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Human Embryonic Stem Cell Derived Vascular Progenitor Cells Capable of Endothelial and Smooth Muscle Cell Function

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Abstract

OBJECTIVE—Previous studies have demonstrated development of endothelial cells (ECs) and smooth muscle cells (SMCs) as separate cell lineages derived from human embryonic stem cells (hESCs). We demonstrate CD34⁺ cells isolated from differentiated hESCs function as vascular progenitor cells capable of producing both ECs and SMCs. These studies better define the developmental origin and reveal the relationship between these two cell types, as well as provide a more complete biological characterization.

MATERIALS AND METHODS—hESCs are co-cultured on M2-10B4 stromal cells or Wnt1 expressing M2-10B4 for 13–15 days to generate a CD34⁺ cell population. These cells are isolated using a magnetic antibody separation kit and cultured on fibronectin coated dishes in EC medium. To induce SMC differentiation, culture medium is changed and a morphological and phenotypic change occurs within 24–48 hours.

RESULTS—CD34⁺ vascular progenitor cells give rise to ECs and SMCs. The two populations express respective cell specific transcripts and proteins, exhibit intracellular calcium in response to various agonists, and form robust tube-like structures when co-cultured in Matrigel. Human umbilical vein endothelial cells (HUVEC) cultured under SMC conditions do not exhibit a change in phenotype or genotype. Wnt1 overexpressing stromal cells produced an increased number of progenitor cells.

CONCLUSIONS—The ability to generate large numbers of ECs and SMCs from a single vascular progenitor cell population is promising for therapeutic use to treat a variety of diseased and ischemic conditions. The step-wise differentiation outlined here is an efficient, reproducible method with potential for large scale cultures suitable for clinical applications.

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Keywords

hESC; vascular progenitor cell; endothelial cell; smooth muscle cell

INTRODUCTION

Human embryonic stem cells (hESCs) provide an optimal cell population to study human vascular development and serve as a promising source for cell-based therapies of ischemic diseases. Previous studies demonstrate separate development of vascular components derived from mouse, primate, and human embryonic stem cells^{1–6}. While vascular progenitor cells from mouse embryonic stem cells have been primarily differentiated via embryoid body formation and selected by Flk1 expression⁷, hESCs differentiated by both embryoid body (EB) formation and stromal cell coculture can generate populations of endothelial cells (ECs) and smooth muscle cells (SMCs)^{8–14}. While generation of ECs using EBs is reproducible, there is still a great degree of variability in EB formation and differentiation efficiency. Using an OP9 stromal cell co-culture system with hESCs, Sone et al derived a Flk⁺CD34⁺CD31⁺ vascular progenitor that produced ECs and SMCs in culture, but as distinct, unrelated populations¹⁵. Using hemangioblasts from hESCs, Lu et al showed that ECs, SMCs, as well as hematopoietic cells could be generated, though without detailed phenotypic and functional analysis¹⁶. Vascular progenitors from hESCs have also been selected by CD31 and CD34 expression^{8, 12}. Both CD31 and CD34 are expressed at different time points in hematoendothelial differentiation, but CD31 is more commonly associated with a more mature EC phenotype and not in SMCs. Vascular progenitor cells isolated using either marker have been shown to express alpha-smooth muscle actin and an SMC phenotype upon culture with PDGF-BB. Other groups have generated SMCs from mouse ESCs and hESCs cells using retinoic acid^{17, 18}. Another method of producing mature functional smooth muscles cells was described by Ross et al. using rat, murine, porcine, and human multipotent post-natal cells in serum free conditions using TGF- β 1 and PDGF-BB¹⁹. Despite multiple methods of SMC differentiation, the exact lineage relation between ECs and SMCs has not been elucidated. hESC derived vascular components, as well as others, have been phenotyped and proven to function both in vitro and in vivo in a similar fashion as vascular counterparts isolated from post-natal sources. Several groups have shown ECs and SMCs placed in an ischemic hind limb mouse model re-organized to form vasculature and improve blood flow^{15, 16, 20}. A review by Gerecht et al describes the potential for differentiation of hESCs into vascular components using three dimensional natural or synthetic scaffolds²¹, which further details the potential of these cells to function in native environments as part of tissue engineered patches or vessels.

Here, we use hESC to demonstrate their potential to differentiate into endothelial and smooth muscle cells in a defined stepwise fashion via a novel three phase culture system. Large populations of CD34⁺ cells derived from hESCs can be differentiated into this population of CD34⁺ progenitor cells via co-culture with either S17 or M2-10B4 stromal cells, minimizing cell death and variability issues associated with EB differentiation. These cells can be expanded in culture and induced to maintain distinct phenotypic and functional characteristics of both ECs and SMCs as demonstrated by flow cytometry, q-RT-PCR, and immunohistochemistry.

