Directing Human Embryonic Stem Cells towards Functional Endothelial Cells Easily and without Purification

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Hemangioblasts or blood islands only arise in early development thereby the sources to obtain these bi-potential cells are limited. While previous studies have isolated both lineages *in vitro* through the hemangioblast, derivation efficiency was rather low due to cellular damage attributed by enzyme usage and fluorescent activated cell sorting (FACS). This study focused on avoiding the use of damaging factors in the derivation of endothelial cells (ECs). Single cell H9-human embryonic stem cells (hESCs) were obtained by using a mild dissociation protocol then human embryoid body (hEB) formation was performed under hemangioblast differentiation conditions. The hEBs were subjected to a two-stage cytokine treatment procedure. Subsequent culture of the adhesive cells in day 4 hEBs gave rise to a seemingly pure population of ECs. The hESC-derived ECs were characterized by identifying signature endothelial gene and protein markers as well as testing for *in vitro* functionality. Furthermore, *in vivo* functionality was also confirmed by transplanting the cells in hindlimb ischemic murine models. We demonstrate that the genetic change required for EC derivation precedes blast colony formation. Furthermore, cell damage was prevented by abating enzyme usage and FACS, resulting in a high yield of ECs upon adhesion. Under this method, confluent cultures of ECs were obtainable 4 days after hEB formation which is significantly faster than previous protocols.

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INTRODUCTION

In humans, hemangioblast or blood island first appear in the mesoderm surrounding the yolk sac and later in the lateral plate mesoderm on third week of development [1,2]. These cells function as a bi-potential precursor with the ability to form both hematopoietic stem cells and endothelial cells (ECs) [3-5]. Almost all tissue in the body depends on blood supply which in turn de-

pends on ECs that compose the vessel network carrying blood cells [6].

ECs are versatile cells that compose the endothelium which lines the vascular system. While the endothelium acts a protective semi-permeable barrier that controls the flow of small and large molecules, ECs in essence are far more dynamic and conduct a variety of metabolic and synthetic functions involving the regulation of vascular tone, blood flow, thrombolysis, platelet adherence as well as immune and inflammatory responses [7]. ECs are thought to arise from the splanchnopleuric mesoderm formed during embryogenesis which is the inner layer of the mesoderm that adheres to the endoderm [6-8]. Its substantial influence on vital functions associated with the vascular and immune system continues to be an attraction in cell biology and regenerative medicine as researchers strive to derive human ECs through various methodologies to understand these cells in recognition of its future therapeutic potential [9-11].

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Unlike somatic sources, human pluripotent stem cells are a promising source for the derivation of unlimited ECs due to its ability to self-renew and proliferate [12]. Endothelial and hematopoietic lineages appear in early development from a common multipotent precursor, the hemangioblast [1,13]. Previous studies have demonstrated the derivation of ECs from human embryonic stem cell (hESC)-hemangioblasts but low yield and cell line dependency limited efficiency and practicality [14]. Furthermore, the isolation process involved the use of dissociation enzymes (trypsin) and fluorescent activated cell sorting (FACS) which can often damage and influence cellular character [15]. Recently, we showed that a Dulbecco's phosphate buffered saline (DPBS)-dissociation method could increase hemangioblast yield in comparison standard methods by reducing cell damage associated with enzymatic and sorting stress. Furthermore, the derivation process was applicable to several hESC cell lines including H9 and CHA-15 [16].

The potential therapeutic application of hESC-derived endothelial cells (hESC-ECs) and cord blood-derived endothelial progenitor cells (CB-EPCs) has been suggested because of their ability to induce neovascularization in ischemic murine models [17,18]. Previous studies have demonstrated that hESC-EC transplantation is more beneficial in terms of vascularization and angiogenesis in comparison to their somatic counterparts such as CB-EPCs and human umbilical vein endothelial cells [17]. From this perspective, hESC derived progenitors or ECs have a clear advantage due to their immortality, proliferative capacity, and therapeutic outcome.

