VEGF Induces Differentiation of Functional Endothelium From Human Embryonic Stem Cells
Implications for Tissue Engineering

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Objective—Human embryonic stem cells (hESCs) offer a sustainable source of endothelial cells for therapeutic vascularization and tissue engineering, but current techniques for generating these cells remain inefficient. We endeavored to induce and isolate functional endothelial cells from differentiating hESCs.

Methods and Results—To enhance endothelial cell differentiation above a baseline of ≈2% in embryoid body (EB) spontaneous differentiation, 3 alternate culture conditions were compared. Vascular endothelial growth factor (VEGF) treatment of EBs showed the best induction, with markedly increased expression of endothelial cell proteins CD31, VE-Cadherin, and von Willebrand Factor, but not the hematopoietic cell marker CD45. CD31 expression peaked around days 10 to 14. Continuous VEGF treatment resulted in a 4- to 5-fold enrichment of CD31+ cells but did not increase endothelial proliferation rates, suggesting a primary effect on differentiation. CD31+ cells purified from differentiating EBs upregulated ICAM-1 and VCAM-1 in response to TNFα, confirming their ability to function as endothelial cells. These cells also expressed multiple endothelial genes and formed lumened vessels when seeded onto porous poly(2-hydroxyethyl methacrylate) scaffolds and implanted in vivo subcutaneously in athymic rats. Collagen gel constructs containing hESC-derived endothelial cells and implanted into infarcted nude rat hearts formed robust networks of patent vessels filled with host blood cells.

Conclusions—VEGF induces functional endothelial cells from hESCs independent of endothelial cell proliferation. This enrichment method increases endothelial cell yield, enabling applications for revascularization as well as basic studies of human endothelial biology. We demonstrate the ability of hESC-derived endothelial cells to facilitate vascularization of tissue-engineered implants. (Arterioscler Thromb Vasc Biol. 2010;30:80-89.)

Key Words: human embryonic stem cells ■ endothelial cells ■ VEGF ■ tissue engineering ■ angiogenesis

A persisting challenge to the application of cell-based therapies is the sourcing of specific cells of interest. Because many mature tissues cannot be rebuilt using adult cells derived from biopsies, human embryonic stem cells (hESCs) could be instrumental in regenerative tissue engineering. Their immense proliferative and differentiation potential could provide extensive banks of cells—in quantity as well as type—for therapeutic applications.

Natural and engineered tissues more than ≈200 μm thick require suitable vascular support to survive and function properly. Although growth of host vessels into tissue engineering scaffolds has been achieved via controlled release of angiogenic molecules, this strategy requires many days to produce mature vessels. Further, host-derived vessel formation may be compromised by conditions that reduce angiogenesis such as diabetes and radiation therapy.1 As angiogenesis is directed by a series of cytokines in a precise temporal sequence, adding 1 or even 2 cytokines may lead to incomplete blood vessel formation. Providing scaffolds with exogenous vascular cells before implantation may increase both the speed and extent of vascularization of engineered tissues. Before this approach can be successful, it will be critical to develop reliable methods to generate sufficient endothelial cells.

Differentiation of hESCs in embryoid bodies treated with serum proceeds in a complex and largely uncontrolled manner. Methods to guide hESC differentiation into a specific lineage would therefore provide a higher yield of cells of interest without increasing the overall culture size. In the present study we describe induction of endothelium from hESCs using vascular endothelial growth factor (VEGF). We also demonstrate the

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ability of hESC-derived endothelial cells to facilitate angiogenesis in vivo. Enhancing endothelial differentiation with VEGF thus provides an efficient cell source for tissue engineering and cell transplantation applications.

Methods

Additional details can be found in the supplemental materials (available online at http://atvb.ahajournals.org).

Human Embryonic Stem Cell Culture and Differentiation

Human ESCs, H7 line passage 37 to 92 and H1 line passage 45 to 60 (WiCell, Madison, Wisc), were grown in feeder-free conditions as previously described.2-5 To initiate differentiation, confluent cultures of undifferentiated hESCs were dissociated into small clumps and placed into ultra low–attachment plates in the presence of 20% fetal bovine serum to induce the formation of embryoid bodies (EBs).3,6

The EGM-2MV culture method, hESCs were seeded onto a MEF feeder layer and then grown in EGM-2MV medium.

Vascular Endothelial Growth Factor

Recombinant human vascular endothelial growth factor 165 (VEGF) was generously donated by Genentech. It was stored at 4°C in 5 mM/L succinate buffer containing 275 mM/L trehalose, 0.01% Tween-20, pH 5.0 at 5 mg/mL. An intermediate stock was prepared by diluting the 5 mg/mL stock to 0.1 mg/mL in a 5 mM/L succinate buffer, pH 5.0, with 0.1% BSA. VEGF was added to hEB medium at a final concentration of 50 ng/mL unless otherwise noted.

Western Blots

Protein lysates were analyzed by Western blotting for CD31 (1:1000; Dako), von Willebrand Factor (vWF, 1:100; Dako), CD45 (1:1000; Dako), VE-Cadherin (1:500; R&D Systems), and β-actin (1:1000; Cell Signaling Technology).

FACS Analysis, Purification, and Expansion

VEGF-induced and control embryoid bodies were dispersed into single cells (Blendsyme IV [Roche Applied Science], 37°C, 45 to 60 minutes) and stained with fluorescently conjugated antibodies for flow cytometry. The antibodies used included CD31-PE (BD Biosciences), VE-Cadherin (FITC-conjugated; Serotec; unconjugated followed by Alexa Fluor 488-conjugated secondary, R&D Systems; Alexa Fluor 488-conjugated monoclonal, ebioscience), CD45 (allo-phycocyanin [APC]-conjugated, ebioscience), c-kit (FITC-conjugated, Chemicon; APC-conjugated, R&D Systems), CD14 (FITC-conjugated, BD Biosciences), CD34 (PE-conjugated, BD Biosciences), KDR (PE-conjugated, R&D Systems), CD115 (PE-conjugated, eBioscience), and CD133 (APC-conjugated, Miltenyi Biotec). Stained cells were analyzed on FC500 or FC500MPL flow cytometry analyzers (Beckman Coulter). Human umbilical vein endothelial cells (HUVECs) served as positive control for endothelial cell antigens, and the U937 human promonocyte cell line was used as positive control for hematopoietic cell antigens.

CD31-positive and CD31-negative cells were collected with a sterile cell sorter6 (FACS Vantage, BD Biosciences) and propagated in EGM or EGM-2 (Lonza) or hEB medium,6 respectively.

Proliferation Studies

Embryoid bodies were grown in the presence or absence of VEGF for 4 or 14 days, pulsed for 24 hours with the thymidine analogue 5-bromo-2-deoxyuridine (BrDU, 10 μmol/L; Roche Applied Science), and embedded in paraffin for histological analysis. Sections were double stained for CD31 (1:20; Dako) and BrDU (1:40; Roche Applied Science) and proliferating endothelial cells (CD31+/BrdU+) were quantified in blinded fashion.

TNFα Stimulation

Monolayers of cells (HUVECs and CD31+ cells) were serum-starved overnight and stimulated with 0.1 to 10 ng/mL TNFα for 4 hours. RNA was collected and probed for ICAM-1, VCAM-1, CD31, and GAPDH transcripts via quantitative RT-PCR.

Matrigel Tube-Forming Assay

Matrigel (BD Biosciences) was diluted 1:2 with EBM-2 on ice and then added to 24-well plates and allowed to gel in a thin layer at 37°C. Cells (HUVECs and CD31+ cells; 25,000 per well) were immediately seeded onto the gels and photographed after 5, 10, 24, 48, and 72 hours.

Methyccoliuose Assay

hESC-derived CD31+ cells (105) were mixed with methylcellulose containing IL-1, IL-3, IL-6, G-CSF, GM-CSF, KL at 10 ng/mL and erythropoietin at 3 U/mL. Positive control long-term culture hematopoietic progenitor cells were included to ensure the colony forming ability of the assay. Cultures were observed for 6 days, and colony formation was recorded for each cell type.

Collagen/Geltrex Constructs

CD31+ cells were mixed with human mesenchymal stem cells at a ratio of 2:1. Three million cells were gently combined with 100 μL of cold pregel mixture (1.25 mg/mL Collagen Type I [Invitrogen], 25 mM/L NaOH, 11% v/v basement membrane extract [Geltrex, Invitrogen], and 57% medium [EGM-2, Lonza]) and allowed to form a tethered gel for 1 hour at room temperature using the Flexcell Tissue Train culture plates (Flexcell International). The constructs were then allowed to float freely in culture medium and were cultured for 5 days in EGM-2 in static culture under standard tissue culture conditions.

