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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

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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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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,

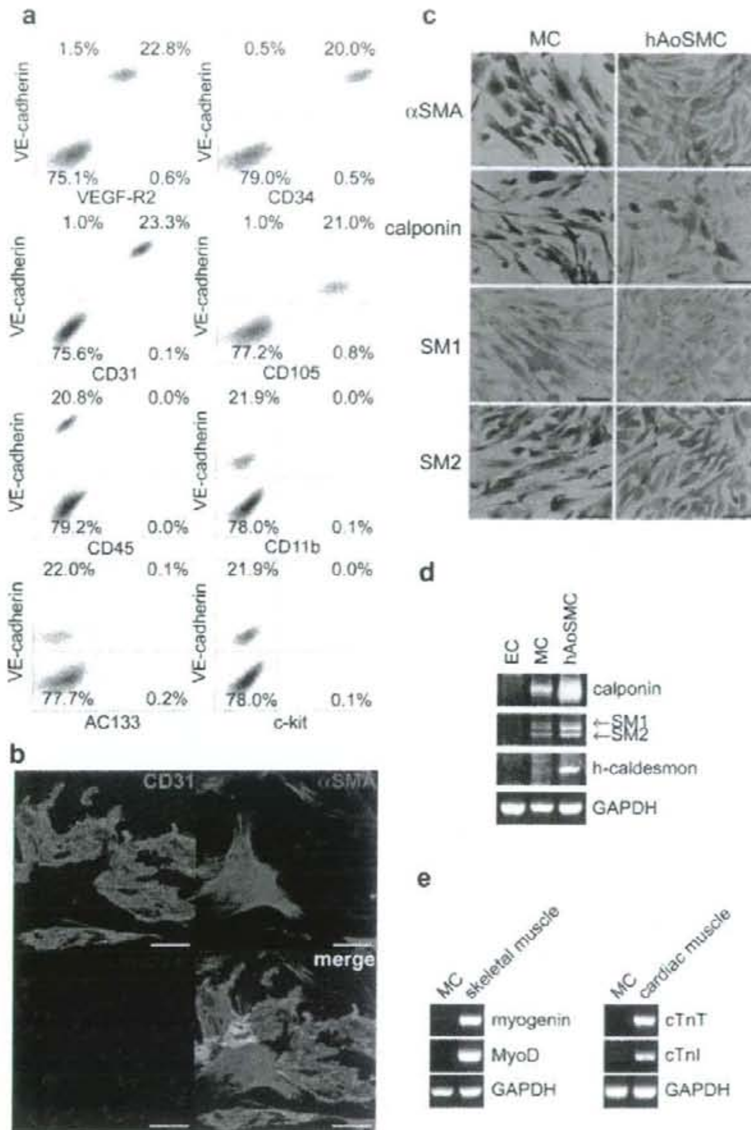

Figure 1. Characterization of transplanted human VPC-derived vascular cells. a) Flow cytometric analysis of cell surface markers on expanded human VPC-derived VEGF-R2$^+$VE-cadherin$^+$ cells (= EC). b) Immunofluorescence image of CD31 (green) and αSMA (red) with nuclear staining (blue) in expanded EC. 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

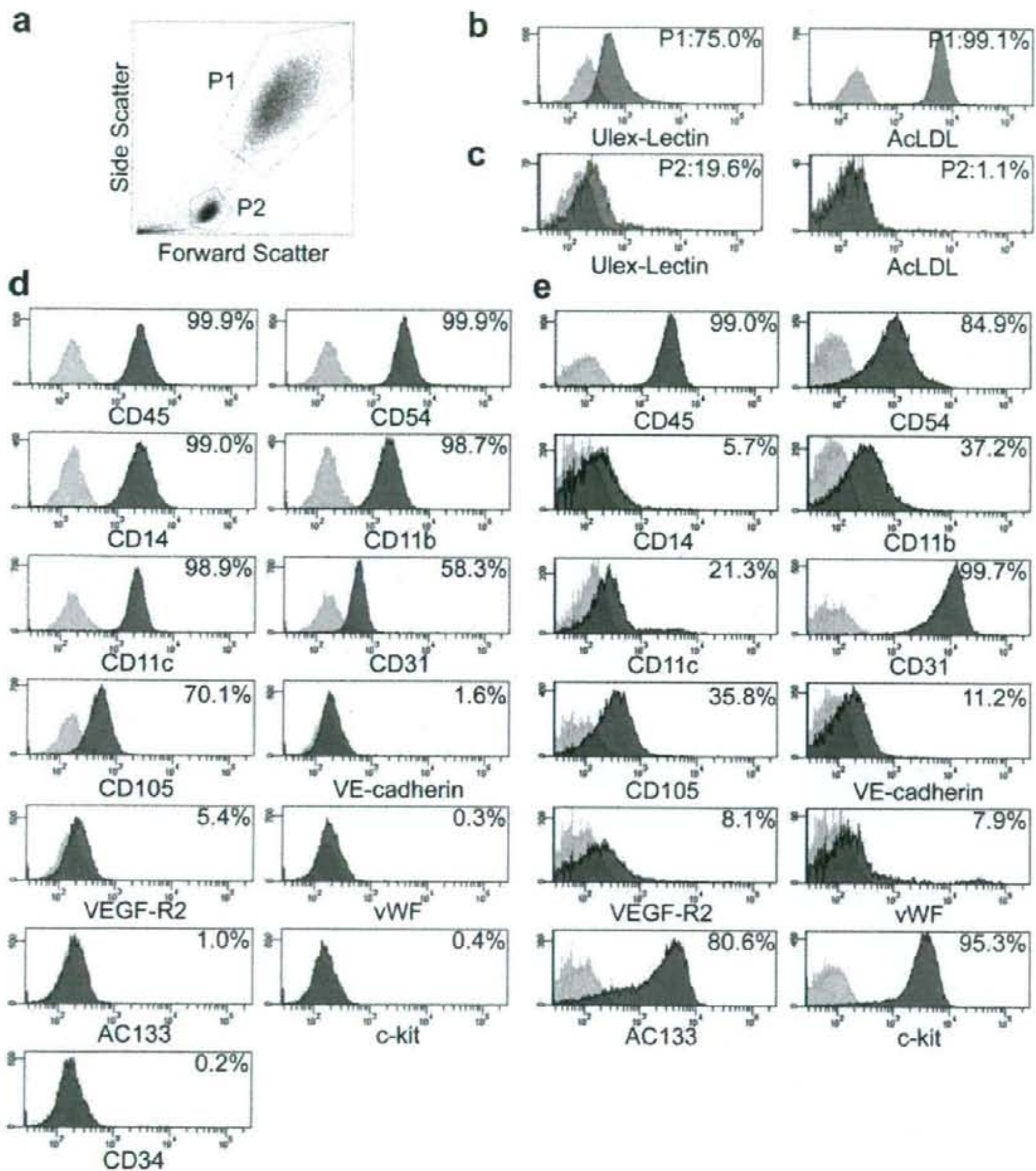

Figure 2. Characterization of peripheral blood and umbilical cord-derived EPC (pEPC and uEPC, respectively) by flow cytometer. a) Representative forward and side scatter profile of cultured pEPC. b-d) Flow cytometric analysis of ulex-lectin binding/acLDL uptake (b, c) and various cell surface markers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

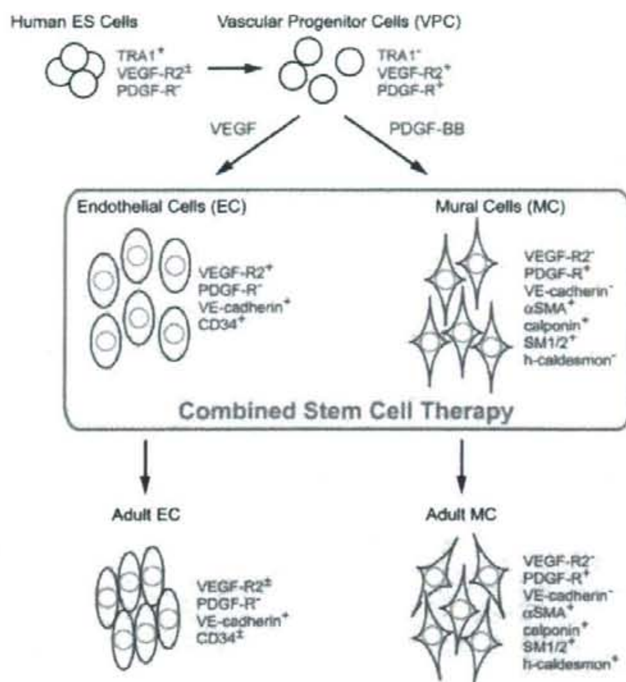

Figure 3. Possible differentiation pathway of vascular cells from human ES cells via VPC.
doi:10.1371/journal.pone.0001666.g003

