular progenitors (VPs) from mesoderm progenitors (Fig. 1A). Our qRT-PCR data showed that FGF2 alone significantly reduced the expression of the pluripotent marker OCT4; however, the combination of all three growth factors substantially reduced OCT4 expression (Fig. 1B). The expression of VEGFR2 (also named KDR or FLK-1), a marker for VPs, was markedly induced by either BMP4 or VEGF, and further enhanced by the combination of BMP4 and VEGF (Fig. 1B). Interestingly, FGF2 by itself did not increase the expression of VEGFR2 and even decreased VEGF-induced expression of VEGFR2 (Fig. 1B), but FGF2 did not reduce the expression of VEGFR2 in the presence of BMP4 or both BMP4 and VEGF (Fig. 1B). We found that VEGF alone only moderately induced the expression of endothelial lineage markers, PECAM1 and VE-cadherin. The combination of BMP4 and VEGF further increased the expression of PECAM1 and VE-cadherin (Fig. 1B). FGF2 alone failed to induce the expression of these endothelial lineage markers. However, the highest expression of PECAM1 and VE-cadherin was achieved with the combination of VEGF, BMP4, and FGF2, which is significantly higher than those with VEGF and BMP4 combined (Fig. 1B). None of these growth factors were capable of potently inducing the expression of smooth muscle cell marker α-smooth muscle actin (α-SMA) or hematopoietic cell marker CD45 in these differentiating cells (Fig. 1B), suggesting the specificity of these growth factors on endothelial lineage differentiation. Thus, our data demonstrated that VEGF alone marginally induced the differentiation of VPs from hiPSC-derived mesoderm, and the combination of VEGF, BMP and FGF2 substantially induced VP generation from hiPSCs.

To characterize the VPs at day 4 of the differentiation in the presence of all three growth factors, we noticed that the morphology of the majority of the cells derived at this stage exhibited cobblestone-like morphology, while some cells at the center of the original hiPSC colonies were smaller in size (Fig. 1C). The cobblestone-like peripheral cells could be easily separated from the smaller central cells by a shorter time exposure to Accutase. Flow cytometry analysis of these cells revealed that very small fractions (<5%) of cells expressed either CD31 (PECAM1) or CD144 (antibody recognize VE-cadherin) but not both (Fig. 1D). The percentage of CD31 and CD144 double positive cells in the entire cell population was 67.8% (Fig. 1D). The CD31+ CD144+ population decreased to 55.6% in the central cells but increased to 84% in the peripheral cells (Fig. 1D). Since the peripheral cells were enriched in vascular progenitor cells, they were used for further differentiation to ECs.

Differentiation of Vascular Progenitors from Mesoderm Requires the MAPK Kinase and PI3K Pathways

It has been established that VEGF and FGF2 activate the MAPK and the PI3K pathways [9, 10]. To understand how the downstream signaling pathways of VEGF and FGF2 induced the differentiation of VPs from hiPSC-induced mesoderm, we treated the hiPSC-derived mesoderm cells with inhibitors of these pathways for 2 days and assessed cell differentiation by qRT-PCR analysis of various vascular lineage markers. Inhibition of either ERK by U0126, p38 by SB202190 (SB), JNK by SP600125 (SP), or PI3K by LY294002 (LY) substantially reduced the expression of the endothelial lineage marker PECAM1 (Fig. 2). The greatest inhibition of PECAM1 expression was...
elicited by the ERK and the p38 inhibitor (Fig. 2). Similarly, the expression of the EST family transcription factor ERG, FLI1, and EST1 was also markedly reduced by the ERK or the p38 inhibitor and moderately reduced by the JNK or the PI3K inhibitor (Fig. 2). Transcription factors FOXC2 and ETV2 have been shown to play an important role in the formation of vasculature [11–13]. We found that the p38 inhibitor moderately reduced the expression of FOXC2, ETV2 as well as
To evaluate whether these inhibitors affect proliferation, the expression of a proliferation marker Ki67 in the cells treated with or without the inhibitors was analyzed by qRT-PCR. We found that only the ERK and the p38 inhibitors significantly reduced Ki67 expression (Fig. 2). However, the proliferation of the cells treated with the inhibitors was maintained at levels as high as those in hiPSCs. Similar results were observed using a different set of inhibitors for these pathways (Supporting Information Fig. 1). Thus, our data suggest that both the MAP kinase and PI3K pathway are critical for generating vascular progenitor cells from hiPSCs, likely through regulation of the EST family transcription factors.

