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Improvement of Postnatal Neovascularization by Human Embryonic Stem Cell–Derived Endothelial-Like Cell Transplantation in a Mouse Model of Hindlimb Ischemia

Seung-Woo Cho, PhD*; Sung-Hwan Moon, MS*; Soo-Hong Lee, PhD*; Sun-Woong Kang, MS; Jumi Kim, MS; Jae Min Lim, MS; Hyo-Soo Kim, MD, PhD; Byung-Soo Kim, PhD†; Hyung-Min Chung, PhD†

Background—We established an efficient preparation method to obtain endothelial-like cells (ECs) from human embryonic stem cells (hESCs) and tested whether these hESC-ECs would show therapeutic potential for treatment of hindlimb ischemia.

Methods and Results—ECs differentiated from hESCs were obtained by mechanical isolation and cell sorting for von Willebrand factor. The isolated hESC-ECs maintained endothelial cell–specific characteristics such as endothelial marker expression and capillary formation. One day after surgical induction of hindlimb ischemia in athymic mice, hESC-ECs were injected intramuscularly into ischemic limbs. Four weeks after treatment, hESC-EC treatment significantly increased limb salvage (36%) compared with treatment with medium (0%). In addition, laser Doppler imaging showed that the ratio of blood perfusion (ischemic to normal limb) was increased significantly ($P<0.01$) by hESC-EC treatment (0.511 ± 0.167) compared with medium injection (0.073 ± 0.061). Capillary and arteriole densities were $658\pm190/mm^2$ and $30\pm11/mm^2$ in the hESC-EC group, respectively, whereas those in the medium group were $392\pm118/mm^2$ and $16\pm8/mm^2$, respectively ($P<0.01$). Reverse-transcription polymerase chain reaction with human-specific primers revealed mRNA expression of human endothelial markers and human angiogenic factors in ischemic mouse tissues. The transplanted hESC-ECs were localized as capillaries near muscle tissues in ischemic regions or incorporated in the vessels between muscle tissues, as confirmed by human nuclear antigen staining with platelet/endothelial cell adhesion molecule or von Willebrand factor.

Conclusions—This study demonstrates that hESC-EC transplantation improves blood perfusion and limb salvage by facilitating postnatal neovascularization in a mouse model of hindlimb ischemia. Thus, hESC-ECs might be useful as an alternative cell source for angiogenic therapy. **(*Circulation*. 2007;116:2409-2419.)**

Key Words: angiogenesis ■ stem cell transplantation ■ endothelial cells ■ ischemia ■ embryonic stem cells

Recently, angiogenic stem cell transplantation has been used to induce neovascularization in animal models of limb and myocardial ischemia. Transplantation of endothelial progenitor cells (EPCs) isolated from peripheral blood,[1] bone marrow,[2] or cord blood[3] induces collateral blood vessel development in ischemic limbs and myocardium. In an animal model of severe limb ischemia, therapy with adipose tissue–derived stromal cells was found to enhance neovascularization through a paracrine effect by angiogenic factor secretion.[4] In spite of the angiogenic potential of these adult stem cells for treatment of ischemic diseases, these cell sources have limitations for therapeutic angiogenesis. Isolation of EPCs from peripheral blood or bone marrow requires the harvesting of a large amount of autologous blood to obtain a sufficient number of EPCs.[5] This may cause complications in some patients with severe cardiovascular disease such as myocardial infarction or atherosclerosis. Although adult stem cells derived from cord blood and adipose tissue are favorable with regard to obtaining the number of cells required for transplantation, few transplanted stem cells have

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From the Department of Bioengineering (S.C., B.K.) and Department of Chemical Engineering (S.K., J.M.L.), Hanyang University, Seoul, Korea; Department of Chemical Engineering (S.C.), Massachusetts Institute of Technology, Cambridge, Mass; Stem Cell Research Laboratory (S.M., S.L., J.K., H.C.), CHA Stem Cell Institute & CHA Biotech, Pochon CHA University, Seoul, Korea; and Department of Internal Medicine (H.K.), Seoul National University College of Medicine, Seoul, Korea.

*Drs Cho, Moon, and Lee contributed equally to this work.

†Drs Kim and Chung contributed equally to this work.

The online-only Data Supplement, consisting of an expanded Methods section, is available with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.106.687038/DC1.

Correspondence to Dr Hyung-Min Chung, CHA Stem Cell Institute & CHA Biotech, Pochon CHA University, 606-16 Yoeksam 1-dong, Gangnam-gu, Seoul 135-081, Korea (e-mail safe33msh@gmail.com), or Dr Byung-Soo Kim, Department of Bioengineering, Hanyang University, 17 Haengdang-dong, Seongdong-gu, Seoul 133-791, Korea (e-mail bskim@hanyang.ac.kr).

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been found to differentiate into endothelial cells (ECs) and incorporate into vascular structures in ischemic sites.[6,7]

Recently, embryonic stem cells (ESCs) have been highlighted as an alternative cell source for therapeutic angiogenesis. ESCs are known to exhibit unlimited proliferation and pluripotency, and they are promising resources for regenerative medicine, which requires large numbers of a particular cell type, including ECs.[8] During the early stages of ESC development, vasculogenesis takes place through a multistep process in which EC precursors differentiate, expand, and coalesce to form a network of primitive tubules. There have been several reports indicating that ESCs can spontaneously differentiate into ECs in vitro or in vivo.[9,10] Furthermore, ECs can be obtained from Flk-1– or Sca-1–positive cell fractions in ESCs in vitro.[11,12] ESC-derived ECs express EC–specific genes and proteins and have been found to form mature vascular, capillary-like structures in a 2D and 3D in vitro culture, which indicates that they have functional EC-specific characteristics.[13,14] Thus, ESCs could be used as a source of ECs for the treatment of ischemia or for cardiovascular tissue engineering. Indeed, a previous study reported that transplantation of mouse ESC–derived ECs accelerated reendothelialization of injured arteries and reduced neointimal hyperplasia.[12] However, no study has yet reported on the therapeutic potential of human ESC-ECs (hESC-ECs) for the treatment of ischemic diseases.

In the present study, we first investigated whether a large population of pure ECs could be obtained from human ESCs (hESCs). We found that platelet/endothelial cell adhesion molecule (PECAM)–positive cells were located mainly at the centers of embryoid bodies (EBs) during differentiation of hESCs. After attachment of EBs on culture dishes, PECAM-positive cells in the center region of the attached EBs were collected efficiently from the outgrowth of EB cells by mechanical isolation. This technique allowed us to acquire a large enough number of cells to be transplanted into ischemic hindlimbs. We subsequently tested the hypothesis that hESCs could show therapeutic potential to treat ischemic disease. Endothelial-like cells isolated from hESCs were expanded in vitro and transplanted into limb muscles of a mouse hindlimb ischemia model. The therapeutic efficacy of hESC-EC transplantation was evaluated by measurement of blood perfusion in ischemic limbs. The angiogenic efficacy of hESC-EC therapy was examined by immunohistochemical analyses, reverse-transcription polymerase chain reaction (RT-PCR) assay, and microvessel density determination in the ischemic tissues.

Methods

An expanded Methods section is provided in the online-only Data Supplement.

hESCs Culture and Endothelial Differentiation

The undifferentiated hESCs (cell line CHA3-hESC) was cultured as described previously.[15] Every 5 to 6 days, hESCs were detached with dissecting pipettes and transferred to dishes with mitomycin C–treated STO feeder cells (ATCC, Manassas, Va), as described previously.[16] For EB formation, hESCs were cultured in suspension in basic fibroblast growth factor–free hESC culture medium for 9 days. The resulting EBs were attached to dishes and cultured for 7 to 9 days in DMEM (Gibco, Gaithersburg, Md) supplemented with 10% fetal bovine serum (HyClone, Logan, Utah). The center regions of attached EBs were isolated mechanically with dissecting pipettes from the surrounding outgrowth cells, and the isolated cells were cultured in defined medium for EC culture (EGM-2; Cambrex, Walkersville, Md). To isolate von Willebrand factor (vWF)–positive cells from cells in the center region, cell sorting with mouse anti-human monoclonal vWF antibodies (Chemicon, Temecula, Calif) was performed with a FACS Vantage flow cytometer (BD Bioscience, San Jose, Calif). The sorted cells were cultured with EGM-2 up to 5 passages to obtain sufficient cells for in vivo injection ($\approx 3.5\times10^7$ cells).

Mouse Limb Ischemia

Female athymic mice at 4 weeks of age (body weight 15 to 18 g; Jungang Laboratory Animal, Seoul, Korea) were anesthetized with xylazine (20 mg/kg) and ketamine (100 mg/kg). The femoral artery and its branches were ligated through a skin incision with 5-0 silk (Ethicon, Somerville, NJ). The external iliac artery and all of the above arteries were then ligated. The femoral artery was excised from its proximal origin as a branch of the external iliac artery to the distal point where it bifurcates into the saphenous and popliteal arteries.[17] All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (National Institutes of Health publication No. 85-23, revised 1996).

Treatments of Limb Ischemia

One day after arterial dissection, athymic mice were randomly assigned to 1 of 2 experimental groups. The control group (n=10) was injected with medium. hESC-ECs (3.0×10^6 cells per mouse) were suspended in 200 μL of EGM-2 (hESC-EC group, n=11) and injected intramuscularly into 4 sites of the gracilis muscle in the medial thigh with 29-gauge tuberculin syringes.

Laser Doppler Imaging Analysis

Laser Doppler imaging analysis was performed as described previously.[18] A laser Doppler perfusion imager (Moor Instruments, Devon, United Kingdom) was used for serial noninvasive physiological evaluation of neovascularization. Mice were monitored by serial scanning of surface blood flow of hind limbs on days 0, 14, and 28 after treatment. The digital color-coded images were analyzed to quantify the blood flow in the region from the knee joint to the toe, and mean values of perfusion were calculated.

Statistical Analysis

Quantitative data are expressed as mean±SD. Statistical analysis was performed with the unpaired Student *t* test with InStat software (InStat 3.0, GraphPad Software Inc, San Diego, Calif). A value of $P<0.05$ was considered statistically significant.