All animal experiments were approved by the University of Washington Animal Care and Use Committee and were in accordance with federal guidelines for the care and use of laboratory animals. Male athymic (nude) rats (nu/nu; Charles River, Charles River, Mass) underwent myocardial ischemia/reperfusion as previously described.5 Seven weeks after injury, the cultured cell/gel constructs were sutured directly onto the epicardium across the infarcted region. Rats were given subcutaneous cyclosporine A (0.75 mg/d; Wako Pure Chemicals) from days 1 to 7. Hearts were collected on day 7 and fixed and processed for histology as described below.

Porous Poly(2-Hydroxyethyl Methacrylate) Scaffolds

Porous poly(2-hydroxyethyl methacrylate) (polyHEMA) scaffolds were prepared by a sphere-templating process as described,11,12 with one significant modification. The scaffolds used here were created with uncrosslinked poly(methyl methacrylate) beads that were sieved through fine screens to produce sharply defined size fractions. The scaffolds made from these beads had precisely controlled 60-μm spherical pores interconnected by pore throats 24 to 30 μm in diameter. The hydroxyl groups on the porous material surface were chemically activated with carbonyl di-imidazole and then covalently coupled to type I collagen to create a cell-adhesive substrate.13 Scaffolds were 5 mm in diameter and 400 to 700 μm thick.

hESC-derived endothelial cells and endothelial-depleted cells (106 cells per scaffold) were seeded by centrifugation and cultured for 2 days in vitro before implantation.

Scaffold Implantation

Cell-scaffold constructs were rinsed in EBM and kept briefly on ice before implantation. Athymic nude rats (Harlan) were anesthetized with isoflurane (initially 5% isoflurane in an induction chamber followed by 1.5% to 2% isoflurane in 98% O2 at a flow of 2 L/min via a nose cone). Two small dorsal subcutaneous pockets were made near each hindlimb. One scaffold was placed into each pocket, and...
the incision was closed with a 4-0 nonabsorbable suture. Rats recovered in room air in a warm chamber.

Ten days after implantation, the rats were euthanized by pentobarbital overdose. Cell-scaffold constructs were removed from the subcutaneous space with some surrounding tissue, fixed in methyl Carnoy’s fixative (60% methanol, 30% chloroform, 10% glacial acetic acid), and processed for histology.

**Immunohistochemistry**

hESC-derived endothelial cells were stained for CD31 (Dako), eNOS (BD Biosciences), smooth muscle α-actin (Dako), Ulex europaeus agglutinin-1 lectin (Ulex; Vector Laboratories), VE-Cadherin (R&D Systems), and vWF (Dako) as described previously.14,15 Antibody/lectin-positive cells were stained with species-specific secondary antibodies, visualized with 3,3′-diaminobenzidine (DAB; Sigma-Aldrich), and counterstained with hematoxylin. In addition, cells were incubated with 10 μg/mL Dil-AcLDL or AlexaFluor594-conjugated AcLDL (Invitrogen) for 4 hours and counterstained with Hoechst 33342 (Sigma-Aldrich). HUVECs served as positive controls for all endothelial stains and assays.

Sections of the cell/gel construct–implanted hearts were stained with CD31 (1:10; Dako) followed by an AlexaFluor488-conjugated goat antimouse secondary antibody (1:100, Invitrogen). Red blood cells were visible by innate autofluorescence and did not require additional staining. Nuclei were counterstained with Hoescht 33342, and slides were coverslipped with Vectashield (Vector Labs). Slides were imaged on an Axio Observer inverted microscope (Carl Zeiss MicroImaging) and captured with an AxioCAM MRm camera (Carl Zeiss MicroImaging).

**Confocal Microscopy**

Fluorescent images of immunohistochemistry stains were obtained on a Zeiss LSM META confocal microscope (Carl Zeiss MicroImaging).

**Results**

**VEGF Induces Endothelium From hESCs**

Human ESCs (H7 line, passage 44) were grown under 4 different culture conditions. Two groups were allowed to grow as embryoid bodies (EBs) in media containing 20% FBS, one of which was given 50 ng/mL VEGF every 2 to 3 days. A third group of cells was grown in coculture with OP9 stromal cells. The last group of cells was grown in EGM-2MV medium (Figure 1). Protein was collected after 14 days and immunoblotted for CD31, VE-Cadherin, von Willebrand Factor (vWF), CD45, and β-actin expression (Figure 1A and Supplemental Figure 1A). The control EBs had a moderate level of CD31 expression, no VE-Cadherin expression, a small amount of vWF expression, and no CD45 expression. EBs treated with VEGF showed a marked increase in CD31, VE-Cadherin, and vWF, but still had no CD45 expression. OP9 cocultures had a moderate amount of CD31 expression but little to no VE-Cadherin or vWF expression, and no CD45 expression. EGM-2MV–treated cultures had CD31 levels similar to control EBs, and no VE-Cadherin or CD45 expression. Interestingly, hESCs differentiated in EGM-2MV had very high levels of vWF compared to EBs or OP9 cocultures. Immunofluorescence of EBs showed that CD31 illuminates vessel-like structures within the EB and colocalizes with VE-Cadherin (Figure 2A and 2B).

Analysis of transcripts for CD31, VE-Cadherin, KDR, and cytokeratins 8 and 18 by quantitative RT-PCR revealed that VEGF treatment of EBs markedly induced the expression of CD31 and VE-Cadherin while having no significant effect on the overall levels of KDR or cytokeratin transcripts (supplemental Figure II).

**Dose Response and Time Course of VEGF Treatment**

To determine the optimal dose of VEGF, EBs were grown in 0 to 100 ng/mL VEGF for 14 days. Western blotting for CD31, VE-Cadherin, vWF, and CD45 revealed that increasing the VEGF dose to 50 ng/mL had a positive effect on the expression of CD31, VE-Cadherin, and vWF, and that this effect reached a plateau beyond 50 ng/mL (Figure 1B). CD45 expression remained low throughout, with vanishingly small amounts detected at 50 and 100 ng/mL VEGF. To establish the time course of endothelial cell differentiation, EBs grown in the presence of 50 ng/mL VEGF or vehicle were harvested from days 1 to 21 of differentiation. Protein samples were again probed for CD31, VE-Cadherin, vWF, and CD45.
CD31 expression was absent in day 1 EBs, barely detectable in day 4 EBs treated with VEGF but not controls, and increased in both control and VEGF-treated EBs through day 14, with a decrease in both groups at day 21. The decrease at day 21 is likely attributable to proliferation of nonendothelial cell types in the cultures. The overall level of CD31 in VEGF-treated EBs was higher than control EBs at all time points in 3 independent experiments. The pattern of expression of VE-Cadherin followed a similar trend: VEGF treatment resulted in higher and more sustained levels of VE-Cadherin expression in EBs, with the highest level seen in day 7 VEGF-treated EBs. vWF expression was seen in day 1 EBs for both control and VEGF treatment. In both groups of EBs, vWF expression rose rapidly at 4 days and then stayed elevated through day 10 before declining at days 14 and 21. Expression of vWF in VEGF-treated EBs was higher than control EBs from days 4 to 21. CD45 expression was not detected at any time in either control or VEGF-treated EBs.

**VEGF Treatment Induces Endothelial and Stem Cell Markers**

FACS analysis was performed to evaluate the distribution of cells expressing endothelial (CD31, VE-Cadherin) and hematopoietic cell markers (CD45, c-kit). FACS analysis showed that VEGF-treated EBs had more CD31-positive (7.9% ± 2.1%, VEGF; 1.7% ± 0.7%, vehicle), VE-Cadherin–positive (15% ± 1.2%, VEGF; 5.6% ± 1.3%, vehicle), and c-kit–positive (4.8% ± 1.3%, VEGF; 1.6% ± 0.6%, vehicle) cells compared to control EBs (P<0.05 for all markers; Figure 2C). c-kit, a stem cell marker found on hematopoietic stem cells and endothelial progenitors, was not coexpressed on the CD31+ cells to any appreciable extent (data not shown). The stem cell marker

![Image](http://www.ahajournals.org/content/76/11/83/)
CD133, though highly expressed in both control and VEGF-treated cells, was not coexpressed with CD31 (data not shown). Cells from VEGF-treated and control EBs triply stained with CD31, VE-Cadherin, and CD45 showed that VEGF treatment increased the number of CD31+/H11001+ cells at days 10 and 14 of differentiation (Figure 2D). There were no detectable CD45+ cells in either condition. Importantly, VEGF treatment also increased the percentage of CD31+ and VE-Cadherin+ cells in the H1 hESC line (supplemental Figure III), suggesting these results are not line-specific.