Here, we also demonstrate more complete characterization of the hESC-derived ECs (hESC-ECs) and hESC-derived smooth muscle cells (hESC-SMCs) using calcium imaging to define distinct responses to a panel of nine agonists. Transmission electron micrograph images of hESC-ECs confirmed the release of microparticles from the surface of the ECs which is associated with functional endothelium. Additionally, these two populations are shown to interact in a functional Matrigel assay to form enhanced vascular-like structure formations. While many studies have utilized ECs in vitro and in vivo to study vasculogenesis, we find the

use of SMCs in this assay more accurately simulates the formation of vessels *in vivo* by acting as physiologically equivalent support structures. We also show an increase in the number and quality of CD34⁺ vascular progenitor cells derived using Wnt1 expressing M210 stromal cell layers during the initial differentiation period. This population of CD34⁺ cells produced a higher percentage of endothelial progenitors in a limiting dilution assay.

This hESC-EC and hESC-SMC model provides important insight into human vascular development as well as a source of preliminary data for future design of clinical vascular regenerative therapies. Most importantly, this system further elucidates the relationship between ECs and SMCs.

Materials and methods

Cell culture

Undifferentiated hESCs (H9 cell line obtained from Wicell, Madison, WI) were cultured as previously described^{11, 22}. Briefly, undifferentiated ES cells were maintained by co-culture with mitomycin C (Bedford Laboratories, Bedford, Ohio) inactivated mouse embryonic fibroblasts (MEF) in DMEM/F12 media (Invitrogen Corporation, Carlsbad, CA) supplemented with 15% knockout serum replacer (KOSR, Invitrogen), 1% MEM- nonessential amino acids (Invitrogen), 2 mM L- glutamine (Sigma, St Louis, MO), 0.1 mM β -mercaptoethanol (Sigma), 8 ng/ml basic fibroblast growth factor (bFGF, NCI). Undifferentiated cells were fed daily with fresh media and passaged onto new MEFs approximately every 5–7 days. hESCs expressing mCherry florescent protein were generated using lentiviral transduction technique and hESCs expressing GFP were generated using sleeping beauty transduction method (Amaxa, Gaithersburg, MD).

To promote differentiation, hESC were cultured as previously described^{23, 24}. Briefly, the undifferentiated hESCs are passaged onto mitomycin C inactivated mouse bone marrow-derived stromal cell line S17 (kindly provided by Dr. Ken Dorshkind, UCLA) or M210B4 cells (ATCC, Manassas, VA) in RPMI 1640 media (Invitrogen) supplemented with 15% fetal bovine serum (FBS) (Hyclone, Logan, UT), 1% MEM-nonessential amino acids (Invitrogen), 1% L- glutamine, 0.1% β -mercaptoethanol (Sigma) for 11–13 days. Differentiated hESCs were dissociated with 1mg/mL collagenase IV (Invitrogen), followed by 0.05% trypsin/0.53mM EDTA (Cellgro, Mediatech), a single cell suspension was generated, and the subpopulation of CD34⁺ cells were isolated using magnetic nanoparticle technology (EasySep Selection Kit, StemCell Technologies, Vancouver, BC, Canada).

The CD34⁺ population was cultured on fibronectin (Sigma) coated tissue culture flasks in EGM2 complete media (Lonza, Gaithersburg, Maryland) consisting of basal media (EBM2) supplemented with an EC cytokine cocktail (Lonza; 5% FBS, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), heparin, and ascorbic acid). This population, hESC-ECs, were grown to 80% confluence and serially passaged in parallel with HUVEC (Lonza) cells which functioned as a positive control in all experiments.

To obtain smooth muscle cells, hESC-SMCs, a portion of the hESC-EC cells established in culture were placed in high glucose Dulbecco modified Eagle media (DMEM) (Invitrogen) containing 5% FBS, 5 ng/mL platelet-derived growth factor-BB (PDGF-BB, PeproTech Inc, Rocky Hill, NJ) and 2.5 ng/mL transforming growth factor-beta 1 (TGF- β 1, PeproTech Inc). Media was changed every 3–4 days.

Flow Cytometry

hESC-ECs were trypsinized using 0.05% Trypsin-0.53mM EDTA and washed with basal medium containing 5% FBS and resuspended in FACS buffer (PBS without Ca^{2+} and Mg^{2+} (Cellgro, Herndon, VA) supplemented with 2% FBS and 0.1% sodium azide). The cells were aliquoted and stained with fluorescently labeled antibodies against: CD34-APC, CD31-PE, Flk1-PE, TIE2-PE, CD144-PE (VE-Cadherin) (BD Pharmingen, San Diego, CA) and the lectins *Ulex europeaus*-FITC, *Helix pomatia*-FITC, and *Griffonia simplicifolia*-FITC (EY Laboratories, San Mateo, CA). Appropriate isotype-matched controls labeled with the same fluorochromes were used to determine the degree of non-specific staining or using the corresponding competitive sugar (EY Laboratories) when lectin binding was examined. Cells were analyzed using a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) with Cell Quest Pro and FlowJo software.

