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Objective—Endothelial damage is an early requisite step for atherosclerosis after vascular injury. It has been reported that vascular wall cells can develop from induced pluripotent stem (iPS) cell–derived fetal liver kinase-1–positive (Flk-1$^+$) cells. Here, we investigated the efficacies of intravenously administered iPS cell–derived Flk-1$^+$ cells on reendothelialization and neointimal hyperplasia in a mouse model of vascular injury.

Approach and Results—Femoral arteries of KSN nude mice were injured using a steel wire. Mouse iPS cell–derived Flk-1$^+$ or Flk-1$^-$ cells were intravenously injected into those mice at 24 hours after vascular injury. Delivery of iPS cell–derived Flk-1$^+$ cells significantly attenuated neointimal hyperplasia compared with controls. Evans blue staining of the injured vessel revealed that administration of iPS cell–derived Flk-1$^+$ significantly enhanced reendothelialization compared with the Flk-1$^-$ cell control group. Recruitment of PKH26-labeled iPS cell–derived Flk-1$^+$ cells to the site of injury was also detectable. Expression level of CXCR4 in iPS cell–derived Flk-1$^+$ cells was 7.5-fold higher than that of iPS cell–derived Flk-1$^-$ cells. Stromal cell-derived factor-1α treatment significantly enhanced adhesion and migration of iPS cell–derived Flk-1$^+$ cells to the endothelia, but these were not observed in Flk-1$^-$ cells.

Conclusions—Intravenously administered iPS cell–derived Flk-1$^+$ cells are recruited to the site of vascular injury, thereby enhancing reendothelialization followed by suppression of neointimal hyperplasia. Administration of iPS cell–derived Flk-1$^+$ cells is a potentially useful therapeutic means for vascular dysfunction and prevention of restenosis after angioplasty. **(*Arterioscler Thromb Vasc Biol*. 2013;33:2218-2221.)**

Key Words: hyperplasia ■ induced pluripotent stem cells ■ vascular system injuries

Vascular injury induces a variety of pathological consequences such as loss of endothelia, followed by thrombus formation, and neointimal thickening.[1–3] Thus, reendothelialization is an important therapeutic strategy for achieving vascular repair in subjects with coronary artery disease undergoing mechanical revascularization. We and others have previously shown that in vivo administration of endothelial progenitor cells (EPCs) can induce both reparative angiogenesis and reendothelialization.[4–6]

Induced pluripotent stem (iPS) cells were first generated from mouse skin fibroblasts by introducing 4 transcriptional factors.[7] iPS cells can differentiate into various cell types, including endothelial cells and vascular smooth muscle cells, and vascular endothelial growth factor receptor fetal liver kinase-1–positive (Flk-1$^+$) cells can give rise to vascular wall cells.[8,9] Thus, iPS cell–derived Flk-1$^+$ cells are called vascular progenitor cells that can supply EPCs. Recently, we demonstrated that transplantation of iPS cell–derived Flk-1$^+$ cells enhanced ischemia-induced angiogenesis in vivo.[10,11] However, the therapeutic efficacy of iPS cells for reendothelialization and prevention of neointimal formation has not been examined.

Accordingly, we investigated the effects of intravenously transfused iPS cell–derived Flk-1$^+$ cells on reendothelialization and neointimal formation using a well-established mouse model of vascular injury.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Mouse Model of Vascular Injury

Male KSN athymic nude mice were obtained from SLC Co Ltd (Nagoya, Japan). Study protocols were approved by the Institutional Animal Care and Use Committee of Nagoya University. Eight-week-old mice were subjected to arterial wire injury as previously described.[12] Briefly, the left femoral arteries of these mice were injured using a 0.38-mm steel wire (No. C-SF-15-15, Cook, Bloomington, IN) inserted from the lumen. The wire was left in place for 1 minute to denude and dilate the artery. iPS cell–derived Flk-1$^+$ cells (1×10^5 or 1×10^6 cells/mouse), Flk-1$^-$ cells (1×10^6 cells/mouse), and phosphate-buffered saline as a control were injected via the jugular vein 1 day after vascular injury.

