

## Directed and Systematic Differentiation of Cardiovascular Cells From 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<sup>+</sup> 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<sup>+</sup> 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),<sup>1</sup> and recently they were also successfully generated from human skin fibroblasts.<sup>2,3</sup> 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.

### Editorial p 472 Clinical Perspective p 506

Previously, we established a novel ES cell differentiation system that can reproduce the early process of cardiovascular development in vitro.<sup>4–6</sup> 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.<sup>7,8</sup> We induced Flk1<sup>+</sup> cells from ES cells, purified them by fluorescence-activated cell sorting (FACS), and recultured the purified cells.<sup>9</sup> We succeeded in inducing cardiovascular cells such as vascular ECs, mural cells (pericytes and vascular smooth muscle cells),<sup>4,10</sup> and cardiomyocytes<sup>5</sup> from common progenitors, Flk1<sup>+</sup> cells. We also identified a novel cardiac progenitor population during ES cell differentiation.<sup>5</sup> More recently,

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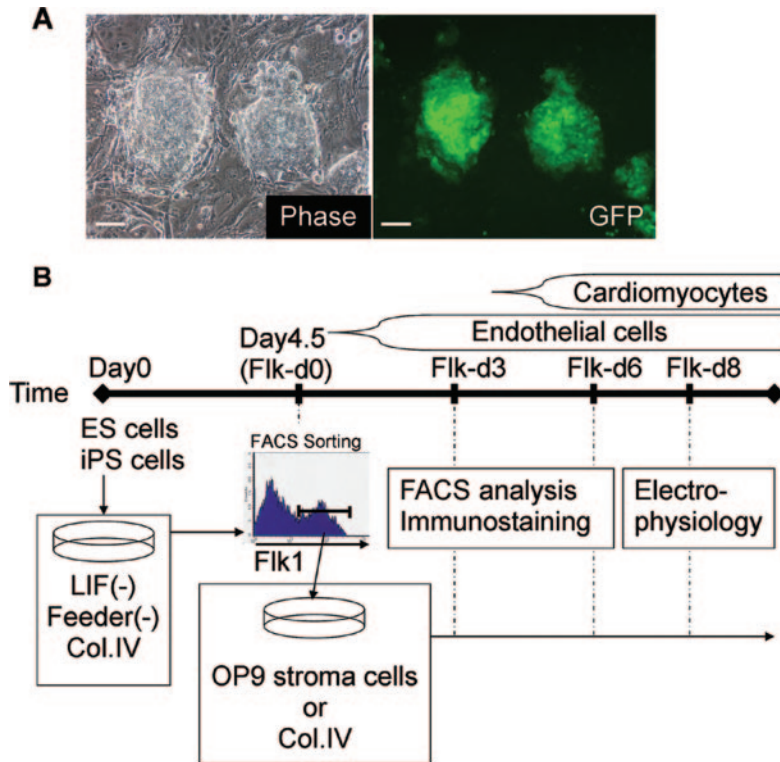
**The online-only Data Supplement, which contains Figures I through V, Movies I and II, and a supplemental Methods section, is available with this article at <http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.108.769562/DC1>.**

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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  $\mu$ m. 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<sup>+</sup> cells. FACS-purified Flk1<sup>+</sup> 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<sup>+</sup> cells.<sup>11,12</sup> This system is therefore useful to systematically induce various cardiovascular cells from common progenitors and dissect their differentiation processes.<sup>6,10,13</sup>

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.<sup>8,14</sup> 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  $\alpha$ -smooth muscle actin (SMA) (1:1000) and  $\alpha$ -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 SM22 $\alpha$  (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.<sup>5,11,15</sup> EMG7 ES cells were generated by introduction of  $\alpha$ -myosin heavy chain (MHC) promoter-driven EGFP gene to EB5 ES cells.<sup>5</sup> Cardiomyocytes induced from EMG7

