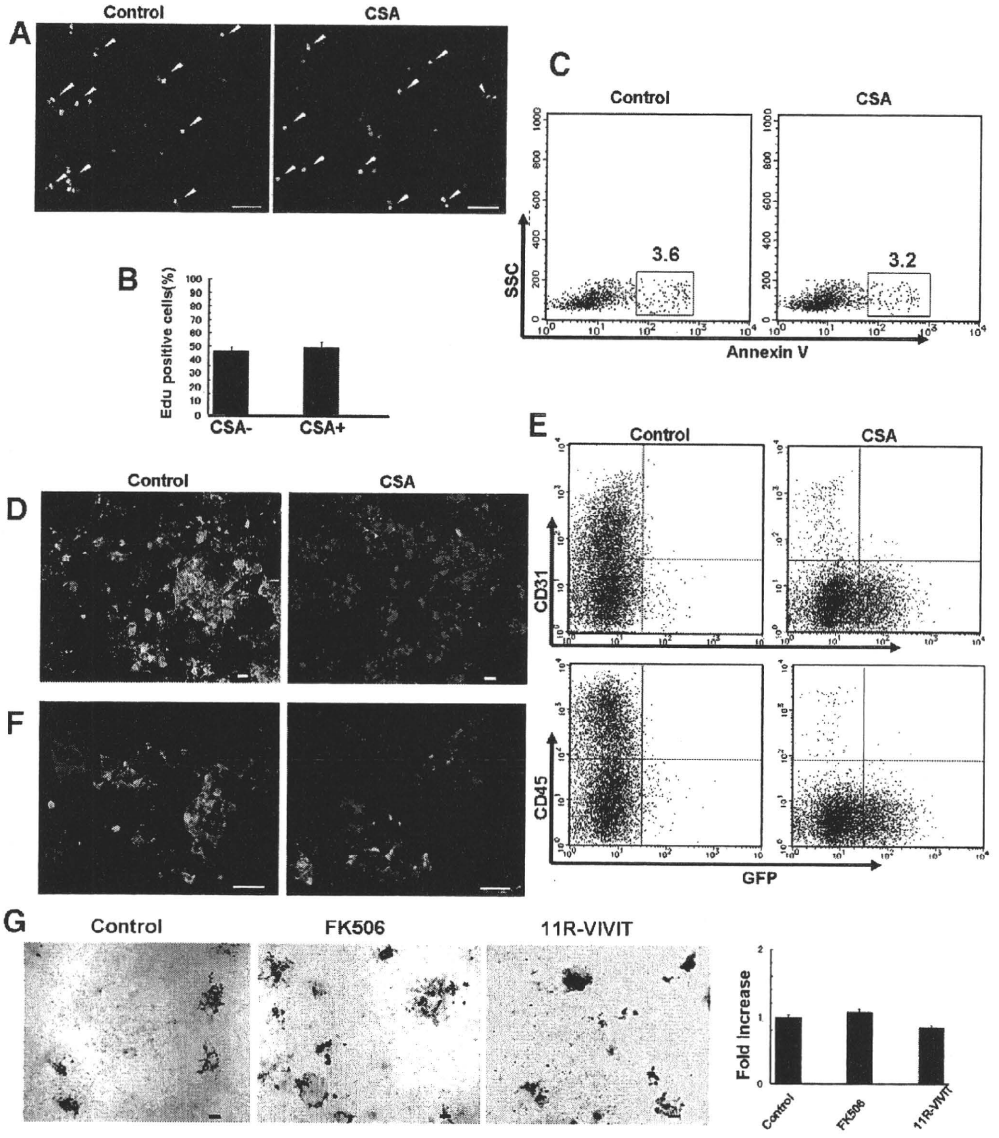

Fig. 3. Cellular and molecular mechanisms of CSA. (A,B) EdU incorporation. EdU-treated FCV cells were plated onto glass slides by cytocentrifugation. (A) Double staining for EdU (green) and DAPI (blue). Left panel: control. Right panel: CSA treatment. Double positive nuclei are indicated by arrowheads. Scale bar = 100 μm. (B) Quantitative evaluation of EdU positive FCV cells. Percentages of EdU positive cells in total cells are indicated (200 nuclei each, $n = 3$). (C) FACS analysis for annexin V expression. Gated FCV cell populations are shown. Percentages of apoptotic cells (annexin V^+, red box) are indicated. (D) Reciprocal appearance of ECs and cardiomyocytes by CSA treatment (experiment 1). Double immunostaining for CD31 (pan-ECs; green) and cTnT (red). Left panel: control. Right panel: CSA treatment. Scale bars = 400 μm. (E) Reciprocal appearance of ECs (upper panels) or blood cells (lower panels) with cardiomyocytes by CSA treatment. FACS analysis at Flk-d6 (experiment 1). Left panel: control. Right panel: CSA treatment. *X* axis: GFP (cardiomyocytes). *Y* axis: CD31 (pan-ECs), CD45 (pan-white blood cells). All $Flk1^+$ cell-derived population was analyzed. (F) Mesoderm-specific treatment of CSA (Flk-d0-2: experiment 5). Double immunostaining for CD31 (pan-ECs; green) and cTnT (red). Scale bars = 400 μm. (G) Effects of FK506 and NFAT inhibitor (11R-VIVIT) on cardiomyocyte differentiation (experiment 1). Left panels: gross appearance of cardiomyocytes stained with cTnT (brown), treated with vehicle (control), FK506, or 11R-VIVIT. Scale bars = 100 μm. Right panel: quantitative evaluation of cardiomyocyte induction by fluorescent intensity of cTnT staining. Relative fluorescent intensity is indicated ($n = 3$).

appearance, are, to our knowledge, the first identified distinct cardiac progenitor population [8] and so far the nearest upstream cardiac progenitors to cardiomyocytes. Recently, several kinds of multipotent cardiac progenitor populations were reported [19–21]. Cardiac progenitors reported by Kattman et al. were identified at an earlier stage of differentiation (i.e. at 4.25 days after the differentiation) than FCV cells [19]. Other $Nkx2.5^+$ or $islet1^+$ cardiac progenitors were reported at 4–6 days of ES cell differentiation [20,21]. In our FCV population, approximately 42% of cells were $Islet1^+$ (42 ± 18%, 1000 cells each, $n = 3$), 24% were $Nkx2.5^+$ (24 ± 5%), and 14% were double positive for Islet1 and Nkx2.5 (14 ± 1%). FCV cells, thus, should be an overlapped population with $Nkx2.5^+$ and/or $Islet1^+$ cardiac progenitors.

Recently, novel ES cell-like pluripotent stem cells, iPS cells, were generated from adult somatic cells by transduction of defined transcription factors [1–4]. We have just succeeded in establishing an iPS cell differentiation system for various cardiovascular cells similar to the ES cell system [22]. This CSA-mediated cardiac cell induction method would be extended and applied to iPS cells, and that would broadly contribute to exploring cardiac regeneration strategies.

Competing interests statement

The authors declare that they have no competing financial interests.

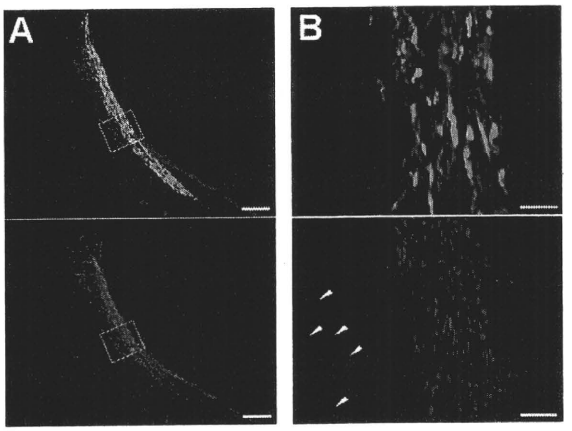

Fig. 4. Cardiagenic potential of expanded FCV cells in vivo. (A,B) Representative data of FCV cell transplantation (4×10^5 cells) to rat myocardial infarction model. Double immunostaining for GFP and cTnT. Upper panels: GFP (donor cell-derived cardiomyocytes, green) and DAPI (blue). Lower panels: cTnT (pan-cardiomyocytes, red) and DAPI. (A) Gross appearance of transplanted cell contribution to infarct area. Scale bars = 400 μm. (B) Higher magnification views of boxed area. Arrowheads show $cTnT^+/GFP^-$ endogenous cardiomyocytes. Scale bars = 50 μm.

Acknowledgments

We thank to Novartis Pharma for cyclosporin, Astellas Pharma for FK506, Dr. M. Takahashi for critical reading of the manuscript. This study was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan, the Ministry of Health, Labour and Welfare, the New Energy and Industrial Development Organization (NEDO) of Japan, the Project for Realization of Regenerative Medicine.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.12.019.

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Circulation 2008;118;498-506; originally published online Jul 14, 2008;
DOI: 10.1161/CIRCULATIONAHA.108.769562
Circulation is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 0009-7322. Online ISSN: 1524-4539

Directed and Systematic Differentiation of Cardiovascular Cells From Mouse Induced Pluripotent Stem Cells

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Background—Induced pluripotent stem (iPS) cells are a novel stem cell population induced from mouse and human adult somatic cells through reprogramming by transduction of defined transcription factors. However, detailed differentiation properties and the directional differentiation system of iPS cells have not been demonstrated.

Methods and Results—Previously, we established a novel mouse embryonic stem (ES) cell differentiation system that can reproduce the early differentiation processes of cardiovascular cells. We applied our ES cell system to iPS cells and examined directional differentiation of mouse iPS cells to cardiovascular cells. Flk1 (also designated as vascular endothelial growth factor receptor-2)-expressing mesoderm cells were induced from iPS cells after ≈4-day culture for differentiation. Purified Flk1$^+$ cells gave rise to endothelial cells and mural cells by addition of vascular endothelial growth factor and serum. Arterial, venous, and lymphatic endothelial cells were also successfully induced. Self-beating cardiomyocytes could be induced from Flk1$^+$ cells by culture on OP9 stroma cells. Time course and efficiency of the differentiation were comparable to those of mouse ES cells. Occasionally, reexpression of transgene mRNAs, including c-myc, was observed in long-term differentiation cultures.

Conclusions—Various cardiovascular cells can be systematically induced from iPS cells. The differentiation properties of iPS cells are almost completely identical to those of ES cells. This system would greatly contribute to a novel understanding of iPS cell biology and the development of novel cardiovascular regenerative medicine. (***Circulation.* 2008;118:498-506.**)

Key Words: differentiation ■ endothelium ■ myocardium ■ stem cells

Embryonic stem (ES) cells have been considered potent candidates for regenerative medicine with their prominent properties of pluripotency and capacity for self-renewal. Novel ES cell–like pluripotent cells, termed *induced pluripotent stem* (iPS) *cells*, were generated from mouse skin fibroblasts by introducing 4 transcription factors (Oct3/4, Sox2, Klf4, c-myc),[1] and recently they were also successfully generated from human skin fibroblaststs.[2,3] These iPS cells opened a new gate for cell transplantation–based regenerative medicine by overcoming the ethical controversy over ES cells. Differentiation and selection methods for target cells were thus required for regenerative medicine with the use of iPS cells. However, the differentiation properties of iPS cells such as time course, potentials, and efficiency of the differentiation in vitro are still unclear, and a directed differentiation method for iPS cells has not been demonstrated.