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

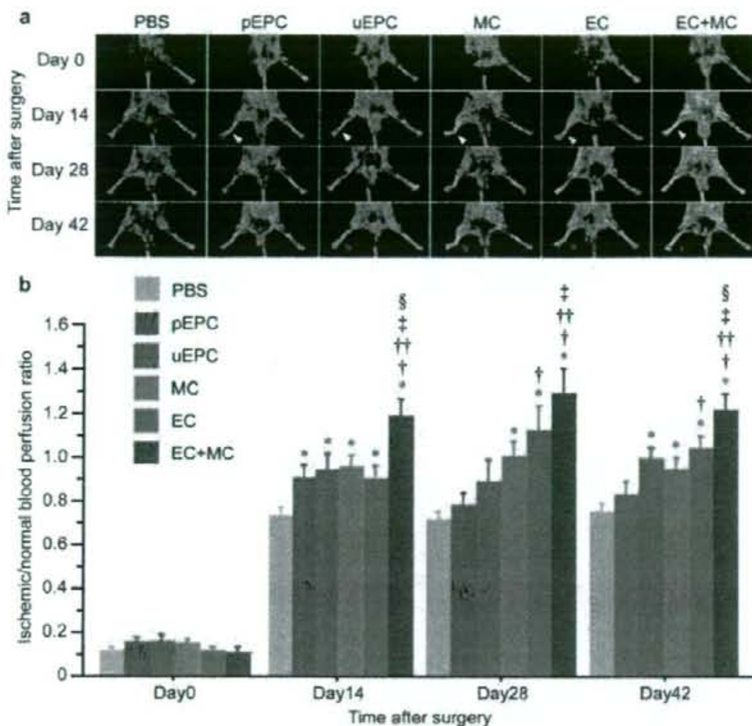

Figure 4. Augmented vascular regeneration by intra-arterial transplantation of human VPC-derived vascular cells in a murine hindlimb ischemia model. a) Serial LDPI analysis in hindlimb ischemia mice. At day 14, the blood flow of ischemic limbs in all cell transplanted groups increased significantly compared to the control group (white arrowhead). After 42 days, significant blood flow recovery was observed in the uEPC and human VPC-derived EC and/or MC-transplanted groups (red arrowhead), but not in pEPC. b) Quantitative analysis of hindlimb blood flow by calculating the ischemic/normal limb perfusion ratios after the induction of hindlimb ischemia. $^{*}P<0.05$ vs. control, $^{\dagger}P<0.05$ vs. pEPC, $^{\dagger\dagger}P<0.05$ vs. uEPC, $^{\ddagger}P<0.05$ vs. MC, $^{\S}P<0.05$ vs. EC.
doi:10.1371/journal.pone.0001666.g004

stage successfully promoted vascular regeneration in the setting of tissue ischemia. After the expansion of human VPC-derived EC and MC, when intra-arterially administered, these cells significantly augmented neovascularization in an animal model of experimentally-induced hindlimb ischemia, compared to human peripheral blood and umbilical cord-derived EPC (pEPC and uEPC). Furthermore, the combined transplantation of human VPC-derived EC and MC could markedly induce vascular regeneration, compared to the single fraction transplantation of VPC-derived vascular cells (EC or MC). We also succeeded in demonstrating that transplanted human VPC-derived vascular cells were incorporated into the host circulation as both EC and MC. These results indicate that the combined transplantation of human VPC-derived EC and MC may have utility as a novel strategy for vascular regenerative medicine.

In the present study we used human VPC-derived VEGFR2^{+} VE-cadherin^{+} cells for the expansion and transplantation of EC. VEGFR2^{+}VE-cadherin^{+} cells, obtained at day 10 of differentiation, were also positive for CD34 and therefore considered to be EC at the early differentiation stage (Figure 3) [9]. Even after 6 passages, 20~40% of these cells exhibited the expression of VEGFR2, VE-cadherin, and CD34, which indicated that they still retained the phenotype of EC at the early differentiated stage. Compared to EPC, transplantation of these EC significantly augmented ischemia-induced neovascularization. In contrast, we found that ischemia-induced neovascularization was not improved in mice receiving human aortic endothelial cells [4]. Therefore, human VPC-derived EC at the early differentiation stage might possess vascular regenerative capacity and these EC can be a valuable source for promoting vascular regeneration.

After expansion of human VPC-derived VEGFR2^{+}VE-cadherin^{+} cells, about 70% of the expanded cells were αSMA positive. However, these cells were negative for the mature mural cell markers, including calponin, SM1, SM2, and h-caldesmon (data not shown). In contrast, expanded VEGFR2^{+} VE-cadherin^{-} cells obtained from human VPC under platelet derived growth factor (PDGF)-BB stimulation were positive for αSMA, calponin, SM1, and SM2, but negative for h-caldesmon. HAoSMC was positive for all of the mature MC markers, including h-caldesmon. In another series of our experiments, the mice receiving hAoSMC transplantation exhibited no significant improvement of neovascularization after the induction of ischemic hindlimbs (data not shown). Because h-caldesmon and calponin were reported to be expressed relatively late in SMC differentiation [10], human VPC-derived MC might be at a rather early "immature" differentiation

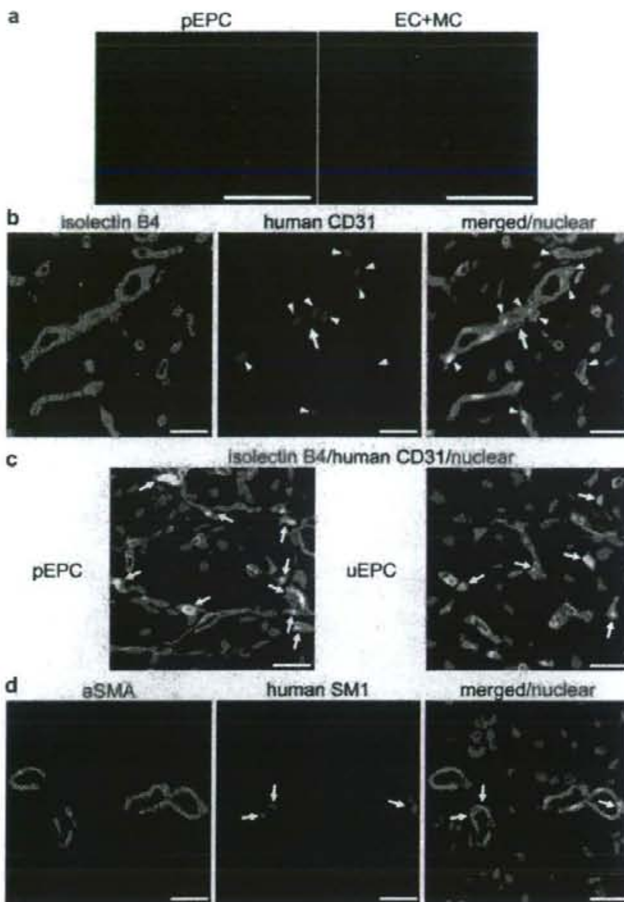

Figure 5. Incorporated human VPC-derived vascular cells at the sites of vascular regeneration. a) Transplanted CM-DiI (red) labeled pEPC or VPC-derived vascular cells in ischemic hindlimbs at day 7 were detected by the fluorescence stereomicroscope. Scale bar: 500 µm. b, c) Immunostaining of frozen sections harvested from ischemic limb tissues at day 14. Fluorescence staining of GSL I-isolectin B4 (green) and human CD31 (blue) with nuclear staining (red) in human VPC-derived EC+MC (b), pEPC, and uEPC (c) transplanted mice. Scale bar: 20 µm. d) Immunostaining of αSMA (green)/human SM1 (blue) with nuclear staining (red) in human VPC-derived EC+MC-transplanted mice at day 14. Scale bar: 20 µm.
doi:10.1371/journal.pone.0001666.g005

stage compared to hAoSMC, and thus, MC could be incorporated into the site of neovascularization.

Recently, Ferreira et al. reported that transplantation of human ES cells-derived EC into nude mice using Matrigel as scaffold contributed for the formation of blood vessels [11]. However, they did not show the direct integration of transplanted human ES cells-derived EC into host blood vessels. Judging from the double staining using intravenously injected isolectin B_4 and anti-human specific CD31 antibody, we found that the transplanted human VPC-derived EC incorporated into host circulating vessels. These transplanted EC could solely form de novo capillaries. In addition, by the double immunostaining of human SM1 and αSMA, we confirmed that transplanted human VPC-derived MC was also incorporated into host vessel walls. Therefore, transplanted human VPC-derived EC and MC structurally contributed to form new vessels in the process of vascular regeneration.