In this study, ECs were derived from H9-hESCs on the fourth day of human embryoid body (hEB) culture. Cell damage was prevented by abating enzyme usage and FACS, resulting in a high yield of ECs-like cells upon adhesion. We showed that an EC committed state can be achieved upon adhesion of hEBs under conditioned medium and that hemangioblast differentiation is not necessary for the derivation of ECs. Under this method, confluent cultures of ECs were obtainable 4 days after hEBs formation which is significantly faster than previous protocols.

MATERIALS AND METHODS

hESC culture

H9 hESCs (Wicell Research Institute, Madison, WI, USA) were grown on mitotically inactivated mouse embryonic fibroblasts (MEFs) under standard protocols described in detail elsewhere [19]. Briefly, hESC colonies were dissected with a glass Pasteur pipette and were transferred on to Mytomycin C (Sigma-Aldrich, St. Louis, MO, USA) inactivated CF1 MEFs in DMEM/F12 (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 20% (v/v) serum replacement (SR;

Thermo Fisher Scientific, Waltham, MA, USA), 100 mM β -mercaptoethanol (Thermo Fisher Scientific, Waltham, MA, USA), 1% (v/v) non-essential amino acid (Thermo Fisher Scientific, Waltham, MA, USA) and 4 ng/mL basic fibroblast growth factor (bFGF) (Peprotech, Rocky Hill, NJ, USA). Cell culture media was changed daily and cultures were passaged weekly through mechanical dissection. Cell cultures were incubated in a thermostat incubator at 37°C under 5% CO₂ in air with 95% saturated humidity.

EB induction and EC derivation

For hEB induction, hESCs were treated with DPBS (without calcium and magnesium chloride) (Thermo Fisher Scientific, Waltham, MA, USA) for 5–10 minutes then collected by centrifugation for filtration through a 40 μ m cell strainer (BD Biosciences, San Diego, CA, USA) [16]. The derivation of hemangioblast was described in detail in our previous study [16]. For EC derivation, hEBs were obtained by culturing in Stemline II™ basal medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 1% GlutaMAX™-I (Thermo Fisher Scientific, Waltham, MA, USA), 50 ng/mL bone morphogenetic protein (BMP)-4 and vascular endothelial growth factor (VEGF) (Peprotech, Rocky Hill, NJ, USA) for 2 days. Subsequently, single cell dissociation was performed and half of the culture media was exchanged for Stemline II™ basal medium supplemented with 50 ng/mL BMP4, VEGF, stem cell factor (SCF), thrombopoietin (TPO), fms-like tyrosine kinase-3 (Flt-3) ligand, and bFGF (Peprotech, Rocky Hill, NJ, USA) for an additional 2 days of culture in order to obtain hESC-EC. The cells were collected through a 40 μ m cell strainer then seeded onto a collagen type IV (Stem Cell Technologies, Vancouver, BC, Canada) culture dish with EGM-2MV™ (Lonza, Basel, Switzerland) media at a density of 5×10^5 cells/21 cm² (60 mm dish) (Fig. 1A).

To expand the selected hESC-EC cultures, 80–90% confluent cultures were digested and separated with 0.05% trypsin-ethylene diamine tetra acetic acid (EDTA) which was inactivated by defined trypsin inhibitor (Thermo Fisher Scientific, Waltham, MA, USA) for sub-culture.

RNA extraction and RT-PCR analysis

Total RNA (1 μ g) was extracted using Trizol® (Thermo Fisher Scientific, Waltham, MA, USA) and by a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) for messenger RNA expression analysis. Reverse-transcription was used to obtain complementary DNA by using a Maxim RT premix kit (iNtRon biotechnology, Seongnam, Korea). Reverse transcription polymerase chain reaction (RT-PCR) analysis was performed for undifferentiated H9-hESCs, hESC-ECs, and CB-EPCs. The cycling parameters were 95°C for 30 sec-