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Clinical Perspective p 506

Previously, we established a novel ES cell differentiation system that can reproduce the early process of cardiovascular development in vitro.[4–6] Flk1 (also designated as vascular endothelial growth factor [VEGF] receptor-2) is the earliest differentiation marker for endothelial cells (ECs) and blood cells and a marker for lateral plate mesoderm.[7,8] We induced Flk1$^+$ cells from ES cells, purified them by fluorescence-activated cell sorting (FACS), and recultured the purified cells.[9] We succeeded in inducing cardiovascular cells such as vascular ECs, mural cells (pericytes and vascular smooth muscle cells),[4,10] and cardiomyocytes[5] from common progenitors, Flk1$^+$ cells. We also identified a novel cardiac progenitor population during ES cell differentiation.[5] More recently,

Received January 28, 2008; accepted May 6, 2008.

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The online-only Data Supplement, which contains Figures I through V, Movies I and II, and a supplemental Methods section, is available with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.108.769562/DC1.

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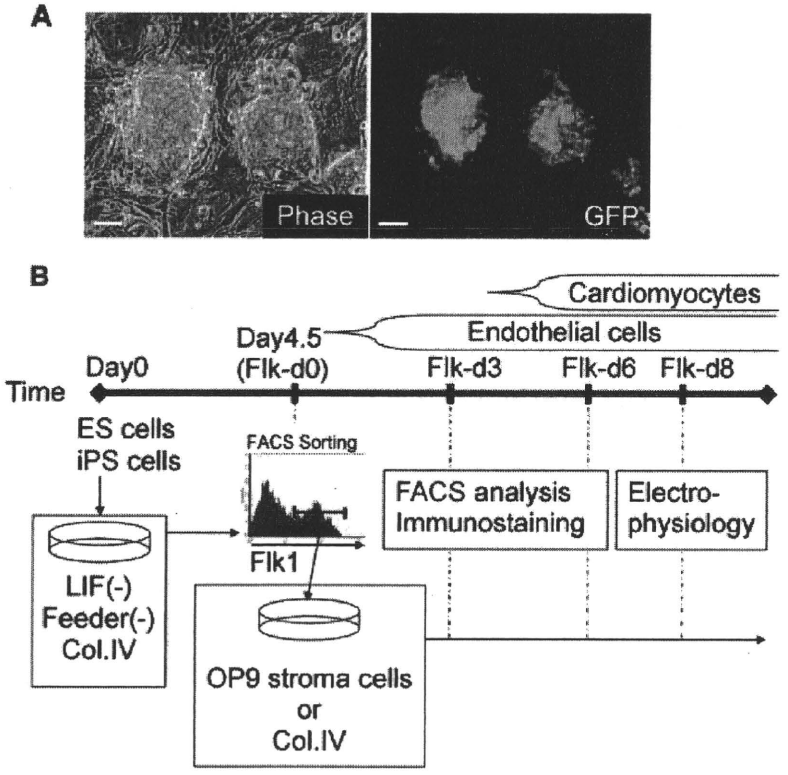

Figure 1. Experimental design of this study. A, Appearance of undifferentiated mouse Nanog-iPS cells. Left, Phase contrast. Right, Nanog promoter-driven GFP. Bars=100 μm. B, Procedure and time course of cardiomyocyte differentiation. Undifferentiated ES or iPS cells were cultured for 4.5 days on collagen (Col.) IV–coated dishes in differentiation medium (see Methods) to induce differentiation to Flk1$^+$ cells. FACS-purified Flk1$^+$ cells were plated onto collagen IV dish or OP9 stroma cells to induce further differentiation to ECs and cardiomyocytes (designated as Flk1-d0). ECs and cardiomyocytes appeared from Flk-d1 and Flk-d4-5, respectively. Differentiation of ECs and cardiomyocytes was evaluated by immunostaining and/or FACS analysis at Flk-d3 and Flk-d6. Electrophysiological study was done at Flk-d8 with the use of TMRM-purified cardiomyocytes (see Methods).

we succeeded in inducing arterial, venous, and lymphatic ECs from Flk1$^+$ cells.[11,12] This system is therefore useful to systematically induce various cardiovascular cells from common progenitors and dissect their differentiation processes.[6,10,13]

We applied this system to iPS cells and examined cardiovascular cell differentiation of iPS cells. In the present report, we show that all of the cardiovascular cells can be systematically induced from iPS cells and that the differentiation properties of iPS cells are largely comparable to those of ES cells.

Methods

Antibodies

Monoclonal antibodies for murine Flk1 (AVAS12) and murine vascular endothelial (VE)–cadherin (VECD1, for FACS) were described previously.[8,14] Monoclonal antibodies for murine CD31 (1:500), VE-cadherin (for immunostaining, 1:200), and CXCR4 were purchased from Pharmingen (San Diego, Calif). Monoclonal antibodies for murine α-smooth muscle actin (SMA) (1:1000) and α-actinin (sarcomeric) (1:800) were from Sigma (St Louis, Mo). The antibody for cardiac troponin-T (cTnT) (1:200) was from NeoMarkers (Fremont, Calif). Polyclonal antibodies for murine LYVE1 (1:500) and prox1 (1:50) were from Angiobio (Del Mar, Calif) and Reliatech (Braunschweig, Germany), respectively. Antibodies for SM22α (1:400) and calponin (1:500) were from Abcam (Cambridge, UK). Anti-HCN4 (1:200) and anti-Cav3.2 (1:200) antibodies were from Chemicon (Temecula, Calif). Anti-Kir2.1 (1:200) and anti-connexin 43 (1:200) antibodies were from Alomone (Israel) and Invitrogen (Carlsbad, Calif), respectively.

Cell Culture

Murine ES cell lines EB5, EMG7, and D3 and OP9 stroma cells were maintained as described.[5,11,15] EMG7 ES cells were generated by introduction of α-myosin heavy chain (MHC) promoter-driven EGFP gene to EB5 ES cells.[5] Cardiomyocytes induced from EMG7 cells could be detected by GFP expression (MHC-GFP). Germline-competent mouse iPS cell lines 20D17, 38C2, and 38D2, carrying Nanog promoter-driven GFP/IRES/puromycin-resistant gene (Nanog-iPS cells), were established and maintained as described previously.[16] Briefly, iPS cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 15% FCS, nonessential amino acids, 1 mmol/L sodium pyruvate, 5.5 mmol/L 2-mercaptoethanol, 50 U/mL penicillin, and 50 mg/mL streptomycin on feeder layers of mitomycin-C–treated mouse embryonic fibroblast cells carrying stably incorporated puromycin-resistance gene. All experiments were performed with the use of 3 Nanog-iPS cell lines. 20D17 was used as the iPS cell representative in all experiments unless stated otherwise.

Induction of cell differentiation was performed as described with the use of differentiation medium[4] (DM) (α-minimum essential medium (Invitrogen) supplemented with 10% fetal calf serum and 5×10^{-5} mol/L 2-mercaptoethanol). Flk1$^+$ mesoderm cells were induced by 96- to 108-hour culture of ES or iPS cells (plated at 1.7×10^3 cells/cm^2) in DM in the absence of leukemia inhibitory factor (LIF) on type IV collagen–coated dishes (BD Biosciences, San Jose, Calif). To induce vascular ECs, FACS-purified Flk1$^+$ cells (1×10^4 cells/cm^2) were cultured on type IV collagen–coated dishes with DM supplemented with 100 ng/mL human $VEGF_{165}$ (R&D Systems, Minneapolis, Minn) in the presence or absence of 8-bromoadenosine-3′:5′-cyclic monophosphate sodium salt (8bromo-cAMP) (0.5 mmol/L) (Nacalai Tesque, Kyoto, Japan).[11] Lymphatic endothelial cell was induced by coculture of Flk1$^+$ cells (5×10^3 cells/cm^2) on confluent OP9 stroma cells[12] with DM. After 3-day culture of Flk1$^+$ cells, induced ECs were subjected to FACS or immunostaining. Cardiomyocytes were induced by coculture of Flk1$^+$ cells (1.5×10^4 cells/cm^2) on OP9 cells for 4 to 6 days[5] with DM.

Three-Dimensional Culture

Three-dimensional culture was performed as described previously.[4] Briefly, Flk1$^+$ cells (4×10^5 cells/mL) were incubated in DM containing 100 ng/mL VEGF on uncoated petri dishes for 12 hours to induce aggregation. Aggregates were resuspended in 2× DM and mixed with an isovolume of collagen I-A gel (3 mg/mL) (Nitta Gelatin, Osaka, Japan). We plated 250 μL of this mixture onto a

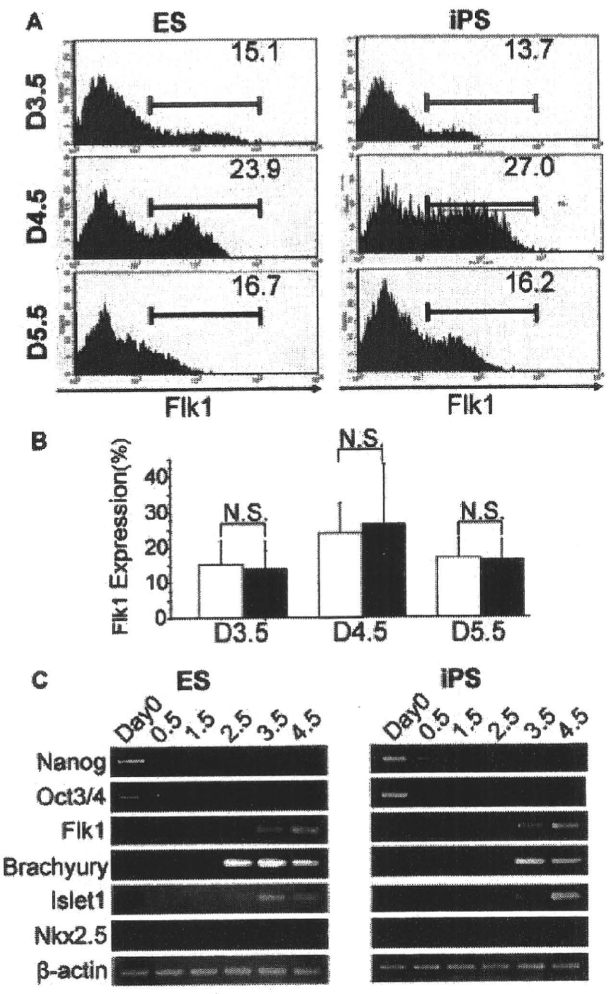

Figure 2. Differentiation of ES and iPS cells to mesoderm. A, Representative results of expression profiles of Flk1 on differentiation day 3.5 to 5.5 by FACS analysis. ES cells (EB5; left) and iPS cells (20D17; right) are shown. Percentages of $Flk1^+$ cells in total cells are indicated. B, Quantitative evaluation of $Flk1^+$ cell differentiation from ES cells (open column) and iPS cells (closed column) (n=4). N.S. indicates not significant. $P=0.79$ (D3.5), $P=0.96$ (D4.5), $P=0.90$ (D5.5), ES cells (EB5) vs iPS cells (20D17). C, Reverse transcription polymerase chain reaction analysis for gene expression of early differentiation markers during mesoderm differentiation (differentiation day 0 to 4.5). ES cells (EB5; left) and iPS cells (20D17; right) are shown.

lucent insert disk, Cell Disk (Sumitomo Bakelite, Tokyo, Japan), in 24-well dishes. After 30 minutes at 37°C to allow polymerization, we added 500 μL DM with VEGF (final, 100 ng/mL). To monitor vascular formation, collagen-embedded $Flk1^+$ cell aggregates were cultured in a temperature- and gas-controlled chamber (37°C, 5% CO_2), and phase-contrast images were acquired every 15 minutes with Metamorph software (Molecular Devices, Tokyo, Japan) for up to 4 days.