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

Efficient Isolation and Culture of hESC-ECs

Figure 1 shows the overall strategy to efficiently acquire ECs from hESCs. First, hESCs were prepared on feeder cells by conventional hESC culture techniques (Figure 1A). Second, EBs were spontaneously formed by suspension culture after removal of the feeder cells. Surprisingly, immunocytochemical staining for PECAM of differentiated EBs showed that a majority of PECAM-positive cells were localized at the centers of EBs (Figure 1B). In the hESC line (CHA3-hESC, online-only Data Supplement Figure I) used in the present study, a considerable amount of PECAM expression was observed in the EBs after 9 days of culture (online-only Data

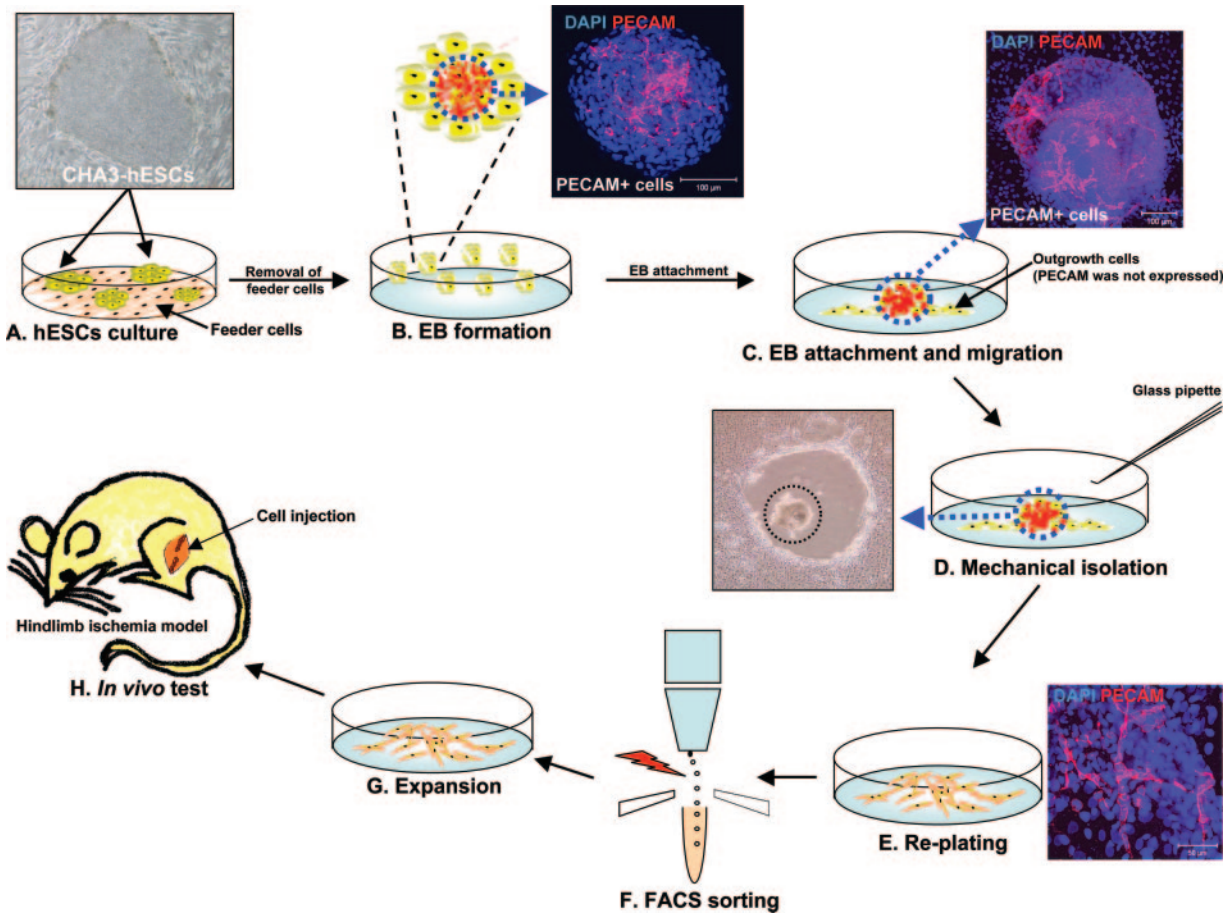


Figure 1. Overall strategy to isolate and culture hESC-ECs and transplant them into a hindlimb ischemia mouse model.

Supplement Figure IIB and IIC). In addition, the EBs maintained morphological similarity for up to 9 days after differentiation (online-only Data Supplement Figure IIA). Third, differentiated EBs were attached on culture dishes, which allowed the outer cells of EBs to spread more rapidly toward the outer edges than cells in the centers of the EBs. Attached EBs had 2 different regions in terms of PECAM expression. PECAM was mainly found at the center regions of attached EBs (Figure 1C). Therefore, we were easily able to acquire a large population of PECAM-positive cells by isolating the center regions of attached EBs from surrounding cells through mechanical isolation (Figure 1D). Online-only Data Supplement Figure III shows the proportions of vWF- and PECAM-positive cells in the EBs at each isolation stage. When the isolated cells were plated again onto gelatin-coated dishes, they exhibited vascular-like structures, which confirmed the existence of a large population of ECs (Figure 1E). Subsequently, cultured isolated cells were sorted with vWF antibodies by fluorescent-activated cell sorter (FACS) analysis (Figure 1F). The sorted cells were expanded (Figure 1G) and injected into the hindlimb-ischemia mouse model for an in vivo function test (Figure 1H).

Characterization of hESC-ECs

Expressions of an undifferentiated marker gene (Oct-4) and endothelial-specific genes (PECAM, VE-cadherin, Tie-2, and Flk-1) were analyzed at different stages of hESC differentiation with RT-PCR (Figure 2A). In accordance with progress in the isolation process (hESC differentiation), Oct-4 expression decreased, whereas expression of all endothelial markers increased, which indicated that ECs were isolated effectively. FACS sorting for vWF revealed that the vWF-positive cell population was 41.81% of the total cell population in mechanically isolated cells (Figure 2B). The sorted cells showed a cobblestone shape, a morphology typical of ECs (Figure 2C). RT-PCR analysis of hESC-ECs used for transplantation showed expression of multiple endothelial markers (PECAM, VE-cadherin, Tie-2, Flk-1, and vWF) in hESC-ECs (Figure 2D). The hESC-ECs were stained positively for various EC markers (vWF, PECAM, and VE-cadherin; Figure 2E). PECAM was expressed at the junction of cells, whereas vWF was largely expressed in the cytoplasm (Figure 2E). FACS data for vWF in hESC-ECs used for in vivo injection indicated that a majority of hESC-ECs (91.35%) were positive for vWF (Figure 2F). The expression of other EC markers (Tie-2, Flt-1, and KDR) was observed in cells over 60% of the total hESC-EC population (Figure 2F).

hESC-ECs were examined further in view of EC-specific functions. When the sorted cells were incubated with 1, 1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI)–labeled acetylated low-density lipoprotein (LDL), its fluorescence (red color) was observed in the cytoplasm of cells, showing the uptake of ac-LDL. The overlapped fluorescent image of acetylated LDL and DAPI confirmed that acetylated

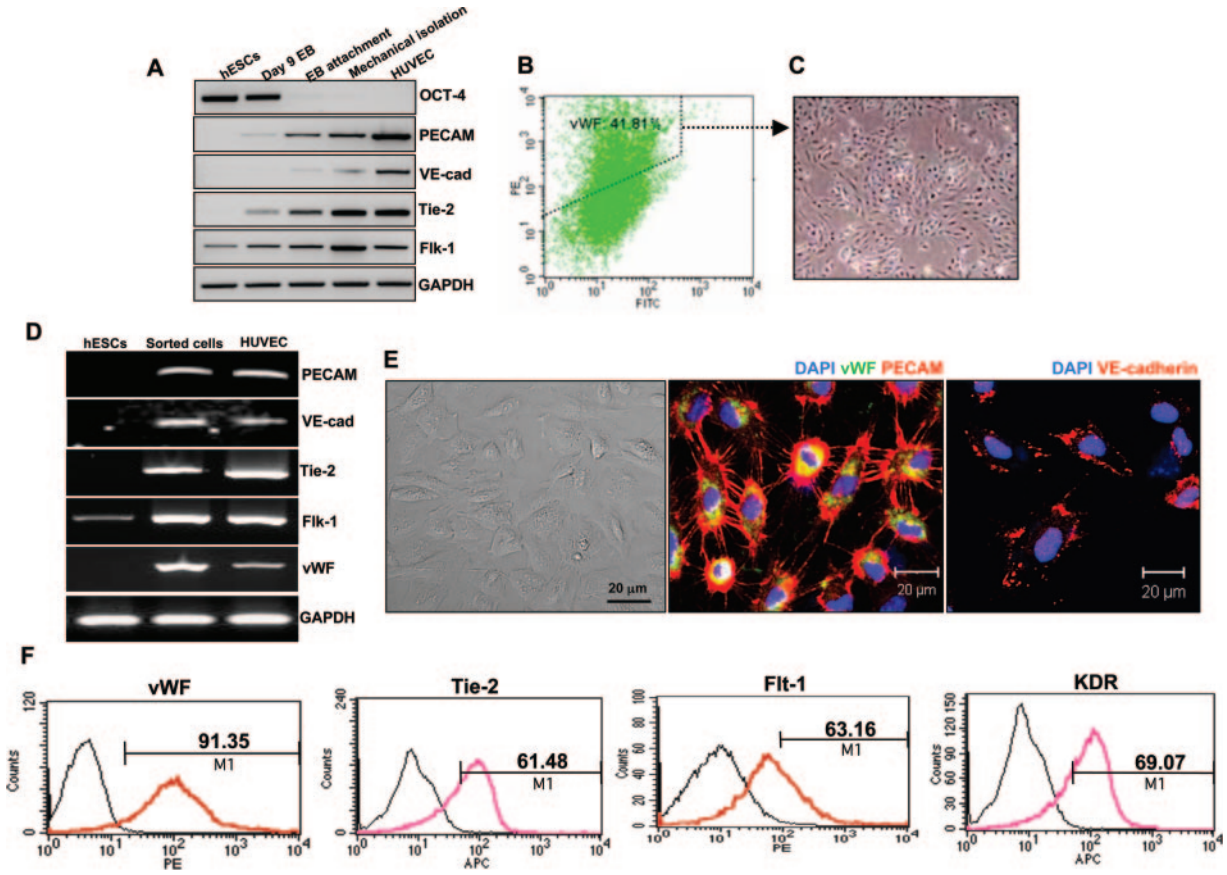


Figure 2. hESC-EC isolation and characterization. A, RT-PCR for examination of EC-specific marker expression in EBs during hESC differentiation. B, FACS analysis for vWF of cells isolated mechanically from EBs. C, Morphology of hESC-ECs sorted in Figure 2B. Sorted cells were expanded up to 5 passages for in vivo injection. D, RT-PCR for various endothelial markers of hESC-ECs. Human umbilical vein ECs (HUVECs) and undifferentiated hESCs were used as a positive and negative control, respectively. E, Immunocytochemical staining of hESC-ECs for multiple EC markers. Cultured hESC-ECs showed cobblestone morphology (left), a typical EC morphology, and were stained positively with endothelial markers (vWF/PECAM [middle] and VE-cadherin [right]). F, FACS analyses of hESC-ECs used for in vivo injection. hESC-ECs continued to express EC markers highly during the culture.