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Results

iPS Cell–Derived Flk-1$^+$ Cells Attenuate Neointimal Hyperplasia After Vascular Injury

To first investigate the impact of iPS cell–derived Flk-1$^+$ on thickening of the neointima induced by vascular injury, KSN mice were treated intravenously with iPS cell–derived Flk-1$^+$ cells (1×10^5 or 1×10^6 cells/mouse), Flk-1$^-$ cells (1×10^6 cells/mouse), and phosphate-buffered saline as a control followed by arterial wire injury. We detected no tumor formation in mice transfused with iPS cell–derived Flk-1$^+$ cells and Flk-1$^-$ cells throughout the 60-day observation period. Figure 1A shows representative hematoxylin–eosin–stained sections of femoral arteries from iPS cell–derived Flk-1$^+$-treated, iPS cell–derived Flk-1$^-$-treated, and control mice at 21 days after vascular injury. iPS cell–derived Flk-1$^+$-treated mice (1×10^6 cells/mouse) showed a significant decrease in the intima:media ratio, compared with either iPS cell–derived Flk-1$^-$-treated or phosphate-buffered saline-treated control mice (Figure 1B).

Because reendothelialization after vascular injury plays an important role in the prevention of neointimal thickening,[3] we assessed the extent of endothelial repair in injured arteries on postoperative day 7 by means of Evans blue staining. Figure 1C shows representative photomicrographs of femoral arteries stained with Evans blue at 7 days after vascular injury from iPS cell–derived Flk-1$^+$-treated (1×10^6 cells/mouse), iPS cell–derived Flk-1$^-$-treated (1×10^6 cells/mouse), and control mice (Figure 1C). Quantitative analysis of the areas stained and not stained with Evans blue dye revealed that iPS cell–derived Flk-1$^+$ treatment significantly accelerated reendothelialization of the injured arteries, as compared with iPS cell–derived Flk-1$^-$ treatment and the control (Figure 1D). Furthermore, we analyzed the extent of reendothelialization by staining for the endothelial cell marker CD31 in arterial lumen at day 7 after injury (Figure 1E). Quantitative analysis of CD31$^+$ cells in the lumen of injured vessels revealed reendothelialization was significantly enhanced by iPS cell–derived Flk-1$^+$ treatment compared with iPS cell–derived Flk-1$^-$ treatment and the control (Figure 1E).

CXCR4 Expression in iPS Cell–Derived Flk-1$^+$ Cells Contributes to Adhesion and Migration Activities

To test whether transfused iPS cell–derived Flk-1$^+$ cells contribute to reendothelialization, PKH26-labeled iPS cell–derived Flk-1$^+$ or Flk-1$^-$ cells were injected into the jugular vein 1 day after vascular injury. Immunohistochemical