Flow Cytometry and Cell Sorting

FACS analysis was performed as described previously.[4,5,11] After induction of $Flk1^+$ cells, cultured cells were harvested and stained with allophycocyanin-conjugated AVAS12. Living $Flk1^+$ cells excluding propidium iodide (Sigma) were sorted by FACS Vantage (Becton Dickinson). After 3 days of $Flk1^+$ cell differentiation (Flk-d3), cultured cells were harvested and stained with a combination of monoclonal antibodies for CD31 and CXCR4 or VE-cadherin, then subjected to FACS analysis. Induced cardiomyocytes were purified with the use of tetramethyl rhodamine methyl ester (TMRM) (Invitrogen),[17,18] a fluorescent probe to monitor the membrane potential of mitochondria. In brief, cells were dissociated with 0.25% trypsin/EDTA, then incubated in DM with 50 nmol/L TMRM at 37°C for 15 minutes. Stained cells were washed twice and subjected to FACS sorting. A TMRM-high population was considered as purified cardiomyocytes in iPS cells (Figures I and II in the online-only Data Supplement).

Immunostaining

Immunostaining for ECs and cardiomyocytes was performed as described previously.[4,5,11] Briefly, 4% paraformaldehyde-fixed cells were blocked by 2% skim milk (BD Biosciences) and incubated overnight with primary antibodies at 4°C. For immunohistochemistry, anti-mouse IgG conjugated with alkaline phosphatase and anti-rabbit IgG horseradish peroxidase (Invitrogen) were used as secondary antibodies. For immunofluorescent staining, anti-mouse, -rat, -rabbit, or -goat IgG antibodies conjugated with Alexa488 or Alexa546 (Invitrogen) were used for secondary antibodies. Nuclei were visualized with DAPI (Invitrogen). EphrinB2 expression was examined by the binding of EphB4-Fc chimeric protein (R&D).[11] Stained cells were photographed with inverted fluorescence microscopy with Eclipse TE2000-U (Nikon, Tokyo, Japan) and the digital camera system AxioCam HRc with the use of AxioVision Software (Carl Zeiss, Jena, Germany) or confocal microscopy (LSM510; Carl Zeiss). To quantify the numbers of induced EC or cardiomyocyte colonies, positively staining colonies were counted in 8 randomly selected fields.

Immunostaining for 3-Dimensional Cultured Vascular Structures

Immunostaining for vascular structures in type I collagen gel was performed after the whole-mount immunostaining procedure. Briefly, gels were fixed with 4% paraformaldehyde and blocked by 1% skim milk/0.1% Triton X/PBS solution and incubated with anti-CD31 and SMA antibodies. Alexa488-conjugated anti-rat IgG and Alexa546-conjugated anti-mouse IgG were used as secondary antibodies. Stained cells were photographed with a confocal microscope (LSM510; Carl Zeiss).

Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated from various kinds of cell populations with the use of RNeasy Mini or Micro Kit (QIAGEN, Valencia, Calif). cDNA was synthesized by the SuperScript III First-strand Synthesis System (Invitrogen). Polymerase chain reaction was performed with the use of Taq polymerase or KOD Plus (Toyobo, Tokyo, Japan). Primers that were used are indicated in Table I in the online-only Data Supplement.

Electrophysiological Studies

The FACS-purified TMRM-high population was seeded on gelatin-coated coverslips. The myocytes were cultured for 2 days under this condition before use. The coverslips were then transferred to a patch clamp recording chamber, and electrophysiological measurements were performed with the use of Axopatch200B (Axon Instruments/Molecular Devices Corp, Union City, Calif).

Composition of Solutions

Physiological bathing solution contained the following (in mmol/L): 140 NaCl, 5.4 KCl, 0.33 NaH_2PO_4, 0.5 $MgCl_2$, 1.8 $CaCl_2$, and 5 HEPES (pH=7.4 with NaOH). Standard high-K^+ pipette solution contained the following (in mmol/L): 110 L-aspartic acid, 30 KCl, 5 MgATP, 0.1 NaGTP, 5 K_2 creatine phosphate, 2 EGTA, 10 HEPES, and 10 NaOH (pH=7.2 with KOH). All experiments were performed at 37°C.

Statistical Analysis

All data were obtained from at least 3 independent experiments. Statistical analysis of the data was performed with Student *t* test or ANOVA. $P<0.05$ was considered significant. All data are shown as mean±SD.

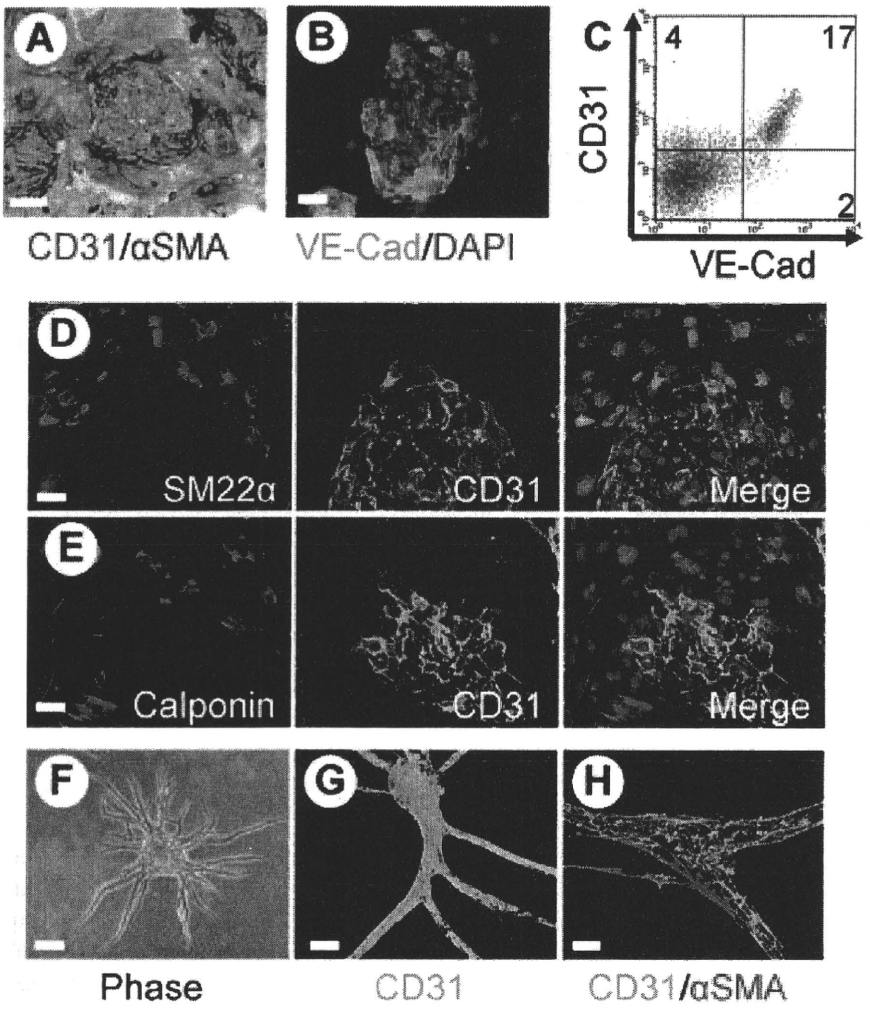

Figure 3. Differentiation of iPS cells to vascular cells. A to E, Two-dimensional culture after 3-day culture of iPS cell–derived Flk1$^+$ cells on collagen IV–coated dishes. A, Double immunostaining for CD31 (pan-ECs; purple) and SMA (mural cells; brown). B, Immunostaining for VE-cadherin (VE-Cad) (green) and DAPI (nuclei, blue). C, FACS analysis for CD31 and VE-cadherin. Percentages of quadrant populations are indicated. D, E, Double immunostaining for mural cell markers SM22α (D) and calponin (E) (left panels, red) and CD31 (middle panels, green). Right panels, Merged images with DAPI (blue). F to H, Three-dimensional culture. Flk1$^+$ cell aggregates were 3-dimensionally cultured in type I collagen gel for 3 days. F, Gross appearance of vascular-like structure. G, In-gel immunostaining for CD31 (green). H, Double in-gel staining for CD31 (green) and SMA (red). Attachments of mural cells (red) were observed. Bar=100 μm (A, F), 50 μm (B, D, E, G), 25 μm (H).

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

iPS Cell Differentiation to Mesoderm Cells

Undifferentiated mouse iPS cell colonies maintained on a feeder layer showed an appearance similar to that of mouse ES cells and coexpressed Nanog promoter-driven EGFP (Figure 1A). Figure 1B schematically summarizes our differentiation methods to induce cardiovascular lineage cells. We applied this system to iPS cells and examined the directional differentiation of iPS cells to cardiovascular cells.

First, we induced mesoderm cell differentiation from ES and iPS cells. Undifferentiated ES or iPS cells were cultured on type IV collagen–coated dishes with DM (see Methods) to induce mesoderm differentiation (Figure 1B). We examined the time course and efficiency of Flk1$^+$ mesoderm cell appearance. As demonstrated previously,[9] ES cell–derived Flk1$^+$ cells appeared from $\approx$3 days of differentiation and reached a maximum at day 4.5 of differentiation (Figure 2A). Similarly, Flk1$^+$ cells were induced from iPS cells under the same culture conditions. Time course and efficiency of Flk1$^+$ cell induction were comparable between ES and iPS cells (Figure 2A and 2B). As shown in Figure 2C, undifferentiated ES cell markers Nanog and Oct3/4[19–21] were expressed in both ES and iPS cells and started to decrease after differentiation. The mesoendoderm marker Brachyury[22] started to be observed from differentiation day 2.5. The mesoderm marker Flk1 appeared from day 3.5. Islet1, a cardiac progenitor marker,[23] was expressed from day 3.5, whereas another cardiac marker, Nkx2.5,[24,25] was not observed until day 4.5. These results were compatible with our previous results.[5] iPS cells therefore differentiate into mesoderm cells with a time course and efficiency similar to those of ES cells.

iPS Cell Differentiation to Vascular Cells

Next we induced ECs from Flk1$^+$ cells. FACS-purified Flk1$^+$ cells were recultured on type IV collagen–coated dishes with VEGF and serum (designated as Flk-d0) (Figure 1B). Three days after the differentiation (Flk-d3), CD31$^+$ ECs and SMA$^+$ mural cells were selectively induced from Flk1$^+$ cells (Figure 3A). Almost all of CD31$^+$ ECs were also positive for another EC marker, VE-cadherin (Figure 3B and 3C). Induced SMA$^+$ mural cells, which were negative for CD31, expressed other smooth muscle markers, SM22α (Figure 3D) and calponin (Figure 3E). When iPS cell–derived Flk1$^+$ cell aggregates were cultured 3-dimensionally in type I collagen gel, a vascular-like structure was formed successfully (Figure 3F; Movie I in the online-only Data Supplement). The vascular-like structure con-