Interaction between EC and MC is essential for vascular development and maintenance of vascular stability [12,13]. Compared to only EC or MC-transplanted mice, the mice transplanted with the combined transplantation of EC and MC showed significant improvement after the induction of ischemic hindlimb. At day 42, the blood flow in the EC+MC group was significantly higher compared to only the EC or MC-transplanted groups. Not only mouse and/or human CD31 but also αSMA positive capillary density at day 42 significantly increased in the EC+MC group. We also found that the density of αSMA positive arterioles also significantly increased in the EC+MC group. These results indicated that combined transplantation of human VPC-derived EC and MC could synergistically contribute to vascular

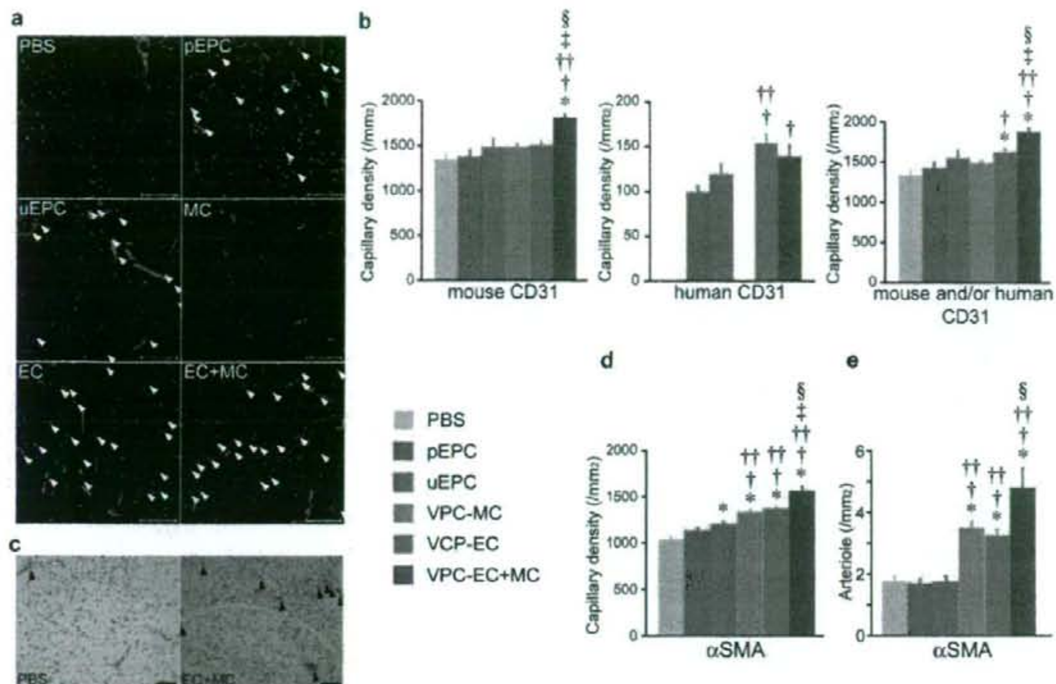

Figure 6. Immunohistochemical analysis of human VPC-derived vascular cells-transplanted murine hindlimb tissues. a) Representative fluorescent photographs of ischemic hindlimb stained for human (red) and mouse (green) CD31 at day 42. Overlapped-stained capillaries are shown in arrowhead. Scale bar: 100 μm. b) Quantitative analysis of the endothelial cell marker positive capillary density in ischemic hindlimbs at day 42. c) Representative αSMA immunostaining (brown) of ischemic hindlimbs at day 42. Scale bar. 100 μm. d) Quantitative analysis of αSMA positive capillary density in ischemic hindlimbs at day 42. e) Quantitative analysis of αSMA positive arterioles (black arrowhead) at day 42. *$P<0.05$ vs. control, †$P<0.05$ vs. pEPC, ††$P<0.05$ vs. uEPC, ‡$P<0.05$ vs. MC, §$P<0.05$ vs. EC.
doi:10.1371/journal.pone.0001666.g006

regeneration, and these MC could make mature blood vessels with adequate MC coating.

VEGFR2 is one of the most specific markers involved in the earliest stage of vascular endothelial and hematopoietic differentiation [14]. Recent reports suggest that VEGFR2$^+$ mesodermal progenitor cells also contribute muscle lineages including vascular smooth, skeletal, and cardiac muscles [1,15]. This evidence indicates the possibility that human VPC-derived MC, which were expanded from VEGFR2$^+$TRA1$^-$VE-cadherin$^-$ cells, might contain skeletal or cardiac muscle cells. However, 40-cycle RT-PCR was confirmed negative for skeletal and cardiac specific markers in expanded human VPC-derived MC. We cultured VPC-derived MC on dishes coated with collagen type IV, which is the major component of basement membrane. Previous reports described that basement membrane played an essential role in endothelial and smooth muscle cell differentiation [16]. Recently, Xiao et al. demonstrated that pretreatment of mouse ES cells with antibodies against collagen IV significantly inhibited smooth muscle cell differentiation [17]. They also demonstrated PDGF receptor-β signaling pathway plays a crucial role in ES cell-derived smooth muscle cell differentiation using PDGF receptor-β siRNA knockdown studies. Therefore, we suspected that, under the presence of collagen type IV and PDGF-BB, our human VPC-derived VE-cadherin negative cells could only differentiate to MC.

Human VPC-derived EC+MC-transplanted KSN nude mice showed considerable blood flow recovery, which led to more than 1.2 in the perfusion ratio of ischemic/non-ischemic limb. When we transplanted human VPC-derived vascular cells to immunosuppressed C57BL/6 mice, the perfusion ratio elevated to nearly 1 (data not shown). Therefore, the tendency of the blood flow recovery in C57BL/6 mice was consistent with the data of KSN nude mice, the absolute value of blood flow ratio after hindlimb ligation was different. Because both KSN nude and C57BL/6 mice received the same procedure for hindlimb ischemia, the degree of perfusion recovery after induction of hindlimb ischemia between these mice might reflect their difference in genetic background for angiogenesis, as reported by Fukino et al [18]. They demonstrated that the VEGF and VEGFR1/2 expression in response to ischemia was impaired in BALB/c mice, compared to other mouse strains (i.e, C57BL/6J or C3H/He mice). These results indicate that, because of the difference in genetic background, spontaneous collateral formation might be accelerated in our KSN nude mice compared to other strain mice.

In transplantation experiments, the number of mouse and/or human CD31 and mouse CD31-positive capillary density in the EC group was $1601.4\pm51.4/\text{mm}^2$ and 1470.1 ± 41.6/mm2, respectively. This difference in capillary density ($1601.4-1470.1=131.3$) was consistent with the number of human CD31-positive capillary density ($149.9\pm12.3/\text{mm}^2$). However,

compared to the EC group, the EC+MC group showed significant augmentation in mouse and/or human CD31 positive capillary density without the increase of human CD31 positive capillary density. One possible reason for this discrepancy is paracrine effects of transplanted human VPC-derived vascular cells might accelerate angiogenesis in ischemic tissues. We demonstrated that cultured human VPC-derived vascular cells expressed several angiogenic factors including VEGF, bFGF, HGF and PDGF-BB, and the release of VEGF from human VPC-derived vascular cells was significantly upregulated after transplantation (data not shown) [4]. Therefore, in addition to the structural contribution of transplanted human VPC-derived vascular cells into the host vascular network, the paracrine effects of these cells might enhance vascular regeneration in tissue ischemia.

Several reports described the contribution of pEPC or uEPC to neovascularization in tissue ischemia [6,7]. However, it has not been clearly demonstrated whether transplanted EPC augment neovascularization through differentiation and proliferation into mature EC or indirectly through paracrine stimulation of resident EC proliferation. Rehamn et al. demonstrated that the majority of pEPC, which were positive for acLDL and ulex-lectin, expressed monocyte/macrophage markers, and only a minority cell fraction expressed the specific endothelial or stem/progenitor markers [8]. They also demonstrated that pEPC did not proliferate, but released several potent angiogenic growth factors. In this study, we confirmed that a low percentage of cultured pEPC and uEPC expressed endothelial makers. A considerable number of pEPC or uEPC were localized inside the capillary lumen, not in the vessel wall. In addition, we found that VEGF mRNA expression in transplanted EPC was significantly higher compared with before transplantation (data not shown). These results suggest that the majority of EPC might have little ability to proliferate or differentiate to endothelial linage, and their angiogenic effects could be attributed to angiogenic factors secreted from transplanted EPC.

In conclusion, we have shown that human VPC-derived cells could effectively differentiate and be expanded to EC and MC. Combined transplantation of these "immature" VPC-derived vascular cells, unlike "mature" somatic EC and MC, augmented reparative neovascularization and contributed to make newly formed vessels in the murine hindlimb ischemia model far more effectively compared to EPC transplantation. Thus, human ES cells-derived EC and MC can be used as the new promising cell source for therapeutic vascular regeneration in patients with tissue ischemia in order to realize a novel combined stem cell therapy.