onds, 55–60°C for 30 seconds, 72°C for 30 seconds, for 35 cycles with AccuPower PCR premix (Bioneer, Daejeon, Korea). The PCR cycles were preceded by an initial denaturation of 5 minutes at 95°C and followed by a final extension of 10 minutes at 72°C. The RT-PCR primer sequences are listed in the Table 1. RT-PCR products were analyzed on ethidium bromide stained with 1.5% agarose gel (Promega, Madison, WI, USA).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde and permeabilized with 0.03% Triton X-100 in phosphate buffered saline (PBS; Sigma-Aldrich, St. Louis, MO, USA) for 5 minutes. After treatment with 5% normal goat serum for 30 minutes, the cells were incubated overnight at 4°C with primary antibodies anti-human platelet endothelial cell adhesion molecule (PECAM) (BD Bioscience, San Diego, CA, USA) and anti-human von Willebrand factor (vWF) (BD Bioscience, San Diego, CA, USA). The cells were washed two times then Alexa Fluor® 488 and 594 secondary antibodies (Molecular Probes Inc., Sunnyvale, CA, USA) were used to visualize the vWF and PECAM staining signals. All images were acquired using a fluorescence microscope (Nikon, Eclipse Ti, Chiyoda, Japan). Finally, cell nuclei were stained and visualized by using primary antibody 4',6-diamidino-2-phenylindole (DAPI; DAKO, Carpinteria, CA, USA).

Flow cytometry analysis

For flow cytometry analysis, the derived-ECs were treated with 0.05% trypsin-EDTA for 2–3 minutes to achieve single cell

dissociation. The cells were then washed with PBS containing 2% (v/v) fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA) for 20 minutes at 4°C. Subsequently, the cells were incubated with antibodies, including phycoerythrin (PE)-conjugated mouse anti-human stage-specific embryonic antigen-4 (SSEA-4), TEK tyrosine kinase-endothelial (Tie-2) and PECAM (BD Biosciences, San Diego, CA, USA). The isotype-matched IgG was used as a control and a FACS Calibur (BD Biosciences, San Diego, CA, USA) and CellQuest software (BD Biosciences, San Diego, CA, USA) was used to analyze the data.

Matrigel assay

A total of 200 µL of Matrigel (Corning, NY, USA) was solidified at 37°C for 30 minutes in tissue culture dish. For induction of vascular formation, 200 µL of suspensions containing 1×10^5 cells were plated on the Matrigel for incubation at 37°C overnight. Tubule structures formation was observed by phase contrast microscopy and performed immunocytochemistry was performed using human PECAM, vWF, and VE-cadherin.

Ac-LDL uptake

Acetylated low density lipoprotein labeled (Ac-LDL) (Bio-medical Technologies, Stoughton, MA, USA) uptakes was examined by incubating the, derived hESC-ECs with 10 µg/mL DiI-labeled Ac-LDL for 4 hours at 37°C. After incubation, cells were washed three times with PBS, fixed with 4% paraformaldehyde for 30 minutes. Images were analyzed with fluorescence microscope (Nikon, Eclipse Ti, Chiyoda, Japan).

Table 1. Human-specific primers for each gene

Gene name	Forward reverse	Primers sequence (5' → 3')	Temperature (°C)	Size (bp)
<i>gapdh</i>	F	ACCACAGTCCATGCCATCAC	55	450
	R	TCCACCACCCTGTTGCTGTA		
<i>oct-4</i>	F	AACTCGAGCAATTTGCCAAGCTCC	55	328
	R	TTCGGGCACTGCAGGAACAAATTC		
<i>nanog</i>	F	CAAAGGCAAACAACCCACTT	55	158
	R	TCTGCTGGAGGCTGAGGTAT		
<i>flt-1</i>	F	TTCCCTGCAAGATTACAGGCACCTA	60	700
	R	AGTCCGGCACGTAGGTGATTCTT		
<i>tie-2</i>	F	ACGGGCCAGATTGTAAGCTTAGGT	57	322
	R	ATCCGGTGGATGGTGAATATGGCT		
<i>ve-cad</i>	F	TGTGGGCTCTCTGTTTGTGAGGA	57	393
	R	TATCCAATGCTATGCCAGCCGCA		
<i>vwf</i>	F	AGAAAGCCCATTGCTGAGCCTTG	57	374
	R	TCCAGAGCACAGTTTGTGGAGGAA		
<i>kdr</i>	F	TCAGCTATGCTGGCATGGTCTTCT	57	376
	R	ATCCGGTGGATGGTGAATATGGCT		