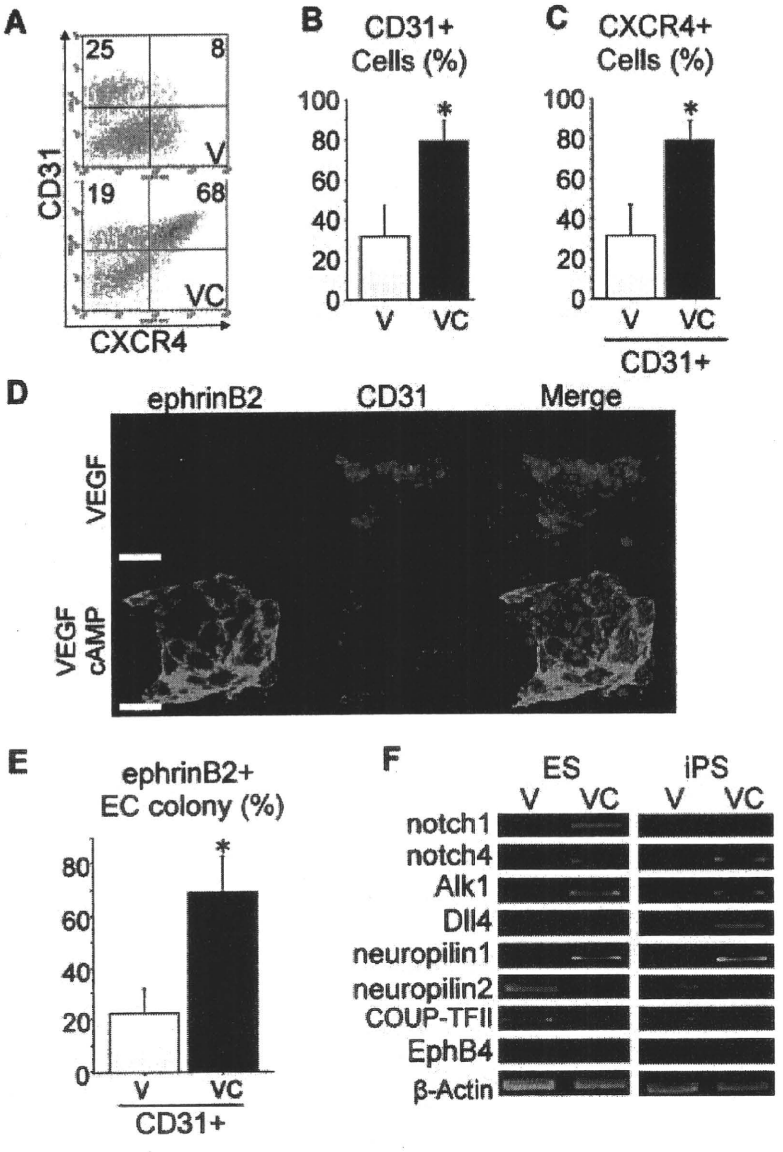

Figure 4. Arterial and venous EC induction from iPS cell–derived Flk1$^+$ cells. A, Representative results of FACS analysis for CD31 and CXCR4 (arterial EC marker) at Flk-d3. VEGF treatment alone (100 ng/mL) (V; top panel) and VEGF with 8bromo-cAMP (0.5 mmol/L) (VC; bottom panel) are shown. Percentages of venous ECs (CD31$^+$/CXCR4$^-$ population) and arterial ECs (CD31$^+$/CXCR4$^+$ population) are indicated. B, Percentages of CD31$^+$ cells (total ECs) in total Flk1$^+$ cell–derived cells (n=3; *P=0.02). C, Percentages of CD31$^+$/CXCR4$^+$ cells (arterial ECs) in total CD31$^+$ cells (n=3; *P=0.01). D, Double immunostaining for ephrinB2 (arterial ECs) (left panels, green) and CD31 (middle panels, red). Right panels show merged images with DAPI (blue). VEGF treatment alone (top) and VEGF with 8bromo-cAMP (bottom) are shown. E, Percentages of ephrinB2$^+$ arterial EC colonies in CD31$^+$ EC colonies (n=4; *P=0.009). F, Gene expressions of arterial and venous EC markers (reverse transcription polymerase chain reaction) in purified CD31$^+$/VE-cadherin$^+$ ECs. Scale bars=100 μm. ES cells (EB5; left) and iPS cells (20D17; right) are shown.

sisted mainly of CD31$^+$ ECs (Figure 3G). Attachment of SMA$^+$ mural cells to endothelial tubes was observed (Figure 3H; Figure III in the online-only Data Supplement), similar to our previous report with the use of ES cells.[4]

Arterial and Venous EC Induction From iPS Cells

We further examined arterial and venous EC differentiation. We recently reported that whereas VEGF treatment alone on Flk1$^+$ cells mainly induced venous ECs, activation of the cAMP pathway increased the total appearance of ECs and potently induced arterial ECs.[11] Figure 4A shows the results of the FACS analysis of Flk1$^+$ cell–derived cells at Flk-d3. VEGF treatment alone mainly induced CD31$^+$/CXCR4$^-$ venous ECs (top panel). In contrast, addition of 8bromo-cAMP, an analogue of cAMP, together with VEGF increased the total CD31$^+$ EC population as well as CD31$^+$/CXCR4$^+$ arterial ECs (bottom panel). The percentage of total ECs (CD31$^+$) in total Flk1$^+$ cell–derived cells (Figure 4B) and percentage of arterial ECs (CD31$^+$/CXCR4$^+$) in total ECs (CD31$^+$) (Figure 4C) were increased by ≈2.5-fold after 8bromo-cAMP treatment, respectively. The efficiency of total EC and arterial EC induction from iPS cell–derived Flk1$^+$ cells was comparable to that in ES cells.[11] Expression of another arterial EC marker, ephrinB2, was examined by immunostaining with the use of EphB4-Fc chimeric protein.[11] 8Bromo-cAMP treatment induced ephrinB2$^+$ arterial ECs (Figure 4D). The number of EC colonies including ephrinB2$^+$ arterial ECs increased ≈2.5-fold by 8bromo-cAMP treatment (Figure 4E). Gene expressions of other arterial and venous EC markers in FACS-purified ECs (CD31$^+$/VE-cadherin$^+$ population) further confirmed arterial and venous EC differentiation (Figure 4F), that is, an increase in arterial EC markers, such as notch1,4,[26] Alk1,[27] Dll4,[28] and neuropilin-1,[29] and a reciprocal decrease in venous EC markers, such as neuropilin-2,[30] COUP-TFII,[31] and EphB4,[32] were observed in VEGF and 8bromo-cAMP–induced ECs, paralleling ES and iPS cells. These results indicate that iPS cells possess the same properties as ES cells for arterial and venous EC differentiation.

iPS Cell Differentiation to Lymphatic ECs

We recently succeeded in inducing lymphatic ECs from mouse ES cells by culturing Flk1$^+$ cells on OP9 stroma cells

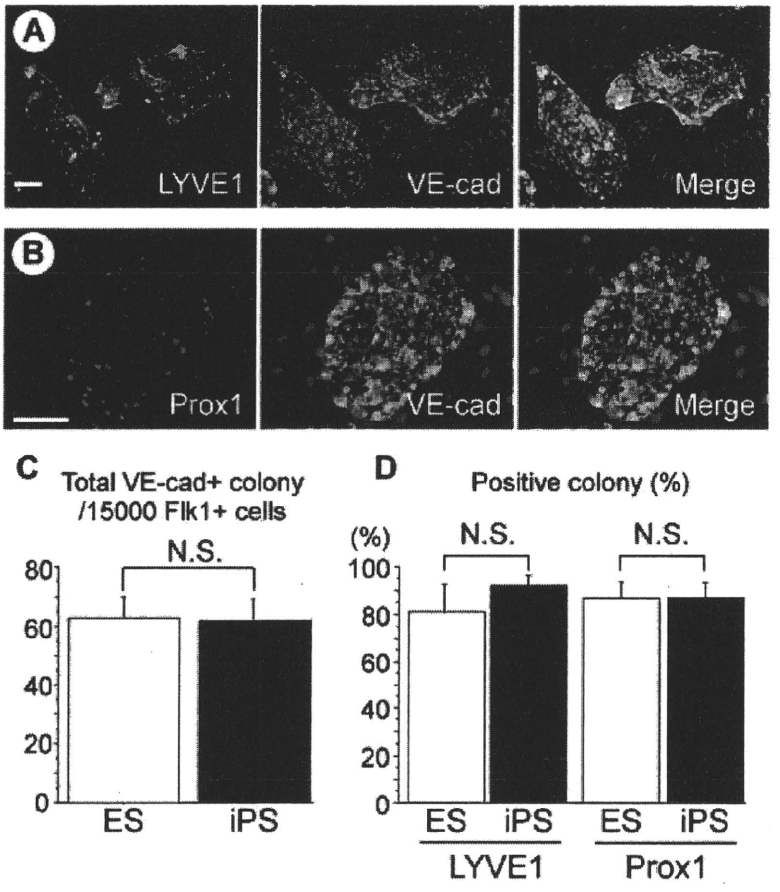

Figure 5. Differentiation of lymphatic ECs from iPS cells. A and B, Double immunostaining of LYVE1 (A) or prox1 (B) (lymphatic ECs; left panels, orange) and VE-cadherin (pan-ECs; middle panels, green) at Flk-d3 on OP9 cells. Right panels, Merged images with DAPI (blue). Bars=100 μm. C, Colony numbers of VE-cadherin (VE-cad)$^+$ ECs induced from 15 000 Flk1$^+$ cells (n=6). N.S. indicates not significant. P=0.86, ES cells (EB5) vs iPS cells (20D17). D, Percentages of EC colonies including lymphatic EC marker–positive cells in total EC colonies (n=3). P=0.89 (LYVE1), P=0.94 (Prox1), ES cells (EB5) vs iPS cells (20D17).

for 3 to 4 days[12] (Figure 1B). When iPS cell–derived Flk1$^+$ cells were cultured on OP9 cells, VE-cadherin$^+$ EC sheets appeared at Flk-d3. Most of the EC colonies that appeared were positive for lymphatic EC markers LYVE-1[33] and prox1[34] (Figure 5A and 5B). The induction efficiency of total EC colonies (VE-cadherin$^+$) (Figure 5C) and lymphatic EC$^+$ colonies (LYVE1$^+$ or prox1$^+$) (Figure 5D) on OP9 cells was comparable between ES and iPS cells, indicating that lymphatic ECs could also be similarly induced from iPS cells.

iPS Cell Differentiation to Cardiomyocytes

Finally, we performed induction of cardiomyocytes from iPS cells. To induce cardiomyocytes, we applied our 2-dimensional cardiomyocyte induction system of ES cells[5] to iPS cells. Similar to ES cells, when purified iPS cell–derived Flk1$^+$ cells were cocultured on OP9 cells, self-beating cardiomyocyte colonies appeared from Flk-d4-5 (Figure 1B) (Movie II in the online-only Data Supplement). Some beating colonies continued to beat for >2 months of culture. These beating colonies were positive for cTnT (Figure 6A). Although the numbers of beating colonies at Flk-d4–6 that appeared from 3 Nanog-iPS cell lines were slightly fewer than those from the EB5 ES cell line,[5] they were still more than those from another ES cell line, D3 cells (Figure 6B; Tables II and III in the online-only Data Supplement). The induction efficiency of cardiomyocytes from iPS cells was further quantitatively evaluated by FACS with the use of TMRM fluorescent dye.[17,18] Approximately half of MHC-GFP$^+$ cardiomyocytes induced from EMG7 ES cells could be detected as a TMRM-high population (Figure IA in the online-only Data Supplement). Being parallel to the beating colony number, the percentages of TMRM-high population induced from Flk1$^+$ cells with the use of EMG7 ES cells, 20D17 iPS cells, and D3 ES cells were 7.9%, 2.5%, and 0.9%, respectively (Figure IB and IC in the online-only Data Supplement). These results indicate that comparable levels of cardiomyocytes could be induced from iPS cells with ES cells. The difference of cardiogenic potentials is likely to be due to variation among cell clones and not due to features of iPS cells per se. Various cardiac marker genes, such as broad cardiomyocyte markers Nkx2.5 and α-MHC, atrial and ventricular myosin light chain 2, pacemaker marker, hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4), and the conduction system marker connexin 40 appeared similarly in differentiation culture of ES and iPS cells on OP9 cells (Figure 6C). Various other features of cardiomyocytes were also observed in purified TMRM-high cells from iPS cells (Figure II in the online-only Data Supplement). Apparent sarcomere formation was detected by actinin staining (Figure 6D). A gap junction protein, connexin 43, was confirmed to be coexpressed with cTnT (Figure 6E). HCN4 and the T-type calcium channel Cav3.2, which were expressed in mouse sinoatrial node and important for automaticity of ES cell–derived cardiomyocytes,[35] also coexisted in cTnT$^+$ cardiomyocytes (Figure 6F and 6G). An atrial and ventricular ion channel, Kir2.1, was observed in cTnT$^+$ cells (Figure 6H). Some of the purified TMRM-high cardiomyocytes showed typical pacemaker-like potential and depolar-