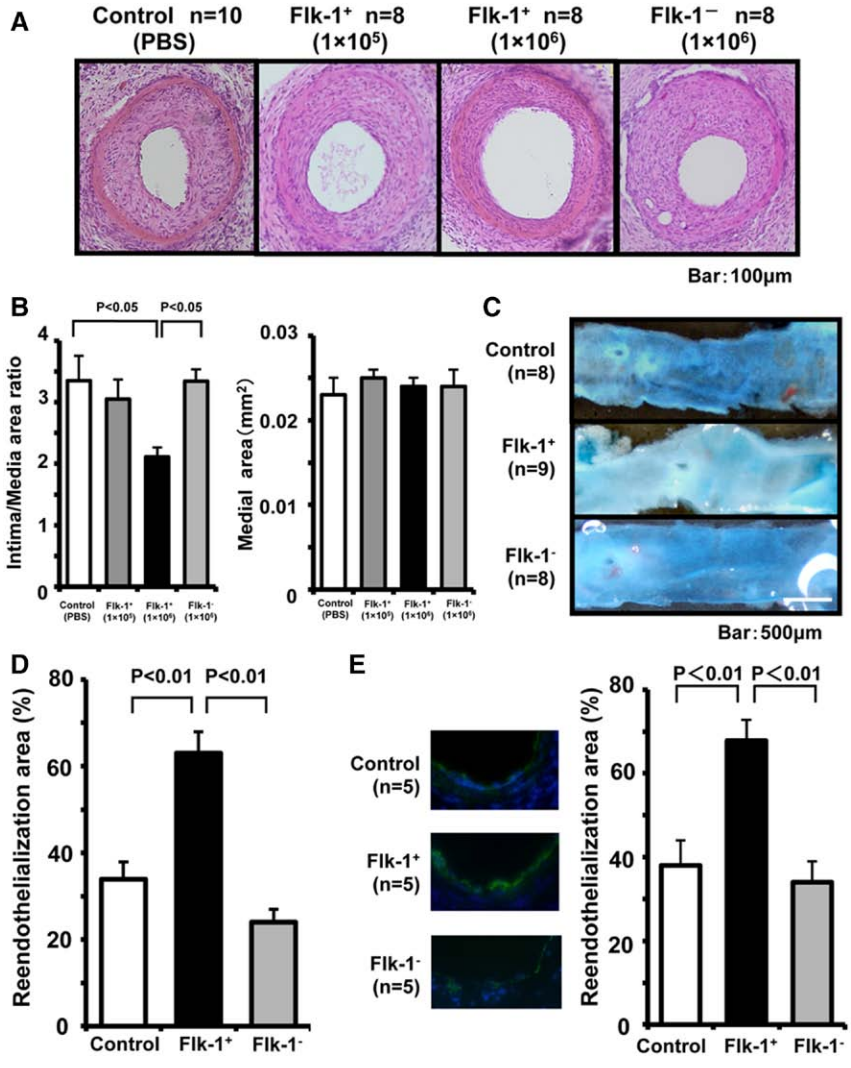

Figure 1. Systemic delivery of induced pluripotent stem (iPS) cell–derived fetal liver kinase-1–positive (Flk-1$^+$) cells attenuates neointimal hyperplasia after vascular injury. **A**, Representative hematoxylin–eosin sections of femoral arteries from iPS cell–derived Flk-1$^+$ cell–treated (1×10^5 or 1×10^6 cells/mouse), Flk-1$^-$ cell–treated (1×10^6 cells/mouse), and phosphate-buffered saline (PBS)-treated (control) KSN mice at 21 days after vascular injury. **B**, Quantitative analysis of the I/M ratio (the ratio of intimal area/medial area; **left**) and medial area (**right**). Results are shown as mean±SEM. **C**, Representative photomicrographs of femoral arteries stained with Evans blue at 7 days after vascular injury. The area stained blue corresponds to the area not reendothelialized. **D**, Quantitative analysis of the percent reendothelialization. Results are shown as mean±SEM. **E**, Reendothelialization by staining for the endothelial cell marker CD31 in arterial lumen. Representative photomicrographs of staining for CD31 in arterial lumen from iPS cell–derived Flk-1$^+$ cell–treated (1×10^6 cells/mouse), Flk-1$^-$ cell-treated (1×10^6 cells/mouse), and PBS-treated (control) KSN mice at 7 days after vascular injury (**left**). Quantitative analysis of the luminal stained with CD31 (**right**). Total nuclei were identified by 4',6-diamidino-2-phenylindole (DAPI) counterstaining (blue).

analysis revealed PKH26-labeled iPS cell–derived Flk-1$^+$ cells at the site of endothelial injury, lining the intraluminal margin of the neointima at day 7 after vascular injury (Figure 2A). In contrast, few iPS cell–derived Flk-1$^-$ cells were detectable in the injured vessels. These cells likewise were not observed in the uninjured parts or the contralateral vessel. We also examined whether in vivo transfused iPS cell–derived Flk-1$^+$ cells can differentiate into endothelial cells using cell tracking method by PKH26. PKH26-labeled iPS cell–derived Flk-1$^+$ cells seemed to be incorporated into CD31$^+$ endothelial cells (Figure I in the online-only Data Supplement).