**Duration of VEGF Treatment**

To determine whether VEGF was needed for the duration of differentiation or for only a short window of time, EBs were treated with VEGF for various intervals. After 14 days EBs were dissociated and CD31 expression was assessed via flow cytometry. As shown in Figure 3A, EBs that received VEGF for only a portion of the 14 day differentiation had fewer CD31+ cells than those that received VEGF throughout. VEGF treatment from days 0 to 4 did not increase the percentage of CD31+ cells. Treatment from days 0 to 7, 4 to 7, and 7 to 14 resulted in a modest increase in the proportion of CD31+ cells. Ten days of treatment, from days 4 to 14, produced an intermediate number of CD31+ cells. These data indicate that increasing the duration of VEGF treatment has a positive effect on the number of CD31+ cells that arise. The lower absolute value of the percentage of CD31+ cells in this experiment, relative to the experiments shown in Figure 2C, demonstrates the inherent variability of the embryoid body system.

**VEGF Does Not Affect Endothelial Proliferation in EBs**

To examine whether VEGF-induced enrichment is attributable to enhanced proliferation of CD31+ endothelial cells,
EBs were labeled with BrdU for 24 hours at 4 and 14 days and stained for CD31 and BrdU (Figure 3B through 3E). Global BrdU incorporation rates of Day 4 EBs for both vehicle- and VEGF-treated cultures were remarkably high, with 80%±4.2% of cells in vehicle-treated EBs and 83%±2.0% of cells in VEGF-treated EBs containing BrdU+ nuclei (Figure 3B and 3F; P>0.05). Day 14 cultures had a much lower overall proliferative index, with 27%±3.5% of cells in vehicle-treated EBs and 28%±1.5% of cells in VEGF-treated EBs containing BrdU+ nuclei (Figure 3C through 3E, 3F; P>0.05).

Examination of the proliferation rates in CD31+ cells for vehicle- and VEGF-treated EBs at day 4 and 14 of differentiation revealed similar results. At day 4, CD31+ cells in vehicle- and VEGF-treated EBs were 67%±15% and 67%±14% BrdU+, respectively. At day 14, the CD31+ cells in VEGF- and vehicle-treated EBs had similar proliferative indexes: 27%±1.3% BrdU+ in VEGF EBs and 23%±0.4% BrdU+ in vehicle EBs (Figure 3G; P>0.05). The similar rates of DNA synthesis for CD31+ cells and the entire cell population indicate that there is no preferential endothelial cell proliferation in either treatment group at 4 or 14 days. Identical analysis using vWF as the marker for endothelial cells also showed no difference in proliferation rates (data not shown). These data suggest that preferential proliferation of endothelial cells does not play a role in the increase of endothelial cells in VEGF-treated EBs.

Histological analyses also showed that treatment of EBs with VEGF resulted in many CD31+ vessel-like structures (arrows, Figure 3D and 3E), whereas CD31+ cells in control EBs were typically found clustered together with scant vascular organization (arrowheads, Figure 3C). This observation suggests development of endothelial networks in EBs treated with VEGF and is consistent with a role for VEGF as a morphogen in differentiating EBs.

Selection and Characterization of hESC-Derived Endothelial Cells
FACS-sorted CD31+ hESCs were expanded in vitro and characterized by flow cytometry and immunocytochemistry. As shown in Figure 4A and supplemental Figure II B, CD31+ cells (95%) were positive for VE-Cadherin (78%), CD34 (65%), and KDR (65%). They were also stained with a panel of hematopoietic antibodies and were found to be negative for CD45, CD14, and CD115 (Figure 4A and supplemental Figure IC through IE). c-kit, a marker found on hematopoietic stem cells, labeled 0.5% of the CD31+ cells.

To further confirm the cells’ identity and demonstrate the cellular morphology, CD31+ cells were grown in chamber slides and stained for immunocytochemistry. The cells took on a cobblestone pattern in cell culture characteristic of endothelial cells (Figure 4B). They were also found to be positive for the endothelial-specific markers CD31, VE-Cadherin, vWF, Ulex, and endothelial nitric oxide synthase. hESC-derived endothelial cells took up Acetylated LDL in culture, indicating further their endothelial phenotype and function (Figure 4E). CD31+ cells were found to be negative for smooth muscle alpha actin, a marker of smooth muscle cells and myofibroblasts (Figure 4B and supplemental Figure IB). These results demonstrate that FACS-based sorting can be used to purify and expand endothelial cells from EBs.

hESC-Derived CD31+ Cells Upregulate ICAM-1 and VCAM-1 in Response to TNFα, Form Tubes on Matrigel, and Do Not Form Hematopoietic Cell Colonies In Vitro
After stimulation with 0.1, 1, or 10 ng/mL of the inflammatory cytokine TNFα for 4 hours, hESC-derived CD31+ cells and HUVECs both upregulated the inflammatory cell adhesion molecules ICAM-1 and VCAM-1, whereas CD31 expression remained the same for all treatments (Figure 5 and supplemental Figure IV). hESC-derived CD31+ cells and HUVECs were plated onto Matrigel and examined by phase contrast microscopy for 72 hours. Both cell types rapidly assembled into networks of tubular structures, forming robust networks by 10 hours of culture. The networks formed by each cell type subsequently degraded to varying degrees by 72 hours (supplemental Figure VB).

hESC-derived CD31+ cells were grown in methycellulose to test for hematopoietic progenitor colony-forming cells, as previously described.10 After 6 days of culture, positive control bone marrow cells had formed hematopoietic colonies whereas hESC-derived CD31+ cells were adherent to the surface of the culture dish but had no visible hematopoietic colonies (supplemental Figure VA).

hESC-Derived Endothelial Cells Form Robust Perfused Vascular Networks In Vivo
To determine whether FACS-sorted CD31+ hESCs expanded in vitro were able to form a functional vascular network once delivered in vivo, hESC-derived endothelial seeded collagen gel constructs were implanted in 7-week-old ischemia/reperfusion infarcted hearts.5 As shown in Figure 6A, an extensive vascular network positive for human CD31 was generated throughout each construct. Furthermore, the presence of autofluorescent red blood cells within the human CD31+ vessel structures clearly demonstrates that these are functional human vessels that have anastomosed with the host vasculature (Figure 6B through 6D).

Vessel Formation on polyHEMA Tissue-Engineering Scaffolds
Unique polyHEMA tissue engineering scaffolds12 were seeded with hESC-derived endothelial cells and hESC-derived endothelial cell-depleted cells in vitro (supplemental Figure VI). In situ hybridization for human pancentromeric sequences confirmed that every cell stained positively with this probe on preimplantation cell/scaffold constructs, establishing it as a reliable way to track these cells in vivo after implantation (supplemental Figure VIC and VIF).

After 10 days in vivo, human cells were readily detected by human pancentromeric probe in situ hybridization on all scaffolds (supplemental Figure VII G and VII H). In the hESC-derived endothelial cell-seeded scaffolds, CD31+/Ulex+ lumen-containing human vessels were seen around the edges of the scaffolds (supplemental Figure VII E: Ulex; supplemental Figure VII G: CD31). No human vessels were seen in the scaffolds.
seeded with the endothelial cell–depleted population (supplementary Figure VIIIH). The human vessels were distributed exclusively within 100 to 150 μm of the edges. In contrast, CD31-negative cells (for both the hESC-derived endothelial cell and endothelial cell depleted population-seeded scaffolds) were found both at the periphery as well as at the center of the scaffolds (supplemental Figure VIIIH).

Figure 4. hESC-derived CD31+ cells express markers of mature endothelium and do not express hematopoietic cell markers. A, CD31+ isolated cells from VEGF-treated EBs were sorted, expanded, and stained for various markers by flow cytometry. These cells remained 95% CD31+ and were 78% VE-Cadherin+, 65% CD34+, 65% KDR+, and 0.5% c-kit+, but were CD45, CD14, and CD115 negative. B, Phase contrast shows CD31+ cells have typical endothelial cobblestone morphology. C through I, Immunocytochemistry shows that the CD31+ cells stained positively (DAB, brown color) for CD31, VE-Cadherin, vWF, Ulex, and eNOS, but were negative for smooth muscle alpha actin. CD31+ cells also took up AcLDL, as demonstrated by the red color in the cytoplasm. Scale bars, 100 μm.