Materials and Methods

Differentiation of Human VPC-derived EC and MC

Maintenance of human ES cell line (HES3) was as previously described [19]. To induce VPC, undifferentiated ES cells were cultured on an OP9 feeder cell line as reported [3,4]. To obtain human VPC-derived EC, VEGFR2$^+$TRA1$^-$VE-cadherin$^+$ cells were sorted by fluorescence activated cell sorter (FACSAria; Becton Dickinson, Bedford, MA) at day 10 of differentiation, and cultured on type IV collagen-coated dishes (Becton Dickinson) in the presence of 10% FCS and 50ng/ml VEGF (human VEGF165, Peprotech Inc, Rocky Hill, NJ). After 6 passages of these cells, we re-sorted VE-cadherin$^+$ cells for transplantation of human VPC-derived EC. To expand human VPC-derived MC, sorted VEGFR2$^+$TRA1$^-$VE-cadherin$^-$ cells derived from VPC at day 8 were re-cultured on type IV collagen-coated dishes with 1% FCS and 20ng/ml human PDGF-BB (Peprotech Inc). We transplanted these human VPC-derived MC after 6 passages.

Preparation of Human EPC

Peripheral MNC-derived EPC (pEPC) were obtained from healthy volunteer, as previously described [6]. To confirm EPC phenotype, cells were detached with cell dissociation buffer (Invitrogen, Carlsbad, CA) and incubated with DiI-labeled acLDL (Invitrogen) and FITC-labeled Ulex europaeus agglutinin I (ulex-lectin) (Sigma-Aldrich, St. Louis, MO) for 1 hour. These cells were analyzed by FACSAria to be confirmed as EPC [6,8].

Umbilical cord blood-derived CD34$^+$ EPC (uEPC) were isolated from human umbilical cord blood, which were obtained from Cell Bank, RIKEN BioResource Center (Tukuba, Japan). CD34$^+$ cells were separated by a magnetic bead separation method using autoMACS system with direct CD34$^+$ progenitor cell isolation kit (Miltenyi Biotec GmbH, Gladbach, Germany) [7]. Protocols for using human umbilical cord blood were approved by the Ethics Committee of Kyoto University Graduate School of Medicine.

Characterization of VPC-derived Vascular Cells and EPC

To evaluate the surface marker phenotype of VPC-derived vascular cells and EPC, these cells were detached by cell dissociation buffer with or without collagenase (Wako Pure Chemical Industries, Osaka, Japan) and labeled for 15 minutes at 4°C with various fluorescence-conjugated monoclonal antibodies (Table 1) [20]. Cells were washed and analyzed on FACSAria flow cytometer with ≥30,000 events stored.

For the staining of cultured VPC-derived vascular cells on dishes, cells were stained with anti-human CD31 (WM59) (Becton Dickinson) antibody and several smooth muscle specific markers, as shown in Table 2. Cultured hAoSMC (Cambrex, East Rutherford, NJ) were used to obtain positive control staining.

For RT-PCR analysis, total RNA was prepared with RNeasy Mini Kit (QIAGEN Inc., Valencia, CA), and RT-PCR was performed by TaKaRa One Step RNA PCR Kit (TaKaRa Bio Inc., Otsu, Japan). Total RNA from human heart and skeletal muscle were purchased from Clontech (Mountain View, CA). Primers are listed in Table 3 [21–23].

Hindlimb Ischemia Model and Cell Transplantation

After 8-week-old male KSN/Slc nude mice (Japan SLC, Shizuoka, Japan) were anesthetized with pentobarbital (80mg/kg, i.p.), the right femoral vein was ligated. To transplant vascular cells intra-arterially, we injected these cells in 100 µl PBS into the right femoral artery. Immediately after the cell injection, the right femoral artery and vein were ligated and excised [24]. Animal procedures were performed according to Kyoto University standards for animal care.

Assessment of Transplanted Animals

The measurement of hindlimb blood flow was performed with a LDPI analyzer (Moor Instruments, Devon, United Kingdom), as previously described [24].

At arbitrary time points, biotin conjugated Griffonia simplicifolia lectin (GSL) I-isolectin B_4 (Vector Laboratories, Burlingame, CA) in 100 µl PBS was injected into the portal vein 15 minutes before sacrifice. Cryostat sections (10 µm thick) of the ischemic lower legs were stained with anti-mouse/human CD31 (clone WM59/Mec13.3) (Becton Dickinson) or anti-αSMA/human SM1 (clone 1A4/3F8) (DakoCytomation, Glostrup, Denmark/Yamasa Co., Tokyo, Japan) antibodies. For biotinylated isolectin B_4 staining to detect circulating vessels, sections were incubated with streptoavidin conjugated Alexa Fluor dye (Invitrogen).

Capillary densities were examined by counting the number of capillaries stained with anti-human and/or mouse CD31 or anti-

Table 1. Fluorescence-conjugated monoclonal antibodies used for FACS analysis

Antibody	Specificity	Clone	Conjugated fluorescence	Supplier
VEGF-R2	Endothelial cells	KM1998	Alexa Fluor 647	A generous gift of Prof. M. Shibuya, Tokyo University (Ref.20)
VE-cadherin	Endothelial cells	55-7H1	FITC or PE	Becton Dickinson, Bedford, MA
von Willebrand Factor (vWF)	Endothelial cells	2F2-A9	Alexa Fluor 488	Becton Dickinson, Bedford, MA
CD31 (PECAM1)	Endothelial cells or Monocytes	WM59	Alexa Fluor 488	eBioscience, San Diego, CA
CD105 (Endoglin)	Endothelial cells or Monocytes	266	Alexa Fluor 647	Becton Dickinson, Bedford, MA
CD11b (Mac1)	Monocytes	ICRF44	PE	eBioscience, San Diego, CA
CD11c	Monocytes	B-ly6	FITC	Becton Dickinson, Bedford, MA
CD14	Monocytes	M5E2	APC	Becton Dickinson, Bedford, MA
CD45	Panleukocytes	HI30	PE	Becton Dickinson, Bedford, MA
CD54 (ICAM-1)	Panleukocytes	581	PE	Becton Dickinson, Bedford, MA
AC133	Stem/Progenitor cells	AC133	PE	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
c-kit	Stem/Progenitor cells	YB5.B8	APC	Becton Dickinson, Bedford, MA
CD34	Stem/Progenitor cells	581	FITC	Becton Dickinson, Bedford, MA

doi:10.1371/journal.pone.0001666.t001

Table 2. Smooth muscle specific antibodies used for analysis

Antibody	Specificity	Clone	Supplier
Alpha smooth muscle actin (αSMA)	Human & mouse	1A4	DakoCytomation Denmark A/S, Glostrup, Denmark Sigma-Aldrich, St. Louis, MO
Calponin	Human	CALP	DakoCytomation Denmark A/S, Glostrup, Denmark
Smooth muscle myosin heavy chain 1 (SM1)	Human	3FB	Yamasa Co., Tokyo, Japan
Smooth muscle myosin heavy chain 2 (SM2)	Human & mouse	1G12	Yamasa Co., Tokyo, Japan

doi:10.1371/journal.pone.0001666.t002

Table 3. Primers for reverse transcription-polymerase chain reaction

Gene		Sequence	Length (bp)
Calponin[1]	Sense	5'-CTTCATGGACGGCCTCAAAGA-3'	713
	Antisense	5'-GTAGTTGTGTGCGTGGTGGTT-3'	
Smooth muscle myosin heavy chain 1 (SM1) and 2 (SM2)[1, 2]	Sense	5'-ATGAGGCCACGGAGAGCAACGA-3'	178 (SM1)
	Antisense	5'-CCATTGAAGTCTGCGTCTCGA-3'	217 (SM2)
h-caldesmon[1]	Sense	5'-AGACAAGGAAAGAGCTGAGGCA-3'	395
	Antisense	5'-GCTGCTTGTTACGTTTCTGCTC-3'	
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[1]	Sense	5'-ACCACAGTCCATGCCATCAC-3'	452
	Antisense	5'-TCCACCACCCTGTTGCTGTA-3'	
Myogenin[3]	Sense	5'-GTGGGCGTGTAAGGTGTGTA-3'	141
	Antisense	5'-TGGTTGGGGTTGAGCAGGGT-3'	
MyoD[3]	Sense	5'-CCAAATGTAGCAGGTGTAAC-3'	142
	Antisense	5'-AGAGATAAATACAGCCCCAG-3'	
Cardiac troponin T (cTnT)[4]	Sense	5'-GGCAGCGGAAGAGGATGCTGAA-3'	150
	Antisense	5'-GAGGCACCAAGTTGGGCATGAACGA-3'	
Cardiac troponin I (cTnI)[4]	Sense	5'-CCCTGCACCAGCCCCAATCAGA-3'	250
	Antisense	5'-CGAAGCCCAGCCCGGTCAACT-3'	

[1]Ref. 21.
[2]We used a single pair of PCR primers that cover the sequence specific to SM2, because these two isoforms are produced from a single gene by alternative splicing.
[3]Ref. 22.
[4]Ref. 23.
doi:10.1371/journal.pone.0001666.t003

αSMA antibodies. Twenty (for CD31) or ten (for αSMA) random fields on two different sections (approximately 3mm apart) from each mouse were photographed and analyzed by NIH image as previously described [24].