Differentiation of ECs from HiPSCs and HESCs

To derive ECs from hiPSCs or hESCs, we first differentiated hiPSCs and hESCs to VPs using our optimized protocol described in the prior section and then grew these VPs in endothelial medium ECGM-MV2 supplemented with extra 50 ng/ml of VEGF for 4-6 days (Fig. 3A). At the end of the differentiation, the hiPSC or hESC-derived ECs (hiPSC-ECs or hESC-ECs) at the end of the differentiation exhibited cobblestone-like EC morphology (Fig. 3B). These cells expressed endothelial lineage markers, CD31/PECAM1 and a mature EC marker VWF as revealed by immunostaining (Fig. 3B). In fact, 94%-97% of these cells expressed CD31 as demonstrated by flow cytometry analysis (Fig. 3C). Among these CD31 cells, 73%-81% cells were CD34 and 78%-83% were CD144 (Fig. 3C). Passaging of these ECs once using the same EC medium led to the loss of CD34 expression but yielded a nearly pure population of ECs (99.7% of CD31 and 96.8% CD144) (Fig. 3D), the highest purity without cell sorting or magnet bead separation, to our knowledge.

Uptaking Dil-acetylated low density lipoprotein (Dil-Ac-LDL) is a functional assay for ECs. Similar to human umbilical vein ECs (HUVECs), the pluripotent cell-derived ECs were capable of uptaking Dil-Ac-LDL, while the hiPSCs were unable to uptake Dil-Ac-LDL (Fig. 3E). Furthermore, when seeded on matrigel, these ECs could assemble into tube-like structures (Fig. 3F), another functional assay of ECs. Therefore, the ECs derived from pluripotent stem cells exhibited not only EC specific gene expression but also EC functions.

To understand how ECs were progressively induced from hiPSCs, the expression of multiple pluripotent stem cell markers, endothelial markers, transcription factors implicated in vasculature formation as well as function, and smooth muscle cell markers were analyzed at different time points from
day 0 to passage 4 (p4) by qRT-PCR. We found that the pluripotent stem cell marker octamer-binding transcription factor 4 (OCT4) and NANOG were progressively reduced in the first 4 days of differentiation and further reduced in passage 3 (p3) and 4 (p4) to a level lower than that in HUVECs (Fig. 4A). VEGFR2 expression peaked at day 3 followed by a moderate decrease but still maintained at a much higher level than that in hiPSCs or HUVECs (Fig. 4B). A small increase of VEGFR1 was observed from day 3-8 (Fig. 4B), suggesting VEGFR1 is not as crucial for EC differentiation as VEGFR2. This is consistent with the animal studies indicating that VEGFR2/−/−/− murine embryos displayed defects in yolk sac blood islands, blood vessels, and endocardium and die around embryonic day 9.5, while VEGFR1 knockout mice develop abnormal vascular channels without affecting EC differentiation [14].

Transcription factor ETS variant 2 (ETV2) was progressively induced in the first 3 days, decreased moderately at day 4 and further decreased below the level of that in hiPSCs thereafter. The expression of FOXC2 was progressively induced in the first 2 days and maintained at the elevated level till day 8 followed by a drop at p3 and p4 (Fig. 4C). The ETS family transcription factors FLI1 and ERG were shown to be important for EC development [15–17]. Knockdown of ERG by shRNA during ESC differentiation resulted in marked reduction in the number of ECs but did not alter the VEGFR2 expressing cells [18]. We found that transcription factors ERG, FLI1, ETS1, and TAL1 were all induced at day 3 and maintained more or less at the elevated level thereafter (Fig. 4D).