Transmission Electron Microscopy (TEM) Assessment of hESC-ECs

hESCs derived ECs were seeded at 4×10^4 cells/cm², onto fibronectin (Becton Dickinson)-coated 0.4 μm polycarbonate membranes (Nalgen Nunc International/Thermo Fisher, Rochester, NY) at ~20,000 cells/membrane. Cells were suspended in EGM2 media supplemented with a cocktail that included hEGF, VEGF, hFGF-B, IGF, hydrocortisone, ascorbic acid and heparin at 200 μL /well. Cells were incubated at 37°C with 5% CO_2 /21% O_2 for 72 to 96-hours post seeding. Cells were plated in triplicate for two separate experiments. After incubation, cells were fixed in 3% glutaraldehyde (in cacodylate buffer). Specimens were post-fixed with 1% osmium tetroxide, dehydrated with a graded alcohol series, and embedded in PolyBed 812 resin. Semi-thin sections (1 μm) were cut and stained with toluidine blue, and examined by light microscopy to select regions containing cells. Thin sections (80 nm) were cut from the regions of the membrane containing cells using a diamond knife. Sections were stained with uranyl acetate, counter-stained with Reynold's lead citrate, and examined by TEM using a Philips CM 100 (FEI, Hillsboro, OR). All fixative and staining reagents purchased from Polysciences (Warrington, PA). Images are representative from random micrographs obtained from the two separate time points assessed.

Matrigel Tube Formation Assay

12 well plates were pre-warmed in incubators (30 minutes) set at 37°C, 5% CO_2 , before adding 200ul per well of Matrigel (Becton Dickinson). After Matrigel solidified (approximately 30 minutes), a total of 1×10^5 cells (hESC-ECs, hESC-SMCs, or 50/50 mixture of ECs/SMCs) suspended in a 50/50 mixture of EC/SMC media were seeded on top of the Matrigel. Subsequent co-culture studies were conducted using fluorescent hESC-ECs (derived from GFP expressing hESCs) and hESC-SMCs (derived from mCherry expressing hESCs). Tube formation was visualized after 1–2 days and images were captured using a phase microscope (Olympus, Center Valley, PA) or an inverted fluorescent microscope (Zeiss, Thornwood, NY) equipped with an Olympus camera, Apotome (Zeiss), and AxioVison software (Zeiss).

Immunohistochemical staining

For hESC-ECs analysis for the acetylated LDL receptor was performed by incubation of the cells in media with 5% FBS containing dil-acetylated low-density lipoprotein (dilAcLDL - Molecular Probes, Eugene, OR) for 4 hours. After washing, the cells were observed by fluorescence microscopy. HUVECs were used as a positive control, hESC-SMCs as a negative control. For detection of von Willebrand factor (vWF) and endothelial nitric oxide synthase (eNOS) cells were fixed, permeabilized, and incubated with primary antibody (vWF – rabbit anti-human, 1:100, Dako, Carpinteria, CA; eNOS – mouse anti-human, 1:50, Beckton Dickinson) for one hour followed by incubation with secondary antibody Alexa Fluor 488 (vWF – goat anti-rabbit, eNOS – goat anti-mouse, Molecular Probes) for 30 minutes. After a

final wash, cells were observed by fluorescence microscopy. CD31 and VE-cadherin were detected using mouse anti-human antibodies (eBioscience, San Diego, CA and BD Pharmingen) diluted 1:100 and 1:50 respectively. Alexa Fluor 488 goat anti-mouse secondary antibody (Invitrogen) diluted 1:600 was used for final detection and visualization.

For hESC-SMCs we examined expression SM22, calponin and alpha-smooth muscle actin using primary goat anti-human SM22 (AbCam, Cambridge, MA) and mouse anti-human calponin (Sigma) and detected with the species matched secondary antibodies labeled with Alexa Fluor 488. Alpha-SMC actin was detected with mouse anti alpha-SMC actin Cy 3 conjugated antibody (Sigma). Mouse and goat IgG isotypes (BD Pharmingen) detected with corresponding secondary Alexa Fluor488 conjugated antibodies were used as negative controls. Cells were fixed, permeabilized, blocked and incubated at room temperature for 1 hour with primary antibodies. For SM22 and calponin, cells were washed and incubated at room temperature for 45 minutes with fluorescently-labeled secondary antibodies. ProlongGold + Dapi (Invitrogen) was utilized for slide preparation and nuclear visualization via fluorescent microscopy.

Reverse transcriptase-polymerase chain reaction (RT-PCR) and quantitative RT-PCR

Total RNA was extracted from HUVEC, undifferentiated ES cells, hESC-ECs and hESC-SMCs using an RNeasy kit (Qiagen, Valencia, CA) with homogenization via Qiashredder (Qiagen, Valencia, CA) according to the manufacturer's instructions. mRNA was reverse transcribed (RT) using an Omniscript RT kit (Qiagen) following the manufacturer's instructions. Simultaneous RT reactions without reverse transcriptase were performed to control for the transcription of contaminating genomic DNA. cDNA was amplified with a HotStarTaq PCR kit (Qiagen) under the following conditions: initial 95 C for 15 minutes, followed by cycles consisting of 94 C for 1 minute, annealing at variable temperature as noted in the table for 1 minute, 72 C for 1 minute, and 72 C for 10 minutes after the final cycle. Thirty cycles were executed for Flk1, 38 cycles for other endothelial primers, and 35 cycles for all SMCs primers (Supplementary Table 1). The amplified products were separated on 1.5% agarose gels and visualized via ethidium bromide staining. Relative quantization of genes expression compared to beta-actin was evaluated by the comparative threshold cycle (C_T) method using Applied Biosystems SDS analysis software (version 1.9.1).