Discussion

To induce the differentiation of endothelial cells, we compared several protocols, based on published ESC literature and the tenets of embryonic development. Of the 4 methods tested, VEGF treatment of EBs resulted in the highest expression of the endothelial cell-surface markers CD31 (PECAM1) and VE-Cadherin without inducing any detectable levels of hematopoietic cells, as determined by CD45 staining. VEGF treatment for 14 days at 50 ng/mL resulted in a 4.7-fold increase in the number of differentiating endothelial cells. Proliferation analysis revealed no differences in DNA synthesis rates of CD31+ cells in vehicle- and VEGF-treated EBs, suggesting that VEGF plays a critical role in inducing endothelial cell differentiation or enhanced survival. Furthermore, CD31+ selected cells formed blood-filled vessels in the infarcted heart and neovessel-like structures when
seeded onto polyHEMA scaffolds. Thus treatment with VEGF followed by purification can facilitate the efficient generation of hESC-derived endothelial cells for tissue engineering and cell grafting applications as well as basic vascular biology applications.

Before the commencement of this study, several groups had described methods of differentiating ESCs into endothelium, but none had specifically addressed the induction of human ESC-derived endothelial cells. By coculturing hESCs with OP9 macrophage colony stimulating factor–deficient mouse stromal cells, Vodyanik et al demonstrated the efficient differentiation of hematopoietic lineages from hESCs.\textsuperscript{7} Despite the close linkage between the hematopoietic and endothelial cell lineages during development, we found this method was inefficient for endothelial differentiation (Figure 1B). One recent report indicates that sorting OP9 cocultured hESCs for KDR (VEGF receptor-2)/\textsuperscript{Traf1–60} cells followed by treatment with VEGF may be necessary for effective endothelial cell differentiation.\textsuperscript{17} In another study, rhesus monkey ESCs grown in a medium optimized for endothelial cells (EGM-2MV)\textsuperscript{8} uniformly expressed many endothelial cell markers after 30 days. When we tested the effect of EGM-2MV medium on hESCs, we found that it effectively induced cells expressing vWF but not CD31, VE-Cadherin, or CD45 (Figure 1A). In contrast, we found that cell purification followed by culture in endothelial growth medium was required to generate significantly homogeneous endothelial cell cultures. Further studies focused on methods that increased the number of CD31\textsuperscript{+} cells, because vWF is intracellular and thus not amenable to cell sorting. Based on our time course studies (Figure 1C) we propose that vWF expression may mark a more immature population of human endothelium than does CD31. In contrast, VE-Cadherin likely identifies a more mature population than CD31, as the former was not expressed until day 7 of differentiation (Figure 1C). Lastly we posited that addition of VEGF to EBs would augment the differentiation of endothelial cells. We found that VEGF treatment resulted in a robust increase the proportion of CD31\textsuperscript{+}/VE-Cadherin\textsuperscript{-}/CD45\textsuperscript{-} endothelial cells in our EB cultures (Figures 1 and 2C and 2D).

VEGF is a well-established mitogen, survival factor, and differentiation factor for endothelial cells.\textsuperscript{18} It is absolutely required for vascular development, as VEGF-null mice die early in development because of a lack of blood vessels.\textsuperscript{19,20} Formation of endothelium in mouse EBs was successfully induced by the addition of VEGF,\textsuperscript{21} but similar results with human EBs have not been reported. Indeed, recent studies have indicated that VEGF plays an important role in the development of the hemangioblast from both EBs\textsuperscript{22} and 2-dimensional cultures,\textsuperscript{23} as well as stimulating the differentiation of hESC-derived CD34\textsuperscript{+} vascular progenitor cells into endothelial cells.\textsuperscript{24} Despite an early report that VEGF does not induce formation of endothelial cells in H9 human EBs,\textsuperscript{9} our data demonstrate that VEGF does indeed induce endothelial formation in 2 hESC lines, H1 and H7.

Evidence from the differentiation of mouse ESCs into hemangioblasts indicates that VEGF is only required during a short window of time to exert its effect on the expansion of KDR\textsuperscript{+} cells.\textsuperscript{25} Differentiation driven by other factors, such as Wnts,\textsuperscript{26} has also demonstrated that treatment for a brief time during early differentiation can result in large differences in the mature cell population, indicating that a cell-fate decision (as opposed to proliferation or survival) was affected by the treatment protocol. To address whether a shorter exposure to VEGF would have the same effect as continuous treatment, we administered VEGF for various intervals during 14 days of differentiation (Figure 3A). In contrast with these other systems, our results indicate that there was no particular time that was especially inductive, and continuous treatment with VEGF resulted in the largest number of endothelial cells.

Three parallel mechanisms can account for the expansion of endothelial cells during EB culture under VEGF treatment. Preferential proliferation, inhibition of death, or increase in progenitor cell differentiation would all result in a larger pool of endothelial cells. The equivalent rates of DNA synthesis in control and VEGF-treated EB CD31\textsuperscript{+} cells (and in vWF\textsuperscript{+} cells; data not shown) at days 4 and 14 presented here (Figure 3G) excludes a role for enhanced endothelial proliferation. Inhibition of endothelial cell death may play a role, as VEGF is a known survival factor for endothelium\textsuperscript{18}; this mechanism will be examined in future studies. Differentiation of mesodermal progenitors into endothelial cells is a likely mechanism as well. We propose that VEGF may stimulate differentiation of endothelial cells from KDR\textsuperscript{+} precursors. Because continuous VEGF treatment is superior to short windows during early and late differentiation, the inductive effect of VEGF must operate throughout the maturation of these cultures. Although KDR is expressed by multiple mesodermal lineages during development, only vascular endothelium and bone marrow–derived endothelial progenitor cells maintain expression of this marker in adult tissues. Thus, the activation of KDR by VEGF may instruct the cell to maintain expression of the receptor and thereby encourage its differentiation into vascular endothelium. Expansion of the KDR\textsuperscript{+} population before CD31 expression is another possible ex-
planation. Future accounting studies of multiple mesodermal lineages will help illuminate the precise mechanisms of the role of VEGF during EB differentiation.

VEGF is well recognized as a potent vasculogenic and angiogenic molecule during development and in adult tissues. Its critical role in mesoderm development is substantiated by the presence of the VEGF receptor KDR on mesodermal precursor cells in mouse embryos and ESCs. A recent report using a KDR-LacZ reporter mouse strain indicates that KDR is expressed in many more mesodermal lineages than previously thought, including skeletal and cardiac myocyte precursors in the early embryo. Interest-ingly, KDR is also expressed on undifferentiated hESCs and is highly expressed in differentiating EBs (supplemental Figure II). Additionally, Keller’s group has shown that KDR cells emerging during early mESC and hESC differ-entiation can become hematopoietic cells, endothelial differentiation can become hematopoietic cells, endothelial cells, smooth muscle cells, and cardiomyocytes. Administration of VEGF promotes endothelial cell differentiation in mESCs, erythroid differentiation from human ESCs, as well as KDR cell expansion after mesoderm induction. Thus appropriately timed VEGF treatment may expand these populations of cells in hESC cultures and could provide a uniquely useful cell source for cardiac tissue engineering applications. Further investigation of the lineages induced with VEGF treatment in EBs is thus warranted.

Endothelial cell identity is established not only by expression of relevant markers but also by functional cell behavior. Endothelial cells form tubes on Matrigel in vitro, upregulate ICAM-1 and VCAM-1 in response to inflammatory cytokines, and form blood vessels when implanted in vivo. For example, Yoder et al have shown that 2 populations of endothelial progenitor cells derived from blood have distinctly different phenotypes, and only endothelial colony forming cells (ECFCs), but not endothelial colony-forming units (CFU-ECs), were endothelial in nature. The ECFCs expressed CD31, VE-Cadherin, vWF, KDR, bound Ulex, and took up AcLDL but were negative for CD14, CD45, and CD115. In contrast the CFU-ECs expressed CD14, CD45, and CD115 but had relatively lower expression of CD31, VE-Cadherin, vWF, KDR, Ulex binding, and AcLDL uptake. Our hESC-derived CD31 isolated cells match the profile of ECFCs, and not CFU-ECs, for every marker or assay examined. Furthermore, when stimulated with the inflammatory cytokine TNFα, both hESC-derived endothelial cells and HUVEC controls upregulated the cell adhesion molecules VCAM-1 and ICAM-1, but did not upregu-late CD31, as expected (Figure 5).