CXCR4 is reportedly a key molecule in regulating the homing of vascular progenitor cells.[13] CXCR4 expression in iPS cell–derived Flk-1$^+$ or Flk-1$^-$ cells was assessed by flow cytometry. The surface expression of CXCR4 was greater in iPS cell–derived Flk-1$^+$ cells than in iPS cell–derived Flk-1$^-$ cells (Figure 2B). Quantitative analysis also revealed the CXCR4 mRNA level in iPS cell–derived Flk-1$^+$ cells was 7.5-fold greater than that in iPS cell–derived Flk-1$^-$ cells (Figure 2C). There were no significant differences in the proportions of CD31$^+$ and integrin β1–positive cells between Flk-1$^+$ cells and Flk1$^-$ cells as previously reported (Figure II in the online-only Data Supplement).[10]

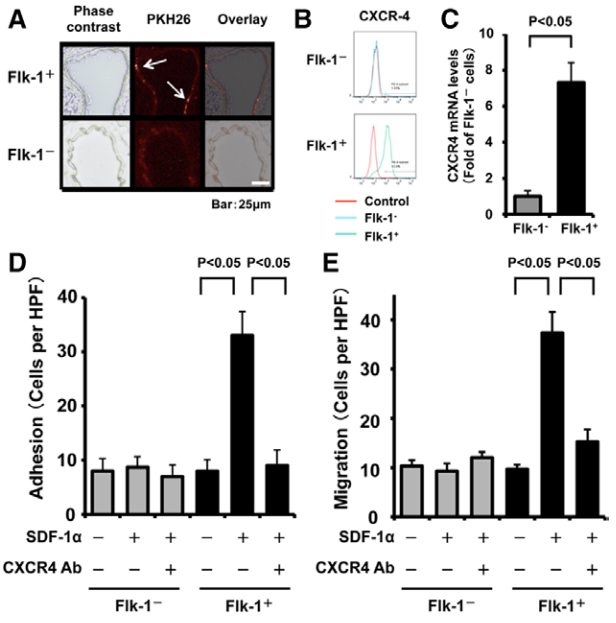

Figure 2. CXCR4 expression in induced pluripotent stem (iPS) cell–derived fetal liver kinase-1–positive (Flk-1$^+$) cells contributes to adhesion and migration activities. **A**, Representative phase contrast images (**left**), fluorescent images (**middle**), and merged images (**right**) are shown. **B**, Detection of CXCR4 on the cell surfaces of iPS cell–derived Flk-1$^+$ cells or Flk-1$^-$ cells by flow cytometry. **C**, CXCR4 mRNA levels in iPS cell–derived Flk-1$^+$ cells or Flk-1$^-$ cells by real-time polymerase chain reaction. Results are expressed as CXCR4 mRNA levels relative to GAPDH mRNA levels (n=3 in each group). **D**, Quantification analysis for stromal cell-derived factor (SDF)-1α–induced adhesion of iPS cell–derived Flk-1$^+$ cells or Flk-1$^-$ cells after preincubation with or without anti-CXCR4 antibody. **E**, Quantification analysis for SDF-1α–induced migration of iPS cell–derived Flk-1$^+$ cells or Flk-1$^-$ cells after preincubation with or without anti-CXCR4 antibody (n=3 in each group). HPF indicates high power-field.

To further investigate the relevance of CXCR4 surface expression in the homing of iPS cell–derived Flk-1+ cells, we performed an adhesion assay. Basal adhesion capacity did not differ between iPS cell–derived Flk-1$^+$ and Flk-1$^-$ cells. The adhesion capacity of iPS cell–derived Flk-1$^+$ cells was dramatically increased by stimulation with the CXCR4 ligand, stromal cell-derived factor (SDF)-1α, whereas SDF-1α stimulation had no effect in the adhesion capacity of iPS cell–derived Flk-1$^-$ cells (Figure 2D). The SDF-1α–induced adhesion activity of iPS cell–derived Flk-1$^+$ cells was blocked by preincubation with an anti-CXCR4 neutralizing antibody (Figure 2D). In addition, we confirmed that SDF-1α is expressed in media and neointima at day 7 after vascular injury in vivo as previously described (Figure III in the online-only Data Supplement).[13,14]