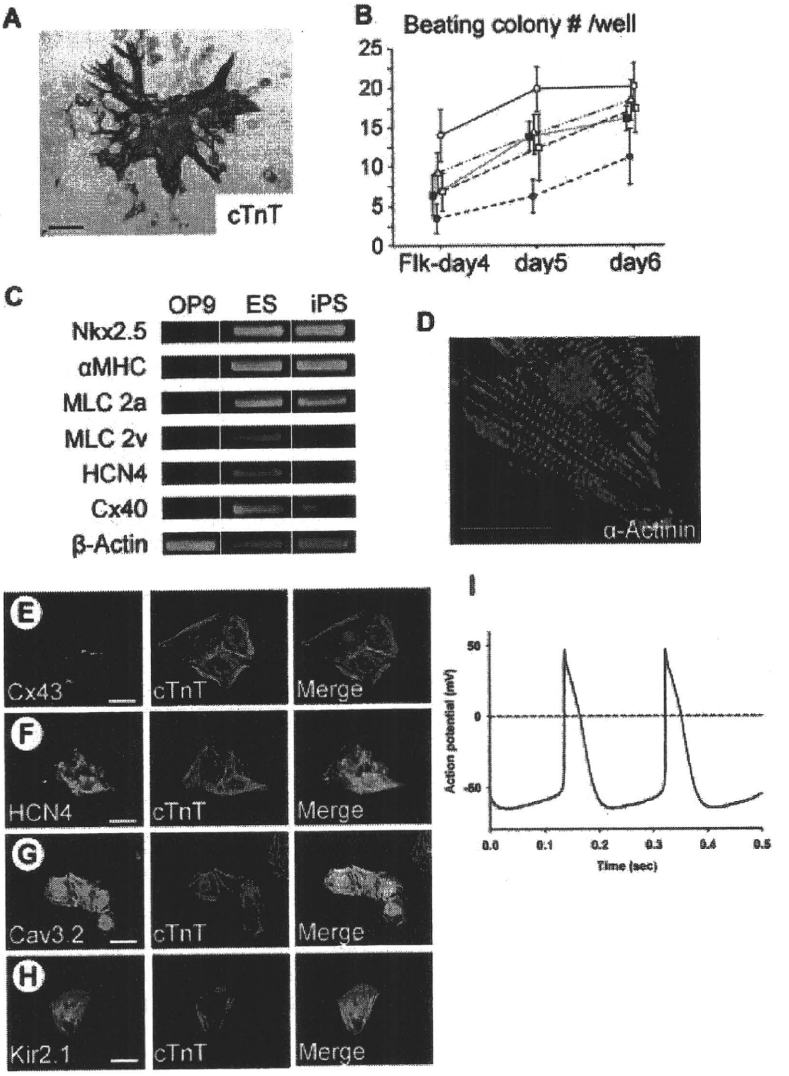

Figure 6. Cardiomyocyte differentiation from iPS cells. A, iPS cell–derived cardiomyocytes on OP9 cells at Flk-d6; cTnT staining (brown). B, Numbers of beating colonies at Flk-d4–6. EB5 ES cells (open circle), D3 ES cells (closed circle), 20D17 iPS cells (closed square), 38C2 iPS cells (open square), and 38D2 iPS cells (open triangle) (n=7) are shown. Averages and statistical analysis are shown in Tables II and III in the online-only Data Supplement. C, Gene expressions of various cardiomyocyte markers in cells at Flk-d6 on OP9 cells (reverse transcription polymerase chain reaction). OP9 cells (left), ES cells (EB5; middle), and iPS cells (20D17; right) are shown. D, Sarcomeric organization in TMRM-purified cardiomyocytes at Flk-d8. Immunostaining with anti-sarcomeric α-actinin antibody (red) and DAPI (blue). E to H, Double immunostaining of connexin43 (Cx43) (E), HCN4 (F), Cav3.2 (G), or Kir2.1 (H) (left, green) and cTnT (middle, orange) in TMRM-purified cardiomyocytes at Flk-d8. Right panels, Merged images with DAPI (blue). I, Representative action potential of iPS cell–derived self-beating cardiomyocytes. Bar=100 μm (A), 25 μm (D), 50 μm (E to H).

ized spontaneously (Figure 6I). Beating rate was 3.59±1.08 Hz, minimum potential was −66.89±6.07 mV, and maximum potential was 44.34±3.58 mV assessed by whole-cell patch clamp method (n=8). These results indicate that iPS cells also can differentiate into various cardiac cell types similar to ES cells.

Discussion

In this report, we showed that various cardiovascular cells could be directionally and systematically induced from mouse iPS cells. $CD45^+$ hematopoietic cells were also induced from iPS cell–derived $Flk1^+$ cells by coculturing with OP9 cells for 3days (Figure IV in the online-only Data Supplement). Thus, we succeeded in establishing a systematic culture system of iPS cells to reproduce differentiation and diversification processes of cardiovascular cells in vitro (Figure 7), which is useful in dissecting the process at the cellular level. The molecular mechanisms of iPS cell differentiation, including genetic and epigenetic aspects, can be examined with this system. This system would greatly contribute to the understanding of iPS cell biology and the development of new cardiovascular regenerative medicines.

During cardiovascular cell differentiation in this system, the properties of ES cells and iPS cells for time course, potential, and efficiency of differentiation were comparable. The physiological function of induced cardiomyocytes also showed no apparent difference between ES and iPS cells. These results suggest that iPS cells were completely reprogrammed and obtained pluripotent differentiation capacities as ES cells. In terms of the differentiation study, ES cells and iPS cells are principally identical, and methodologies obtained from ES cell studies can be applicable to iPS cells and vice versa.

iPS cells were originally induced by retrovirus-mediated transduction of 4 transcription factors, Oct3/4, Sox2, Klf4, and c-myc. It was reported that tumor formation was often observed in iPS cell–derived mice through the reexpression of the c-myc transgene.[16] When we examined mRNA expressions of the transgenes during long-term differentiation culture of $Flk1^+$ cells on OP9, the expression patterns were not constant. Occasionally, we observed upregulation of transgene mRNAs including c-myc after 1- or 2-month cultures (Figure V in the online-only Data Supplement). No apparent reappearance of undifferentiated or tumor cell–like structures were observed in the in vitro culture. Regulation of transgene

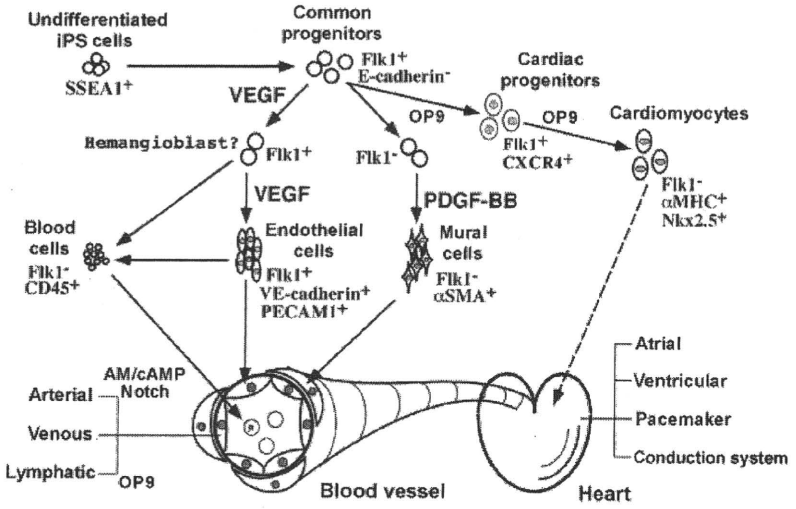

Figure 7. Systematic differentiation system of cardiovascular cells from iPS cells. Mouse iPS cell–derived Flk1$^+$ cells give rise to ECs in the presence of VEGF. VEGF alone induces ephrinB2$^-$ (EphB4$^+$) venous ECs. When cAMP signaling is activated together with VEGF, ephrinB2$^+$ arterial ECs are induced. For lymphatic EC differentiation, OP9 stroma cells are required. When Flk1$^+$ cells are cultured on OP9 stroma cells for 4 to 5 days, cardiomyocytes are induced as a mixture of various cardiac cell types: atrial, ventricular, pacemaker, and conduction system cells. CD45$^+$ blood cells are also induced from Flk1$^+$ cells cultured on OP9 cells. Various cardiovascular cells are therefore systematically induced from common progenitor Flk1$^+$ cells of mouse iPS cells. SSEA1 indicates stage-specific embryonic antigen.

expression should be different among iPS cell lines and modified by various factors, such as integration sites, copy numbers, epigenetic modifications of transgenes, and culture conditions. Although novel iPS cell induction methods devoid of the c-myc transgene have been reported recently,[36,37] retroviral transduction causes genomic insertion of transgenes, which may trigger tumor formation. The safety of iPS cells in regard to tumor formation should therefore be confirmed in each iPS cell line both in vitro and in vivo.

We successfully developed a cardiovascular differentiation system for mouse iPS cells and compared the differentiation properties of ES and iPS cells. Recently, we developed a similar vascular cell differentiation system using human ES cells[38] and applied that to vascular regeneration.[39] These differentiation systems for pluripotent stem cells would be applicable to human iPS cells and would greatly contribute to the generation of new-mode cardiovascular regenerative medicine with the use of iPS cells.

Acknowledgments

We thank Dr K. Takahashi for supporting and supervising iPS cell culture and Dr M. Takahashi for critical reading of the manuscript.

Sources of Funding

This study was supported by grants from the Ministry of Education, Science, Sports, and Culture of Japan; New Energy and Industrial Development Organization of Japan; Novartis Foundation for the Promotion of Science; Terumo Life Science Foundation; Yasuda Medical Foundation; and the Astellas Foundation for Research on Metabolic Disorders.

Disclosures

None.

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CLINICAL PERSPECTIVE

The recent invention of skin fibroblast-derived pluripotent cells, induced pluripotent stem (iPS) cells, opened a new gate for regenerative medicine. Establishment of iPS cells from adult human tissue is further facilitating development of cell transplantation–based regenerative strategies by avoiding the legal and ethical controversy over human embryonic stem cells. This study discusses a directed and systematic differentiation method of using mouse iPS cells for various cardiovascular cells, which would provide a scientific and technological basis for human iPS cell differentiation. As well as the direct application to cell-based regenerative medicine, this study also examines and elucidates the cellular and molecular mechanisms of cardiovascular cell differentiation, thereby contributing to identify novel targets for gene therapy and drug discovery. Furthermore, this system could be directly involved in screening of small molecules to find cardiovascular regenerating substances. This study therefore may greatly contribute to the clinical application of iPS cells and develop novel regenerative medicine for cardiovascular diseases.