Because of the close developmental relationship between endothelium and blood, it is also important to demonstrate that primitive endothelial cells do not retain hematopoietic cell mark-ers. To test our cultures for hematopoietic elements, we exam-ined the expression of CD45 protein (Figures 1, 2, and 4), CD14, and CD115 (Figure 4; positive controls shown in supplemental Figure I). The expression of CD45 was vanishingly low in differentiating EBs, and CD31 cells did not express CD45 at day 10 or day 14 of differentiation (Figure 2). Isolated CD31 cells were completely negative for CD45, CD14, and CD115 (Figure 4). Quantitative RT-PCR analysis of CD115 confirmed that CD31 isolated cells contained no transcript for this gene (supplemental Figure IF). Furthermore, hematopoietic progeni-tor cells have the ability to generate colonies in methylcellulose culture conditions. When we plated our isolated CD31 cells into these conditions, no hematopoietic colonies formed (supplementary Figure V).

A recent report by Kennedy et al demonstrated the induction and isolation of KDR cells and identified these cells as hemangioblasts, with the potential to become either hematopoietic cells or endothelial cells. Their data showed, as does ours, that endothelial cells derived from hESCs do not express CD115 (colony stimulating factor 1 receptor, also known as c-fms), demonstrating that macrophages were not present in either cell population (Figure 4A and supplemental Figure IF). Another report from the same group demonstrated that KDR cells isolated from early differentiating embryoid bodies grown in the presence of growth factors including 10 ng/mL VEGF (from day 4 to 6 of differentiation) have the potential to become endothelial cells, smooth muscle cells, or cardiomyocytes. The low proportion of CD31 cells (4%) in these cultures increased dramatically when the cells were grown in the presence of bFGF for 10 to 12 days (30%). A related population of KDR cells isolated at the same time as the tripotential cells expressed several markers of endothelial cell phenotype (CD31, VE-Cadherin, and KDR) without the addition of bFGF; these cells most closely approximate the population described herein.

hESC-derived ECs may have practical use in biomedical applications. Indeed, Cho et al and Yamahara et al have recently shown improved neovascularization of hindlimb ischemia by transplantation of hESC-derived ECs. Another application for these cells is the vascularization of scaffolds for tissue engineering and tissue repair purposes. When combined with collagen gels, our hESC-derived ECs were able to organize into vascular networks in vivo (Figure 6). Further, their ability to robustly engraft, anastamose with host vasculature, and conduct host blood confirms the endothelial identity and functionality of these cells. Lastly, seeding of synthetic polyHEMA scaffolds with CD31 cells also resulted in the formation of human vessel structures (supplemental Figure VII). Such preseeding could be readily incorpo-rated as a support technology to aid tissue engineering, thus highlighting the potential utility of hESC-derived ECs for neovascularization therapies.

Acknowledgments
The authors thank Kira Bendixen, Jennifer Deem, Dr Sarah Fer-nandes, James Fugate, Paul St. Laurent, Kenny Lin, Veronica Muskheli, Mark Saiget, and Kellii Schurb for expert technical assistance. We thank Dr Hans Reinecke for numerous helpful discussions. Fred Lewis and Michelle Black provided support for FACS sorting.

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Disclosures
None.
References


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Supplemental Methods (Online)

Media formulations

**EGM**: Endothelial Basal Medium (EBM) containing 2% fetal bovine serum (FBS), epidermal growth factor (EGF), hydrocortisone, 0.4% bovine brain extract, gentamicin, and amphotericin-B (Lonza, Basel, Switzerland).

**EGM-2**: Endothelial Basal Medium-2 (EBM-2) containing 2% FBS, basic fibroblast growth factor (bFGF), EGF, insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), hydrocortisone, ascorbic acid, heparin, gentamicin, and amphotericin-B (Lonza).

**EGM-MV**: EBM containing 5% FBS, EGF, hydrocortisone, 0.4% bovine brain extract, gentamicin, and amphotericin-B (Lonza).

**EGM-2MV**: EBM-2 containing 5% FBS, bFGF, EGF, IGF-1, VEGF, hydrocortisone, ascorbic acid, gentamicin, and amphotericin-B (Lonza).

**hEB medium**: KO-DMEM (Invitrogen, Carlsbad, CA) supplemented with 20% FBS (Hyclone, Logan, UT or Biomeda, Burlingame, CA), 1% non-essential amino acids, 1.25 mM L-glutamine, and 0.1 mM β-mercaptoethanol (Invitrogen).

**Mesenchymal Stem Cell Growth Medium**: Mesenchymal stem cell basal medium (Lonza) supplemented with mesenchymal stem cell growth supplement, L-glutamine, gentamicin, and amphotericin-B (Lonza).

**Mouse embryonic fibroblast (MEF)-conditioned medium**: KO-DMEM (Invitrogen) supplemented with 20% knockout serum replacement, 1% non-essential amino acids, 1.25 mM L-glutamine, 0.1 mM β-mercaptoethanol, and 4 ng/ml bFGF (Peprotech, Rocky Hill, NJ); conditioned by irradiated confluent MEFs for 24 hours and sterile filtered.
**OP9 medium:** αMEM (Invitrogen; from powder) supplemented with 10% FBS (Biomeda), 1% L-glutamine, and 100 µM monothioglycerol (Sigma-Aldrich, St. Louis, MO).

**U937 growth medium:** RPMI1640 (containing L-glutamine, Invitrogen) supplemented with 10% FBS (Hyclone), penicillin and streptomycin (Invitrogen).

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**Human Embryonic Stem Cell Culture and Differentiation**

**Undifferentiated cell culture**

Undifferentiated human embryonic stem cells (H7 line passage 37-92 and H1 line passage 45-60 (WiCell, Madison, WI)) were maintained in MEF-conditioned medium supplemented with 4 ng/ml bFGF on Matrigel-coated (BD Biosciences, San Jose, CA) tissue culture polystyrene dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ). Cells were passaged upon reaching 90% confluence by digestion with collagenase IV (200 U/ml; Invitrogen, Carlsbad, CA) for 7 minutes at 37°C followed by mechanical dissociation with a cell scraper and then replated at 1:6, 1:12, or 1:24 ratios (ratio for subculturing determined empirically depending on cellular growth rate and morphology).

Cytogenetic analysis was performed on G-banded metaphase cells at several times throughout the experiments described herein. Results obtained with cells that were later shown to have an abnormal karyotype were subsequently confirmed to be valid in karyotypically normal cells.

**Embryoid body formation and culture**

To initiate differentiation, cells were digested with collagenase IV, scraped off the plate in small clusters of cells with a cell scraper, and placed into ultra low attachment cluster plates (Corning,
Corning, NY) for 4 days, as previously described\textsuperscript{1-3}. Differentiation was carried out in hEB medium. In these conditions the cells formed 3-dimensional clusters called embryoid bodies (EBs). After 4 days in suspension, EBs were plated onto 0.67% gelatin-coated dishes and grown for 10-14 additional days. Medium was changed every two to three days thereafter.

Alternate Differentiation Protocols

Undifferentiated H7 hESC and OP9 cells were grown in coculture as described\textsuperscript{4}. OP9 cells were kindly donated by Dr. C. Anthony Blau (University of Washington), and were originally obtained from Dr. Toru Nakano (Osaka University, Japan). OP9 cells were passaged with 0.1% trypsin (Invitrogen) and replated at a ratio of 1:5. For coculture, H7 human ESCs were dissociated into single cells after a 7-minute digestion in collagenase IV followed by incubation in Versene (0.5 mM EDTA and 1.1 mM dextrose in calcium-free PBS) and robust trituration, and then added to a confluent OP9 feeder layer at 2.9 x 10\textsuperscript{4} cells/cm\textsuperscript{2}.

For endothelial growth medium culture\textsuperscript{5}, undifferentiated hESCs grown in MEF-conditioned medium were passaged at 1:6 (∼2.1 x 10\textsuperscript{4} cells/cm\textsuperscript{2}) onto an irradiated MEF feeder layer (2.0 x 10\textsuperscript{4} cells/cm\textsuperscript{2}). The following day, the medium was switched to EGM-2MV. The cells were fed every 3 days with fresh EGM-2MV for 14 days.