Measurement of [Ca²⁺] in response to calcium signaling agonists

Cells were grown on fibronectin coated 22×22 mm glass cover slips for 2 days in standard culture media. Cells were then loaded with fura-2 acetoxymethyl ester (fura-2 AM, Invitrogen) in standard culture media at 37 C for 60 minutes. After incubation, the cells were washed with HEPES buffered physiological salt solution (10 mM HEPES, 1.25 mM NaH₂PO₄, 146 mM NaCl, 3 mM KCl, 2 mM MgCl₂·6H₂O, 10 mM glucose, 2 mM CaCl₂, 1 mM Sodium Pyruvate, pH 7.4) and placed on an inverted microscope (Olympus) where they were continuously perfused with HEPES buffered solution for 20 minutes before imaging. The following panel of drugs was tested: Norepinephrine 100 uM, Carbachol 100 uM, Oxytocin 1 uM, Endothelin-1 100 nM, 5-Hydroxytryptamine 10 uM, Vasopressin 100 nM, ATP 10 uM, Bradykinin 1 uM. Each drug was applied for 30 seconds with recovery time between drug applications varied by cellular response. Images were acquired with a 40× oil immersion objective, a CoolSNAP (Roper Scientific, Trenton, NJ) digital camera using an exposure time of 25 ms. Fura-2 was excited alternately at 340 and 380 nm using a computer-controlled filter wheel and shutter (Lud Electronics Corporation, Hawthorne, NY) Pairs of images were acquired at 2–30 sec. intervals. Background-corrected images were ratioed (340/380) and analyzed using MetaMorph/MetaFluor image acquisition and analysis software (Molecular Devices, Sunnyvale, CA). Changes in ratio in individual cells were measured and plotted versus time using graphing software (Excel, Microsoft, Redmond, WA).

Limiting Dilution Assay

To test the role of Wnt proteins on hESC differentiation, two genetically modified M2-10B4 cells lines were used (one over expressed Wnt1 and the other over expressed Wnt5, kindly provided by Dr. Randall Moon, Howard Hughes Medical Institute)²⁵. On day 14–15, CD34⁺ cells were selected from each stromal cell co-culture and plated at limiting dilution in 96 well plates with 4×10^3 , 1.3×10^3 , 4×10^2 , 1.3×10^2 , 40, 13, and 4 cells per well that were pre-coated with fibronectin and cultured in hESC-EC media. Cells received media changes every 4–5 days and after 15 days wells were scored for growth. Progenitor frequencies were calculated and reported graphically as progenitor cells per 10000 cells.

RESULTS

Characterization of hESC-ECs

H9 hESCs were supported to differentiate toward vascular progenitors through an initial step utilizing stromal cell co-culture. We found no significant difference in ability to induce differentiation between M2-10B4 and S17 two cell lines²³. After culture for 13–15 days, CD34⁺ cells were isolated via magnetic sorting and assessed via flow cytometry for vascular and endothelial surface markers (Figure 1A). A significant percentage of this population co-expresses typical endothelial markers CD31 and Flk1. For expansion and further differentiation, these cells were placed on fibronectin coated culture flasks and cultured in EGM2 media supplemented with a cytokine and growth enhancing cocktail including hEGF, VEGF, hFGF-B, IGF, hydrocortisone, ascorbic acid, and heparin. Under these culture conditions, the differentiated cells assumed a typical EC phenotype and could be readily passaged and expanded. After expansion of the cells for 2–5 passages, further assessment and characterization was performed using flow cytometry, immunohistochemistry (IHC) and Q-RT-PCR. hESC-ECs demonstrated typical EC surface antigen expression of CD31, VE-cadherin (CD144), CD146, FLK1, lectins *Helix pomatia*, *Griffonia simplicifolia*, and *Ulex europaeus*, and intracellular markers vWF and endothelial cell nitrous oxide synthase (eNOS) (Figure 1B). IHC confirmed EC morphology and expression of endothelial proteins: CD31, VE-Cadherin, vWF, eNOS, as well as uptake of dil-ac-LDL, another characteristic of ECs (Figure 1C). RT-PCR was done to demonstrate expression of Flk1, CD31, CD34, vonWilldebrand factor (vWF), VE-cadherin, eNOS, and Tie2 (Figure 1D), and the expression level of these transcripts was quantified via Q-RT-PCR (Table 1).