Statistical Analysis

Results are presented as means±S.E.M. The serial changes of the hindlimb blood flow were assessed by repeated measures ANOVA, followed by Bonferoni's multiple comparison test. Comparisons among groups were tested by one-way ANOVA followed by Bonferoni's multiple comparison test. A P value <0.05 was considered significant.

Acknowledgments

The human ES cells (HES3) were provided by ES Cell International Pre Ltd., Singapore.

Author Contributions

Conceived and designed the experiments: KY HI KN MS JY TY TC KH DT KM KP NO NS NT YF. Performed the experiments: KY MS KH. Analyzed the data: KY. Wrote the paper: KY HI.

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Pathway for Differentiation of Human Embryonic Stem Cells to Vascular Cell Components and Their Potential for Vascular Regeneration

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Objective—We demonstrated previously that mouse embryonic stem (ES) cell–derived vascular endothelial growth factor receptor-2 (VEGF-*R2*)–positive cells can differentiate into both vascular endothelial cells and mural cells. This time, we investigated kinetics of differentiation of human ES cells to vascular cells and examined their potential as a source for vascular regeneration.

Methods and Results—Unlike mouse ES cells, undifferentiated human ES cells already expressed VEGF-*R2*, but after differentiation, a VEGF-*R2*-positive but tumor rejection antigen 1-60 (TRA1-60)–negative population emerged. These VEGF-*R2*-positive but tumor rejection antigen 1-60–negative cells were also positive for platelet-derived growth factor receptor α and β chains and could be effectively differentiated into both VE-cadherin$^+$ endothelial cell and α-smooth muscle actin$^+$ mural cell. VE-cadherin$^+$ cells, which were also CD34$^+$ and VEGF-*R2*$^+$ and thought to be endothelial cells in the early differentiation stage, could be expanded while maintaining their maturity. Their transplantation to the hindlimb ischemia model of immunodeficient mice contributed to the construction of new blood vessels and improved blood flow.

Conclusions—We could identify the differentiation process from human ES cells to vascular cell components and demonstrate that expansion and transplantation of vascular cells at the appropriate differentiation stage may constitute a novel strategy for vascular regenerative medicine. **(*Arterioscler Thromb Vasc Biol.* 2007;27:2127-2134.)**

Key Words: angiogenesis ■ developmental biology ■ embryonic stem cells ■ vascular biology ■ endothelium

Pluripotent embryonic stem (ES) cells are gaining attention as promising cell sources for regenerative medicine, especially after the establishment of human ES cells.[1] Because the knockout animal research approach is not applicable to humans, investigating human cell development/differentiation using human ES cells is more helpful. These cells possess a number of characteristics distinct from those of mouse ES cells, such as surface antigens, leukemia inhibitory factor independency, and long doubling time.[1] We demonstrated previously that mouse ES cell–derived vascular endothelial growth factor (VEGF) receptor-2 (VEGF-*R2*)–positive cells can differentiate into both vascular endothelial cells (ECs) and mural cells (MCs), the latter composed of pericytes and vascular smooth muscle cells. We termed these mouse VEGF-*R2*$^+$ cells "vascular progenitor cells" (VPCs).[2] We also showed that VEGF and the vasodilating peptide adrenomedullin, which, as we reported previously, enhances angiogenesis,[3] play important roles in EC differentiation from these mouse VEGF-*R2*$^+$ cells.[4] However, recent studies have shown that, in undifferentiated human ES cells, unlike in mouse ES cells, VEGF-*R2* is expressed and continues to be expressed during differentiation associated with embryoid body formation.[5,6] To further clarify the vascular differentiation process in human beings and to determine the possible clinical application of ES cells to vascular regeneration, investigation of human ES cells is essential. It was also found that CD31$^+$ cells could be isolated from human embryoid bodies, indicating that they can act as ECs.[6] However, a precise analysis of the differentiation process

Original received February 27, 2007; final version accepted June 9, 2007.

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DOI: 10.1161/ATVBAHA.107.143149

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from human ES cells to EC or other vascular cell components, such as MC, in the embryoid body differentiation system has so far not been possible.

In the study reported here, we identified the differentiation kinetics of human ES cells to vascular cell components by using our in vitro 2D differentiation system. Furthermore, we succeeded in establishing new human cell sources derived from human ES cells, which may be used for therapeutically effective transplantation in vivo.

Methods

Cell Culture

ES cells were maintained as described.[1,7] OP9 feeder cell lines were established and maintained as described,[8] whereas their growth was inactivated by mitomycin C.

To induce differentiation, ES cells were dissociated into small colonies with the aid of 0.1% collagenase (Wako) and cultured on an OP9 feeder layer in a differentiation medium (minimal essential medium, GIBCO) supplemented with 5×10^{-5} M 2-mercaptoethanol with 10% FCS. Sorted cells were recultured on a collagen IV–coated dish in the differentiation medium with the addition of 10% FCS, VEGF (100 ng/mL; PeproTech EC Ltd), or platelet-derived growth factor (PDGF)-BB (10 ng/mL) (PeproTech EC Ltd).

Flow Cytometry and Cell Sorting

Cells were detached by cell dissociation buffer (GIBCO) with or without collagenase and labeled with various fluorescence-conjugated monoclonal antibodies (please see http://atvb.ahajournals.org). Flow cytometry analysis and cell sorting were performed as described.[2,4,8]

Immunohistochemistry

Cultured cells were stained with various monoclonal antibodies (please see http://atvb.ahajournals.org) as described.[2,4] The immunofluorescence photographs were taken with a confocal laser-scanning microscope (LSM5-Pascal, Carl Zeiss).

Hindlimb Ischemia Model

Eight-week–old KSN/Slc and BALB/c Slc nude mice were purchased from SLC Japan. After anesthetization with pentobarbital (80 mg/kg IP), the right femoral vein was ligated. We injected 5×10^5 cells in 100 μL of PBS or 100 μL of PBS only into the right femoral artery. Immediately after the cell injection, the right femoral artery was ligated and excised.[9] Experimental procedures were performed in accordance with Kyoto University standards for animal care. Hindlimb blood flow was measured with a laser Doppler perfusion image analyzer (Moor Instruments Ltd), as described.[9] Biotin-conjugated Griffonia simplicifolia lectin I-isolectin B_4 (Vector Laboratories) was injected into the portal vein 15 minutes before sacrifice. After fixation with 4% paraformaldehyde, the ischemic lower legs were embedded in optimal cutting temperature compound (Sakura Finetechnical Co Ltd) and frozen. Capillary numbers were examined by counting the number of capillaries stained with anti-human and/or mouse CD31 antibody. Ten random fields on 2 different sections (≈3 mm apart) from each mouse were photographed and analyzed by National Institutes of Health imaging as described.[9] The vessel area and length were measured quantitatively with the Kurabo angiogenesis image analyzer (Kurabo).

Teratoma Formation Study

We transplanted 5×10^5 cells in 50 μL of PBS under the dorsal back skin of SCID mice (CLEA Japan, Inc), which are commonly used for teratoma formation for human ES cells.[1,7] After 5 months, 1 cm^2 of the skin around the point of injection was harvested, and the excised tissue was serially sectioned (10 μm) at 200-μm intervals.

Analysis of Angiogenic Factor mRNA Expression

Total cellular RNA was isolated from VPCs or ECs in the early differentiation stage (eECs) with RNAeasy Mini kit (Qiagen KK). The mRNA expression was analyzed with the One-Step RNA PCR kit (TaKaRa). Primer pairs were purchased from R&D Systems Inc. PCRs were performed as manufacturers protocols.

Measurement of Angiogenic Factors in VPC- or eEC-Conditioned Media

Cells (1×10^6) of VPCs or eECs were plated on 10-cm dishes and incubated with 5 mL of media (minimal essential medium with 0.5% bovine serum) for 72 hours. The concentrations of human VEGF, basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF) were measured by SRL, Inc. Human PDGF-BB was measured with the Human PDGF-BB Quantikine ELISA kit (R&D Systems Inc).

Statistical Analysis

Results are presented as mean±SEM. In the hindlimb ischemia model, the statistical significance was evaluated using ANOVA followed by Fisher's analysis for comparisons between 2 means. $P<0.05$ was considered significant.

Results

Differentiation Pathway of Human ES Cells to Vascular Cell Components

First, we examined the expression of VEGF-*R*2 and some putative stem cell markers on a human ES cell line, HES3, which was established at Monash University in Australia.[1] Approximately 50% of undifferentiated human ES cells expressed VEGF-*R*2, whereas these cells were also positive for AC133 and c-Kit but negative for CD34, respectively (Figure 1A). We also analyzed the expression of tumor rejection antigen (TRA) 1-60 on these human ES cells. The TRA1 antigen is expressed on the surface of human tetracarcinoma stem cells, human embryonic germ cells, and human ES cells. Thus, we used it as a marker of the human ES cell. The VEGF-*R*2$^+$ population of human ES cells was also positive for TRA1-60 (Figure 1B).