Ischemic hindlimb model and cell transplantation

Hindlimb ischemic murine models were developed by following a previous procedure explained in detail elsewhere [17]. Briefly, mice (6 weeks of age, body weight 15 to 18 g; Orientbio, Seoul, Korea) were anesthetized and a skin incision was performed to expose the femoral artery which was then carefully separated from the femoral vein. Double knots were made with a suture at the distal and proximal femoral ligation sites. Subsequently, the femoral segments were excised and the incision was closed, resulting in mice with hindlimb ischemia. A day after arterial dissection, ischemic mice were randomly assigned to 1 of 2 experimental groups. Intramuscular transplantation was conducted by injecting each group into 4 sites of the gracilis muscle in the medial thigh with 29-gauge tuberculin syringes [17]. The saline group (n=10) was injected with medium and the experimental group received-hESC-ECs (3×10^5 cells per mouse) suspended in 200 μ L of EGM-2 MVTM (hESC-ECs group, n=10).

Laser doppler analysis

A laser Doppler blood perfusion imager (Moor Instruments, Devon, UK) was used for serial noninvasive physiological evaluation of vascularization. Mice were monitored by serial scanning of surface blood flow of hindlimbs on days 0, 7, 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 [17].

Micro computed tomography

Murine blood vessels were fixed Microfil® (MV-120, Flow Tech Inc., Carver, MA, USA) following the use of a blood perfusion system (Thermo Fisher Scientific, Waltham, MA, USA). Transplantation sites were fixed with 4% formalin for 28 days and assessed via radiographic analysis with a micro-CT scanner (Skyscan1172, Bruker-microct, Kontich, Belgium). The scanned CT images of specimens were taken at a resolution of 50 μ m (100 kV and 100 mA) and three-dimensionally (3D) reconstructed with computer software (NRecon; Skyscan, Aartselaar, Belgium). The regenerated microvessel and muscle volume and defect coverage in the defect site were calculated from the 3D reconstructed images using CTAn software (Skyscan, Aartselaar, Belgium) [20].

Histological and immunohistochemistry analysis

For tissue staining, mice were sacrificed after 4 weeks and ischemic limb tissues were retrieved. Specimens were fixed in 10% (v/v) buffered formaldehyde, dehydrated with a graded ethanol series, and embedded in paraffin. Specimens were sliced into 4 μ m sections and stained with hematoxylin and eosin (H&E). Masson's trichrome (MT) collagen staining was also

performed to examine fibrosis in ischemic tissues. To detect the presence of human cell, tissue sections were immunohistochemically stained using anti-human smooth muscle actin (SMA) (BD Bioscience, San Diego, CA, USA) and anti-human PECAM antibodies (BD Bioscience). The staining signal was visualized with avidin-biotin complex immunoperoxidase (Vectastain® ABC kit) and 3,3'-diaminobenzidine (DAB) substrate solution kits (Vector Laboratories, Burlingame, CA, USA).

Fluorescence *in situ* hybridization analysis

First, specimen slides were deparaffinized in xylene at room temperature then dehydrated with 100% ethanol before air dry. Paraffin pretreatment solution was pre-heated to 95°C in a heating mantle and the slide was immersed for 30 minutes followed by immersion in 2X standard saline citrate (SSC) twice for 5 minutes each. Protease solution was prepared by adding 500 μ L protease in 50 mL protease buffer which was pre-heated to 37°C for slide immersion for 20 minutes. The slides were again immersed in 2X SSC then dehydrated in 70% ethanol for 1 minute and 100% ethanol for another minute. After air dry, a diamond pen was used to mark the hybridizing area and the chromosome enumeration probe (CEP) 17 DNA probe (2 μ L, Vysis, Downers Grove, IL, USA) was applied before being sealed under glass coverslips with rubber cement. Subsequently, the probe was denatured at 75°C for 5 minutes while being protected from light and hybridization was performed overnight at 37°C in a humidified chamber. After hybridization, the rubber cement was removed and the section slides were washed for 10 minutes with 50% (v/v) formamide/2X SSC at 42°C and placed in 4X SSC with 0.1% NP for 5 minutes. The sections were air-dried in the dark and counter-stained with DAPI.

Statistical analysis

Data are presented as mean value $\pm$ standard deviation for statistical comparison. Significance of differences was assessed by Student t-test, where $p < 0.05$ was considered significant. Histograms of the obtained data were fitted using the Microsoft Excel 2010 (Microsoft Inc., Redmond, WA, USA). All experiments were independently performed thrice, each in triplicate.