Transmission electron micrographs (TEM) of hESC-ECs showed the presence of microparticles being released from the membrane surface of the cell (Figure 1E). This is a key characteristic of endothelial cells, as microparticles play an important role in endothelial cell function^{26–28}. The TEM images also revealed that these endothelial cells are in a metabolically active state, displaying an abundance of mitochondria and endoplasmic reticulum as well as nuclear euchromatin.

Characteristics of hESC-SMCs

Studies of several human and mouse progenitor cell sources demonstrate that SMC survival and growth is promoted by TGF- β 1 and PDGF-BB^{29, 30}. To generate SMCs, hESC-ECs (passage 3 or 4) are removed from endothelial cell culture media and cultured in media containing TGF- β 1 and PDGF-BB. 24 to 36 hours after this change, a complete morphologic change can be observed. The cells flatten and acquire intracellular fibrils as seen in other SMC cultures (Figure 2A). Immunofluorescent staining revealed robust expression of SM22, calponin, and alpha-smooth muscle actin in hESC-SMCs (Figure 2B) and the absence of these markers in hESC-ECs (Figure 2C). Notably, HUVEC cells cultured under SMC conditions (with TGF- β 1 and PDGF-BB) did not convert to SMC morphology. Also, direct culture of the initial hESC-derived CD34⁺ population under SMC conditions did not yield stable cultures

with SMC morphology or characteristics⁸. hESC-SMCs generated from hESC-ECs continued to proliferate for 2–3 passages in culture.

RT-PCR demonstrates transcripts of typical smooth muscle cells genes including alpha-SMC actin, calponin, SM22, SMI, smoothelin, myocardin (Figure 2C). Concomitant expression of APEG-1 and CRP2/SMLIM was also noted (Figure 2C). These two genes are preferentially expressed in arterial SMCs, indicating these SMCs may be a more specific sub-type of supportive vasculature.

Next, we used Q-RT-PCR to more accurately compare expression of transcripts specific for smooth muscle cells with expression of transcripts for endothelial genes in the populations of hESC-ECs and hESC-SMCs, respectively (Table 1). When compared to the hESC-EC cell population, the hESC-SMC cells exhibited a remarkable increase in expression of transcripts specific for SMC genes (alpha-smooth muscle actin, calponin, SM22, SMI and myocardin) with a concomitant decrease in endothelial gene transcripts. In a corresponding manner, in the hESC-EC population, a high level of endothelial gene transcripts were measured at the same time as a decrease in SMC gene transcripts. In contrast, while HUVECs could survive and proliferate in SMC conditions (media containing TGF- β 1 and PDGF-BB), HUVECs under these conditions did not change morphology and they did not show an increased expression of SMC genes (Table 1).

Enhanced Endothelial Progenitors from Wnt Expressing Stromal Cells

Studies by our group and others have shown that various stromal cell lines provide lineage specific support for differentiation^{31, 32}. To better define conditions that support or enhance differentiation of ECs or SMCs from hESCs, M2-10B4 stromal cells over-expressing either Wnt1 and Wnt5 were used to induce differentiation in hESCs as described²⁵. CD34⁺ cells were isolated and assessed quantitatively for their ability to produce endothelial progenitors. Not only were a greater number of CD34⁺ cell obtained, the limiting dilution assay also revealed that CD34⁺ cells isolated from Wnt1 or Wnt5 expressing stromal cells yielded a higher number of ECs that could be subsequently induced to form SMCs, as an indication of vascular progenitor cells (Figure 3). Statistical analysis by unpaired t-test generated the following p values: regular M2-10B4 stromal cells vs Wnt1 expressing M2-10B4 (p=0.0279) and vs.Wnt5 M2-10B4 (p=0.0309).

Functional characterization of hESC-derived ECs and SMCs

To evaluate the functional characteristics of hESC-ECs and SMCs, nine different pharmacological agonists were used to measure the ability of these cells to respond to stimuli by release with a change in intracellular calcium concentration. Fura-2 labeled hESC-ECs and hESC-SMCs were tested and differences in responsiveness to various agonists were evaluated (Figure 4). The majority of the SMC population responded to bradykinin, oxytocin and endothelin-1 and fewer cells demonstrated a response to histamine, ATP, serotonin, vasopressin, norepinephrine and carbachol. In contrast, the hESC-ECs responded to endothelin-1, histamine, bradykinin, as well as carbachol, though there was little response to oxytocin or the other agonists (Figure 4). Not only do these results support the notion that hESC-ECs and hESC-SMCs are distinct populations, but also indicate their ability to function in a physiologically appropriate manner. Undifferentiated hESCs were also tested and were found only to have a uniform Ca-response to carbachol, ATP, and ET-1. While the three populations each have different response profiles, the lack of response by hESC-ECs and hESC-SMCs to certain agonists indicates these cells have not advanced to a fully mature phenotype.