Western Blots

Protein was collected from EBs in sample buffer (50 mM Tris-HCl, 1% SDS, 10% glycerol) and homogenized by passing through a 22G needle 10 times. Protein concentration was quantified with a MicroBCA kit (Pierce Biotechnology, Rockford, IL). Equal amounts of protein (10 or 25
µg) were boiled for 3 minutes, separated on 7.5% or 8.5% polyacrylamide gels (BioRad, Hercules, CA), and transferred to Hybond-ECL nitrocellulose (Amersham Biosciences, Piscataway, NJ) or Immobilon polyvinylidene fluoride (Millipore, Billerica, MA) membranes. Membranes were blocked and then incubated with primary antibodies at 4°C overnight. Horseradish peroxidase (HRP) or infrared fluorescent molecule-conjugated secondary antibodies specific for the host species of the primary antibody were applied to the membranes. Membranes were then washed and scanned on the Licor Odyssey infrared imaging system (Lincoln, NE) according to the manufacturer’s instructions or developed with SuperSignal Pico West or Dura West (Pierce Biotechnology) and exposed to film (Hyperfilm ECL, Amersham Biosciences).
Western Blot conditions:

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Membrane</th>
<th>Primary antibody source and dilution</th>
<th>Secondary antibody and dilution</th>
<th>Blocking buffer</th>
<th>Primary antibody incubation buffer</th>
<th>Secondary antibody incubation buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD31 (PECAM)</td>
<td>Nitrocellulose</td>
<td>Dako; 1:1000</td>
<td>Alexa Fluor 695- goat anti mouse; 1:10,000</td>
<td>0.1% casein in 0.2x PBS</td>
<td>0.1% casein in 0.2x PBS + 0.1% Tween-20</td>
<td>0.1% casein in 0.2x PBS + 0.1% Tween-20 and 0.01% SDS</td>
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<tr>
<td>VE-Cadherin</td>
<td>PVDF</td>
<td>R&amp;D Systems; 1:500</td>
<td>HRP-conjugated rabbit anti-goat; 1:8000</td>
<td>1% milk + 0.1% Triton-X 100 in TBS</td>
<td>1% milk in TBS</td>
<td>1% milk in TBS</td>
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<tr>
<td>vWF</td>
<td>PVDF</td>
<td>Dako (HRP-conjugated primary); 1:100</td>
<td>N/A</td>
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<td>5% milk + 0.1% Tween-20 in TBS</td>
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<td>CD45</td>
<td>PVDF</td>
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<td>HRP-conjugated sheep anti-mouse; 1:2000</td>
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<td>1% milk + 0.1% TritonX-100 in TBS</td>
<td>1% milk + 0.1% TritonX-100 in TBS</td>
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<td>β-actin (Licor)</td>
<td>Nitrocellulose</td>
<td>Cell Signalling Technology; 1:2000</td>
<td>IrDye800-conjugated donkey anti-rabbit; 1:20,000</td>
<td>0.1% casein in 0.2x PBS</td>
<td>0.1% casein in 0.2x PBS + 0.1% Tween-20</td>
<td>0.1% casein in 0.2x PBS + 0.1% Tween-20 and 0.01% SDS</td>
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<tr>
<td>β-actin (Chemiluminescence)</td>
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<td>Cell Signalling Technology; 1:1000</td>
<td>HRP-conjugated goat anti-rabbit; 1:20,000</td>
<td>5% milk + 0.1% Tween-20 in TBS</td>
<td>5% milk + 0.1% Tween-20 in TBS</td>
<td>5% milk + 0.1% Tween-20 in TBS</td>
</tr>
</tbody>
</table>

**FACS Analysis**

Embryoid bodies were washed for 5 minutes in PBS at 37°C and then enzymatically dispersed using 0.56 U/ml Liberase Blendzyme IV (Roche Applied Science) and 150 U/ml DNase I
Supplement - 6

(Roche Applied Science) for 30-45 minutes. Single cell suspensions were generated by forceful triturations through a P1000 pipette tip or through 5 or 10 ml pipettes. Cells were washed in Versene + 1% FBS, counted, and then stained with fluorescently conjugated antibodies for flow cytometry in a final volume of 100 µl of Versene + 1% FBS. The antibodies used included CD31-PE (1:5; clone WM-59, BD Biosciences), VE-Cadherin (1:10 FITC conjugated - Serotec, Raleigh, NC; 1:10 polyclonal goat IgG unconjugated (R&D Systems) followed by Alexa Fluor 488-conjugated donkey anti-goat secondary (Invitrogen); 1:5 Alexa Fluor 488-conjugated monoclonal, eBioscience), c-kit-FITC (1:10; clone YB5.B8, Chemicon, Temecula, CA), CD133-APC (1:10; clone AC133, Miltenyi Biotec, Auburn, CA), and CD45 (1:5 APC-conjugated, eBioscience). Cells were stained with one, two, or three antibodies and analyzed on FC500 or FC500MPL flow cytometry analyzers (Beckman Coulter, Fullerton, CA).

Proliferation Studies

EBs were grown in the presence or absence of VEGF for 4 or 14 days. The thymidine analogue 5-bromo-2-deoxyuridine (BrdU, 10µM; Roche Applied Science) was added during the final 24 hours of cell culture to mark cells in the S phase of the cell cycle. EB outgrowth cultures were then scraped with a cell lifter (Corning), embedded in Histogel (Richard-Allan Scientific, Kalamazoo, MI), fixed in Methyl Carnoy’s fixative (60% Methanol, 30% chloroform, 10% glacial acetic acid), and paraffin embedded. Five-micron thick sections were deparaffinized, quenched in 0.3% H2O2/cold Methanol for 30 minutes at room temperature, blocked in 1.5% normal goat serum, and incubated in mouse anti-human CD31 antibody (1:20, Dako) overnight at 4°C. The following day sections were incubated with biotinylated goat anti-mouse secondary antibody (1:500, Vector Laboratories, Burlingame, CA) for 1 hour at room temperature followed
by a 30 minute incubation with an avidin/biotin/alkaline phosphatase complex (ABC-AP kit, Vector Laboratories). The CD31 signal was visualized with Vector red alkaline phosphatase substrate (Vector Laboratories). Nuclear epitopes were then unmasked by 15 minute incubation in 1.5N HCl at 37°C, followed by 2 neutralization washes in 0.1M sodium tetraborate decahydrate, pH 8.5 (Borax buffer; Sigma-Aldrich). Slides were then blocked again in 1.5% NGS and incubated at 4°C overnight with HRP-conjugated anti-BrdU antibody (1:40; Roche Applied Science). The following day BrdU signal was visualized by developing with 3,3'-diaminobenzidine (DAB, Sigma-Aldrich). Nuclei were counterstained with hematoxylin.

Proliferating cells were counted in blinded fashion by rastering across the entire tissue section and counting every second field (day 4 EBs) or every fifth field (day 14 EBs). Detailed counts of CD31⁺ cells were carried out as well. Multiple slides per sample (>50 µm separation between slides) were stained and counted for the latter metric.

**hESC-derived Endothelial Cell Isolation via Fluorescence Activated Cell Sorting (FACS)**

For initial FACS-sorting experiments, EBs were heat shocked one day prior to harvest by placing them at 43°C for 30 minutes and then returned to 37°C. Heat shock treatment was performed to increase the survival of concomitantly harvested hESC-derived cardiomyocytes for cardiac grafting studies, reported elsewhere². Subsequent studies showed that heat shock did not affect endothelial differentiation or abundance (data not shown). To collect cells, flasks were rinsed with PBS and then incubated with 0.56 U/ml Liberase Blendzyme IV (Roche Applied Science, Indianapolis, IN) for 30-45 minutes at 37°C. Dissociated cells were rinsed and then separated by
density centrifugation using a 40.5% and 58.5% discontinuous Percoll gradient (Sigma-Aldrich) for 30 minutes at 1500g<sup>1-3</sup>.