LDL was localized in the cytoplasm (Figure 3A). Additionally, the sorted cells on Matrigel spontaneously sprouted and branched, which resulted in well-defined networks of capillary structures (Figure 3B). The formation of capillary-like structures was observed within 12 hours after plating. The cells in capillary structures distinctly expressed endothelial-specific PECAM, vWF, and VE-cadherin (Figure 3C through 3E). Lumen structures were observed in vascular networks formed on Matrigel and maintained their stability in Matrigel during in vitro culture for 5 to 7 days (Figure 3D and 3E).

Improvement of Ischemic Limb Salvage by hESC-EC Transplantation

The angiogenic potential of hESC-ECs was evaluated in an athymic mouse model of hindlimb ischemia. At 4 weeks after the injection treatment, the control group (medium injection) showed extensive necrosis of the ischemic hindlimb, which resulted in limb loss by autoamputation (Figure 4A). The intramuscular transplantation of hESC-ECs significantly reduced the rate of limb loss compared with injection with medium alone. All mice receiving medium injection underwent limb loss (90%, 9 of 10) or severe limb necrosis (10%, 1 of 10), with no cases of limb salvage. On the other hand, most of the mice that received hESC-EC transplantation exhibited limb salvage (36.4%, 4 of 11) or had only mild limb necrosis (36.4%, 4 of 11), although some of these mice lost their limbs (27.2%, 3 of 11; Figure 4B).

Inhibition of Muscle Degeneration and Fibrosis in Ischemic Limbs by hESC-EC Transplantation

Histological examinations of the ischemic limbs retrieved at 4 weeks after the injection treatment revealed that hESC-EC transplantation protected limb muscles against necrotic damage caused by ischemia. Hematoxylin and eosin staining of the control group (medium injection) showed massive muscle degeneration in the ischemic regions (Figure 4C). In contrast, muscles in the ischemic limbs of mice in the hESC-EC transplantation group were protected after cell transplantation (Figure 4C). Masson's trichrome staining showed that fibrosis was markedly attenuated after local transplantation of hESC-ECs into the ischemic regions (Figure 4C).

Improvement of Blood Perfusion in Ischemic Limbs by hESC-EC Transplantation

Laser Doppler perfusion imaging analysis revealed that blood perfusion in ischemic limbs was significantly improved in the hESC-EC transplantation group compared with the medium-

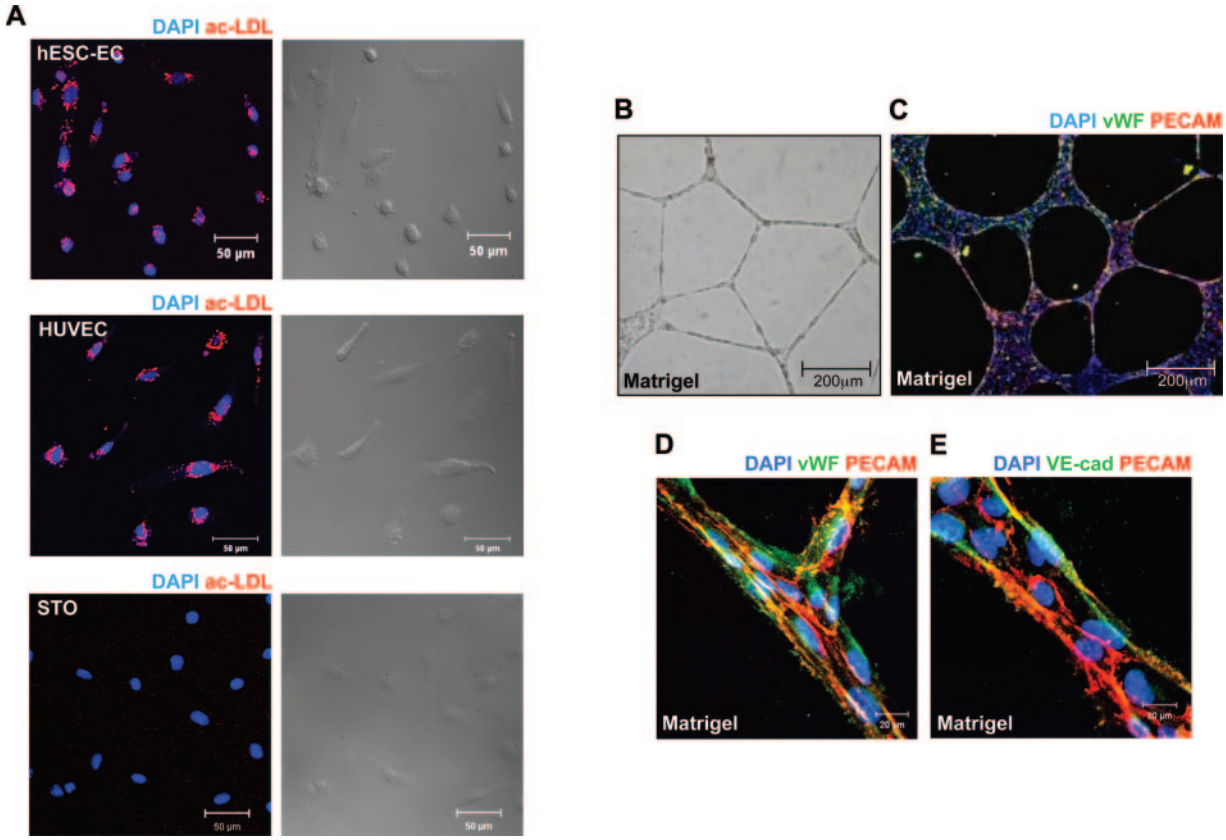
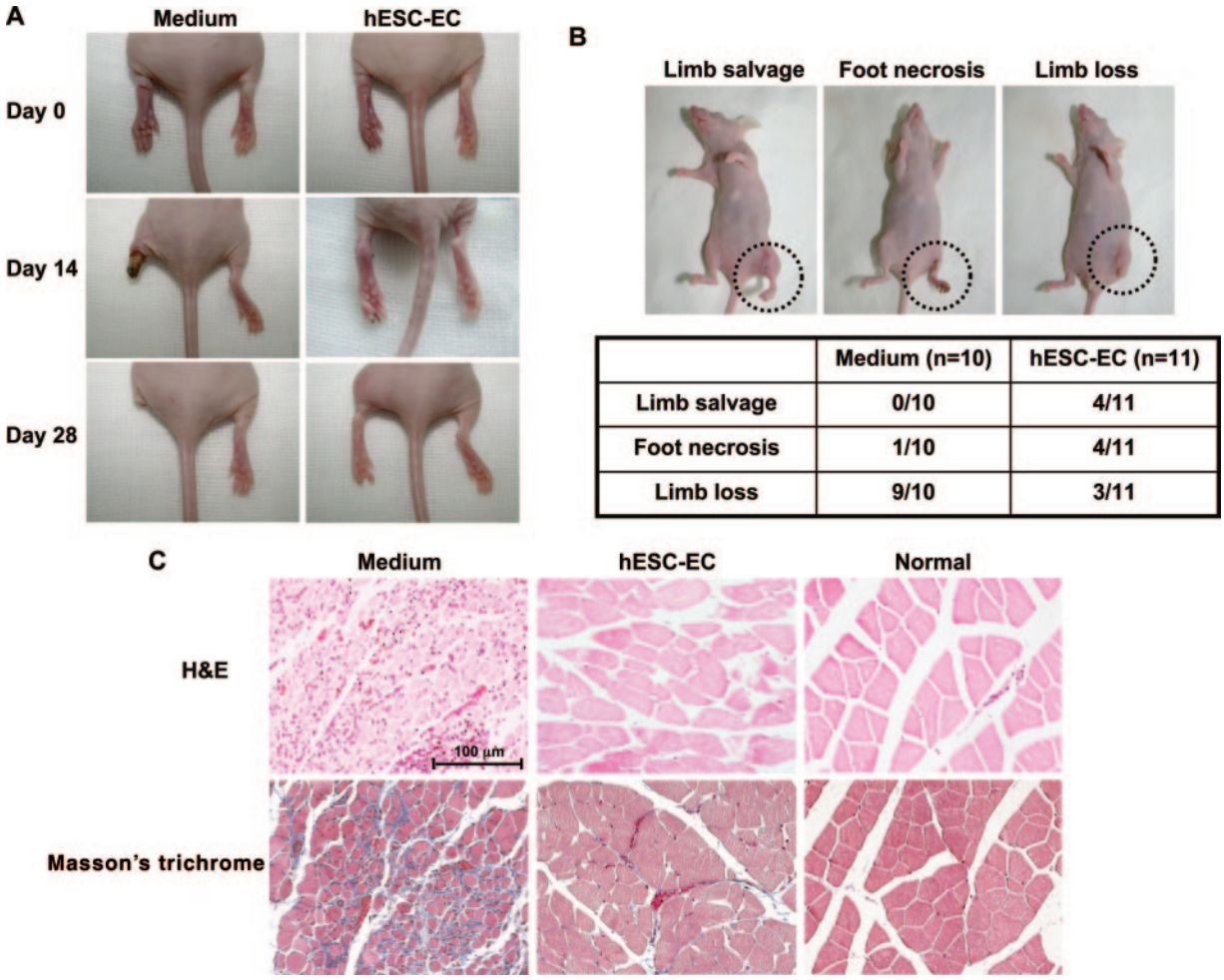