RESULTS

Simple derivation of endothelial cells without purification

First, H9-hESC aggregates were allowed to form embryoid bodies in suspension culture and the hEBs appeared well-made and healthy (Fig. 1B). Differentiation was induced by applying a two-stage treatment procedure involving the use of BMP4 and VEGF for 2 days then TPO, SCF, Flt3, and bFGF for another 2

days of culture. Subsequently, the two-stage cytokine treated day 4 hEB was dissociated by 0.05% trypsin-EDTA and the adhesive cells were plated on a collagen type IV coated culture dish, resulting in cells with an endothelial morphology (Fig. 1C). In addition, suspension culture under similar conditions of day 4 hEB leads to hemangioblast formation (Fig. 1D). This suggests that FACS associated cellular damage can be avoided if the objective is to simply purify cells with an EC morphology.

Characterization of the hESC- ECs

RT-PCR was performed to confirm that differentiation was induced by detecting the downregulation of pluripotency genes (*oct-4* and *nanog*) and subsequent upregulation of endothelial markers. Pluripotent genes were strongly expressed in hESCs as well as day 2 hEBs but were not detected in hESC-derived ECs. Similar to that of the control CB-EPC, the derived EC expressed endothelial progenitor cell (EPC) markers *flt-1*, *tie-2*, *VE-cad*, *vwf*, and *kdr* (kinase insert domain receptor) on day 7 as a consequence (Fig. 2A). Immunocytochemistry confirmed

Figure 1. Experimental scheme for *in vitro* derivation of hemangioblasts and endothelial cells from hESCs. (A) A DPBS-dissociation protocol was used to obtain single cells for (B) embryoid body formation and suspension culture. (C) Following two-stage cytokine treatment, adhesive cells from day 4 hEBs were plated on collagen-coated dishes resulting in hESC-ECs and (D) suspension culture gave rise to the hemangioblast. hESCs: human embryonic stem cells, DPBS: Dulbecco's phosphate buffered saline, hEBs: human embryoid bodies, ECs: endothelial cells.

that endothelial cell specific proteins vWF and PECAM were also strongly expressed (Fig. 2B). Furthermore, protein expression was observed in hESC-ECs by conducting flow cytometry analysis which showed the absence of pluripotent marker SSEA-4 and presence of EPC markers Tie-2 (11.29%) and, PECAM (37.07%) (Fig. 2C). *In vitro* functionality was validated by a tubule formation assay which demonstrated that the derived ECs were capable of forming capillary-like structures expressing PECAM, vWF, and VE-cad and uptake Ac-LDL (Fig. 2D and E). These results indicate that hESCs had undergone differentiation towards an endothelial fate as specific genes were expressed in response to the downregulation of pluripotent genes. In addition, the hESC-ECs possessed signature functional characteristics *in vitro* that were similar to CB-EPC.

Enhanced vascularization by *in vivo* transplantation of hESC-ECs in ischemic mice

In vivo functionality was evaluated by transplanting hESC-ECs in ischemic murine models to examine limb repair in com-

parison to a saline-treated control. Both groups (n=10) were subjected to an observation period of 4 weeks. The intramuscular transplantation of hESC-ECs significantly reduced the rate of limb loss compared to the saline group. All mice receiving saline injection underwent limb loss (80%, 8 of 10) or severe limb necrosis (20%, 2 of 10), with zero cases of limb salvage. However, half of the mice that received hESC-EC transplantation exhibited limb salvage (20%, 2 of 10) or mild limb necrosis (30%, 3 of 10) (Fig. 3A). By day 28, half of the transplanted group displayed restored blood perfusion in the ischemic limb while the control group exhibited limb loss or severe necrosis (Fig. 3B). At 1 week after treatment, the relative ratios of blood flow were 0.272 ± 0.017 in the hESC-EC transplantation group and 0.133 ± 0.003 in the saline injection treatment group. At 2 week after treatment, the relative ratios of blood flow were 0.471 ± 0.045 in the hESC-EC transplantation group and 0.136 ± 0.009 in the saline injection treatment group. At 3 week after treatment, the relative ratios of blood flow were 0.551 ± 0.067 in the hESC-EC transplantation group and 0.14 ± 0.019 in the saline injection treatment