The endothelial marker PECAM and VE-cadherin were markedly induced at day 3 and progressively increased thereafter to levels comparable to that in HUVECs (Fig. 4E). The expression of endothelial markers vWF and NOS3 was progressively induced and reached a plateau at day 4. These ECs had similar levels of NOS3 but much lower levels of vWF comparing to those in HUVECs, suggesting incomplete maturation of the cells. An artery endothelial marker JAG1 was strongly induced at day 3 and continually increased thereafter, while another artery endothelial marker HEY1 was markedly induced at day 3 and reached the highest expression on day 4 (Fig. 4G). Both JAG1 and HEY1 in the ECs were at much higher levels than those in HUVECs. The venous endothelial markers CoupTF2 and NRP2 increased markedly at day 3 and thereafter (Fig. 4H). The level of CoupTF2 in the ECs was lower than that in HUVECs, while NRP2 was expressed at a level comparable or higher than that in HUVECs (Fig. 4H). Therefore, these hiPSC-ECs more closely resemble arterial rather than venous ECs.

Since VPs can also give rise to smooth muscle cells, we also assessed by qRT-PCR the expression of smooth muscle cell markers, α-SMA and calponin, during the differentiation
of hiPSCs. The expression of the α-SMA gene was markedly induced at day 4-6 and then dramatically declined at p3 and p4 to a level lower than that in HUVECs (Fig. 4I). Calponin expression was decreased during the differentiation of hiPSC and reached the lowest level at p3 and p4, which is comparable to that in HUVECs (Fig. 4I). Our data suggest that the protocol we have established progressively induces EC fate but not smooth muscle cell fate.

The ERK Pathway Promotes EC Differentiation but Prevents Smooth Muscle Cell Differentiation

We have demonstrated that the MAPK and the PI3K pathways are required for generation of VPs from hiPSC-derived mesoderm in the prior section. We next investigated whether the MAP kinase and the PI3K pathway regulate the differentiation of ECs from progenitor cells that have already committed to vascular lineage by exposing the differentiating cells to inhibitors of these pathways from day 4 to 8. Inhibition of the PI3K, the ERK, the p38, or the JNK pathway significantly reduced the expression of endothelial markers PECAM1 and vWF, suggesting that the EC fate was repressed in the absence of the PI3K and the MAPK pathways (Fig. 5A, Supporting Information Fig. 2). We found that inhibition of the ERK and JNK MAPK pathways enhanced the expression of the α-SMA gene, but only inhibition of the ERK pathway strongly promoted the expression of another smooth cell marker calponin (Fig. 5B, Supporting Information Fig. 2). The increase of α-SMA expressing cells in the presence of the ERK pathway inhibitor U0126 was further confirmed by immunostaining (Fig. 5C). In contrast, inhibition of the PI3K marginally decreased the expression of calponin and α-SMA (Fig. 5B).

Our data suggest an important role of the MAPK and the PI3K pathways in determining EC fate even when cells have been committed to vascular lineages. In addition, the ERK pathway also blocks smooth muscle cell fate.

ECs Derived from Pluripotent Stem Cells Formed Functional Vessels In Vivo

To characterize the functionality of the ECs derived from pluripotent stem cells in vivo, we first assessed the vasculogenic capacity of the hESC-ECs in mice under physiologic environment. The hESC-derived ECs were injected with matrigel under the skin in the hind limb of immune deficient NSG mice. Immunostaining the matrigel plug for human specific CD31 revealed that these ECs formed multiple vessels with branches in vivo (Fig. 6A).