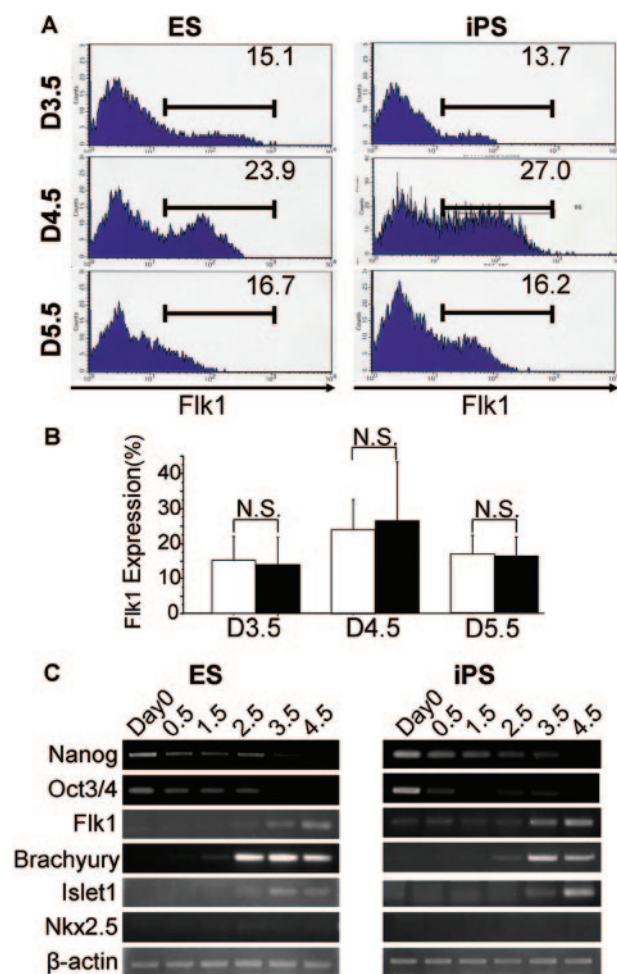
cells could be detected by GFP expression (MHC-GFP). Germline-competent mouse iPS cell lines 20D17, 38C2, and 38D2, carrying Nanog promoter-driven GFP/IRES/puromycin-resistant gene (Nanog-iPS cells), were established and maintained as described previously.<sup>16</sup> Briefly, iPS cells were maintained 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<sup>4</sup> (DM) ( $\alpha$ -minimum essential medium (Invitrogen) supplemented with 10% fetal calf serum and  $5 \times 10^{-5}$  mol/L 2-mercaptoethanol). Flk1<sup>+</sup> mesoderm cells were induced by 96- to 108-hour culture of ES or iPS cells (plated at  $1.7 \times 10^3$  cells/cm<sup>2</sup>) 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<sup>+</sup> cells ( $1 \times 10^4$  cells/cm<sup>2</sup>) were cultured on type IV collagen-coated dishes with DM supplemented with 100 ng/mL human VEGF<sub>165</sub> (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).<sup>11</sup> Lymphatic endothelial cell was induced by coculture of Flk1<sup>+</sup> cells ( $5 \times 10^3$  cells/cm<sup>2</sup>) on confluent OP9 stroma cells<sup>12</sup> with DM. After 3-day culture of Flk1<sup>+</sup> cells, induced ECs were subjected to FACS or immunostaining. Cardiomyocytes were induced by coculture of Flk1<sup>+</sup> cells ( $1.5 \times 10^4$  cells/cm<sup>2</sup>) on OP9 cells for 4 to 6 days<sup>5</sup> with DM.

### Three-Dimensional Culture

Three-dimensional culture was performed as described previously.<sup>4</sup> Briefly, Flk1<sup>+</sup> cells ( $4 \times 10^5$  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  $2 \times$  DM and mixed with an isovolume of collagen I-A gel (3 mg/mL) (Nitta Gelatin, Osaka, Japan). We plated 250  $\mu$ L 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<sup>+</sup> cells in total cells are indicated. **B**, Quantitative evaluation of Flk1<sup>+</sup> 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  $\mu$ L DM with VEGF (final, 100 ng/mL). To monitor vascular formation, collagen-embedded Flk1<sup>+</sup> cell aggregates were cultured in a temperature- and gas-controlled chamber (37°C, 5% CO<sub>2</sub>), 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.<sup>4,5,11</sup> After induction of Flk1<sup>+</sup> cells, cultured cells were harvested and stained with allophycocyanin-conjugated AVAS12. Living Flk1<sup>+</sup> cells excluding propidium iodide (Sigma) were sorted by FACS Vantage (Becton Dickinson). After 3 days of Flk1<sup>+</sup> 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),<sup>17,18</sup> 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.<sup>4,5,11</sup> 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).<sup>11</sup> 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