Figure 3. Endothelial-specific functional characterization of hESC-ECs. A, Acetylated LDL uptake by hESC-ECs. Red in cytoplasm represents DiI-labeled acetylated LDL. Human umbilical vein ECs (HUVEC) and STO fibroblasts were used as a positive and negative control, respectively. B, Vascular tubelike networks formed by hESC-ECs on Matrigel at 5 days after plating. C through E, Immunocytochemical staining of the vascular networks formed on Matrigel for PECAM (red), vWF or VE-cadherin (green), and DAPI (blue). Stable lumen structures were observed in the vascular networks during in vitro culture of 5 to 7 days. VE-cad indicates VE-cadherin.

injection group (Figure 5A). At 2 weeks after treatment, the relative ratios of blood flow (ischemic to normal limb) were 0.235±0.085 in the hESC-EC transplantation group (n=8) and 0.051±0.017 in the medium-injection group (n=7), respectively ($P<0.01$; Figure 5B). Intramuscular transplantation of hESC-ECs significantly improved the relative ratio of blood perfusion (hESC-EC transplantation group 0.511±0.167, n=11, versus medium-injection group 0.073±0.061, n=10; $P<0.01$) by 4 weeks after treatment (Figure 5B). Smaller doses of cells (3.0×10^4 and 3.0×10^5 cells per mouse) also yielded improvements in blood perfusion (3.0×10^4 group [n=3] 0.359±0.036, 3.0×10^5 group [n=4] 0.407±0.032; $P<0.01$ versus medium group 0.116±0.041 [n=5]) in ischemic limbs at 4 weeks after treatment (online-only Data Supplement Figure IVA and IVC). However, mice treated with 3.0×10^4 cells underwent more severe foot necrosis at 4 weeks than mice treated with 3.0×10^5 cells (online-only Data Supplement Figure IVB). Injection of cells negative for vWF (non-EC component of the EBs) after sorting for vWF did not yield improvements in blood perfusion (0.196±0.074, n=3) in ischemic limbs compared with the medium-injection group (0.116±0.041, n=5; $P>0.05$) at 4 weeks after treatment (online-only Data Supplement Figure IVA and IVC).

Enhancement of Neovascularization by hESC-EC Transplantation

Transplanted hESC-ECs enhanced angiogenesis, arteriogenesis, and angiogenic factor expression in ischemic limb tissues. With the use of immunohistochemical staining for vWF (Figure 6A) and quantification of capillary density (Figure 6D), it was revealed that hESC-EC transplantation significantly enhanced capillary formation (658±190/mm^2) compared with medium injection (392±118/mm^2; $P<0.01$). Immunohistochemical staining for PECAM (Figure 6C) confirmed this enhanced capillary formation by hESC-EC transplantation. Immunohistochemical staining for smooth muscle α-actin (Figure 6B) and quantification of arteriole density (Figure 6E) revealed that arteriole formation was significantly enhanced ($P<0.01$) by hESC-EC transplantation (30±11/mm^2) compared with medium injection (16±8/mm^2). RT-PCR analysis with human-specific primers for multiple endothelial markers showed that the expression of human endothelial marker mRNA was detected in ischemic limb tissues only in the hESC-EC transplantation group (Figure 6F). Furthermore, the mRNA expression of human EC markers (hESC-EC group in Figure 6F) was comparable to that of mouse EC markers (hESC-EC group in Figure 6G), which demonstrates that a therapeutic contribution of hESC-ECs to neovascularization was significantly induced in ischemic tissues.



	Medium (n=10)	hESC-EC (n=11)
Limb salvage	0/10	4/11
Foot necrosis	1/10	4/11
Limb loss	9/10	3/11

Figure 4. Improvement of ischemic limb salvage by hESC-EC transplantation. A, Representative photographs of medium-treated (left) and hESC-EC–treated (right) ischemic hindlimbs on days 0, 14, and 28 after treatment. B, Physiological status of ischemic limbs 4 weeks after medium injection or hESC-EC transplantation. Intramuscular transplantation of hESC-ECs improved the salvage of ischemic limbs compared with medium injection. C, Histological analyses of normal limbs and ischemic limbs retrieved 4 weeks after treatment. Staining with hematoxylin and eosin (H&E; original magnification ×400) showed massive muscle degeneration in the ischemic regions of control limbs. Infiltration of numerous granulocytes and neutrophils, indicative of tissue inflammation after ischemia, was observed in the ischemic regions. In contrast, muscles in the ischemic limbs of the hESC-EC transplantation group were protected after cell transplantation. Masson's trichrome staining (original magnification ×400) showed that fibrosis was markedly attenuated after local transplantation of hESC-ECs into the ischemic regions.

Human angiogenic factor expression by hESC-EC transplantation was also observed in mouse ischemic tissues. Immunohistochemical staining for vascular endothelial growth factor and basic fibroblast growth factor showed significant expression of these human factors in the hESC-EC injection group (Figure 7A and 7B). Moreover, RT-PCR analysis with human-specific primers proved that transplanted hESC-ECs induced the expression of human angiogenic growth factor mRNA in ischemic regions. Human vascular endothelial growth factor, basic fibroblast growth factor, and angiopoietin-1 mRNA were distinctly expressed in the ischemic limbs that received hESC-EC transplantation (Figure 7C). The mRNA expression of these human angiogenic factors was not observed in the ischemic limbs that received medium injection (Figure 7C).

Engraftment of Transplanted hESC-ECs Into Vascular Structures in Ischemic Limbs

RT-PCR for human β-actin and fluorescence in situ hybridization staining revealed the presence of human cells in ischemic tissues after transplantation. The expression of human β-actin mRNA was detected in hESC-EC–transplanted limb tissues but not in medium-injected limb tissues (Figure 8A). Fluorescence in situ hybridization staining showed human cells with Y chromosomes (arrows) in the ischemic regions (Figure 8B). PECAM immunostaining with human-specific antibodies showed that the human PECAM-positive vessel contained red blood cells in its lumen (Figure 8C, arrow), which indicates that the neovessels induced by hESC-EC transplantation were apparently functional and indeed contributed to the blood perfusion. Double-immunofluorescent staining for human nuclear antigen and PECAM (Figure 8D) showed that human nuclear antigen– and PECAM-positive cells (transplanted hESC-ECs, arrows) were present in the ischemic regions. The formation of capillary networks between PECAM-positive human cells (transplanted hESC-ECs, arrows) and PECAM-positive mouse cells (recruited mouse ECs, arrowheads) was also

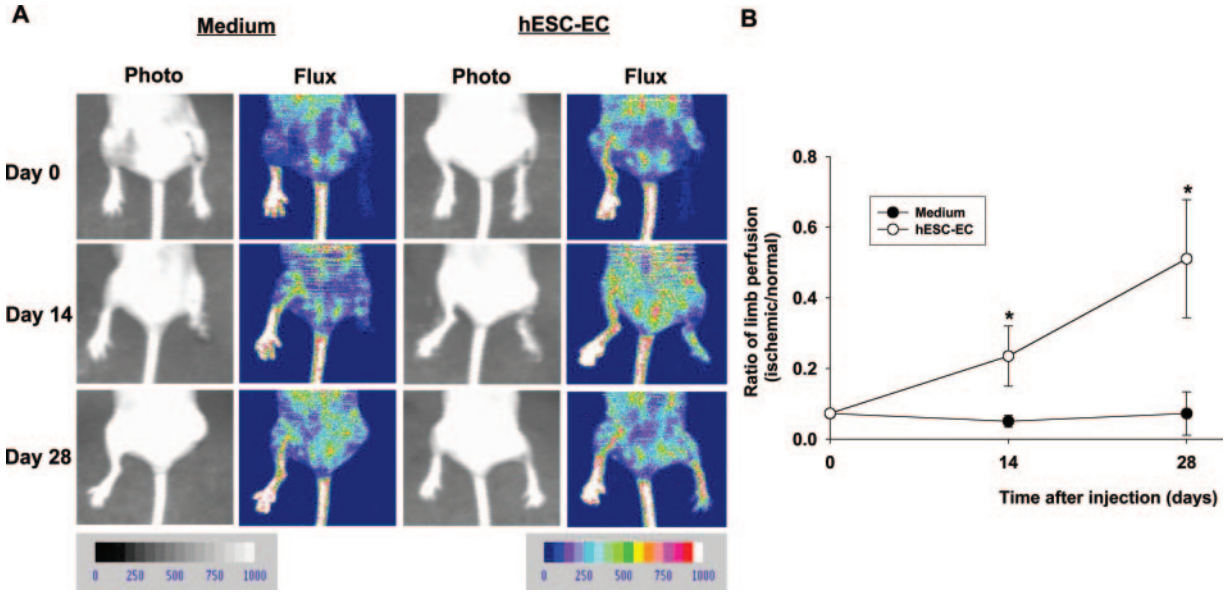


Figure 5. Improvement in blood flow in ischemic hindlimbs after hESC-EC transplantation. A, Serial analysis of laser Doppler perfusion imaging at 0, 14, and 28 days after treatment. A greater increase in limb blood perfusion was observed in the ischemic limbs of mice that received hESC-EC transplantation than in the medium-injection group. B, Blood perfusion ratio of ischemic limbs measured by laser Doppler imaging at 0, 14, and 28 days after treatment. The ratio of ischemic to normal limb blood perfusion was significantly improved in mice that received hESC-EC transplantation compared with the medium-injection group at 2 weeks (n=7 for medium and n=8 for hESC-ECs; *$P<0.001$) and 4 weeks (n=10 for medium and n=11 for hESC-ECs; *$P<0.001$) after treatment.

observed in the ischemic muscles (Figure 8D). The transplanted hESC-ECs were found at capillaries near muscle tissues in ischemic regions (Figure 8D) or incorporated in the vessels between muscle tissues (Figure 8D and 8E). Human nuclear antigen staining with PECAM (Figure 8D) or vWF (Figure 8E) confirmed the presence of hybrid blood vessels composed of transplanted hESC-ECs (arrows) and mouse ECs (arrowheads). The density of hESC-ECs (human nuclear antigen– and PECAM-positive cells) that existed in ischemic tissues was 161 ± 137 cells/mm^2. The densities of mouse EC–derived vessels and hESC-EC–incorporated hybrid vessels were 102 ± 65/mm^2 and 24 ± 36/mm^2, respectively ($P<0.01$; Figure 8F). Blood vessels composed of only transplanted human cells were not detected (Figure 8F). These results suggest that transplanted hESC-ECs survived after transplantation, were engrafted into mouse tissue, and subsequently induced vascular networks in the ischemic muscles.