To further assess the functionality of the ECs derived from pluripotent stem cells under a pathological condition, hiPSC-ECs were tested in a murine critical limb ischemia model. Immunodeficient NSG mice underwent surgery to induce ischemic in hind limbs (n = 5) [19], and then these mice received an injection of 1 × 10^6 hiPSC-ECs 1 day after the surgery. The control mice were injected with saline. Ten weeks after cell transplantation, FITC-Dextran was injected into the mouse blood circulation via tail vein 5-10 minutes before being sacrificed in order to label blood flow. While no human CD31 positive cells were detected in the control mice without cell transplantation (Fig. 6B), the transplanted hiPSC-ECs were abundantly detected in all of the mice receiving hiPSC-ECs as revealed by immunostaining for human specific CD31 (Fig. 6C). The FITC-dextran signal revealed a relatively well
vascularized area on the left and ischemic area on the right of a mouse muscle section (Fig. 6C). Interestingly, hiPSC-ECs were present mostly in the ischemic area adjacent to the area with relatively normal blood flow (Fig. 6C). The colocalization as well as the immediately adjacent localization of FITC-Dextran and hCD31 positive cells indicates that the transplanted hiPSC-ECs formed vessels and connected to the host vasculature, with blood flowing through these hiPSC-EC generated vessels (Fig. 6C). Furthermore, although the ischemic area was still evident, the places with hiPSC-ECs in the tissue section had relatively higher FITC-dextran signals (Fig. 6C), suggesting that these hiPSC-ECs have the capacity to improve blood flow in an ischemic condition. Thus, our data demonstrate that the ECs derived from pluripotent stem cells can form functional blood vessels in vivo and have potential to treat ischemia.

**DISCUSSION**

Differentiation protocols intended to derive a specific cell type from hESCs or hiPSCs often yield mixed cell populations. Significant improvements in EC differentiation have been made in recent years by progressively directing hESCs or hiPSCs to mesoderm progenitors, VPs, and ECs without employing embryoid bodies [20–22]. However, the efficiency of the EC differentiation remains to be improved, and cell sorting or magnetic affinity separation for the early stage vascular progenitor cells was still required to obtain pure population of ECs. To our knowledge, ours is the first protocol that produces nearly pure ECs from pluripotent stem cells without cell sorting or magnetic affinity separation (Fig. 3). This approach not only allows the production of ECs in a timely and cost efficient manner but also provides a better platform to study mechanisms that regulate EC fate. Our time-course analysis of gene expression during EC differentiation from hiPSCs reveals how EC fate is progressively induced. We found that transcription factor ETV2 was induced very early, preceding the induction of ERG, FLI1, EST1, TAL1, and VEGFR2 during the differentiation of hiPSCs toward ECs (Fig. 4). Our data are consistent with Liu’s report that in zebrafish, ERG and FLI1 act cooperatively for later angiogenesis after hemangioblasts are induced by ETV2 [12]. ETV2 was shown to directly bind to the promoter of VEGFR2 and activate VEGFR2 expression [13]. VEGFR2 expression was also shown to be activated by ETS1, Tal-1, and GATA in mice [23], and by ERG and KLF2 in Xenopus [24]. We suggest that one of the main roles of ETV2 in EC differentiation from pluripotent stem cells is to upregulate VEGFR2, while ERG, ETS1, and Tal-1 maintain the low level of VEGFR2 expression at later stage. We show for the first time that transcription factor FOXC2 was induced as early as ETV2, preceding the rise of ERG, FLI1, EST1, and TAL1 during the differentiation of hiPSCs toward ECs (Fig. 4). Our study also suggests that upregulation of not only ETV2 but also FOXC2 initiated vascular progenitor fate, and that ERG, FLI1, ETS1, and TAL1, once induced, were
persistently expressed throughout the differentiation process to enforce the differentiating cells to adapt the EC fate.