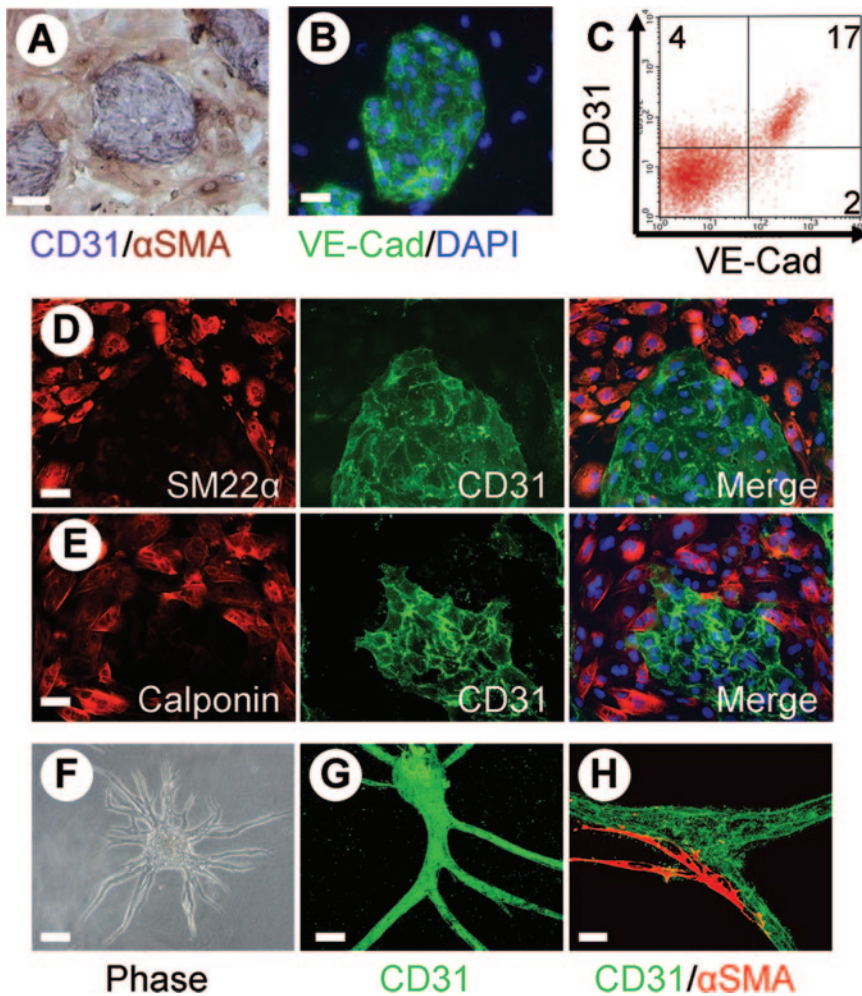
The FACS-purified TMRM-high population was seeded on gelatin-coated coverslips. The myocytes were cultured for 2 days under this condition before use. The coverslips were then transferred to a patch clamp recording chamber, and electrophysiological measurements were performed with the use of Axopatch200B (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<sub>2</sub>PO<sub>4</sub>, 0.5 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, and 5 HEPES (pH=7.4 with NaOH). Standard high-K<sup>+</sup> pipette solution contained the following (in mmol/L): 110 L-aspartic acid, 30 KCl, 5 MgATP, 0.1 NaGTP, 5 K<sub>2</sub> 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 $\pm$ 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<sup>+</sup> 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 SM22 $\alpha$  (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<sup>+</sup> 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  $\mu$ m (A, F), 50  $\mu$ m (B, D, E, G), 25  $\mu$ m (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.