Discussion

ESCs could be an alternative cell source for use in therapeutic angiogenesis to treat ischemic diseases. First, ESCs could provide functional ECs in unlimited amounts that would be available for clinical application. It has been reported that the number of EPCs in adult stem cell sources, such as bone marrow or peripheral blood, is limited.[4] In addition, adult stem cells usually have a limited proliferative capacity, and thus, expansion of sufficient EC populations from these adult stem cells for transplantation remains a major task.[19] In contrast, ESCs have an extended self-renewal activity and can be expanded without limit.[20] Second, ESCs may show low immunogenicity in vivo, although this is rather controversial at this point. It has been reported that ESCs express few immune-related cell-surface antigens[21]; thus, ESCs and their derivatives may be less susceptible to immune rejection than adult cells.[22] Indeed, several studies report the immune tolerance of transplanted mouse ESCs in rat[23] and sheep[24] infarcted myocardium. However, a recent study demonstrated that mouse ESCs were immunologically rejected several weeks after transplantation into mouse ischemic myocardium, which indicates the lack of immune tolerance of ESC derivatives even in allogeneic transplantation.[25] The immune benefit of ESC therapy requires verification by immune-competent animal study.

hESC-ECs could also potentially be used for cell therapy to treat ischemic tissues or for vascular tissue engineering. Unfortunately, in spite of these advantages of hESC-ECs compared with adult stem cells, no studies have investigated the use of hESC-ECs to improve therapeutic angiogenesis for clinical application, presumably because it is difficult to produce hESC-ECs on a large scale and with high purity. Many recent studies have used EBs to acquire ECs differentiated from hESCs, because EBs can be formed simply from hESCs and can be handled easily. However, investigators failed to produce the number of hESC-ECs needed for in vivo study. For example, Levenberg et al[9] isolated an ≈2% yield of ECs by sorting PECAM-positive cells in EBs.

In the present study, we sought to acquire a high yield of hESC-ECs from a majority of PECAM-positive cells located at the center of EBs formed after suspension culture. Surprisingly, most of the PECAM-positive cells remained in the center of the EBs even after attachment to culture dishes, which resulted in 2 regions of EC populations. We also verified a similar distribution of PECAM-positive cells in the center regions of EBs from another hESC line (H9 cell line; data not shown) and efficiently isolated the cells in the center regions, which demonstrates that PECAM-positive cells pref-

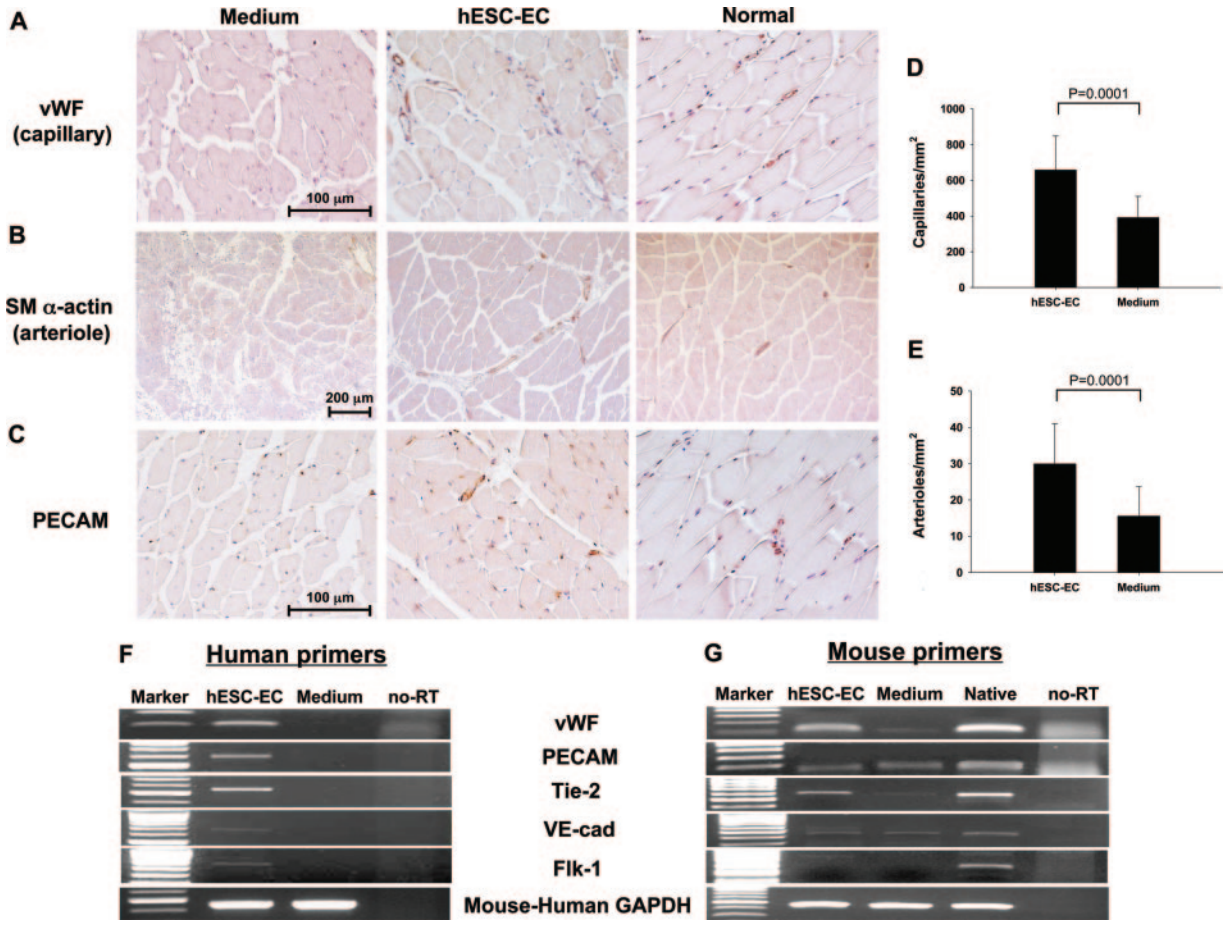


Figure 6. Enhancement of neovascularization in ischemic limb tissues at 4 weeks after hESC-EC transplantation. A through C, Immunohistochemical staining with vWF (A), smooth muscle (SM) α-actin (B), and PECAM (C). D and E, Quantification of capillary density (D) and arteriole density (E) in ischemic regions of control group (medium injection) and hESC-EC–transplanted group. Capillary and arteriole formation were significantly enhanced ($P<0.01$) by hESC-EC transplantation compared with medium injection. F and G, RT-PCR analysis of ischemic limb tissues retrieved 4 weeks after treatment for multiple endothelial markers with (F) human- and (G) mouse-specific primers. The same amounts of PCR products were loaded for human and mouse EC markers.

erentially develop at the center of EBs irrespective of hESC line. After mechanical isolation of the PECAM-positive cell-enriched fraction and sorting for vWF, a high yield of pure ECs were obtained. FACS data for vWF of hESC-ECs used for transplantation showed that a majority of hESC-ECs (91.35%) were positive for vWF (Figure 2F), which was comparable to a FACS result (92.06%) for vWF in human umbilical vein endothelial cells, a homogeneous EC population (online-only Data Supplement Figure IIIE), which indicates the homogeneity of ECs used in the present study.

The present study is the first report demonstrating that hESCs could be feasible as a novel cell source for therapeutic angiogenesis in an animal model of severe ischemia. For expansion of hESC-ECs with high purity, the present study used an efficient EC culture method by combining mechanical isolation and cell sorting with an endothelial-specific marker. Local transplantation of hESC-ECs prepared by this method improved perfusion blood flow in ischemic limbs (Figure 5), which resulted in a lower rate of ischemic limb loss (Figure 4B). The present study demonstrates that the beneficial effect of hESC-ECs on ischemic tissues is attributable not only to their participation in the vascular structures but also to their ability to produce angiogenic factors. Direct incorporation of transplanted cells into vascular structures could contribute to improvements in blood perfusion in ischemic tissues through the induction of angiogenesis. The present in vivo study showed that transplanted hESC-ECs were incorporated into vascular networks in ischemic sites (Figure 8D and 8E). The paracrine effects of factors secreted by transplanted cells could be an additional part of their contribution to the repair of ischemic tissues. RT-PCR analysis revealed that human angiogenic growth factors (vascular endothelial growth factor, basic fibroblast growth factor, and angiopoietin-1) were expressed only in the ischemic limbs that received hESC-EC transplantation and not in the limbs treated with medium (Figure 7C). This result may indicate that additional human angiogenic factors secreted by transplanted hESC-ECs further enhanced angiogenesis by promoting a paracrine effect in ischemic tissues, which leads to functional recovery of ischemic muscles. It has also been reported that embryonic EPCs can secret a wide spectrum of proteins related to cell survival and wound healing, as well as angiogenic factors.[26] Thus, hESC-ECs might prevent apoptosis in muscle tissue after ischemic lesion through the secretion of antiapoptotic factors.