Finally, we investigated the migration capacity of iPS cell–derived Flk-1$^+$ cells in response to SDF-1α in a transwell migration assay. The numbers of spontaneous migrations did not differ between iPS cell–derived Flk-1$^+$ and Flk-1$^-$cells. However, iPS cell–derived Flk-1$^+$ cells showed an increased response to the SDF-1α–mediated chemotactic effect as compared with iPS cell–derived Flk-1$^-$cells (Figure 2E). Preincubation with anti-CXCR4 neutralizing antibody diminished the SDF-1α–mediated migration of iPS cell–derived Flk-1$^+$ cells (Figure 2E).

Discussion

The present study provides the first evidence that iPS cell–derived Flk-1$^+$ cells, or iPS cell–derived vascular progenitor cells, function to prevent the neointimal thickening after arterial injury. Intravenously transfused iPS cell–derived Flk-1$^+$ cells enhanced reendothelialization associated with decreased neointimal hyperplasia in mechanically injured arteries in vivo.

We recently showed that direct local implantation of mouse iPS cell–derived Flk-1$^+$ cells enhanced ischemia-induced angiogenesis in a mouse model of hindlimb ischemia.[10,11] Implanted Flk-1$^+$ cells derived from mouse iPS cells differentiated into endothelial cells in the chronic phase.[10] Intramyocardial transplantation of iPS cell–derived Flk-1$^+$ cells also stimulated angiogenesis and improved cardiac function after myocardial infarction in mice.[15] In the present study, intravenously transfused iPS cell–derived Flk-1$^+$ cells were recruited to the site of vascular injury in mice, resulting in enhanced reendothelialization associated with decreased neointimal hyperplasia. Thus, the sorting of iPS cells using the cell surface marker, Flk-1, is a potentially useful strategy for vascular regenerative medicine, including angiogenesis and reendothelialization.

Previous studies demonstrated that CXCR4 in EPCs is essential for their recruitment and homing.[13] Blockade of CXCR4 function attenuated EPC recruitment to injured arteries.[16] In contrast, adenovirus-mediated overexpression of CXCR4 improved the functional properties of EPCs and enhanced reendothelialization in the injured artery.[17] The ligand of CXCR4, SDF-1α, is expressed in injured arteries with a marked mobilization of EPCs in peripheral blood, resulting in cell homing to the site of reendothelialization.[13] Treatment with an anti–SDF-1α-neutralizing

antibody delayed reendothelialization of injured arteries.[13] In the present study, we demonstrated that iPS cell–derived Flk-1$^+$ cells express high levels of CXCR4, whereas iPS cell–derived Flk-1$^-$ cells do not. Recruitment of transfused iPS cell–derived Flk-1$^+$ cells to the injured artery was confirmed in vivo. Furthermore, iPS cell–derived Flk-1$^+$ cells exhibited more substantial adhesion and migration activities compared with iPS cell–derived Flk-1$^-$ cells in vitro. Collectively, these results indicate that the SDF-1α/CXCR4 axis might be crucial for homing of iPS cell–derived Flk-1$^+$ cells after endothelial injury to induce reendothelialization and to limit neointimal hyperplasia.

In the context of possible iPS cell therapies, coating of polymer stents or systemic vascular grafts with iPS cell–derived Flk-1$^+$ cells may support endothelialization, thereby effectively preventing postangioplasty restenosis and obstructive neointimal graft insufficiency. Thus, iPS cell–derived Flk-1$^+$ cells are a potentially valuable resource for vascular disease treatment in the future.

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Disclosures

None.

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Significance

Endothelial damage is an early requisite step for atherosclerosis. Recently, induced pluripotent stem cell–derived fetal liver kinase-1–positive cells have been shown to serve as vascular progenitor cells and can supply endothelial progenitors. Here, we show that intravenously administered induced pluripotent stem cell–derived fetal liver kinase-1–positive cells can stimulate reendothelialization and suppress neointimal hyperplasia in a mouse model of vascular injury. Administration of induced pluripotent stem cell–derived fetal liver kinase-1–positive cells may be a potentially useful therapeutic means for vascular dysfunction and prevention of restenosis after angioplasty.