Figure 2. Characterization of hESC-derived endothelial cells. (A) Differentiation analyzed by RT-PCR for pluripotent genes (analysis of EC-specific gene (*oct4* and *nanog*) and EPC-specific genes *flt-1*, *tie-2*, *ve-cad*, *vwf*, and *kdr*). (B) Immunocytochemistry for endothelial specific protein marker expression vWF (green) and PECAM (red); DAPI (blue) and Merged (C) flow cytometry analysis of EC-specific protein expression Tie-2 and PECAM in hESC-ECs. hESCs: human embryonic stem cells, RT-PCR: reverse transcription polymerase chain reaction, EC: endothelial cell, EPC: endothelial progenitor cells, vWF: van Willebrand factor, PECAM: platelet endothelial cell adhesion molecule, DAPI: 4',6-diamidino-2-phenylindole. (Continued to the next page)

group. At 4 week after treatment, the relative ratios of blood flow were 0.588 ± 0.014 in the hESC-EC transplantation group and 0.145 ± 0.003 in the saline injection treatment group (Fig. 3C). In addition, a micro CT scan was performed to visualize vessel structure and formation which revealed vessel regeneration in the transplanted group, repairing ischemic limbs closer to normal conditions. The red arrows indicate arterioles and capillaries while the yellow arrows indicate blood vessels (Fig. 3D). These results demonstrated that the derived hESC-ECs were capable of restoring blood flow and salvage the hindlimb by promoting angiogenesis and neovascularization which is

consistent to that of previous EC transplantation studies.

Restored muscle and tissue structure by hESC-EC transplantation

To perform histological analysis of muscle degeneration and fibrosis, ischemic hindlimbs were harvested after 4 weeks post-transplantation. H&E staining was used to visualize tissue structure and determine the extent of muscle repair (H&E) (Fig. 4A). According to the results, the hESC-ECs group displayed improved restoration in comparison to the control group which displayed severe muscular degeneration (hESC-EC) (Fig. 4A). Fur-

Figure 2. (Continued from the previous page) (D and E) *In vitro* functionality confirmed by tubule formation assay on matrigel coated dish, immunocytochemistry, hESC-EC, specific marker, expression, vWF (red), PECAM (red), VE-cadherin (red), DAPI (blue), Merged (D) and Ac-LDL uptake of Dil-labeled hESC-ECs (E). vWF: van Willebrand factor, PECAM: platelet endothelial cell adhesion molecule, DAPI: 4',6-diamidino-2-phenylindole, hESCs: human embryonic stem cells, EC: endothelial cell.

thermore, MT staining revealed that collagen deposition was most progressive in the hESC-ECs group which allowed for tissue to be reconstructed accordingly (MT) (Fig. 4A). In contrast, the control sample displayed severe fibrosis and an inability for muscle repair (saline) (Fig. 4A). Next, to detect and quantify arteriole and capillary volume (mm^2), SMA and PECAM staining was performed, showing that vessel volume was significantly higher in hESC-EC transplanted group. Quantification revealed that neovascularization was significantly enhanced compared with saline injection. The volume of arterioles and capillaries in the saline group was $17 \pm 4 \text{ mm}^2$ and $293.3 \pm 45.1 \text{ mm}^2$ in comparison to $37 \pm 3 \text{ mm}^2$ and $696.6 \pm 49.3 \text{ mm}^2$ in the transplanted group, respectively (Fig. 4B). In addition, FISH analysis was

conducted to confirm that the transplanted EC cells carried human chromosomes (white arrow) (Fig. 4C). Taken together, these results further support the notion that hESC-derived ECs were functional *in vivo* and the newly formed vessels were attributed by hESC-EC transplantation.

DISCUSSION

Despite the progressive advancements made towards understanding and defining the nature of ECs, its diversity and involvement in various biological functions has made it difficult for researchers to reach a definite consensus on its characteristics.