Cells from the two lightest Percoll fractions (Fractions 1&2) were counted, and 5 x 10⁷ cells were removed for labeling. Cells were rinsed twice in 20 ml staining buffer (PBS containing 0.5% BSA and 1 mM EDTA). An R-phycoerythrin (PE)-conjugated anti-CD31 antibody (BD Biosciences) was then added to the cells at a 1:25 dilution (20 µl per 10⁶ cells) and incubated for 30 minutes in the dark on ice<sup>6</sup>. Cells were then pelleted and resuspended in 10 ml staining buffer, to which DNase I (Invitrogen) was added at a final concentration of 171 U/ml. After passing through a 36-µm mesh cell strainer, stained cells were purified on a FACS Vantage cell sorter (BD Biosciences) and collected in EGM. Both CD31<sup>+</sup> and CD31<sup>-</sup> cell fractions were plated in EGM-MV on 0.67% gelatin-coated tissue culture vessels (Sigma-Aldrich). The CD31<sup>-</sup> cells were later switched back to hEB medium to avoid the preferential expansion of any trace CD31<sup>+</sup> cells or endothelial progenitors in endothelial-permissive conditions. When cells reached 80-90% confluence, they were subcultured with 0.025% trypsin and 0.34-0.5 mM EDTA and replated at 5,000 cells/cm<sup>2</sup>.

**Immunohistochemistry**

Cells or slides were blocked with 1.5% NGS at room temperature for 1 hour. Slides were then incubated at 4°C overnight with either primary antibody (CD31, 1:10) or 1.5% NGS (Ulex slides). The following day, slides were rinsed and incubated with biotinylated goat anti-mouse secondary antibody (1:500) (CD31 slides) or biotinylated Ulex (1:400) for one hour at room temperature. After rinsing, avidin/biotin/HRP complex (ABC kit, Vector Laboratories) was
applied to the slides for 30 minutes. Antibody/lectin positive cells were visualized with DAB and counterstained with hematoxylin.

In a separate set of experiments, hESC-derived endothelial cells and unsorted H1 human ES cells and HUVECs (positive control) were grown in gelatin-coated 2-well Permanox chamber slides and fixed in methanol for 10 minutes at 4°C. The cells were stained for von Willebrand factor (vWF) with an HRP-conjugated rabbit polyclonal antibody (neat; Dako), developed with DAB, and counterstained with hematoxylin.

Sections from the explanted cell-scaffold constructs were stained with hematoxylin and eosin (H&E) to show tissue morphology and cell infiltration. Picrosirius red/fast green was used to stain fibrillar collagen red and other tissue elements green. A human-specific CD31 antibody (1:20; Dako) and the human-specific lectin Ulex (20 µg/ml) were used to avoid labeling rat endothelium. In addition, in situ hybridization for a human-specific pan-centromeric probe was also performed, either alone or sequentially with antibody staining as previously described. This highly specific staining allowed for visualization of all human cells including CD31+ or Ulex+ vessels formed by human cells.

Acetylated LDL (AcLDL) Uptake

FACS-sorted hESC-derived endothelial cells, endothelial cell-depleted cells, and HUVECs (positive control) were grown in gel-coated two-well Permanox chamber slides for 2-3 days. Cells were incubated with 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate-conjugated acetylated LDL (10 µg/ml; Dil-AcLDL, Molecular Probes/Invitrogen) for 4 hours in
normal growth medium (EGM or hEB medium). Cells were rinsed in PBS and fixed with 2% PF for 5 minutes at 4°C. Nuclei were counterstained with Hoechst 33342 (Sigma-Aldrich) and imaged with a 20x oil immersion objective on an upright fluorescence microscope (Olympus, Center Valley, PA).

*Immunofluorescence of whole-mount embryoid bodies*

EBs were plated into gelatin-coated Sonic Seal 4-well chamber slides (Nunc, Rochester, NY) after 4 days in suspension and grown for an additional 10 days. Cultures were rinsed in PBS and fixed in 100% cold methanol for 10 minutes. Slides were then rehydrated in PBS and blocked with 1.5% normal goat serum (NGS, Vector Laboratories, Burlingame, CA) stained for CD31 (1:20, Dako), followed by biotinylated goat anti-mouse secondary antibody (1:500, Vector Laboratories) and streptavidin conjugated to Alexa 594 (1:100, Invitrogen). Nuclei were counterstained with Hoechst 33342. Slides were imaged on a Nikon 80i epifluorescence microscope and captured with a Qcapture camera (Olympus America, Center Valley, PA).
Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Primer sequences:

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<th>Gene</th>
<th>Forward primer (5' – 3')</th>
<th>Reverse primer (5' – 3')</th>
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<td>VCAM1</td>
<td>GACCCGGAGATAATCTCACAGT</td>
<td>CATTGAAACACCGATCGAG</td>
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RNA was collected from EBs and isolated cell populations for RT-PCR analysis using Qiagen RNeasy kits. RNA was reverse transcribed to cDNA using random priming and Superscript II (Invitrogen, Carlsbad, CA). RNA, random primers (Promega), and first strand buffer (Invitrogen), were combined, incubated at 90°C for 2 min, cooled to 42°C, and then combined with dNTPs (Promega), DTT (Invitrogen), RNase inhibitor (Invitrogen), and Superscript II at 42°C for 60 minutes. The mixture was then heated to 96°C for 5 minutes and the resulting cDNA was stored at -20°C until further use.

Transcripts were amplified with a SensiMix SYBR Green kit (Quantace, Norwood, MA) on a 7900HT Fast-Real-Time PCR System (Applied Biosystems, Foster City, CA). The temperature profile for the amplification was: 15 minutes at 95°C, 40 cycles of 15 seconds at 95°C, 30 seconds at the annealing temperature, and 30 seconds at 72°C, followed by a 5 minute extension at 72°C. A dissociation curve was also performed to confirm that the PCR reaction resulted in a single amplicon. Results were quantified by the delta Ct method for each transcript, relative to housekeeping genes GAPDH or HPRT.
Porous Poly(2-hydroxyethyl methacrylate) Scaffold Seeding Details

Scaffolds were soaked in FBS overnight to encourage the deposition of additional adhesive proteins, briefly rinsed in PBS, and placed in a 96-well plate for the seeding process. hESC-derived endothelial cells and endothelial-depleted cells were trypsined and concentrated to 2 x 10^7 cells/ml. Fifty microliters (10^6 cells) of each cell suspension were added directly onto each scaffold. The plates were then centrifuged at 1500 rpm for 5 minutes to enhance the movement of the cells into the central region of the scaffold. Following incubation at 37°C for 45 minutes the scaffolds were then turned over within the well, a fresh cohort of cells was infused onto the reverse side, and the scaffolds were centrifuged again. Each cell-scaffold construct was then transferred to a 24-well ultra low attachment plate and fed with EGM2. Two days later, a second batch of cells was prepared in the same way and again seeded onto the scaffolds (4.6 x 10^6 cells/ml).

A subpopulation of each cell type used for the scaffold experiments was plated onto Sonic Seal 4-well chamber slides (Nunc), grown for 2 days, and fixed in 100% cold methanol for 5 minutes. To determine cell purity, the slides were immunostained for CD31 (Dako, 1:20) and developed with DAB as described above. Nuclei were counterstained with hematoxylin. CD31 percentage was obtained by counting cells in several high-power fields until at least 500 total nuclei had been evaluated for each cell population.

One cell-scaffold construct from each group was fixed in Methyl Carnoy’s fixative (60% methanol, 30% chloroform, 10% glacial acetic acid) on the day that the remaining scaffolds were implanted. These scaffolds were processed, paraffin embedded, and sectioned for histology.
Sections from the cell-scaffold constructs at day 3 after seeding and at day 10 after implantation were stained for hematoxylin and eosin (H&E) to show tissue morphology and cell infiltration. Picrosirius red/fast green was used to stain fibrillar collagen red and other tissue elements green\textsuperscript{7}. Human CD31 antibody (1:20; Dako) followed by biotinylated goat anti-mouse secondary antibody (1:500) was used to identify human endothelium because the antibody does not cross-react with rat endothelium. Ulex (20 µg/ml) also specifically labels primate endothelium. Human CD31 and Ulex staining was visualized by either horseradish peroxidase or alkaline phosphatase-conjugated reagents (ABC and ABC-AP kits, respectively, Vector Laboratories) and developed with DAB or Vector Red (Vector Laboratories), respectively.