We revealed the additive and synergistic effects of VEGF and BMP4 on VP generation (Fig. 1B). FGF2 alone failed to induce the expression of PECAM1 and VE-cadherin but could further enhance the expression of these genes in the presence of VEGF and BMP4 (Fig. 1B). The mechanisms for the synergistic effects of these growth factors on the induction of VPs remain to be determined.

We found that the ERK, p38, and JNK MAP kinase pathways are all required not only for differentiation of vascular progenitor cells from hiPSCs but also for the differentiation of...
ECs from VPs. Blocking either of these pathways using a small molecule inhibitor reduced the expression of the endothelial markers in both stages of the differentiation (Figs. (2 and 5), Supporting Information Figs. 1, 2). All three MAPK pathways regulated the expression of ERG and FLI (Fig. 2). It has been shown previously that the knockout of p38α with or without a simultaneous knockout of p38β in mouse ESCs has no effect on the differentiation to ECs or to smooth muscle cells. [25, 26]. Our data clearly showed that the p38 pathway is critical for EC differentiation from human iPSCs. This apparent inconsistency may be explained either by differences between human and mouse cells or by insufficient knockdown of all p38 isoforms in the mouse cells in those studies. To our knowledge, we are the first to show that both the p38 and JNK signaling pathways are critical for EC differentiation from pluripotent stem cells.

The ERK pathway has been shown to induce the differentiation of multi-potent adult progenitor cells to ECs [27], and also the differentiation of hiPSCs to CD34⁺CD31⁺ vascular progenitor cells [21]. However, the targets of the ERK pathway for EC differentiation have not been reported previously. We have now identified that ERG and FLI1 are the targets of all three MAPK pathways, while ETS1 is targeted by the ERK and p38 pathways. Since ETS1⁻/⁻ mice had normal vasculature [28, 29], the role of ETS1 in EC differentiation is dispensable. Our data suggest that ERG and FLI1 most likely mediate the effect of all three MAPK on EC differentiation.

High doses of PI3K inhibitors have been shown to cause defects in angioblast cell fate in zebrafish, while lower doses of the inhibitors were shown to promote arteriolar specification [30]. We showed here that the PI3K pathway plays a critical role in EC differentiation from hiPSCs because inhibition of this pathway significantly reduced the expression of several EC markers (Figs. (2 and 5), Supporting Information Fig. 2). Myofibroblast differentiation of periodontal ligament-derived endothelial progenitor cells was previously shown to be attenuated by EGF-mediated ERK activation [31]. JNK alone did not affect this process directly, but it enhanced the effect of ERK on it. [31]. We found that the inhibition of JNK moderately upregulated smooth muscle marker α-SMA, but the inhibition of the ERK pathway robustly upregulated both α-SMA and calponin (Fig. 5, Supporting Information Fig. 2). We noticed that the MEK1/2 inhibitor U0126 is a much more potent inducer of smooth muscle cells compared to the ERK1/2 inhibitor GDC-0994 (Fig. 5, Supporting Information Fig. 2). The mechanism that is responsible for the cell fate switch to smooth muscle cells remains to be understood. Apparently, blocking EC fate alone is not sufficient to induce a smooth muscle cell fate, because the inhibition of PI3K blocks the EC fate but does not promote the smooth muscle cell fate (Fig. 5).

We demonstrated that our protocol of EC differentiation from pluripotent stem cells produced functional ECs capable of vasculogenesis. We showed here that these ECs could uptake Dil-Ac-LDL and form tubes both in vitro and in vivo (Figs. (3 and 6)). When transplanted into ischemic mouse limbs, these ECs formed vessels that carried blood and were connected to the host vasculature in vivo (Fig. 6). Although ischemia was not completely reversed, these ECs locally improved the blood flow (Fig. 6). We think that a better approach to deliver ECs to the entire ischemic area may provide more benefits. Alternatively, progressive application of ECs at proper intervals may eventually repair ischemia.