Materials and Methods

Materials

APC streptavidin, PE-conjugated anti-mouse CXCR4 and anti-mouse CD31 antibodies were purchased from BD Pharmingen (San Diego, CA, USA). Anti-mouse Flk-1 Biotin and PE-conjugated Integrin β1 antibodies were purchased from eBioscience (San Diego, CA, USA). Human/Mouse CXCL12/SDF-1 antibody was purchased from R & D Systems, Inc. (Minneapolis, MN, USA). Alexa Fluor 488 and 594-conjugated anti-mouse IgG antibody was purchased from Molecular Probes (Eugene, OR, USA). A PKH26 Red Fluorescent Cell Linker Kit was purchased from SIGMA-ALDRICH (St Louis, MO, USA). Murine recombinant SDF-1α/CXCL12 was purchased from PEPROTECH (Rocky Hill, NJ, USA).

Assessment of intimal hyperplasia

Three weeks after wire injury, the mice were sacrificed, by administering an overdose of sodium pentobarbital, and then perfusion-fixed at 100 mm Hg with 4% paraformaldehyde. The femoral artery was excised and embedded in paraffin after

fixation in 4% paraformaldehyde overnight at 4℃. Sections (5 μm) from the middle of segments were stained with hematoxylin and eosin. The cross sectional area of the blood vessel layers, including the intimal and medial areas, was measured with a computerized digital image analysis system (BZ-8000, Keyence, Osaka, Japan). The average of the four sections was considered to represent intimal hyperplasia[1].

Analysis of reendothelialization

The extent of re-endothelialization in injured arteries was analyzed as described previously[1-3]. A planimetric analysis of re-endothelialization after vascular injury was performed with a computerized digital image analysis system (BZ-8000, Keyence, Osaka, Japan). Mice received an intravenous injection of 0.5% Evans blue dye (20 mg/kg, Sigma) to identify non-endothelialized arterial areas with this blue stain. After perfusion-fixation with 100% methanol, the injured arteries were excised, incised longitudinally, and then photographed with a microscope (AxioVision, Carl Zeiss, Munich, Germany). The initially injured area was defined as the total surface of the harvested arterial segment. The re-endothelialized area was defined as the area not

stained with Evans blue dye. The extent of re-endothelialization (% re-endothelialization) was expressed as a percentage of the initially injured area.

We also performed immunochemical analysis at day 7 after the vascular injury. Frozen tissue sections 8 μm in thickness were prepared from each sample. These sections were incubated with primary antibody against mouse CD31. This was followed by incubation with Alexa Fluor 488-conjugated secondary antibody. The sections were counterstained with DAPI. The reendothelialized area was defined as the area covered with CD31 positive cells. The extent of reendothelialization (% reendothelialization) was expressed as a percentage of the initially injured area.

Cell tracking and immunohistochemistry

To trace transplanted cells in the injured arteries, sorted Flk1^{+} cells were labeled using the PKH26 Red Fluorescent Cell Linker Kit[4, 5], and then injected into the internal jugular vein one day after vascular injury. Infused cells were evaluated by immunohistochemical analysis 7 days after vascular injury. Frozen tissue sections 8 μm in thickness were prepared from each sample. The signals were detected and analyzed by fluorescence

microscopy.

To analyzed the expression of SDF-1α in injured arteries, the arterial sections were incubated with primary antibody reactive to SDF-1α. The sections were then incubated with Alexa Fluor 594-conjugated secondary antibody and nuclei were counterstained with DAPI.