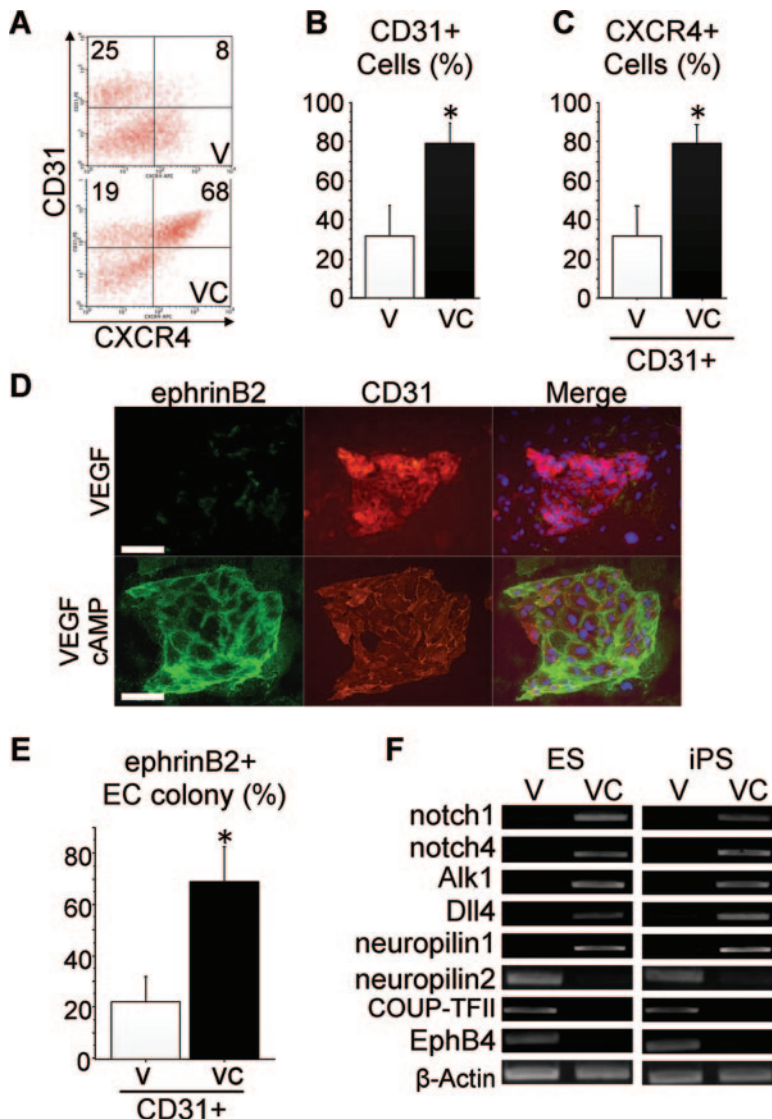
First, we induced mesoderm cell differentiation from ES and iPS cells. Undifferentiated ES or iPS cells were cultured on type IV collagen-coated dishes with DM (see Methods) to induce mesoderm differentiation (Figure 1B). We examined the time course and efficiency of Flk1<sup>+</sup> mesoderm cell appearance. As demonstrated previously,<sup>9</sup> ES cell-derived Flk1<sup>+</sup> cells appeared from  $\approx$ 3 days of differentiation and reached a maximum at day 4.5 of differentiation (Figure 2A). Similarly, Flk1<sup>+</sup> cells were induced from iPS cells under the same culture conditions. Time course and efficiency of Flk1<sup>+</sup> 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<sup>19–21</sup> were expressed in both ES and iPS cells and started to decrease after differentiation. The mesoendoderm marker Brachyury<sup>22</sup> started to be observed from differentiation day 2.5. The mesoderm marker Flk1 appeared from day 3.5. Islet1, a cardiac progenitor marker,<sup>23</sup> was expressed from day 3.5, whereas another cardiac marker, Nkx2.5,<sup>24,25</sup> was not observed until day 4.5. These results were compatible with our previous results.<sup>5</sup> 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<sup>+</sup> cells. FACS-purified Flk1<sup>+</sup> 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<sup>+</sup> ECs and SMA<sup>+</sup> mural cells were selectively induced from Flk1<sup>+</sup> cells (Figure 3A). Almost all of CD31<sup>+</sup> ECs were also positive for another EC marker, VE-cadherin (Figure 3B and 3C). Induced SMA<sup>+</sup> mural cells, which were negative for CD31, expressed other smooth muscle markers, SM22 $\alpha$  (Figure 3D) and calponin (Figure 3E). When iPS cell-derived Flk1<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup>/CXCR4<sup>-</sup> population) and arterial ECs (CD31<sup>+</sup>/CXCR4<sup>+</sup> population) are indicated. **B**, Percentages of CD31<sup>+</sup> cells (total ECs) in total Flk1<sup>+</sup> cell-derived cells (n=3; \*P=0.02). **C**, Percentages of CD31<sup>+</sup>/CXCR4<sup>+</sup> cells (arterial ECs) in total CD31<sup>+</sup> 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<sup>+</sup> arterial EC colonies in CD31<sup>+</sup> EC colonies (n=4; \*P=0.009). **F**, Gene expressions of arterial and venous EC markers (reverse transcription polymerase chain reaction) in purified CD31<sup>+</sup>/VE-cadherin<sup>+</sup> ECs. Scale bars=100  $\mu$ m. ES cells (EB5; left) and iPS cells (20D17; right) are shown.