To evaluate functional interactions between the two cell types, hESC-ECs and hESC-SMCs were cocultured in a Matrigel tube formation assay. Here, distinct difference was evident when the two populations were cultured together as opposed to being cultured as single populations in Matrigel. hESC-ECs cultured alone formed typical relatively thin capillary-like tubes (Figure 5A–C). hESC-SMCs cultured by themselves on Matrigel did not form significant structures (data not shown). However, in the samples consisting of a mixture of hESC-ECs and hESC-SMCs, substantially denser and more robust three-dimensional networks of vasculature like structures were observed (Figure 5D–F). The hESC-ECs and hESC-SMCs could be tracked in culture by their respective fluorochrome expression. The hESC-ECs were derived from a H9 cells with stable GFP expression and the hESC-SMCs were derived from another H9 cell line expressing mCherry. Images show alignment of the cells as well as physical cell-cell interaction of enhanced vascular formation that appears to recapitulate in vivo EC and SMC interactions (Figure 5G).

CONCLUSION

Two methods have been utilized by our group and others to support differentiation of hESCs into ECs: stromal cell co-culture and embryoid body (EB) formation^{3, 10, 12–14, 33, 34}. In contrast to work by Levenberg et al. using EB-mediated differentiation¹⁰, here we used stromal cell co-culture with the mouse bone marrow derived stromal cell lines as an efficient method to derive vascular progenitor cells. Phenotypical and functional characteristics of our hESC-ECs including morphology, cell-surface antigens, uptake of acetylated LDL and tube formation in Matrigel were consistent with the typical EC characteristics. Next, based upon studies to derive SMCs from other cell populations such as mouse ES cells and adult progenitor cells we evaluated the SMC potential of this hESC-EC population. Here, we demonstrated that changing the culture conditions to media containing TGF- β 1 and PDGF-BB resulted in a profound change to SMC morphology, immunophenotype, and gene expression. Additionally, these hESC-SMCs demonstrate a functional response to calcium signaling agonists similar to responses of SMCs in their physiological environments in vivo. Notably, the response of hESC-ECs to these pharmacological agonists is considerably different than the hESC-SMCs. Despite the fact these two populations are derived from a common CD34⁺ vascular progenitor cell population, these studies clearly illustrate the difference between the two cell types and highlight their independent contribution in the structure and function of mature vasculature. Other populations such as mesenchymal stem cells and pericytes have been sited as SMC precursors^{35–37}. The hallmark characteristics of these populations are controversial and in some cases overlap with SMCs. Further studies to allow definitive characterization will be necessary to determine the relationship, if any, between these cell types.

While ECs and SMCs have been previously derived and characterized from hESCs, our results advance these previous results in several important ways. First, we are able to demonstrate the potential of a hESC-derived CD34⁺ cells to produce both ECs and SMCs. Second, this is accomplished in a novel, efficient three phase culture system through the development of hESC-ECs and subsequent differentiation of an hESC-SMC cell population. Third, the method utilized is scalable to produce large populations with little variability. Finally, in addition to demonstrating distinct responses to a variety of pharmacological agents, the two populations were successfully combined in culture to form more robust, enhanced vascular structures.

There are at least two mechanisms that may account for the ability to derive SMCs from a population of hESC-ECs. One possibility is the initial hESC-ECs, generated from the CD34⁺ vascular progenitor population contain a very limited number of SMC progenitor cells that remain relatively suppressed under EC culture conditions. Then, upon changing the culture from EC to SMC conditions, the SMC population rapidly expands, and the EC growth is limited, eventually eliminating the hESC-EC population from the culture. These potential SMC

progenitor cells could be either in the main CD34⁺ cell population, or the residual CD34⁻ cells that remain after sorting. Alternatively, and more likely, the hESC-ECs may be capable of directly converting to an SMC population under the alternative conditions. This second hypothesis is supported by the fact that hESC-ECs cultured under SMC conditions for as little as 24 hours quickly change morphology and no EC-like cells are observed. In attempts to derive hESC-SMC directly from the CD34⁺ population, cells placed under SMC conditions did not give rise to viable cultures. The two main factors that attributed to this fate were low plating efficiency and no detectable cell proliferation of plated cells. Moreover, typical EC characteristics such as the expression of EC markers and tube formation capabilities are also quickly diminished. Further studies are required to better define and validate the mechanisms of differentiation operating in these cultures.

Mouse ESCs have been previously used to model both EC and SMC development including characterization of Flk1⁺ cells capable of producing ECs, SMCs, and hematopoietic cells^{7, 38}. While both ECs and SMCs could be derived from this population, they were culture expanded as separate populations and the SMCs did not differentiate from the EC population³⁸. It is important to note that other more definitive or mature EC populations such as HUVECs are not able to convert to SMCs under the same conditions that induce differentiation from hESC-ECs to hESC-SMCs.