Next, we induced differentiation of human ES cells in an in vitro 2D culture on an OP9 stromal cell line. Although monkey ES cells have effectively differentiated as single cells on an OP9 layer,[10] human ES cells have not survived as single cells. We, therefore, plated small human ES cell colonies on OP9 to induce differentiation. Under these conditions, the TRA1-60$^+$ cell population gradually decreased in number during differentiation. On the other hand, a VEGF-*R*2$^+$ TRA1-60$^-$ population emerged and accounted for ≈15% of all of the cells on day 8 (Figure 1C). We confirmed the differentiation kinetics of human ES cells by using another human ES cell line, KhES-1, established by us.[7] Similar to the HES3 cell line, VEGF-*R*2 was low positive, and the VEGF-*R*2$^+$ cells were also TRA1-60$^+$ in undifferentiated KhES-1 (Figure 1D). After differentiation on an OP9 feeder layer, VEGF-*R*2$^+$ TRA1-60$^+$ cells decreased, and VEGF-*R*2$^+$ TRA1-60$^-$ cells appeared on days 8 (Figure 1E). Next, we analyzed the expression of several cell surface markers on the VEGF-*R*2$^+$ TRA1-60$^-$ population on day 8 of HES3. Flt1 was positive, c-Kit and CXCR4 were both negative. PDGR receptor (PDGFR)α and PDGFRβ were positive, AC133

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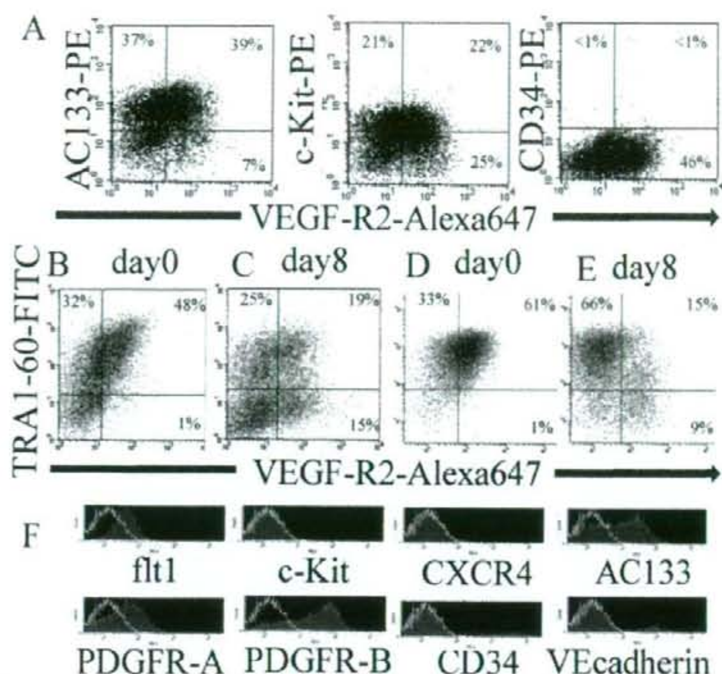

Figure 1. Flow cytometry analysis of differentiation kinetics of human ES cells. A, Expression of cell surface markers on undifferentiated human ES cells (HES3). B through E, TRA1-60 and VEGF-*R2* expression on 2 human ES cell lines (HES3 and KhES-1) during differentiation on an OP9 feeder layer. B and C, HES3; D and E, KhES-1. F, Cell surface marker expression on VEGF-*R2*$^+$ TRA1-60$^-$ cells on day 8.

was still positive, and CD34 and vascular endothelial cadherin (VE-cadherin) were negative on a large population of the VEGF-*R2*$^+$ TRA1-60$^-$ cells (Figure 1F). Monocyte markers, such as CD45, Cd11b, and CD14, were negative.

The VEGF-*R2*$^+$ TRA1-60$^-$ and VE-cadherin–negative cells were sorted by flow cytometry on day 8 and cultured on a collagen IV–coated dish without a feeder cell layer for an additional 8 days in the presence of 10% FCS and VEGF. This cell culturing condition induced the emergence of CD34$^+$, VE-cadherin$^+$, CD31$^+$, and endothelial NO synthase–positive cells (Figure 2A through 2D), which can be categorized as ECs. The rest of the cells negative for CD31 were polygonal in shape and showed α-smooth

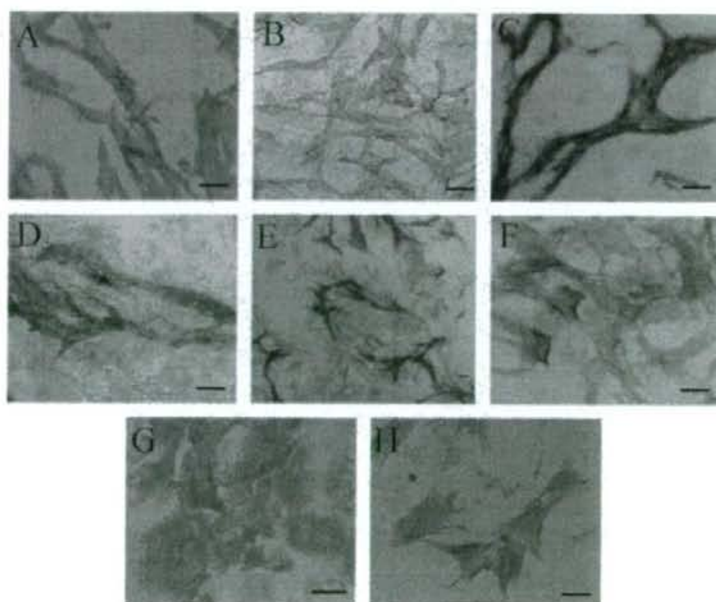

Figure 2. Immunocytological analysis of differentiation of vascular progenitor cells into vascular cells. A through D, Immunostaining for endothelial cell markers on VEGF-*R2*$^+$ TRA1-60$^-$ cells recultured with VEGF and FBS. A, CD34; B, VE-cadherin; C, CD31; D, endothelial NO synthase; E, Double immunostaining for CD31 (blue) and α-smooth muscle actin (brown). F and G, Immunostaining for MC markers on VEGF-*R2*$^+$TRA1-60$^-$ cells recultured with FBS. F, α-Smooth muscle actin; G, Calponin; H, Immunostaining for α-smooth muscle actin with treatment of PDGF-BB on VEGF-*R2*$^+$ TRA1-60$^-$ cells. Scale bars, 50 μm.

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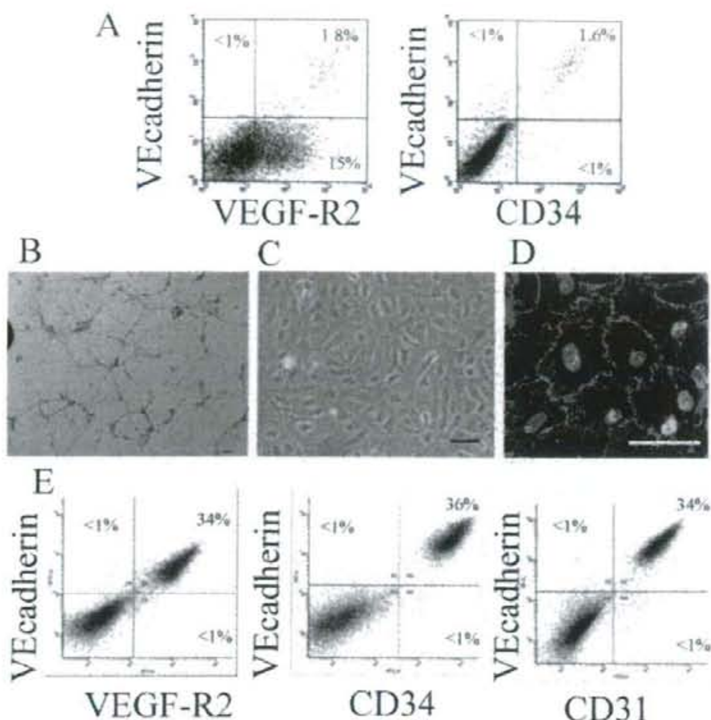

Figure 3. Isolation and expansion of vascular eECs. A, Flow-cytometric analysis of human ES cell–derived cells on an OP9 feeder layer on day 10. B, Network formation of human ES cell–derived VE-cadherin$^+$ cells (eECs) after 24-hour culture on Matrigel. C, Phase-contrast microscopic analysis of recultured VE-cadherin$^+$ cells on a collagen IV–coated dish. D, Immunostaining of recultured VE-cadherin$^+$ cells; red, CD31; green, nuclear staining (SYBR-Green I). E, Flow-cytometric analysis of the cells at the sixth passage expanded from VE-cadherin$^+$ cells. Scale bar, 50 μm.

muscle actin expression (Figure 2E). In the absence of VEGF, VEGF-*R2*$^+$ TRA1-60$^-$ cells did not differentiate into ECs, but almost all of them differentiated into α-smooth muscle actin and calponin-positive cells, which can be categorized as MCs (Figure 2F and 2G). Because these VEGF-*R2*$^+$ TRA1-60$^-$ cells expressed PDGFRβ, PDGF-BB with 0.5% FCS induced MC induction in a similar manner (Figure 2H). We, therefore, concluded that these VEGF-*R2*$^+$ TRA1-60$^-$ cells could be categorized as human VPCs that can differentiate into both ECs and MCs. Next we examined whether VEGF-*R2*$^+$ TRA1-60$^+$ cells after 8 days of differentiation is immature or not. Before differentiation, VEGF-*R2*$^+$ TRA1-60$^+$ cells were positive for flt1, AC133, and c-Kit and negative for CXCR4, PDGFRα, PDGFRβ, CD34, and VE-cadherin. However, after 8 days of differentiation, c-Kit expression decreased, and PDGFRα-positive and/or β-positive cells appeared in VEGF-*R2*$^+$ TRA1-60$^+$ cells (data not shown). Thus, VEGF-*R2*$^+$ TRA1-60$^+$ cells after 8 days of differentiation were not equivalent to the immature ES cells on day 0.