In the present study, we not only developed one of the most efficient protocols to derive ECs from pluripotent stem cells, a protocol that did not require cell sorting or magnetic purification to yield a very pure population, but we have also delineated a detailed mechanistic description of the differentiation process. We revealed novel roles of the p38 and JNK pathways in EC differentiation. We also identified that ERG and FLI1 are the critical targets of all three MAPK and the PI3K pathways that are responsible for induction of EC fate. Furthermore, we demonstrated that pluripotent stem cell-derived ECs are capable of vasculogenesis under ischemic environment. Our study thus shed light on the future development of cell therapy to treat ischemia.

**CONCLUSION**

In conclusion, homogeneous ECs can be derived from hESCs and hiPSCs without cell sorting, and they can engraft and generate functional vasculature in normal and ischemic environments in vivo. The MAPK and PI3K pathways are crucial for vascular progenitor formation and EC fate determination, likely through regulation of ERG and FLI1. Pluripotent stem cell-derived ECs have great potential to treat vascular disease.

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**AUTHOR CONTRIBUTIONS**

A. H.: collection and/or assembly of data, data analysis and interpretation, manuscript writing; N. L. M.: collection and/or assembly of data; J. R. B.: collection and/or assembly of data; D. C.: collection and/or assembly of data; E.C.: collection and/or assembly of data; D. H.: collection and/or analysis of data; A. W.: data interpretation, financial support; J. A. N.: conception, data interpretation, financial support, manuscript writing; P. Z.: conception and design, data analysis and interpretation, financial support, manuscript writing, final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicate no potential conflicts of interest.
REFERENCES