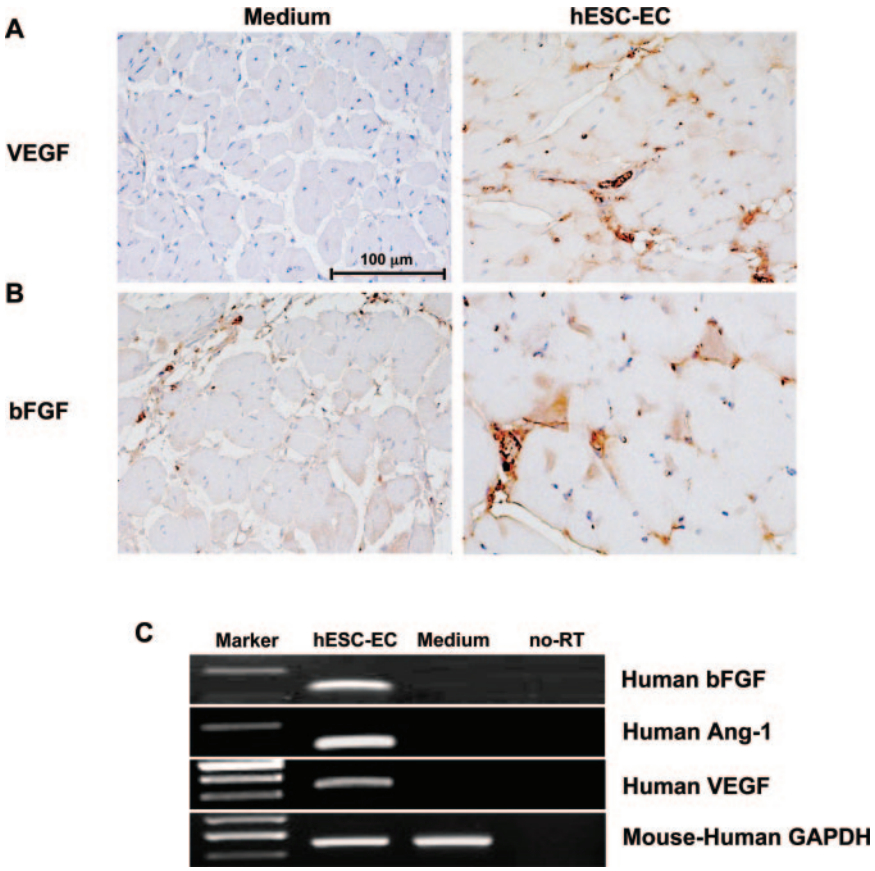


Figure 7. Expression of human angiogenic growth factors in ischemic limb tissues by hESC-EC transplantation. A and B, Immunohistochemical staining for vascular endothelial growth factor (VEGF; A) and basic fibroblast growth factor (bFGF; B) with human-specific antibodies. Significant expression of these human factors was observed in hESC-EC injection group. C, RT-PCR analysis with human-specific primers for angiogenic growth factors. Transplanted hESC-ECs induced the expression of human angiogenic growth factor mRNA in ischemic regions. Ang-1 indicates angiopoietin-1.

The strategy for therapeutic ESC transplantation requires more effective improvement for therapeutic angiogenesis. Although hESC-EC transplantation improved blood perfusion in ischemic limbs compared with control treatment (medium injection) in the present study, the therapeutic efficacy of hESC-EC transplantation was rather low (4 of 11 cases of limb salvage). Recent studies have shown that a combination of stem cell transplantation and angiogenic or antiapoptotic factors or genes synergistically enhanced neovascularization in ischemic tissues compared with each therapy alone.[27,28] Coadministration of several growth factors enhanced ESC engraftment and host-specific differentiation in hearts with myocardial infarction.[29] Thus, combination therapy could be used to enhance the angiogenic efficacy of hESC-EC transplantation. The present study used cell sorting with a mature EC marker (vWF) to remove undifferentiated ESCs or cells of different lineages. Although teratoma formation was not observed in the present study, animals receiving hESC-ECs were followed up for a relatively short period of time (4 weeks), which may be insufficient to allow for the formation of teratomas. Other techniques might be introduced to completely eliminate the possibility of teratoma formation. For example, antibiotic selection enabled by the introduction of a fusion gene comprising an endothelial-specific promoter and antibiotic resistance sequence into ESCs could aid in EC isolation and in the prevention of teratoma formation.[30] Cell transplantation with a matrix may enhance the efficacy of cell therapy by facilitating optimal cell transfer and distribution into ischemic sites. Indeed, ESC transplantation with injectable hydrogel enhanced the efficacy of cell therapy in the treatment of myocardial infarction.[31] The number and type of hESC-ECs used for transplantation should also be optimized to enhance angiogenic efficacy.

In summary, we were able to acquire a high yield of ECs through mechanical isolation of the center regions of attached EBs that differentiated from hESCs and through subsequent cell sorting for vWF. Compared with cells from whole EBs, cells isolated from the center regions of EBs showed higher PECAM expression and spontaneously formed well-defined vascular structures. Thus, this isolation technique can be used to produce a large population of ECs from hESCs. More importantly, the present study provides evidence that transplanted hESC-ECs could act as an alternative source of cells for therapeutic angiogenesis to treat peripheral ischemia. hESC-EC therapy could also be used for treatment of other ischemic diseases such as myocardial or cerebral ischemia. However, because the present study is a preliminary work with small immune-deficient animals, hESC-EC therapy should be further tested in an immune-competent large-animal model as a preclinical study to prove the therapeutic potential and immune benefit of hESCs in the treatment of severe ischemic diseases.

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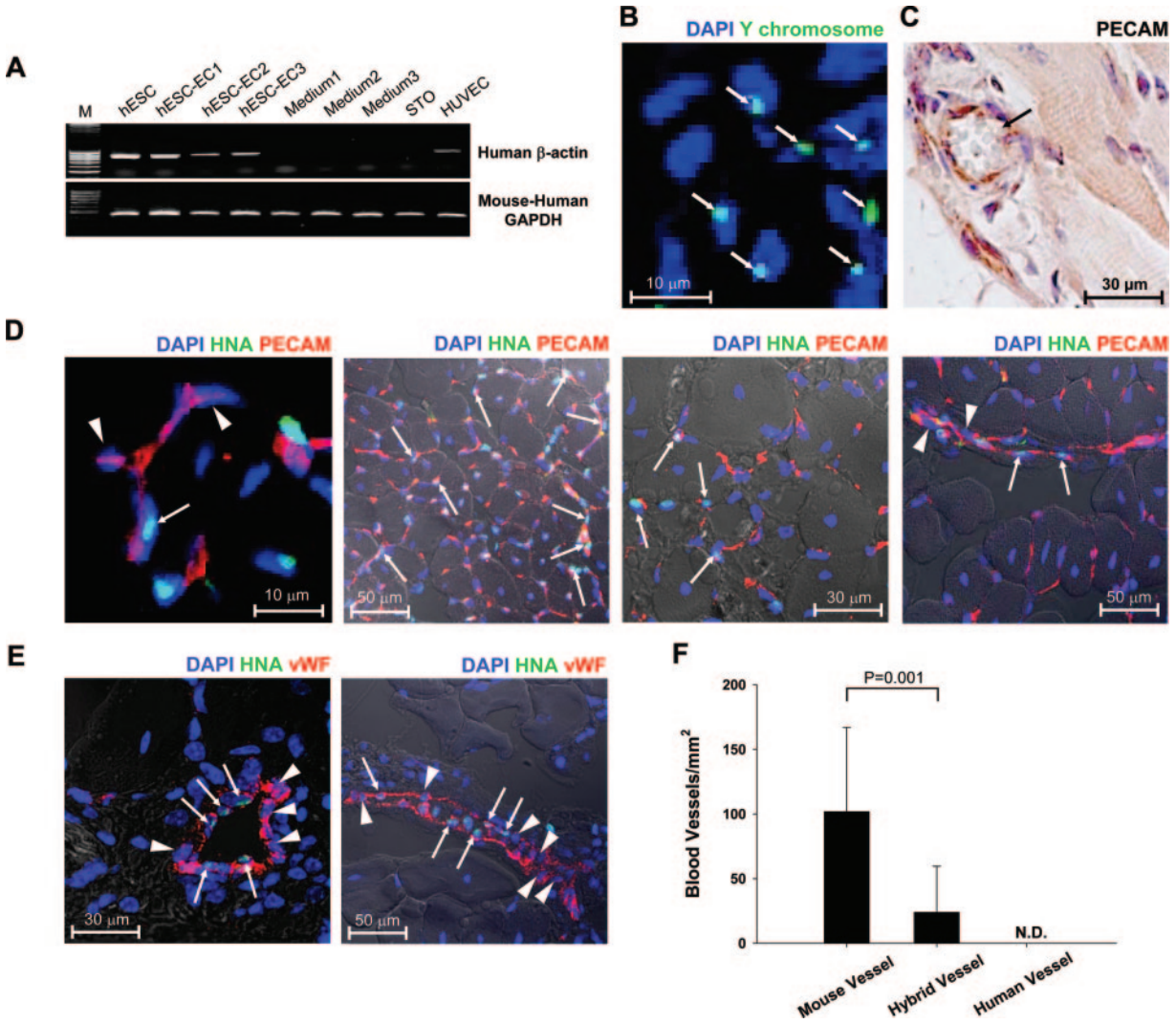

Figure 8. Engraftment of transplanted hESC-ECs into vascular structures in ischemic limb tissues. A, RT-PCR for human β-actin to show the presence of human cells in mouse ischemic tissues. HUVEC indicates human umbilical vein ECs. B, Fluorescence in situ hybridization staining showing human cells with Y chromosomes (arrows) in the ischemic regions. C, Immunohistochemical staining for human PECAM showing newly formed blood vessel containing red blood cells in its lumen (arrow). D, Double-immunofluorescent staining for human nuclear antigen and PECAM. From left to right: first panel, formation of capillary networks between PECAM-positive human cells (transplanted hESC-ECs, arrow) and PECAM-positive mouse cells (mouse ECs, arrowheads) was observed in ischemic muscles; second and third panels, transplanted hESC-ECs (arrows) were found at capillaries near muscle tissues in ischemic regions; and fourth panel, transplanted hESC-ECs (arrows) were incorporated into the vascular endothelium of vessels newly formed in the ischemic regions. E, Double-immunofluorescent staining for human nuclear antigen and vWF. Transplanted hESC-ECs (arrows) were incorporated into blood vessels with mouse ECs (arrowheads) between muscle tissues. F, The density of mouse EC–derived vessels and hESC-EC–incorporated hybrid vessels in ischemic regions. N.D. indicates not detected.

Disclosures

None.