sisted mainly of CD31<sup>+</sup> ECs (Figure 3G). Attachment of SMA<sup>+</sup> 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.<sup>4</sup>

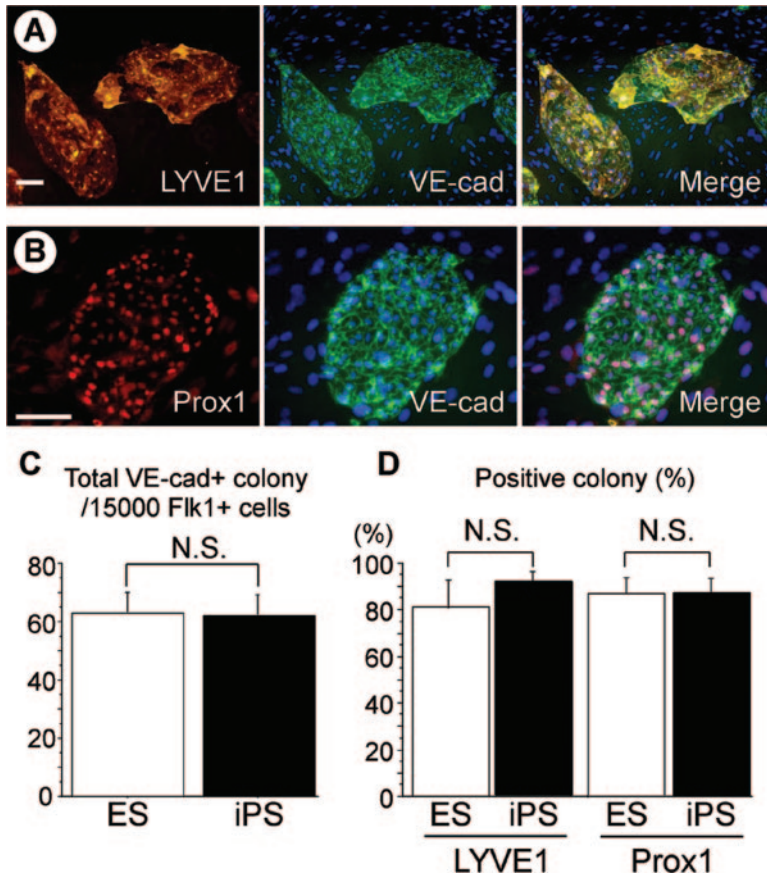
#### Arterial and Venous EC Induction From iPS Cells

We further examined arterial and venous EC differentiation. We recently reported that whereas VEGF treatment alone on Flk1<sup>+</sup> cells mainly induced venous ECs, activation of the cAMP pathway increased the total appearance of ECs and potentially induced arterial ECs.<sup>11</sup> Figure 4A shows the results of the FACS analysis of Flk1<sup>+</sup> cell-derived cells at Flk-d3. VEGF treatment alone mainly induced CD31<sup>+</sup>/CXCR4<sup>-</sup> venous ECs (top panel). In contrast, addition of 8bromo-cAMP, an analogue of cAMP, together with VEGF increased the total CD31<sup>+</sup> EC population as well as CD31<sup>+</sup>/CXCR4<sup>+</sup> arterial ECs (bottom panel). The percentage of total ECs (CD31<sup>+</sup>) in total Flk1<sup>+</sup> cell-derived cells (Figure 4B) and percentage of arterial ECs (CD31<sup>+</sup>/CXCR4<sup>+</sup>) in total ECs (CD31<sup>+</sup>) (Figure 4C) were increased by  $\approx$ 2.5-fold after 8bromo-cAMP treatment, respectively. The efficiency of