Other recent studies using mouse ESCs demonstrate that Flk1 positive and/or Isl1 positive are able to give rise to not only ECs and SMCs, but also cardiomyocyte progenitor cells^{39, 40}. Another study demonstrated that Nkx2.5 positive cells derived from mouse ESCs could form both cardiac tissue (vascular and conductive) and SMCs, though not ECs⁴¹. Also, while many studies have now demonstrated hematopoietic development from hESCs, one recent study demonstrates a putative bi-potential hemato-endothelial (hemangioblast) development from a Flk1⁺ cell population⁴². ECs and SMCs are known to have differences in phenotype and gene expression depending on anatomic location or developmental source⁴³⁻⁴⁵. Therefore, it will now be of interest to evaluate the potential for the hESC-derived populations described in our studies not only for cardiomyocyte potential but also for their potential contributions in other tissues and organs. Furthermore, these cells can now be better evaluated for functional capacity using in vivo models of cardiac or peripheral vascular ischemia^{15, 46}.

A recent study also found separate outgrowth of ECs and SMCs from a CD34⁺ population selected from embryoid bodies (EBs)⁸. While these populations display phenotypes similar to the cell described in this study, it is important to note the differences in methods and in the secondary characteristics of the populations. The method for generating SMCs featured in our work occurs via an hESC-EC intermediate in a step wise differentiation process. While the CD34⁺ population is present during all stages of differentiation, the co-expression of other surface antigens better define the progenitor cell population(s).

Co-culture differentiation is advantageous in efforts to more accurately recreate native stem cell niches, as stromal cell lines can be engineered to express various components that may promote or inhibit lineage specific differentiation²⁵. In this study, the use of Wnt1 and Wnt5 over expressing M210 stromal cells not only increased the quantity of CD34⁺ progenitors but also the co-expression of typical EC surface antigens such as CD31 and Flk1 (also termed KDR or VEGFR2). Moreover, it is possible that additional phenotypic and functional cardiac and vascular progenitors could be produced at different stages of differentiation by utilizing these methods.

The field of cardiovascular regenerative medicine is rapidly progressing. Multiple studies have evaluated the ability of different cell populations to mediate cardiac repair and/or improved function both using model animals and clinical studies⁴⁷⁻⁵². Most of these studies use

heterogenous or poorly defined cell populations such as myocytes, whole bone marrow or MSCs, and the mechanisms that lead to improved function are often not clear. While improvement of cardiac function has been demonstrated in rodent models^{47, 52–54}, these findings do not always translate to similar efficacy in clinical trials^{55–58}. In most cases where there is functional improvement, it is uncertain whether this is due to the exogenous cells generating functional tissue, or if these injected cells stimulate endogenous repair. Use of hESC-derived cells can be utilized to better identify cells most effective at cardiovascular repair. Specifically, use of hESCs with stable expression of fluorescent proteins (GFP, dsRed, or others) as described here, and bioluminescent imaging via luciferase-expressing cells, as previously demonstrated⁵⁹, can be used to better define the contribution of defined cell populations in pre-clinical models of ischemia. Additionally, studies describing hematopoietic^{60, 61}, endothelial⁶¹, and cardiac development⁶² from iPSCs suggests the potential for autologous cell sources for cardiovascular repair.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. CD34⁺ Vascular Progenitor Cells Capable of Endothelial Cell Differentiation
 (A) hESC-derived cells after 15 days of differentiation demonstrating expression of CD34, CD31, and Flk1 both before (presort) and after (postsort) sorting for CD34⁺ cells. (B) After sorting, the CD34⁺ cells are placed in endothelial cell (EC) culture medium on fibronectin coated tissue culture plastic. After 2–4 passages, expression of EC specific surface markers and lectins can be detected by flow cytometry. Histograms demonstrate red plot as isotype control or corresponding competitive sugar control (for lectins) in each panel, and blue plot is stained for surface antigen or lectin, as indicated. hESC-ECs also exhibited uptake of di-ac-LDL. (C) Immunofluorescent staining of hESC-EC: (left to right) CD31, VE-Cadherin, vWF, eNOS, dilac-LDL. Original magnification 100 $\times$ for each plot. Blue signal represents DAPI stained nuclei. (D) RT-PCR of hESC-derived ECs: mRNA expression of 7 transcripts for typical EC genes as indicated above each row. (E) Transmission Electron Micrograph (TEM) images of hESC derived ECs cultured under endothelial cell culture conditions show release of microparticles of approximately 100nm in size (as indicated by arrows) from the cell surface.

Figure 2. Micrographic, Immunofluorescent, and Genotypic Assessment of hESC-SMCs
 (A) Schema of hESC-EC culture in EGM2 media, then development of hESC-SMCs via change of conditions to DMEM with TGF- β 1 and PDGF-BB. Photomicrographs show hESC-ECs with characteristic EC morphology and tube formation on Matrigel. After change to SMC conditions, cells flatten out and show pronounced intracellular fibrils. Genotypic analysis via RTPCR showed specific expression of SMC transcripts. (B) Further phenotypic analysis was conducted via immunofluorescent staining for SMC specific intracellular proteins. Staining of hESC-SMCs (left to right): SM22, alpha-smooth muscle actin (α -SMA), and calponin. Blue signal represents Hoechst 33258 stained nuclei. Original magnification 200 $\times$. (C) Staining of hESC-ECs (left to right): SM22, α -SMA, and calponin. Blue signal represents Dapi counter stain. Original magnification 20 $\times$. (D) RT-PCR of hESC-derived SMCs for eight common SMCs genes (and β -actin control), as indicated above each indicated lane. To control for contaminating genomic DNA, reactions were also done under conditions with no reverse transcriptase. For expression of EC and SMC genes, all values are means and standard deviations of 3 RT-PCR analysis of independent experiments.