ECs are intimately linked with hematopoietic or blood cells

Figure 3. *In vivo* functional analysis in hindlimb ischemic animal models. (A) Physiological status of hindlimbs on day 28 showing the number of salvaged limbs as well as necrotic and lost limbs in each group (n=10). (B) Blood perfusion examined through a laser Doppler image on 0, 14, 21, and 28 days after hESC-EC transplantation. (C) The ratio of limb perfusion increased approximately 60% in the hESC-EC group in comparison to the control (saline). (D) A micro-CT scan of a normal limb in comparison to the saline and hESC-EC transplanted group. hESC-EC: human embryonic stem cell-derived endothelial cell.

during development due to a common precursor, the hemangioblast. These are multipotent cells that arise early during EB formation marked by an increase in expression of kinase insert domain receptor (KDR), stem cell leukemia, CD34, CD31 (PECAM), protein patched homolog 1 (PTCH 1), and glioma-associated oncogene (GLI) family zinc finger 1 genes [21]. *In vitro*, hemangioblasts grow as transient blast colony-forming cells or blast colonies that contain both adherent (endothelial) and round non-adherent (hematopoietic) populations. Isolation and re-plating of the adherent population onto matrigel-containing wells with defined medium will give rise to vascular-like structures expressing PECAM and Ac-LDL uptake, which is a unique property of ECs [3].

Traditionally, hESC EC were isolated from a heterogeneous population by enzyme dissociation and FACS sorting but such procedures often lowers derivation efficiency because it inflicts cellular damage. Furthermore, the derivation of ECs that follows the natural process of embryogenesis has proven to be quite challenging. Previous reports have suggested that the bi-potential expression both hematopoietic and angiogenic markers are transient characteristics in blast-colony forming cells [4,6,8,22-26], which refers to suspension cultured hEBs in defined medium for 7 to 10 days until a hemangioblast morphology appears. These cells were capable of contributing to neovascularization in several animal models when transplanted [17]. While these studies substantiate the existence of the hemangioblast in hEBs, the stage of genetic change that precedes phenotypic and morphological change were not investigated thoroughly.

This study showed that a relatively pure population of ECs can be obtained from two-stage treated hEBs on day 4, suggesting that the genetic composition required for EC derivation can be established before blast-colony formation. Adherent culture of day 4 hEBs rapidly lead to EC specification, resulting in a high yield of EC cells as cellular damage was mitigated by minimizing the use of enzymes and abating FACS sorting. The derived ECs exhibited key endothelial markers, expressing PECAM, vWF and VE-cad in addition to *in vitro* functionality of tubule formation and Ac-LDL uptake. *In vivo* transplantation and micro CT scan of salvaged ischemic limbs also support the notion that the hESC-derived ECs possess angiogenic potential, displaying neovascularization and angiogenesis in ischemic limbs.

Continued suspension culture of the hEB lead to the formation of hemangioblasts (Fig. 1D), which is consistent with our previous report as well as others [16].

The protocol described in this study is a fast and efficient way to derive ECs from H9-hESCs by avoiding the use of damaging factors such as trypsin and sorting to decrease generation-time and increase the yield of endothelial derivatives. This can be useful for accelerating research in order to further understand

Figure 4. Engraftment of transplanted hESC-ECs for the restoration of vascular integrity in ischemic tissues. (A) Hematoxylin and eosin and Masson's trichrome staining for the analysis of muscle degeneration and fibrosis in the ischemic region. (B) Immunohistochemical staining anti-smooth muscle actin (SMA) and anti-PECAM antibodies to quantify volume of the micro vessels (arterioles and capillaries) in tissue sections. (C) Fluorescence *in situ* hybridization (FISH) stains showing the presence of human cells (chromosomes) in sectioned tissue ($p < 0.05$). hESCs: human embryonic stem cells, PECAM: platelet endothelial cell adhesion molecule. Arrow: chromosomes.

the underlying mechanisms that govern endothelial character as well as its interaction with other types of cells in animal models. Previous methods generally require 7 days to derive the hemangioblast which is then plated for adherent culture to give rise to ECs. This study demonstrates that the necessary conditions for endothelial cell derivation can arise at an earlier stage in hEBs than previously thought.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

The animal study was approved by the Institutional Animal Care and Use Committee (IACUC) of Konkuk University.

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