Isolation and Expansion of Vascular eECs

Next, we focused our attention on VE-cadherin$^+$ ECs that were more differentiated than the VPC. On 10 days of differentiation of HES3 on an OP9 feeder layer, VEGF-*R2*$^+$ and VE-cadherin$^+$ cells emerged and accounted for $\approx$1% to 2% of all the cells (Figure 3A). This VE-cadherin–positive cell population was almost identical to the CD34$^+$ population (Figure 3A). We sorted these VE-cadherin$^+$ cells, and, because these cells were also VEGF-*R2*$^+$ and CD34$^+$ (Figure 3A), we used the term "eEC" for these EC in the early differentiation stage. These cells formed a network-like structure on Matrigel in vitro (Figure 3B), showed a cobblestone appearance when they became confluent (Figure 3C), and immunofluorescence staining with CD31 showed a characteristic marginal staining pattern (Figure 3D). These eECs were negative for monocyte makers CD45, CD11b, and CD14 (data not shown) and could be successfully propagated by a factor of $\approx 1.2\times10^2$ (from 2×10^5 cells to 2.4×10^7 cells) after 6 passages on collagen IV–coated dishes. They were cultured with a cell density of 1.5×10^4 cells/cm^2 with VEGF because they did not expand when they were more sparsely plated or cultured without VEGF. Flow-cytometric analysis showed that VE-cadherin$^+$ cells were reduced to $\approx$35% of the total number of cells after 6 passages (Figure 3E), but they were still VEGF-*R2*$^+$, CD34$^+$, and CD31$^+$ at the sixth passage, indicating that the cell differentiation stage had been maintained (Figure 3E). In another series of experiments, we sorted these VE-cadherin$^+$ cells on day 10 and replated them on an OP9 feeder layer or nonfeeder collagen IV–coated dishes for 1 additional week. The VE-cadherin$^+$ cells in these 2 culture groups were then resorted and plated on nonfeeder collagen IV–coated dishes for reculturing. After an additional 3 weeks of reculturing, VE-cadherin expression was examined. The cells that were cultured for 1 additional week on OP9 were 90% positive for VE-cadherin, but the cells kept on nonfeeder dishes were only 44% positive. This suggests that VE-cadherin$^+$ eECs still retain the potential to differentiate

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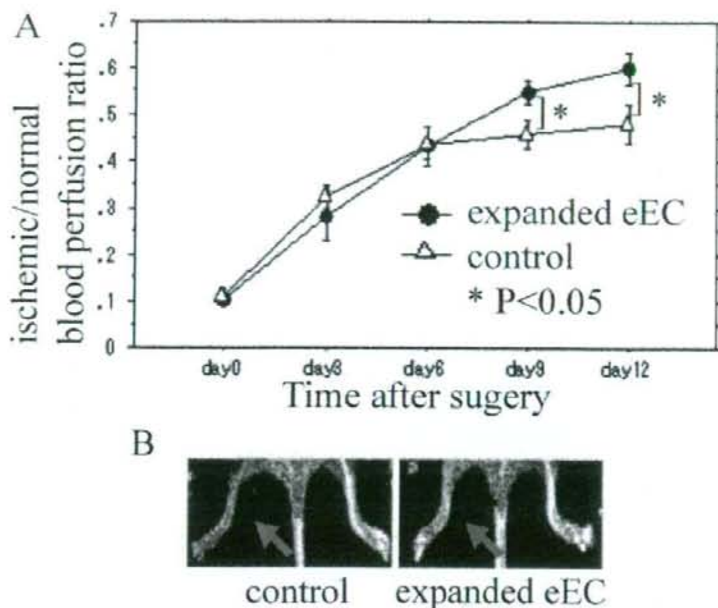

Figure 4. Transplantation of human ES cell–derived vascular cells to the hindlimb ischemia model of immunodeficient mice. A, Quantitative analysis of the hindlimb blood flow by means of calculating the ischemic/normal limb perfusion ratios in mice with ischemic hindlimb. *$P<0.05$. On days 9 and 12, the blood flow in the ischemic limb of the expanded eEC-injected mice (n=8) had increased significantly compared with that of control mice (n=8). B, Representative laser Doppler perfusion images on day 12.

into other cell types, but more differentiated VE-cadherin^{+} ECs may lose this ability.

Transplantation of Human ES Cell-Derived Vascular Cells to the Hindlimb Ischemia Model of Immunodeficient Mice

As the next step, we investigated whether human ES cell–derived vascular cells can be used for vascular regeneration in cell transplantation in the hindlimb ischemia model. KSN nude mice received an intra-arterial injection of cells in PBS or PBS only into the right femoral artery, followed by right femoral artery ligation and removal to create hindlimb ischemia. VEGF-*R2*$^{+}$ TRA1-60^{-} cells, identified as VPCs, were transplanted, but laser Doppler perfusion image analysis on day 14 showed no significant difference in recovery of the blood flow (expressed as the ischemic/normal limb blood perfusion ratio) between the cell-transplanted mice (0.496 ± 0.29; n=10) and the control PBS-injected mice (0.433 ± 0.42; n=14). Next, we used the eECs expanded at passages 4 to 6. As shown in Figure 4A and 4B, the hindlimb blood flows had significantly improved in the cell-injected group 9 and 12 days after injection. For histological analysis, transplanted cells had been labeled with CM-DiI before cell transplantation, and biotin-conjugated isolectin B_4 was intravenously injected to stain ECs before sacrifice on day 14. Although some of the transplanted cells were incorporated as isolectin B4^{+} vascular ECs in the large vessels (Figure 5A), most of the transplanted cells were incorporated as small capillaries (Figure 5B). To quantify the capillary density, sections of the ischemic hindlimbs were stained with anti-mouse and human-specific CD31 antibodies (Figure 5C and 5D). Human CD31^{+} capillaries were detected in the expanded eEC-transplanted mice. The mouse and/or human CD31^{+} total capillary number and area significantly increased in the expanded eEC-transplanted group compared with the control PBS-injected group, whereas there was tendency but no significant difference in the mouse CD31^{+} host capillary number and area (Figure 5E and 5F). On the other hand, there was no significant difference in the capillary length (Figure 5G). Because we cut our sections at right angles with muscle fibers and femoral artery, it might be difficult to estimate the capillary density by vessel length.

Next we performed our transplantation experiments with the same procedure using BALB/c Slc nude mice, in which hindlimb ischemia is more severe than in KSN/Slc nude mice. PBS, VPCs, eECs or human adult aortic ECs were transplanted, and the ischemic hindlimbs were observed on day 14. In the PBS-injected mice, the ischemic hindlimb was autoamputated in 3 of 7 mice, and mild necrosis was observed in 1 of 7. In the VPC-transplanted mice, 3 of 7 were autoamputated and mild necrosis was seen in 2 of 7. In the eEC-transplanted mice, the ischemic hindlimb was not autoamputated, and only mild necrosis was observed in 2 of 8. In the human adult aortic EC-transplanted mice, 4 of 7 were auto-amputated, and mild necrosis was seen in 1 of 7. Furthermore, sections of the ischemic hindlimb in mice without autoamputation were stained with anti-mouse and human-specific CD31 antibodies. Human CD31^{+} capillaries were most abundant in the eEC-transplanted mice, although some human CD31^{+} cells were detected in the VPC or human adult aortic EC-transplanted mice (please see http://atvb.ahajournals.org).