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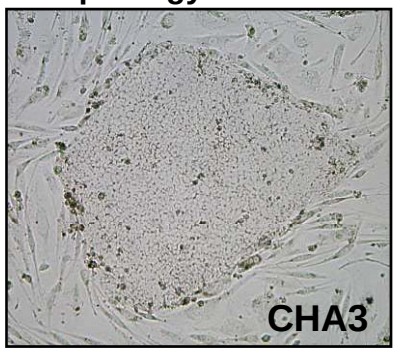
CLINICAL PERSPECTIVE

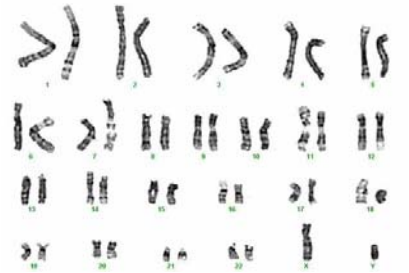
Myocardial infarction, stroke, and coronary artery disease are devastating and life-threatening ischemic diseases. In this study, we confirmed that human embryonic stem cells (hESCs) represent a new cell source for angiogenic therapy to treat ischemic diseases. We differentiated hESCs through human embryoid body formation, and endothelial-like cells (ECs) were obtained by mechanical isolation and cell sorting for von Willebrand factor. The isolated hESC-ECs maintained endothelial cell–specific characteristics such as endothelial marker expression and capillary formation. hESC-ECs were injected intramuscularly into ischemic limbs of mouse models, and 4 weeks after treatment, hESC-EC treatment significantly increased limb salvage (36%) compared with treatment with medium (0%). In addition, the ratio of blood perfusion (ischemic to normal limb) was increased significantly ($P<0.01$) by hESC-EC treatment (0.511 ± 0.167) compared with medium injection (0.073 ± 0.061). Capillary and arteriole densities were $658\pm190/mm^2$ and $30\pm11/mm^2$ in the hESC-EC group, respectively, whereas those in the medium group were $392\pm118/mm^2$ and $16\pm8/mm^2$, respectively ($P<0.01$). Reverse-transcription polymerase chain reaction with human-specific primers revealed mRNA expression of human endothelial markers and human angiogenic factors in ischemic mouse tissues. The transplanted hESC-ECs were localized as capillaries near muscle tissues in ischemic regions or incorporated into the vessels between muscle tissues, as confirmed by human nuclear antigen staining with platelet/endothelial cell adhesion molecule or von Willebrand factor. This study demonstrates that hESC-EC transplantation improves blood perfusion and limb salvage by facilitating postnatal neovascularization in a mouse model of hindlimb ischemia. Thus, hESC-ECs might be useful as an alternative cell source for angiogenic therapy.

In vitro characterization

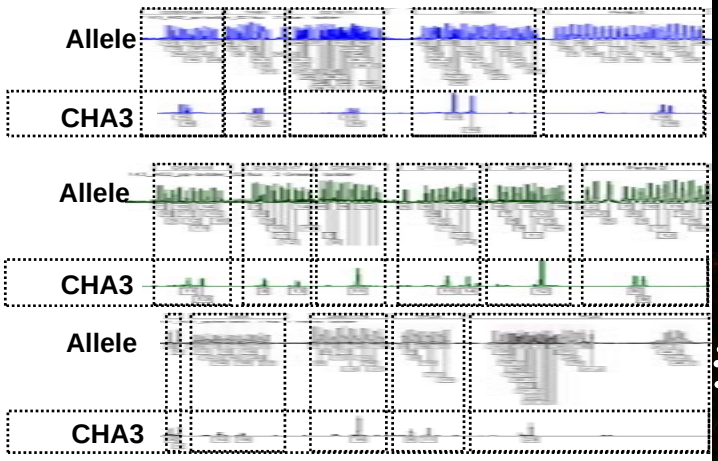
A. Morphology



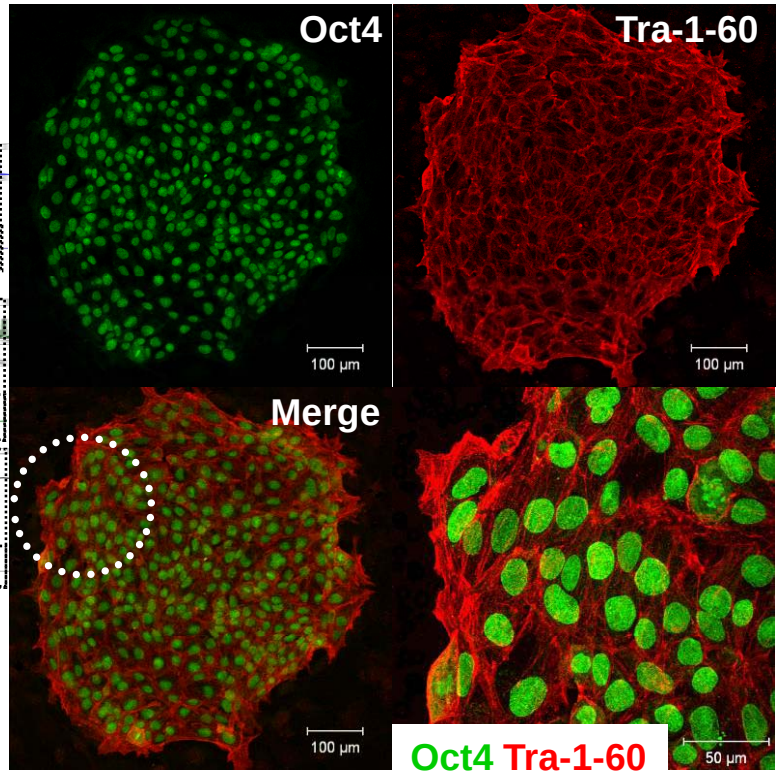
B. Karyotype (44+XY)