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Bioorganic & Medicinal Chemistry Letters 18 (2008) 2982–2984
Bioorganic & Medicinal Chemistry Letters

Evaluation of antiangiogenic activity of azumamides by the in vitro vascular organization model using mouse induced pluripotent stem (iPS) cells

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Received 12 February 2008; revised 3 March 2008; accepted 18 March 2008
Available online 21 March 2008

Abstract—Evaluation of antiangiogenic activity of marine sponge derived azumamides by the in vitro vascular organization model using mouse induced pluripotent stem (iPS) cells was carried out. Azumamide E (**5**) strongly inhibited in vitro angiogenesis from iPS cells at 1.9 μM while azumamide A (**1**) showed only weak inhibition at 19 μM. These results were well correlated with HDAC inhibitory activity of these compounds, revealing the prospect of azumamides as the probe molecules useful for stem cell chemical biology.

The establishment of mouse induced pluripotent stem (iPS) cells[1] followed by the success in reprogramming of differentiated human somatic cells into a pluripotent state[2,3] gave a significant impact on the life sciences not only because of the possibility of regeneration therapy without rejection associated with traditional transplantation but also because of the prospect for application to the assay systems evaluating efficacy and toxicity of drugs against the individual SNP. Therefore, it seems to be an urgent issue to verify the biological activity of the probe molecules found by the assay using ES cells in the iPS systems for further development of stem cell biology by means of chemical biological strategy.

Azumamides A–E (**1**–**5**) are the histone deacetylase (HDAC) inhibitors isolated from the marine sponge *Mycale izuensis*.[4,5] Consistent with the former report that HDACs can be the promising target of antiangiogenic therapy,[6,7] azumamide A (**1**) was also found to inhibit vascularization in the in vitro vascular organization model using mouse ES cells at 19 μM.[8]

Since the following studies[9,10] revealed that the HDAC inhibitory activity of synthetic **1** was not as potent as that of originally reported while synthetic **5** retained the equivalent potency, we re-evaluated inhibitory activities of **1**–**5** against HDAC1, HDAC4, and HDAC6, as well as p21 promotion activities. Then, the antiangiogenic activities of azumamides A (**1**) and E (**5**) by the in vitro vascular organization model using mouse induced pluripotent stem (iPS) cells were tested. As a result, we found a dose-dependent antiangiogenic activity in the iPS cell system of **5**, while **1** showed only a moderate effect.

These results were well correlated with their HDAC inhibitory activities, and therefore, azumamide E (**5**) is presumed to be the promising probe molecule for the chemical biology of angiogenesis and stem cell differentiation.

Azumamides A–E were assayed for HDAC inhibitory activity using HDAC1, HDAC4, and HDAC6 enzymes prepared from 293T cells.[11] Azumamides A (**1**) and D (**4**) showed only a very weak inhibitory activity against

Keywords: iPS cells; Histone deacetylase; Angiogenesis; Chemical biology.
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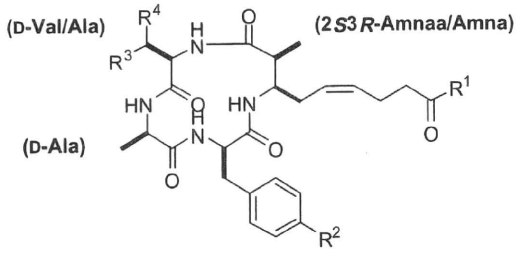
0960-894X/$ - see front matter © 2008 Published by Elsevier Ltd.
doi:10.1016/j.bmcl.2008.03.053

these subtypes at 50 μM, while B (**2**), C (**3**), and E (**5**) showed inhibitory activities against HDAC1 and HDAC4 with IC_{50} values ranging from 1.17 to 3.66 μM. None of **1**–**5** showed remarkable activity against HDAC6. Promotion of p21 expression was also evaluated[12] and only azumamide E (**5**) showed a moderate activity with EC_{1000} value of 17.0 μM (Table 1).



	R^1	R^2	R^3 =R^4
azumamide A (1)	NH_2	H	Me
B(2)	NH_2	OH	Me
C(3)	OH	OH	Me
D(4)	NH_2	H	H
E(5)	OH	H	Me

These results suggested that azumamide E (**5**) is the most active among **1**–**5**, therefore, **1** and **5** were tested for their antiangiogenic activity in the in vitro vascular organization model using mouse iPS cells.[13]

Evaluation of antiangiogenic activity was carried out basically following the procedure using mouse ES cells,[4,8] except that mouse iPS cells derived from mouse skin fibroblasts were used instead of mouse ES cells.

In this model, azumamide A (**1**) did not show or showed only a weak inhibition even at 19 μM (Fig. 1c and g), which is inconsistent to the former result but consistent with the HDAC inhibitory activity (IC_{50} > 50 μM) obtained in this assay.

On the other hand, azumamide E (**5**) showed dose-dependent inhibition of angiogenesis in this model at as low as 0.19 μM (Fig. 2). The observed antiangiogenic activity of **5** was again well consistent with the inhibitory activity against HDAC1 and HDAC4.

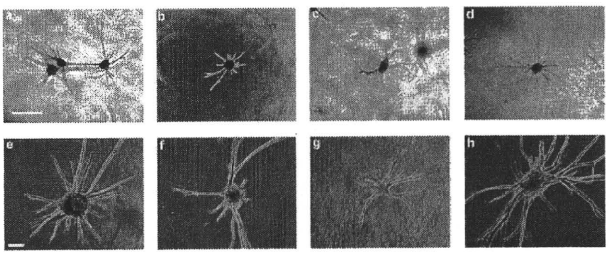


Figure 1. Effects of azumamide A (**1**) on in vitro vascular organization of mouse iPS cells. (a–d) ×4, bar = 500 μm. (e–h) ×10, bar = 100 μm. (a and e) 0.19 μM of **1**. (b and f) 1.9 μM of **1**. (c and g) 19 μM of **1**. (d and h) 0 μM of **1**.

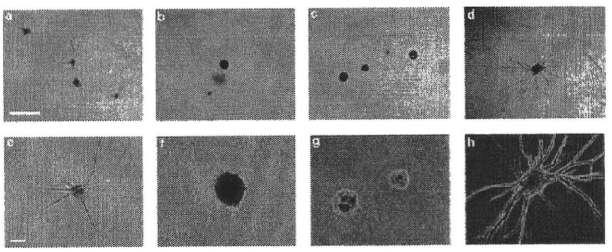


Figure 2. Effects of azumamide E (**5**) on in vitro vascular organization of mouse iPS cells. (a–d) ×4, bar = 500 μm. (e–h) ×10, bar = 100 μm. (a and e) 0.19 μM of **5**. (b and f) 1.9 μM of **5**. (c and g) 19 μM of **5**. (d and h) 0 μM of **5**.

In the present study, we have tested inhibitory activity of azumamides A–E (**1**–**5**) against HDAC1, HDAC4, and HDAC6, as well as p21 promotion activity. In the HDAC inhibitory assay system used in this study, azumamides showed weaker activities than those obtained in the previous report.[4] Inhibitory activities obtained for the synthetic azumamides by Ganesan[10] also showed a weaker activity of **1**. From the results of De Riccardis[9] and Ganesan,[10] it seems that assay conditions may largely affect the HDAC inhibitory activities. However, the results of HDAC inhibition assay for azumamides A and E in the present study were well correlated with the antiangiogenic activity in the iPS cells system.

It will be a valid strategy for the stem cell chemical biology, to compare gene expression or epigenetic states during the cell differentiation processes under the different culture conditions prepared by the administration of positive or negative probe molecules, respectively. Azumamides A (**1**) and E (**5**) can be such a probe molecule

Table 1. Biological activity of azumamides A–E (**1**–**5**)

Compound	HDAC1[a]	HDAC4[a]	HDAC6[a]	p21 promotion[b]
1	>50 μM	>50 μM	>50 μM	>25 μM
2	1.83 μM ± 0.11	3.66 μM ± 1.34	>50 μM	>25 μM
3	1.17 μM ± 0.16	3.16 μM ± 0.21	>50 μM	>25 μM
4	>50 μM	>50 μM	>50 μM	>25 μM
5	1.22 μM ± 0.13	2.28 μM ± 0.16	>50 μM	17.0 μM ± 3.91
TSA[c]	0.0366 μM	0.0629 μM	0.0833 μM	0.0115 μM

[a] IC_{50} values (±standard deviation).
[b] EC_{1000} values.
[c] Trichostatin A.

set ideal for the chemical biological study of stem cell differentiation and angiogenesis using iPS cells.

Acknowledgments

This work was partly supported by the Waseda University Grant for Special Research Projects, the Nissui Research foundation, and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.03.053.

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OPEN ACCESS Freely available online

PLoS one

Augmentation of Neovascularizaiton in Hindlimb Ischemia by Combined Transplantation of Human Embryonic Stem Cells-Derived Endothelial and Mural Cells

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Abstract

Background: We demonstrated that mouse embryonic stem (ES) cells-derived vascular endothelial growth factor receptor-2 (VEGF-R2) positive cells could differentiate into both endothelial cells (EC) and mural cells (MC), and termed them as vascular progenitor cells (VPC). Recently, we have established a method to expand monkey and human ES cells-derived VPC with the proper differentiation stage in a large quantity. Here we investigated the therapeutic potential of human VPC-derived EC and MC for vascular regeneration.

Methods and Results: After the expansion of human VPC-derived vascular cells, we transplanted these cells to nude mice with hindlimb ischemia. The blood flow recovery and capillary density in ischemic hindlimbs were significantly improved in human VPC-derived EC-transplanted mice, compared to human peripheral and umbilical cord blood-derived endothelial progenitor cells (pEPC and uEPC) transplanted mice. The combined transplantation of human VPC-derived EC and MC synergistically improved blood flow of ischemic hindlimbs remarkably, compared to the single cell transplantations. Transplanted VPC-derived vascular cells were effectively incorporated into host circulating vessels as EC and MC to maintain long-term vascular integrity.

Conclusions: Our findings suggest that the combined transplantation of human ES cells-derived EC and MC can be used as a new promising strategy for therapeutic vascular regeneration in patients with tissue ischemia.

Citation: Yamahara K, Sone M, Itoh H, Yamashita JK, Yurugi-Kobayashi T, et al (2008) Augmentation of Neovascularizaiton in Hindlimb Ischemia by Combined Transplantation of Human Embryonic Stem Cells-Derived Endothelial and Mural Cells. PLoS ONE 3(2): e1666. doi:10.1371/journal.pone.0001666

Editor: Tailoi Chan-Ling, University of Sydney, Australia

Received November 5, 2007; **Accepted** January 24, 2008; **Published** February 27, 2008

Funding: This work is supported by a grant-in-aid for scientific research (the Japanese Ministry of Education, Culture, Sports, Science and Technology), a research grant from them 21st COE Program for Integration of Transplantation Therapy and Regenerative Medicine (Japan Society for the Promotion of Science), a research grant from the Project for Development of Regenerative Medicine (the Japanese Ministry of Education, Culture, Sports, Science and Technology), and a research grant from the Japan Foundation for Aging and Health (the Japanese Ministry of Health, Labor and Welfare).

Competing Interests: The authors have declared that no competing interests exist.

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☯These authors contributed equally to this work.

Introduction

Embryonic stem (ES) cells, with their extensive regeneration potential and functional multilineage differentiation capacity, are now highlighted as promising cell sources for regenerative medicine. Previously we reported that mouse ES cells-derived vascular endothelial growth factor receptor-2 (VEGFR2) positive cells could differentiate into both endothelial cells (EC) and mural cells (MC) (pericytes and vascular smooth muscle cells) and reproduce the vascular organization process, which we termed "vascular progenitor cells (VPC)" [1]. Transplanted VPC into tumor-bearing nude mice were incorporated into blood vessels and significantly increased blood flow, which suggests that VPC might be useful for augmenting vessel growth in ischemic tissue [2].