Exclusion of Possible Teratoma Formation by the Expanded eEC

Further experiments were conducted to detect possible teratoma formation by eEC. We conducted long-term follow-ups

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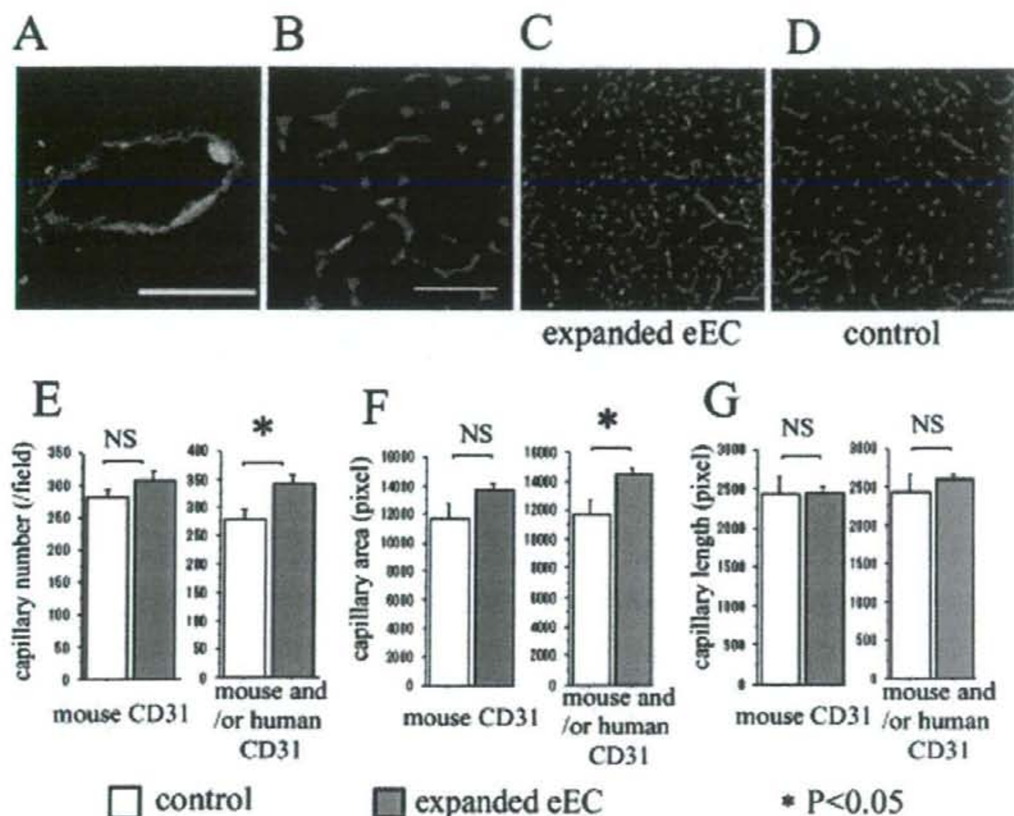

Figure 5. A and B, Histological analysis of vascular regeneration by the intra-arterially injected expanded eEC; green, isolectin B_4^+ endothelial cells; red, CM-Dil$^+$ transplanted cells (representative photographs). C and D, Fluorescent photographs of ischemic hindlimb for capillary density analysis; green, mouse CD31$^+$ capillaries; red, human CD31$^+$ capillaries (representative photographs). E through G, Quantitative analysis of the mouse and human endothelial cell marker–positive capillary densities in the ischemic hindlimb. E, capillary numbers. F, capillary areas. G, capillary lengths. Scale bars, 50 μm.

by transplanting expanded eECs or undifferentiated human ES cells into 3 mice each and following them for 5 months. We transplanted 5×10^5 cells under the dorsal back skin of SCID mice, which are commonly used for teratoma formation for human ES cells. Large tumors had formed after 3 to 5 months in 2 of the 3 mice in the human ES cell–transplanted group, but none had formed in any of the 3 mice in the expanded eEC-transplanted group. In immunohistological analysis, HLA-ABC$^+$ tumors were not observed in the subcutaneous region of eEC transplanted mice, although only a few HLA-ABC$^+$ human cells were remaining (data not shown).

Expression of Angiogenic Factors in Human ES Cell–Derived Vascular Cells

In addition, we investigated whether VPC or eEC produced major angiogenic factors such as VEGF, bFGF, human growth factor, and PDGF-BB. RT-PCR analysis detected mRNA expressions of VEGF, bFGF, and human growth factor in VPCs and PDGF-B and bFGF in eECs (please see http://atvb.ahajournals.org). We measured the protein concentration of these angiogenic factors in culture media by ELISA; however, the concentration of VEGF, human growth factor, and PDGF-BB did not reached the detectable level, and the concentration of bFGF was <30 pg/mL.

Discussion

In this study, we were able to clarify the differentiation process from human ES cells to mature vascular cell components. In adults, VEGF and PDGF receptors are expressed on EC and MC, respectively, and VEGF and PDGF stimulate the growth of the respective cell types. In this study, human ES cell–derived VPCs expressed both VEGF and PDGF receptors. In addition, stimulation with VEGF and PDGF-BB induced 2 differentiation pathways for EC and MC in this cell population. In mouse embryos, VEGF-*R2* and PDGFRα were reported to be expressed in the mesoderm.[11] In whole-mount immunohistochemistry of mouse embryos (E7.5 to 8), VEGF-*R2* was expressed predominantly in the extraembryonic and proximal-lateral mesoderm. PDGFRα was detected mainly in the paraxial embryonic mesoderm. Both VEGF-*R2* and PDGFRα were

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detected in the anterior paraxial mesoderm. It was also reported that vascular endothelial precursors were identified from the cephalic mesoderm of the avian embryo labeled using an antibody against Quek1 (avian homolog of VEGF-*R2*).[12] Our result that VEGF-*R2*$^+$ PDGFR$^+$ VPCs can differentiate into vascular cells may agree with their reports. In our transplantation examination, some human CD31$^-$ ECs were observed in the ischemic hindlimb of VPC-transplanted mice. This suggests that some transplanted VPC (negative for CD31) differentiated into CD31$^-$ ECs in vivo.

In addition, we investigated whether human ES cell–derived vascular cells can be used for vascular regeneration. Transplanted eECs were successfully incorporated into the host circulation and significantly accelerated improvement of local blood flow, whereas VPCs did not. We reported recently that VEGF-*R2*$^+$ cells derived from mouse ES cells could differentiate into not only vascular cells but also cardiomyocytes.[13] Thus, VPCs may be too immature to be used directly as the source for vascular regeneration. It has also been reported that ischemia-induced neovascularization did not improve in mice receiving human mature ECs (eg, human microvascular ECs).[14] Their report is compatible with our result that human adult aortic EC transplantation had no effect for the prevention of ischemic necrosis. The induction and isolation of the cells at the differentiation stage most appropriate for transplantation seem to be important. Judging from our results obtained from histological analysis and capillary density evaluation, at least some of the therapeutic effect of transplantation of expanded eECs could be attributed to vascular regeneration as a result of incorporation of the transplanted cells into the host vessels. Because RT-PCR analysis detected mRNA expression of PDGF-B not in VPCs but in eECs, PDGF-BB secretion might affect the effect of eEC transplantation, although PDGF-BB did not reached the detectable level in culture media. In adults, endothelial progenitor cells (EPCs) reportedly participate in postnatal angiogenesis,[15] whereas other reports suggest that EPCs contribute to neovascularization in tissue ischemia.[14] However, the expansion of EPCs in sufficient quantities to improve blood flow in large animals has not yet been achieved. In addition, some recent reports suggest that adult bone marrow–derived cells, such as EPCs, do not transdifferentiate into ECs under physiological conditions.[16,17] Although the role of EPCs as a modifier of vascular growth awaits further investigation, our findings may help provide an alternative and novel supply of vascular cells for cell therapy as a contribution to vascular regenerative medicine.

Furthermore, the establishment of an in vitro differentiation system of human vascular cell components from human ES cells in this study should also make it possible to dissect out cellular mechanisms in human vascular development and diseased states for which the knockout animal research approach is not practical. Trials on gene expression profiling using our in vitro differentiation system of human vascular cells from human ES cells could assist in the search for novel gene products to develop new therapeutic approaches for vascular regeneration.

Acknowledgments

The human ES cells (HES-3) were contributed by ES Cell International Pte Ltd, Singapore. The anti-human VEGF-*R2* antibody (KM1668) was a generous gift from M. Shibuya, MD, PhD, Department of Cancer Biology, Institute of Medical Science, University of Tokyo.

Sources of Funding

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Health, Labour and Welfare and the Ministry of Education, Culture, Sports, Science and Technology. This work was also supported by a grant from the Japan Smoking Foundation, a Japan Heart Foundation/Pfizer Japan Grant for Cardiovascular Disease Research, and a grant from the Study Group for Molecular Cardiology Research. This work was also supported by Fellowships of the Japan Society for the Promotion of Young Scientists and Establishment of International COE for Integration of Transplantation Therapy and Regenerative Medicine (COE program of the Ministry of Education, Culture, Sports, Science and Technology, Japan).

Disclosures

None.

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