total EC and arterial EC induction from iPS cell-derived Flk1<sup>+</sup> cells was comparable to that in ES cells.<sup>11</sup> Expression of another arterial EC marker, ephrinB2, was examined by immunostaining with the use of EphB4-Fc chimeric protein.<sup>11</sup> 8Bromo-cAMP treatment induced ephrinB2<sup>+</sup> arterial ECs (Figure 4D). The number of EC colonies including ephrinB2<sup>+</sup> arterial ECs increased  $\approx$ 2.5-fold by 8bromo-cAMP treatment (Figure 4E). Gene expressions of other arterial and venous EC markers in FACS-purified ECs (CD31<sup>+</sup>/VE-cadherin<sup>+</sup> population) further confirmed arterial and venous EC differentiation (Figure 4F), that is, an increase in arterial EC markers, such as notch1,<sup>4,26</sup> Alk1,<sup>27</sup> Dll4,<sup>28</sup> and neuropilin-1,<sup>29</sup> and a reciprocal decrease in venous EC markers, such as neuropilin-2,<sup>30</sup> COUP-TFII,<sup>31</sup> and EphB4,<sup>32</sup> 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<sup>+</sup> cells on OP9 stroma cells



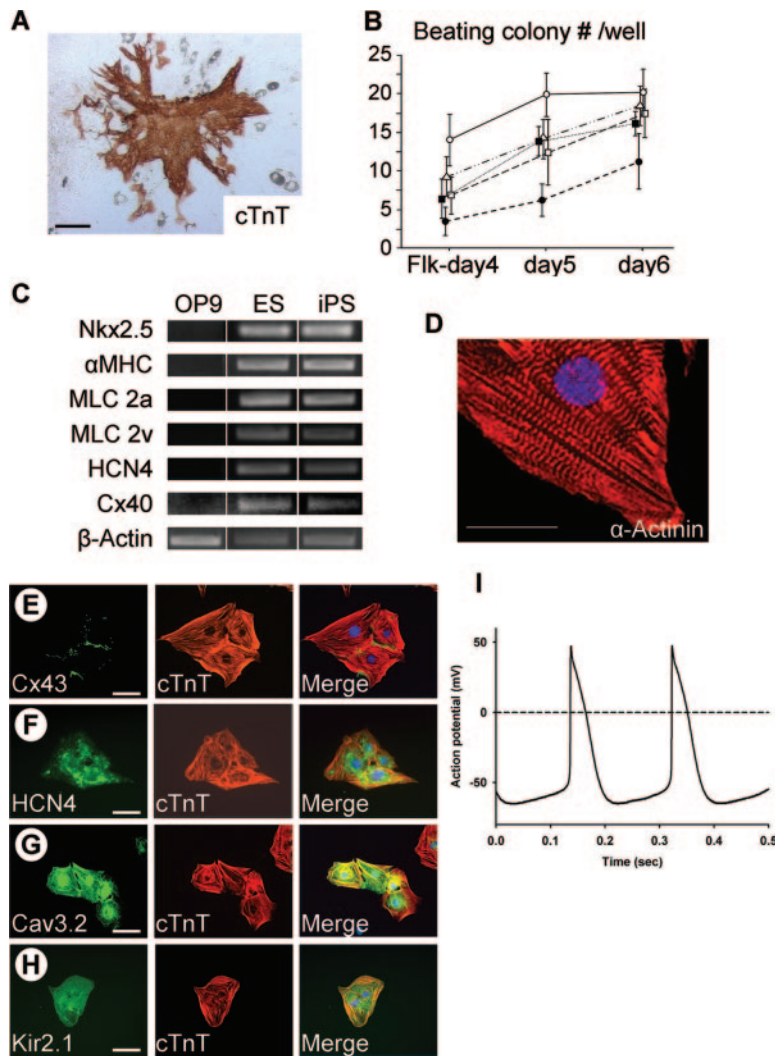
**Figure 5.** Differentiation of lymphatic ECs from iPS cells. A and B, Double immunostaining of LYVE1 (A) or prox1 (B) (lymphatic ECs; left panels, orange) and VE-cadherin (pan-ECs; middle panels, green) at Flk-d3 on OP9 cells. Right panels, Merged images with DAPI (blue). Bars=100  $\mu$ m. C, Colony numbers of VE-cadherin (VE-cad)<sup>+</sup> ECs induced from 15 000 Flk1<sup>+</sup> 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<sup>12</sup> (Figure 1B). When iPS cell-derived Flk1<sup>+</sup> cells were cultured on OP9 cells, VE-cadherin<sup>+</sup> EC sheets appeared at Flk-d3. Most of the EC colonies that appeared were positive for lymphatic EC markers LYVE-1<sup>33</sup> and prox1<sup>34</sup> (Figure 5A and 5B). The induction efficiency of total EC colonies (VE-cadherin<sup>+</sup>) (Figure 5C) and lymphatic EC<sup>+</sup> colonies (LYVE1<sup>+</sup> or prox1<sup>+</sup>) (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<sup>5</sup> to iPS cells. Similar to ES cells, when purified iPS cell-derived Flk1<sup>+</sup> 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,<sup>5</sup> 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.<sup>17,18</sup> Approximately half of MHC-GFP<sup>+</sup> cardiomyocytes induced from EMG7 ES cells could be