We have demonstrated that human as well as monkey ES cells possessed different differentiation kinetics of VPC derived from mouse ES cells [3,4]. In contrast to mouse ES cells, undifferentiated human ES cells already expressed VEGFR2. After the induction of differentiation on OP9 feeder cells, VEGFR2 positive and tumor rejection antigen-1 (TRA1: a marker indicative of undifferentiated cell phenotype) negative cells appeared at day 8. We confirmed that VEGFR2 positive cells at this stage effectively differentiated into both VE-cadherin positive EC and α-smooth muscle actin (αSMA) positive MC to suffice as human VPC.

Human VPC-derived VEGFR2$^+$ VE-cadherin$^+$ cells, which were considered as EC at an early differentiation stage, formed a network structure on Matrigel-coated dishes.

Based upon these works, in the present study we transplanted human VPC-derived vascular cells; that is, EC and MC in a murine hindlimb ischemia model. By transplantation of these EC and MC differentiated from human VPC, we investigated whether and how they could be incorporated as EC and MC into the sites of neovascularization, compared to human peripheral blood and umbilical cord blood-derived endothelial progenitor cell (EPC) transplantation [5–7]. Furthermore, we specifically asked whether the combined transplantation of human VPC-derived EC and MC could induce stable vascular regeneration to achieve long-term vascular integrity.

Results

Characterization of Transplanted Human VPC-derived Vascular Cells

Flow cytometric analysis disclosed that 20–40% of expanded human VPC-derived EC retained the expression of the endothelial cell-related markers, including VE-cadherin, VEGFR2, CD34, CD31 and CD105, and all of the cells were negative for a panleukocyte marker CD45, monocyte/macrophage marker (CD11b), and stem/progenitor makers (AC133 and c-kit) (Figure 1a). By the double immunostaining of CD31 and αSMA, the cells negative for CD31 were exclusively positive for αSMA (Figure 1b), but weak or negative for staining with other MC markers, including calponin, smooth muscle myosin heavy chain 1 (SM1) and 2 (SM2) (data not shown).

Immunocytochemistry of expanded human VPC-derived MC revealed that all these cells were positive for αSMA, calponin, SM1 and SM2 (Figure 1c). Analysis by reverse transcription-polymerase chain reaction (RT-PCR) also confirmed that mRNA expressions of these MC markers were upregulated in human VPC-derived MC and negative in sorted VE-cadherin$^+$ fraction of expanded human VPC-derived EC (Figure 1d). Although cultured human aortic smooth muscle cells (hAoSMC) expressed a high level of h-caldesmon, its expression in human VPC-derived MC was not detected. Furthermore, mRNA for skeletal (myogenin and MyoD) or cardiac (cardiac troponin T (cTnT) and I (cTnI)) specific marker was not detected in human VPC-derived MC (Figure 1e).

Characterization of Transplanted Human EPC

Flow cytometric analysis of pEPC demonstrated that these cells mainly exhibited two light-scattering properties: one was consistent with a relatively large cell size (gate P1) and the other was found in a smaller gate P2 (Figure 2a). The P1-gated cells were positive for DiI-acLDL uptake and ulex-lectin binding (Figure 2b), and exhibited the reported EPC phenotype [6,8]. However, the smaller P2-gated cells were low positive for DiI-acLDL/ulex-lectin (Figure 2c). Therefore, we performed subsequent fluorescence activated cell sorter (FACS) analysis of pEPC on the P1-gated cells.

As shown in Figure 2d, nearly all pEPC expressed the hematopoietic markers CD45 (99.9%) and CD54 (99.9%) and the monocyte/macrophage markers CD14 (99.0%), CD11b (98.7%), and CD11c (98.9%). The monocyte/macrophage or endothelial markers CD31 (58.3%) and CD105 (70.1%) were also expressed. A much lower percentage of these cells expressed the endothelial cell-related markers VE-cadherin (1.6%), VEGFR2 (5.4%), and von Willebrand Factor (vWF) (0.3%), or the stem/progenitor cell markers AC133 (1.0%), c-kit (0.4%), and CD34 (0.2%).

Flow cytometric analysis of magnetic cell separation system (MACS)-sorted uEPC showed more than 80% of these cells were positive for CD34 (data not shown). Similar to pEPC, almost all CD34$^+$ fraction of uEPC expressed the hematopoietic markers CD45 (99.0%) and CD54 (84.9%) (Figure 2e). However, the expression of monocyte/macrophage markers was limited in uEPC (CD14 5.7%, CD11b 99.7%, CD11c 21.3%), and significant number of these cells was positive for the endothelial cell-related markers, including VE-cadherin (11.2%), VEGFR2 (8.1%), and vWF (7.9%). In addition, these CD34$^+$ uEPC expressed the stem/progenitor markers AC133 (80.6%) and c-kit (95.3%).

Long-term Improvement of Blood Flow of Ischemic Hindlimb by Human VPC-derived Vascular Cell Transplantation

To examine the comparative effectiveness of transplanted human VPC-derived vascular cells for vascular regeneration, we set up six groups as follows (Figure 3);

1) EC+MC group (n = 9): the mixture of 0.5×10^6 human VPC-derived EC and 0.5×10^6 MC, with the total cell number of 1×10^6,
2) EC group (n = 20): 0.5×10^6 human VPC-derived EC,
3) MC group (n = 18): 0.5×10^6 human VPC-derived MC,
4) uEPC group (n = 10): 1×10^6 umbilical cord-derived CD34$^+$ cells,
5) pEPC group (n = 16): 1×10^6 peripheral mononuclear cells (MNC)-derived EPC,
6) Control group (n = 17): only 100 μl PBS.

To analyze subcutaneous hindlimb perfusion, laser Doppler perfusion image (LDPI) analysis was performed (Figure 4a). Throughout the 42 day follow-up period, significantly accelerated limb perfusion improvement was observed in the VPC-derived EC+MC-transplanted group, compared to the EPC and control groups (Figure 4b, $P<0.001$ vs. control, pEPC, uEPC, and MC groups, $P = 0.002$ vs. EC group, repeated measures ANOVA followed by Bonferoni's multiple comparison test).

At day 14, blood flow of the mice transplanted with EPC (the ratio of ischemic/non-ischemic blood flow: 0.907 ± 0.058 in pEPC and 0.942 ± 0.075 in uEPC) ($P = 0.035$ and 0.028, compared to the control group), as well as MC (0.957 ± 0.056) ($P = 0.006$) and EC (0.901 ± 0.063) ($P = 0.032$) showed significant increase, compared to the control group (0.730 ± 0.042) (Figure 4b). In the EC+MC group, the ratio of ischemic/non-ischemic blood flow markedly elevated to 1.187 ± 0.083 ($P<0.0001$), compared to other groups.

Blood flow in the pEPC group, however, did not increase thereafter and no significant difference in the blood flow between the pEPC and control group was seen at days 28 and 42 (Figure 4b). In the uEPC group, significant blood flow recovery was seen at day 42 (0.990 ± 0.054) ($P = 0.009$), compared to the control group (0.749 ± 0.039). The blood flow in the VPC-derived vascular cells-transplanted groups progressively increased. At day 42, the calculated perfusion ratio of ischemic to non-ischemic hindlimb significantly elevated to 0.943 ± 0.057 for the MC ($P = 0.013$), 1.038 ± 0.059 for the EC ($P = 0.0002$), and 1.231 ± 0.067 for the EC+MC group ($P<0.0001$) compared to the control group (0.749 ± 0.039). Between the cell mixture transplantation (EC+MC) group and the single cell transplantation (EC or MC) groups, the blood flow of ischemic hindlimbs was significantly different at day 42 ($P<0.05$).

Effective Contribution of Human VPC-derived Vascular Cells for Vascular Regeneration

Fixed tissues harvested from ischemic hindlimbs at day 7 were inspected by the fluorescence stereomicroscope (Leica, Wetzlar,

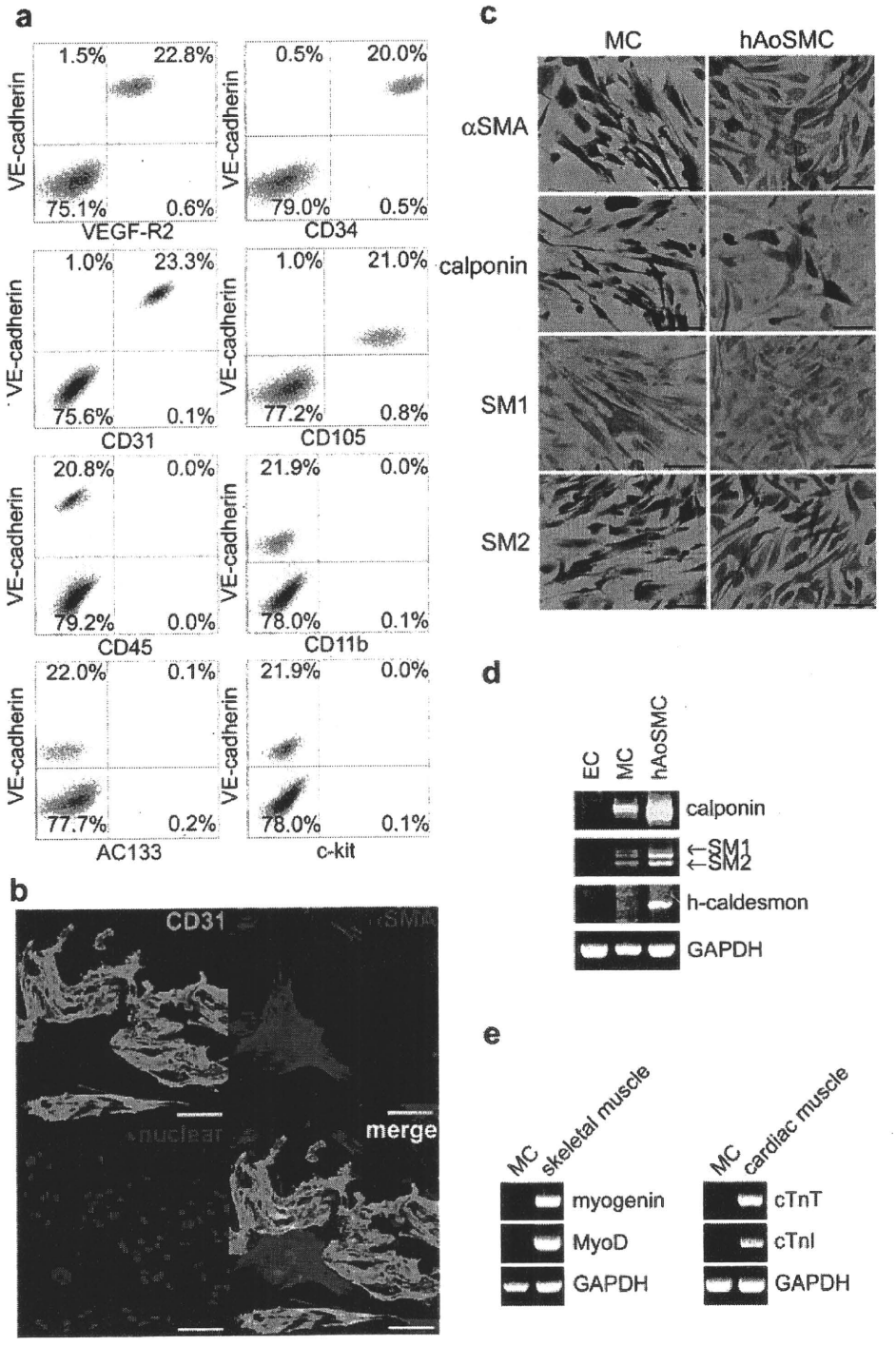

Figure 1. Characterization of transplanted human VPC-derived vascular cells. a) Flow cytometric analysis of cell surface markers on expanded human VPC-derived VEGF-R2$^+$VE-cadherin$^+$ cells (=EC). b) Immunofluorescence image of CD31 (green) and αSMA (red) with nuclear staining (blue) in expanded EC. Scale bar: 100 μm. c) Immunostaining of mural cell markers (brown) with hematoxyline counter-staining of expanded VPC-derived VEGF-R2$^+$VE$^-$cadherin- cells (=MC). Scale bar: 100 μm. d, e) RT-PCR analysis of mural cell (d) and skeletal/cardiac specific (e) markers in human VPC-derived vascular cells.