Cell culture

Germline competent mouse iPS cell lines, termed "iPS-MEF-Ng-20D-17", which had been generated from mouse embryonic fibroblasts by introducing four factors (Oct3/4, Sox2, Klf4 and the c-Myc mutant c-Myc [T58A]) using retroviral vectors, were provided by Riken Cell Bank with the permission of Dr. S. Yamanaka. iPS cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% Knockout Serum Replacement, 1% fetal bovine serum (FBS), nonessential amino acids, 5.5 mmol/L 2-mercaptoethanol, 50 U/mL penicillin and 50 mg/mL streptomycin on feeder layers of mitomycin-C–treated mouse embryonic fibroblasts stably releasing leukemia inhibitory factor (LIF)[6]. Cell differentiation was induced as described previously[4]. In brief,

differentiation medium (DM) (α-minimum essential medium supplemented with 10% FBS and 5 × 10^{-5} mol/L 2-mercaptoethanol) was used to induce iPS cell differentiation. Flk-1^+ mesodermal cells were induced by cultivating iPS cells (plated at 1.7 × 10^3 cells/cm^2) in DM in the absence of LIF on type IV collagen-coated dishes (ASAHI GLASS CO., LTD, Tokyo, Japan).

Cell separation

Cultured cells were harvested after induction of undifferentiated iPS cells cultivated in DM on type IV collagen-coated dishes. Induced cells were stained with biotin conjugated anti-mouse Flk-1 antibody followed by APC streptavidin secondary antibody. Flk-1^+ cells were identified and sorted by fluorescence-activated cell sorting; FACS (BD FACS Aria; Becton Dickinson, Franklin Lakes, NJ, USA)[4].

Adhesion assay

iPS cell-derived Flk1^+ or Flk1^- cells were stimulated with or without SDF-1α (100 ng/mL) for 10 minutes before the assay. Then, 1 × 10^5 cells were seeded onto each well

of a 24-well plate, pre-coated with fibronectin. After 30 minutes of incubation at 37°C, plates were thoroughly washed to remove non-adherent cells. After fixation with 4% paraformaldehyde, adherent cells were stained with the DNA binding dye 4,6-diamino-2-phenylindole (DAPI) (Dojin, Kumamoto, Japan). The number of adherent cells was counted with a fluorescent microscope in five randomly selected microscope fields (×200) per well.

Cell migration assay

iPS cell-derived $Flk1^{+}$ or $Flk1^{-}$ cells (3×10^5 in 100μL) were placed in the upper chamber of the transwell system. Then, 600 μL of serum free basal medium, containing PBS or 100 ng/mL SDF-1α, were applied to the lower chamber. After 6 hours at 37°C, the filter was fixed with 4% paraformaldehyde and the upper side of the filter was carefully cleaned with a cotton swab. The cells on the lower side were stained with May-Grünwald/Giemsa and counted in six randomly chosen fields (×200) under a microscope. In some experiments, these cells were incubated with CXCR4 monoclonal antibody (10 μg/mL) for 30 minutes at 37°C prior to the addition of SDF-1α.

Reverse Transcription PCR Analysis

Total RNA from iPS cell-derived $Flk1^{+}$ or $Flk1^{-}$ cells was extracted using the RNeasy micro kit (Qiagen, Valencia, CA, USA). Reverse transcription was performed with 1 μg of RNA, random primers and MMLV reverse transcriptase (ReverTraAce-α TOYOBO, Osaka, Japan). The PCR procedure was performed with a Bio-Rad real-time PCR detection system using SYBR Green I as a double-standard DNA-specific dye. Primers; mouse CXCR4: sense, 5'-CCTCGCCTTCTTCCACTGTT-3', and antisense, 5'-CTGGGCAGAGCTTTTGAACTTG-3', mouse CD31: sense, 5'-CACAGATAAGCCCACCAGAG-3', and antisense, 5'-TGACAACCACCGCAATG-3', mouse Integrin β1: sense, 5'-AATGTTTCAGTGCAGAGCC-3', and antisense, 5'-TTGGGATGATGTCGGGAC-3'.

Statistical analysis

All analyses were performed using PASW Statistics18 software (SPSS Inc., IL, USA). Student's *t* test was performed for comparisons between two groups. One-way ANOVA was performed for comparisons among multiple groups. Prior to statistical analysis, data were tested for normal distribution using the Shapiro-Wilk test, and shown to exhibit normal distribution. A value of $p<0.05$ was considered to indicate a statistically

significant difference. All data are shown as means ± SEM.

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