detected as a TMRM-high population (Figure 1A in the online-only Data Supplement). Being parallel to the beating colony number, the percentages of TMRM-high population induced from Flk1<sup>+</sup> 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 1B and 1C 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  $\alpha$ -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,<sup>35</sup> also coexisted in cTnT<sup>+</sup> cardiomyocytes (Figure 6F and 6G). An atrial and ventricular ion channel, Kir2.1, was observed in cTnT<sup>+</sup> 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  $\alpha$ -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  $\mu$ m (A), 25  $\mu$ m (D), 50  $\mu$ m (E to H).

ized spontaneously (Figure 6I). Beating rate was  $3.59 \pm 1.08$  Hz, minimum potential was  $-66.89 \pm 6.07$  mV, and maximum potential was  $44.34 \pm 3.58$  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.

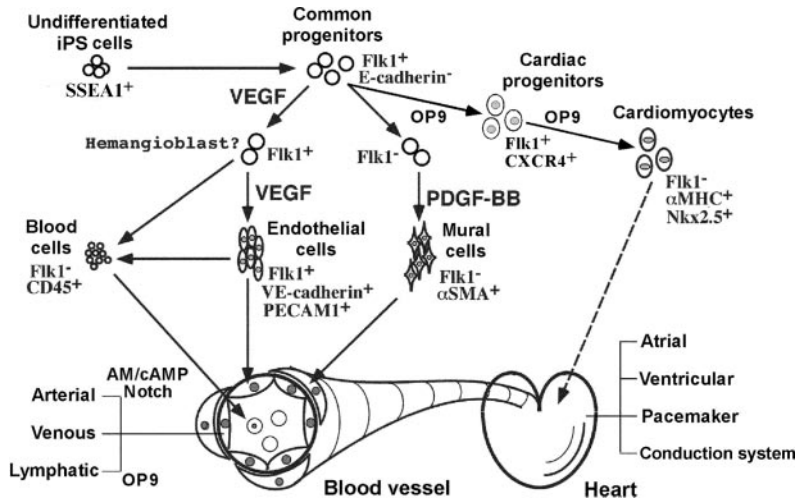
### Discussion

In this report, we showed that various cardiovascular cells could be directionally and systematically induced from mouse iPS cells. CD45<sup>+</sup> hematopoietic cells were also induced from iPS cell-derived Flk1<sup>+</sup> cells by coculturing with OP9 cells for 3 days (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.<sup>16</sup> When we examined mRNA expressions of the transgenes during long-term differentiation culture of Flk1<sup>+</sup> 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<sup>+</sup> cells give rise to ECs in the presence of VEGF. VEGF alone induces ephrinB2<sup>-</sup> (EphB4<sup>+</sup>) venous ECs. When cAMP signaling is activated together with VEGF, ephrinB2<sup>+</sup> arterial ECs are induced. For lymphatic EC differentiation, OP9 stroma cells are required. When Flk1<sup>+</sup> 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<sup>+</sup> blood cells are also induced from Flk1<sup>+</sup> cells cultured on OP9 cells. Various cardiovascular cells are therefore systematically induced from common progenitor Flk1<sup>+</sup> 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,<sup>36,37</sup> 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<sup>38</sup> and applied that to vascular regeneration.<sup>39</sup> 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.

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### 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.