\textit{In situ} hybridization for a human-specific pan-centromeric probe was also performed, either alone or sequentially with antibody staining\textsuperscript{2,8}. The probe was generated by degenerate PCR primer sets targeting human centromere sequences, purified, and digoxigenin-labeled using the DIG-High Prime kit (Roche Applied Science). Following immunohistochemical labeling with alkaline phosphatase and Vector Red, slides were boiled in a citrate buffer (0.01 M citrate buffer pH 4.0-4.5) for 20 minutes, digested in proteinase K (0.1 µg/ml, 37°C) for 20 minutes, rinsed three times in 2x SSC (0.3M sodium chloride, 0.03M sodium citrate, 0.1% Tween-20), and incubated with pre-hybridization buffer (2x SSC, 50% formamide) for 15 minutes at room temperature. Denaturation of the probe (0.45 µl digoxigenin-labeled human pan-centromeric probe/100 µl probe buffer) in probe buffer (2x SSC, 50% formamide, 20% of 10% dextran sulfate, 0.42 mg/ml salmon sperm DNA) was carried out at 98°C for 15 minutes followed by overnight hybridization at 37°C. Following a stringent wash (2x SSC, 50% formamide), sections
were blocked (Roche commercial blocking solution), incubated with an HRP-conjugated anti-digoxigenin antibody (1:100; Roche Applied Science), and developed with DAB to visualize the probe. This highly specific staining allowed for visualization of all human cells including CD31$^+$ or Ulex$^+$ vessels formed by human cells.
Supplemental References

Supplemental Figure Legends:

Figure I: Western Blot, Immunohistochemistry, FACS, and PCR Controls.

A, Positive controls for Western blots (Figure 1). HUVEC served as positive control for CD31, VE-Cadherin, and vWF. U937 cells served as positive control for CD45. B, Rat skeletal muscle served as positive control for smooth muscle alpha actin staining (Figure 5). C-E, U937 cells served as FACS positive controls for hematopoietic markers CD45, CD14, and CD115. F, qRT-PCR data showing presence of CD115 transcript in U937 cells but not CD31+ cells.

Figure II: qRT-PCR Data for Induced EBs and Isolated CD31+ Cells.

A, Quantitative RT-PCR for CD31 and VE-Cadherin demonstrates marked upregulation in VEGF-treated EBs when compared to control EBs (*, p<0.01; #, p<0.05). KDR expression was similar in VEGF-treated EBs and control EBs. Cytokeratin 8 and 18, markers of epithelium, were similar in VEGF-treated and control EBs (p>0.05). B, Quantitative RT-PCR for CD31, VE-Cadherin, and KDR in isolated cell populations and controls. CD31+ cells expressed high levels of CD31, VE Cadherin, and KDR, while CD31− cells expressed low levels of all three markers. U937 cells and neonatal human dermal fibroblasts express low levels of all three markers. HUVECs expressed very high levels of all three markers.

Figure III: VEGF Induces Formation of Endothelial Cells in H1 hESCs.

Human ES cells, H1 line, were differentiated as EBs for 14 days and then examined by FACS analysis. A, Fluorescence density plot for vehicle-treated EBs (median sample). B, Fluorescence density plot for VEGF-treated EBs (median sample). C, Quantification of vehicle-treated and VEGF-treated EBs (n=3 per group). *, p<0.05. Bars represent mean ± SEM. D,
Phase contrast image showing cobblestone morphology of CD31\(^+\) sorted H1 EBs. E, vWF staining of CD31\(^+\) sorted and unsorted H1 EBs showing enrichment of vWF\(^+\) cells in the CD31\(^+\) fraction. F, DiI-AcLDL uptake in CD31\(^+\) sorted H1 cells (Red fluorescence). Nuclei counterstained with Hoechst 33342 (blue).

Figure IV: Relative Abundance of ICAM-1, VCAM-1, and CD31 Transcripts.
Both HUVECs and hESC-derived CD31\(^+\) cells upregulated ICAM-1 and VCAM-1 transcripts in response to stimulation with TNF\(\alpha\) for 4 hours, while the level of CD31 expression remained constant. HUVECs have higher levels of all three transcripts than hESC-derived CD31\(^+\) cells. *, p<0.05 vs. vehicle control.

Figure V: Hematopoietic Colony and Endothelial Functional Assays.
A, Images from methylcellulose colony forming assay after 6 days in culture. Positive control human bone marrow cells have formed colonies on the plate, whereas hESC-derived CD31\(^+\) cells formed none. Only adherent cells on the surface of the dish were visible. B, Both hESC-derived CD31\(^+\) cells and HUVECs formed tubules on Matrigel as shown. Scale bar, 100 µm.

Figure VI: Scaffold Histology after Three Days in vitro.
Following expansion in culture for five passages, immunohistochemical staining for CD31 showed that the cells used to seed the scaffolds were 47% CD31\(^+\) for hESC-derived endothelial cells and 0.87% CD31\(^+\) for the endothelial cell-depleted population. Scaffolds seeded with each cell type showed equivalent cell density for all groups three days after seeding.
A, D, Picrosirius red/fast green staining shows minimal collagen deposition in scaffolds after 3 days in vitro. The appearance of a thin layer of red staining around the inner surface of each pore may be an indicator of the successful matrix coating on these scaffolds as this was also seen in unseeded scaffolds (data not shown). B-C, hESC-derived endothelial cell-seeded scaffolds contained Ulex and human pan centromeric probe+/CD31+ cells, some of which already formed lumen-containing structures. E-F, Endothelial cell-depleted cell-seeded scaffolds remained Ulex− and CD31−. Scale bars, 50 μm.

Figure VII: Human ESC-Derived Endothelial-Seeded Scaffolds form Human Vessels in vivo.
A-B, Hematoxylin and eosin stained constructs demonstrate host tissue ingrowth and encapsulation. C-D, Picrosirius red (collagen) and fast green (all other tissue elements) stains show the collagenous nature of the fibrous capsule. E-F, Ulex staining (brown) reveals human vessels in the scaffolds seeded with hESC-derived endothelial cells (E) but not in the scaffolds seeded with the endothelial cell-depleted fraction (F). G-H, Double staining for human pan-centromeric in situ hybridization probe (brown nuclei) and human CD31 (red cytoplasm) demonstrates the presence of human cells in all experimental groups and human vessels in the hESC-derived endothelial cell group. After in vivo transplantation for 10 days, 43 ± 4.3% of all human cells were CD31+, which closely matched the composition prior to implantation. Cells on the scaffolds seeded with the endothelial cell-depleted population remained essentially CD31-negative (0.2 ± 0.2% CD31+). Scale bars: 1 mm for A-B, 100 μm C-D, 50 μm E-H.
Supplemental Figure I: Western blot, immunohistochemistry, FACS, and PCR controls.

A Western blot positive controls

- CD31
- VECad
- vWF
- HUVEC
- U937
- CD45

B Skeletal muscle positive control for SM α-actin

C U937 cells

- PE channel (unstained)
- CD45 (APC)

D U937 cells

- PE channel (unstained)
- CD14 (FITC)

E U937 cells

- CD115 (PE)
- FITC channel (unstained)

F qRT-PCR for CD115 normalized to HPRT

- 0.0005
- 0.001
- CD31+ U937 ESC
Supplemental Figure II: qRT-PCR data for induced EBs and isolated CD31+ cells

A  Endothelial cell-specific transcripts are upregulated in VEGF-treated EBs

B  CD31+ sorted ESC-derived cells express endothelial cell-specific transcripts; CD31- cells do not.
Supplemental Figure III: Endothelial cell induction and sorting with H1 huESCs.

A. Control H1 EBs

B. VEGF-treated H1 EBs

C. Percent Positive

D. Phase contrast

E. CD31 sorted (H1) | Unsorted (H1)

F. CD31 sorted (H1)

AcLDL uptake (red)
Supplemental Figure IV. Relative abundance of ICAM-1, VCAM-1, and CD31 transcripts.
Supplemental figure V: hESC-derived CD31+ cells do not form hematopoietic colonies when plated into colony formation assay (methylcellulose + cytokine cocktail). CD31+ cells do form tubules when plated onto Matrigel similar to HUVECs.
Supplemental Figure VI.  Cell-seeded scaffolds in vitro.

A  B  C
Picosirius Red &  Ulex (brown)  Human Nuclei (brown)
Fast Green  Human CD31 (red)

D  E  F

Scale bars: 50 µm
Supplemental Figure VII. Human ESC-Derived Endothelial Cell-Seeded Scaffolds form Human Vessels in vivo.

hESC-derived endos

A

H&E

B

hESC endo-depleted

C

Picrosirius Red &

Fast Green

D

E

Human vessels (Ulex)

F

G

Human vessels (CD31/human nuclei)

H