doi:10.1371/journal.pone.0001666.g001

Germany). Extended distribution of DiI-positive transplanted cells was clearly seen in both VPC-derived EC+MC and pEPC-transplanted hindlimbs (Figure 5a). We also detected some DiI-positive vessel-like formation in the lung and spleen, but no obvious tumor-like structures were seen (data not shown). Ischemic hindlimbs at day 14 were sectioned and treated with streptavidin conjugated dye to stain intravenously injected biotinylated isolectin B_4, followed by anti-human CD31 antibody, and scanned for the incorporation of transplanted cells into circulating vessels. In the EC+MC group, we found that human

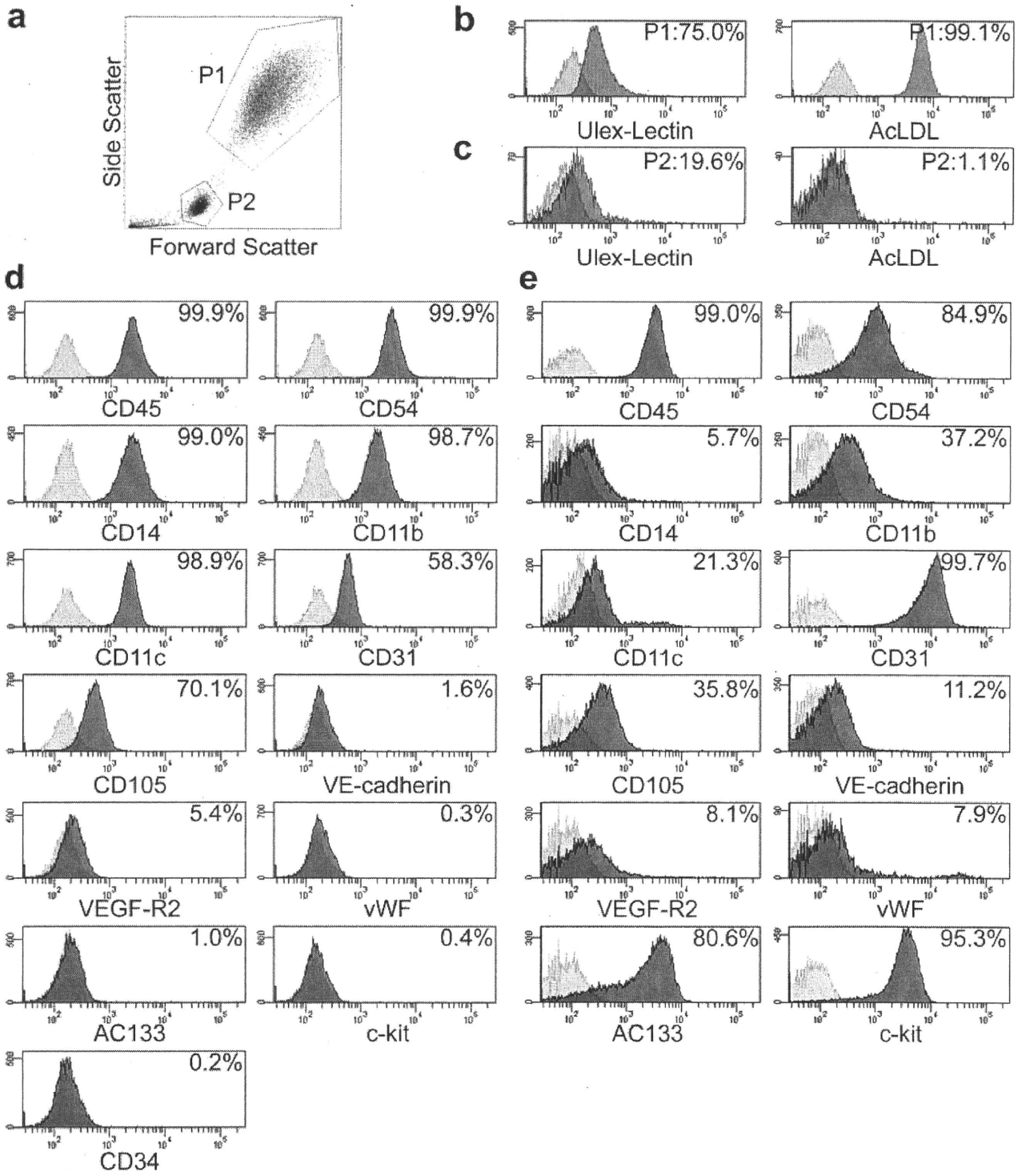

Figure 2. Characterization of peripheral blood and umbilical cord-derived EPC (pEPC and uEPC, respectively) by flow cytometer. a) Representative forward and side scatter profile of cultured pEPC. b-d) Flow cytometric analysis of ulex-lectin binding/acLDL uptake (b, c) and various cell surface markers (d) in pEPC. e) Flow cytometric analysis of cell surface markers in uEPC.
doi:10.1371/journal.pone.0001666.g002

CD31 positive cells formed capillaries with host EC, which were stained with isolectin B_4 (Figure 5b: arrowhead). Furthermore, some human CD31 positive cells solely formed capillary vessel (Figure 5b: arrow), which might indicate de novo vessel formation from human VPC-derived EC. We also detected human CD31 positive cells in the pEPC and uEPC group; however, many of these cells were located within the lumen of host capillaries (Figure 5c, arrow).

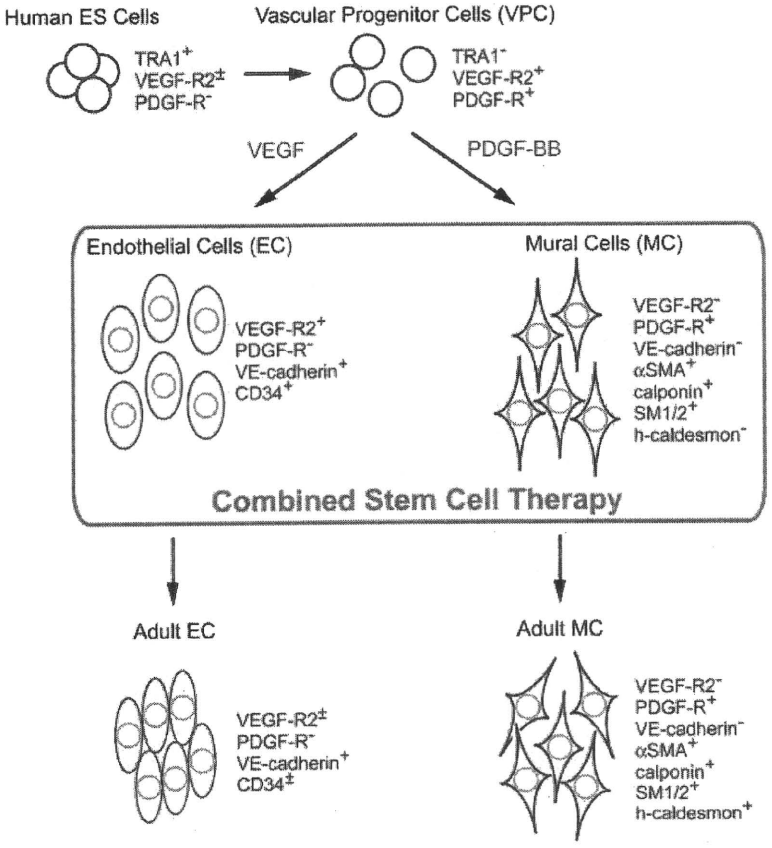

Figure 3. Possible differentiation pathway of vascular cells from human ES cells via VPC.
doi:10.1371/journal.pone.0001666.g003

We further investigated the contribution of transplanted VPC-derived MC to the recruitment of mural cells. We stained the sections of ischemic hindlimbs at day 14 with anti-human SM1 and αSMA antibodies. In EC+MC-transplanted mice, we found some human SM1 and αSMA double positive cells, which were localized within the αSMA positive host vessel wall (Figure 5d: arrow).

Quantification of Transplanted VPC-derived Vascular Cell-induced Vascular Regeneration in Ischemic Hindlimb

The sections of ischemic hindlimbs of the EC+MC group at day 42 were stained with anti-human and mouse CD31 antibodies. Mouse CD31 positive capillary density was significantly high in the EC+MC group ($1775.3\pm54.2/mm^2$), compared to other groups ($P<0.0001$ vs. control group: $1318.6\pm73.0/mm^2$) (Figure 6b). Human CD31 positive capillary density in mice transplanted with human VPC-derived EC (EC ($149.9\pm12.3/mm^2$) and EC+MC ($135.7\pm13.7/mm^2$) was significantly higher than that in mice transplanted with EPC ($95.7\pm8.5/mm^2$ in the pEPC and $115.2\pm12.0/mm^2$ in the uEPC group) ($P<0.05$). Compatible with the result of blood flow measurement, mouse and/or human CD31 positive capillary density markedly increased in mice that received human VPC-derived EC+MC ($1856.8\pm57.0/mm^2$) ($P<0.0001$, compared to the control group ($1318.6\pm73.0//mm^2$)), and also to other groups. Among the single cell transplantation groups, mouse and/or human CD31 positive capillary density increased in the EC group ($1601.4\pm51.4/mm^2$) ($P=0.0016$) compared to the control group, but did not increase in the MC ($1471.8\pm42.4/mm^2$) or EPC groups ($1403.5\pm84.4/mm^2$ in the pEPC and $1524.8\pm108.2/mm^2$ in the uEPC group).

To confirm the maturity of newly formed vessels, we performed the immunostaining of the ischemic tissues with anti-αSMA antibody, which could stain both human and mouse MC (Figure 6c). We confirmed that αSMA positive capillary density was significantly increased in the human VPC-derived vascular cells-transplanted groups (MC ($1317.6\pm45.4/mm^2$), EC ($1357.7\pm27.3/mm^2$) and EC+MC ($1554.9\pm48.8/mm^2$)) ($P<0.0001$), compared to the control group ($1021.3\pm46.3/mm^2$) (Figure 6d). Among the EPC groups, αSMA positive capillary density was significantly increased in the uEPC group ($1185.7\pm42.2/mm^2$) ($P<0.0076$) compared to the pEPC ($1118.9\pm36.8/mm^2$) and control group. We further investigated the extent of arteriogenesis in these groups using αSMA immunostaining sections. Many αSMA positive arterioles with more than 20 μm in diameter were detected in the EC+MC group, but not in the control group (Figure 6c: arrowhead). The number of αSMA positive arterioles significantly increased in the human VPC-derived vascular cells-transplanted groups, especially in the EC+MC group (the MC group:$4.0\pm0.3/mm^2$ and the EC group:$3.7\pm0.2/mm^2$; $P<0.001$, compared to the control group: $2.0\pm0.2/mm^2$, the EC+MC group: $5.5\pm0.7/mm^2$; $P<0.0001$, compared to all other groups) (Figure 6e). However, no significant difference in the number of αSMA positive arterioles was seen between the EPC (the pEPC group:$1.9\pm0.2/mm^2$ and the uEPC group:$2.0\pm0.2/mm^2$) and control groups.

Discussion

The present study demonstrated that the transplantation of human VPC-derived vascular cells at the proper differentiation