1 Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. Nat Med 2000;6:389–395.
2 Weiss N, Miller F, Cazaubon S et al. The blood-brain barrier in brain homeostasis and neurological diseases. Biochim Biophys Acta 2009;1788:842–857.
3 Wilson HK, Canfield SG, Shusta EV et al. Concise review: Tissue-specific microvascular endothelial cells derived from human pluripotent stem cells. Stem Cells 2014;32:3037–3045.
4 Yoder MC. Differentiation of pluripotent stem cells into endothelial cells. Curr Opin Hematol 2015;22:252–257.
5 Zhou P, Hohn S, Olusanya Y et al. Human progenitor cells with high aldehyde dehydrogenase activity efficiently engraft into damaged liver in a novel model. Hepatology 2009;49:1992–2000.
6 Gadea P, Huber TL, Padisson PJ et al. Wnt and TGF-beta signaling are required for the induction of an in vitro model of primitive streak formation using embryonic stem cells. Proc Natl Acad Sci USA 2006;103:16806–16811.
7 Lindsley RC, Gill JG, Kyba M et al. Canonical Wnt signaling is required for development of embryonic stem cell-derived mesoderm. Development 2006;133:3787–3796.
8 Fehling HJ, Lacaud G, Kubo A et al. Tracking mesoderm induction and its specification to the hemangioblast during embryonic stem cell differentiation. Development 2003;130:4217–4227.
9 Zachary I, Gilki G. Signaling transduction mechanisms mediating biological actions of the vascular endothelial growth factor family. Cardiovasc Res 2001;49:568–581.
10 Ornitz DM, Itoh N. The Fibroblast Growth Factor signaling pathway. Wiley Interdiscip Rev Dev Biol 2015;4:215–266.
11 Seo S, Fujita H, Nakano A et al. The forkhead transcription factors, Foxc1 and Foxc2, are required for arterial specification and lymphatic sprouting during vascular development. Dev Biol 2006;294:458–470.
12 Liu F, Patient R. Genome-wide analysis of the zebrafish ETS family identifies three genes required for hematoblast differentiation or angiogenesis. Circ Res 2008;103:1147–1154.
13 Lee D, Park C, Lee H et al. ER71 acts downstream of BMP, Notch, and Wnt signaling in blood and vessel progenitor specification. Cell Stem Cell 2008;2:497–507.
14 Shahaly F, Rossant J, Yamaguchi TP et al. Failure of blood-island formation and vascular development in Fli-1-deficient mice. Nature 1995;376:62–66.
15 Hart A, Melet F, Grossfeld P et al. Fli-1 is required for murine vascular and mesangio- ytic development and is hemizygosely deleted in patients with thrombocytopenia. Immunity 2000;13:167–177.
16 Spyropoulos DD, Pharr PN, Lavenburg KR et al. Hemorrhage, impaired hematopoie- sis, and lethality in mouse embryos carrying a targeted disruption of the Fli1 transcription factor. Mol Cell Biol 2000;20:5643–5652.
17 Vlaeminck-Guille V, Carrere S, Dewitte F et al. The Ets family member Erg gene is expressed in mesodermal tissues and neural crests at fundamental steps during mouse embryogenesis. Mech Dev 2000;91:331–335.
18 Nikolova-Krstevski V, Yuan L, Le Bras A et al. ERG is required for the differentiation of embryonic stem cells along the endothelial lineage. BMC Dev Biol 2009;9:72.
19 Rosova I, Dao M, Capoccia B et al. Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells. Stem Cells 2008;26:2173–2182.
20 Tan JY, Siram G, Rufaihah AJ et al. Efficient derivation of lateral plate and paraxial mesoderm subtypes from human embryonic stem cells through GSKi-mediated differentiation. Stem Cells Dev 2013;22:1893–1906.
21 Lian X, Bao X, Al-Ahmad A et al. Efficient differentiation of human pluripotent stem cells to endothelial progenitors via small-molecule activation of WNT signaling. Stem Cell Rep 2014;3:804–816.
22 Patsch C, Challet-Meylan L, Thoma EC et al. Generation of vascular endothelial and smooth muscle cells from human pluripotent stem cells. Nat Cell Biol 2015;17:994–1003.
23 Kappel A, Schlaeger TM, Flamme I et al. Role of SCF/Tal-1, GATA, and Ets transcription factor binding sites for the regulation of flk-1 expression during murine vascular development. Blood 2000;96:3078–3085.
24 Meadows SM, Salanga MC, Krieg PA. Kruppel-like factor 2 cooperates with the ETS family protein ERG to activate Flik1 expression during vascular development. Development 2009;136:1115–1125.
25 Guo YL, Ye J, Huang F. p38alpha MAP kinase-deficient mouse embryonic stem cells can differentiate to endothelial cells, smooth muscle cells, and neurons. Dev Dyn 2007;236:3383–3392.
26 Chakraborty S, Kang B, Huang F et al. Mouse embryonic stem cells lacking p38alpha and p38delta can differentiate to endothelial cells, smooth muscle cells, and epithelial cells. Differentiation 2009;78:143–150.
27 Xu J, Liu X, Jiang Y et al. MAPK/ERK signaling mediates VEGF-induced bone marrow stem cell differentiation into endothelial cell. J Cell Mol Med 2008;12:2395–2406.
28 Bories JC, Willerford DM, Grevin D et al. Increased T-cell apoptosis and terminal B-cell differentiation induced by inactivation of the Ets-1 proto-oncogene. Nature 1995;377:635–638.
29 Muthusamy N, Barton K, Leiden M. Defective activation and survival of T cells lacking the Ets-1 transcription factor. Nature 1995;377:639–642.
30 Hong CC, Peterson PQ, Hong JY et al. Artery/vein specification is governed by opposing phosphatidylinositol-3 kinase and MAP kinase/ERK signaling. Curr Biol 2006;16:1366–1372.
31 Kimura H, Okubo N, Chosa N et al. EGF positively regulates the proliferation and migration, and negatively regulates the myo- fibroblast differentiation of periodontal ligament-derived endothelial progenitor cells through MEK/ERK and JNK-dependent sig- nals. Cell Physiol Biochem 2013;32:899–914.