C. DNA fingerprinting



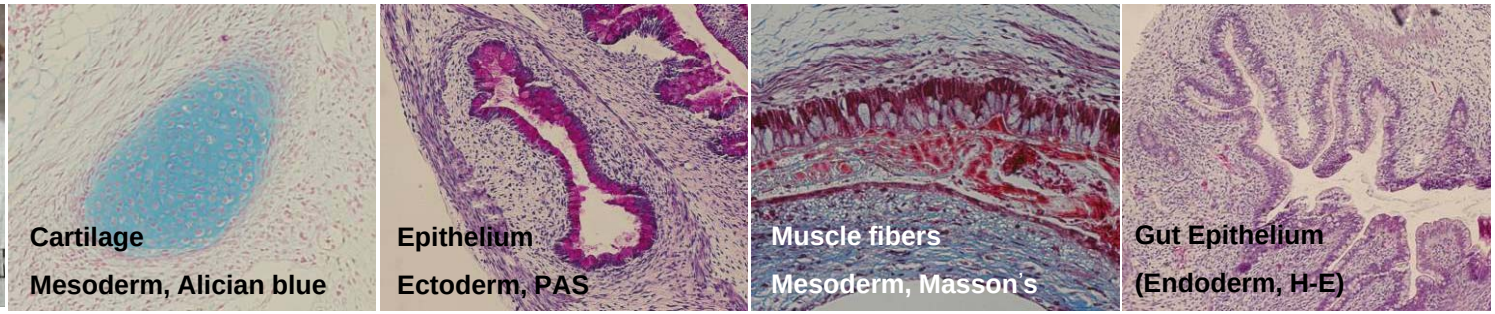
D. Immunocytochemistry



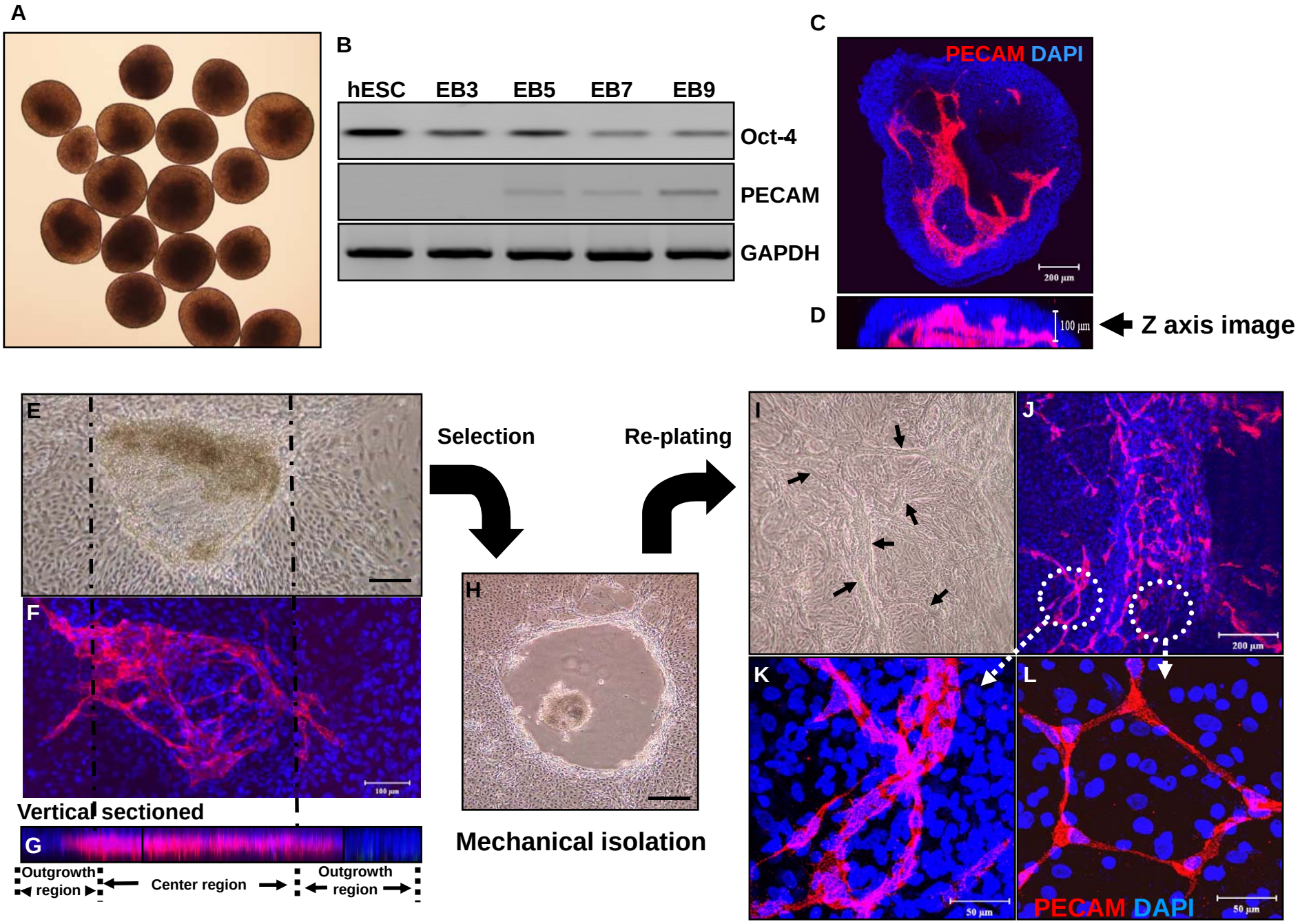
In vivo characterization

A. Teratoma formation

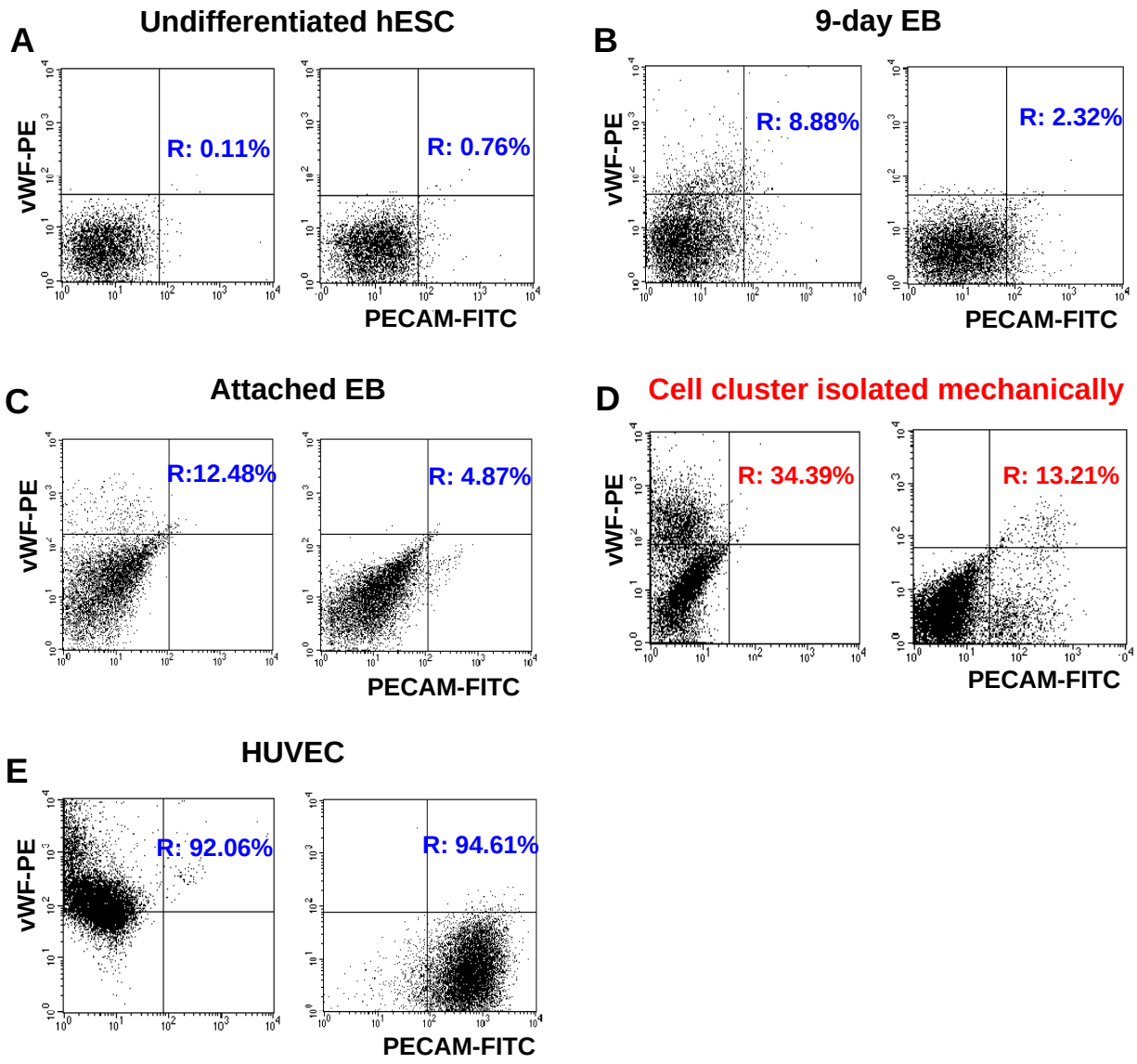
B. Histology



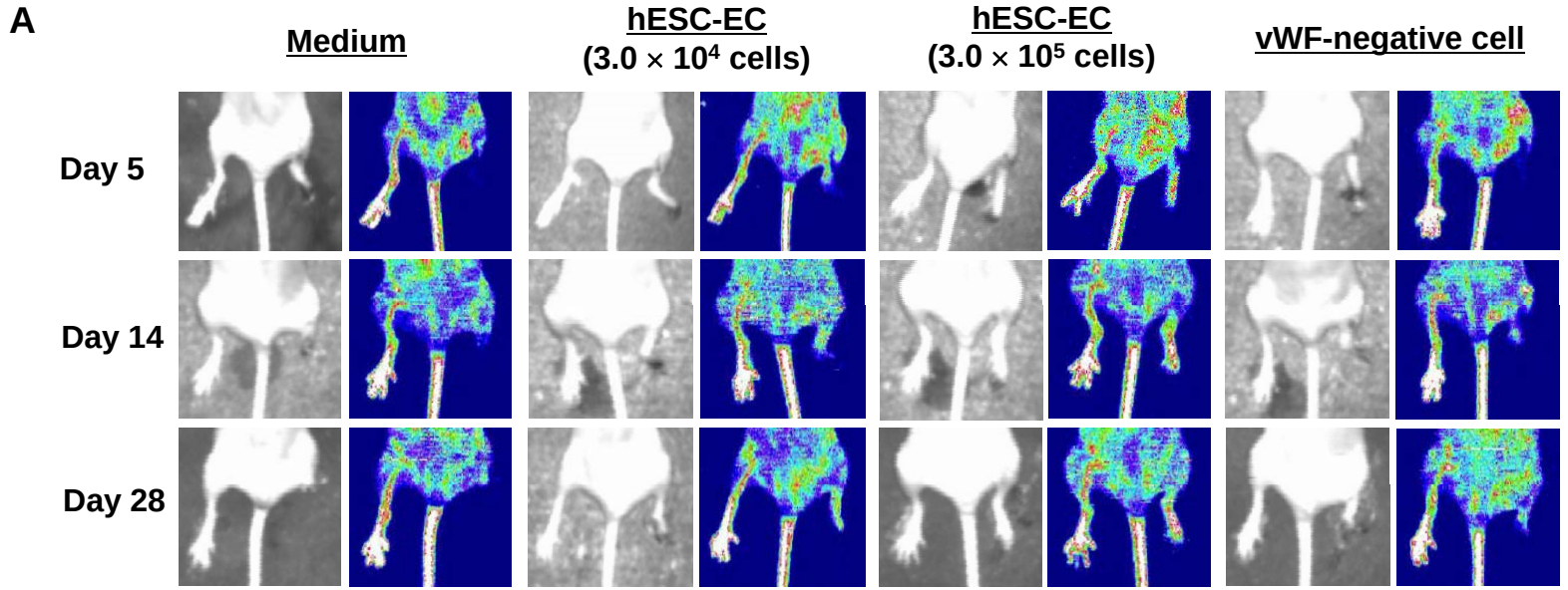
Supplement Figure I

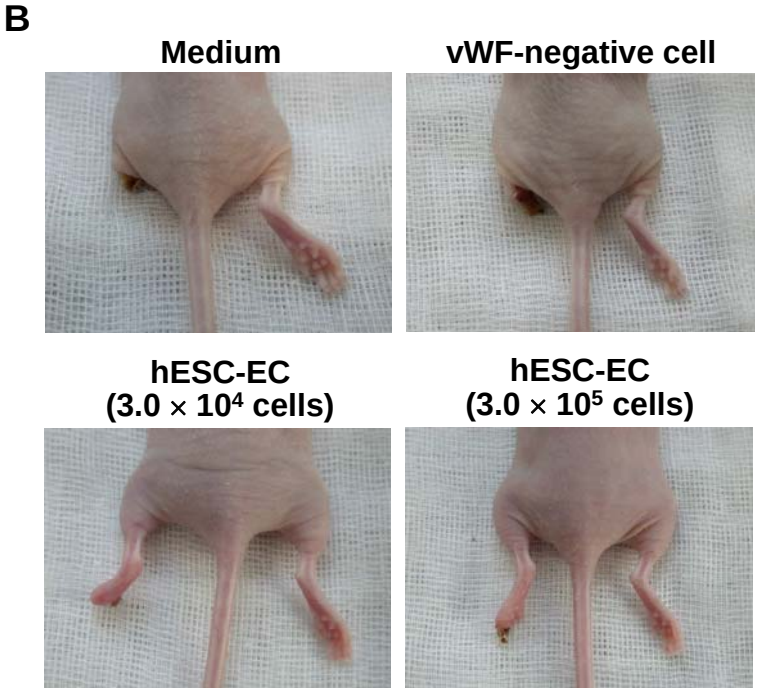


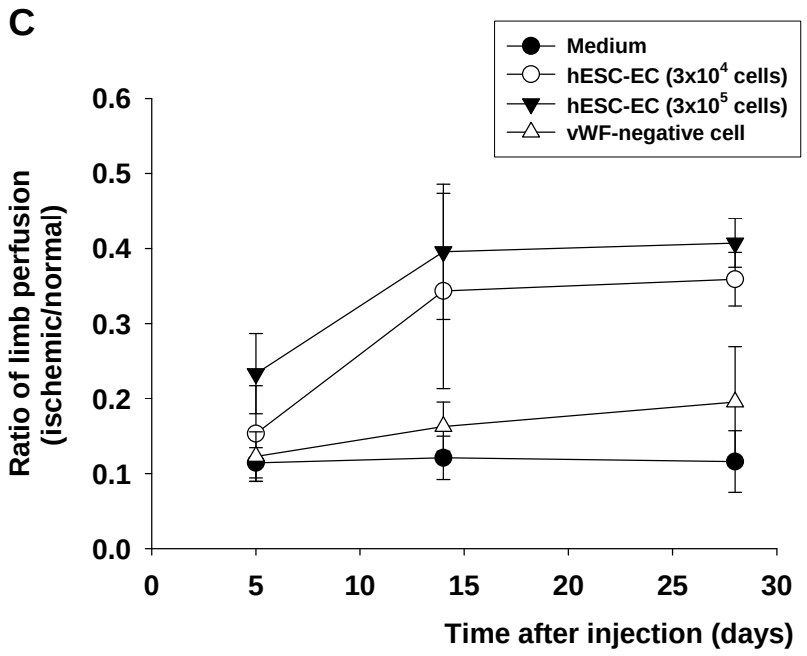
Supplement Figure II



Supplement Figure III







Supplement Figure IV