Figure 3. Increased Development of CD34+ Vascular Progenitor Cells From Wnt1 Expressing Stromal Cell Differentiation

Limiting dilution analysis was done to quantify vascular progenitor cells from hESCs allowed to differentiate on M2-10B4 stromal cells that did not over-express Wnt proteins, or M2-10B4 cells that over-expressed either Wnt1 or Wnt5, as indicated. Numerical values shown as progenitor cells per 10,000 cells. Error bars represent Standard Error of the Mean of $n=4$ individual experiments; * Wnt1 ($p=0.0279$) and * Wnt5 ($p=0.0309$).

Figure 4. Intracellular calcium response of hESC-EC and hESC-SMC to pharmacological agonists Single cell preparations were exposed to pharmacological agonists as indicated. Responses of each cell type were measured via a fluorimetric ratio using fura-2. (A) hESC-SMC phase image (B) Fura2 loaded hESC-SMCs prior to agonist exposure (C) Fluorometric change post-agonist exposure (oxytocin); pseudocolor scale: low ratios indicated by blue color and high ratios indicated by yellow to red color (A–C original magnification 400×). (D) Representative of time course graph of seven individual hESC-SMCs exposed to oxytocin, ET-1, ATP and Bradykinin. Each line represents the ratios obtained from an individual cell in successive image pairs. (E) Graphic summary comparing responses of 100 undifferentiated hESC, 100 hESC-ECs, and 105 hESC-SMCs to specific agonists: bradykinin (BK), endothelin-1 (ET-1), oxytocin (Oxy), histamine (Hist), ATP, serotonin (5-HT), vasopressin (AVP), norepinephrine (NE), and carbachol (Carb). Each population tested was comprised of cells from more than one culture. Not all agonists elicited response in all populations.

Figure 5. In-vitro Matrigel Tube Formation Assay

To assess functional potential of hESC-ECs and hESC-SMCs in vitro, cells were cultured on Matrigel in a 50/50 EC/SMC medium. Vascular tube structures formed in both hESC-EC and hESC-EC/hESC-SMC cultures, with the co-cultured cells interacting to form more robust, denser tube structures. (A–C) hESC-ECs alone (D–F) hESC-ECs co-cultured with hESC-SMCs (G) Fluorescent image of GFP-expressing hESC-ECs co-cultured with mCherry-expressing hESC-SMC demonstrate close interaction between the two cell populations in these in vitro Matrigel cultures. Images in (G) show low power (25×) of both cell populations, as well as higher power (200×) of separate GFP and mCherry expressing cells, as well as co-localization of fluorescent cells with phase image. (Original magnifications: A 40×, B 100×, C 400×, D 40×, E 100× F 400×, G 25× and 200×).

Table 1
Q-RT-PCR of hESC-ECs, hESC-SMCs, and HUVECs

hESC-ECs and hESC-SMCs were analyzed for expression of typical EC and SMC genes. A) hESC-ECs express EC-specific genes, whereas hESC-SMCs express lower levels of these genes. In contrast, hESC-SMCs express SMC-specific genes, and hESC-ECs express lower levels of these genes. B) QRT-PCR was also performed on HUVECs cultured in EC and SMC conditions. Here, only EC-specific genes are expressed by these cells cultured under either EC or SMC conditions, and SMC-specific genes are not expressed under either condition. mRNA levels were normalized against β -actin.

A		
Gene	hESC-ECs	hESC-SMCs
EC Specific Genes		
CD31	46.29 $\pm$ 1.02	0.74 $\pm$ 0.05
Flk1	7.15 $\pm$ 1.56	0.01 $\pm$ 0.00
Tie2	9.48 $\pm$ 0.14	3.53 $\pm$ 2.27
eNOS	7.56 $\pm$ 7.54	2.90 $\pm$ 4.09
VE-cadherin	4.76 $\pm$ 5.23	0.00 $\pm$ 0.00
SMC Specific Genes		
calponin	0.67 $\pm$ 0.02	3.1 $\pm$ 0.40
alpha-SMA	11.24 $\pm$ 0.52	47.93 $\pm$ 20.38
SM22	0.62 $\pm$ 0.06	6.37 $\pm$ 1.05
SM1	1.49 $\pm$ 0.55	2.30 $\pm$ 0.49
myocardin	13.39 $\pm$ 2.59	45.95 $\pm$ 27.85
B		
Gene	HUVECs in EC Culture	HUVECs in SMC Culture
EC Specific Genes		
CD31	12459.86	16612.71
Flk1	4.87	5.91
Tie2	225.97	598.41
eNOS	39.8	37.41
VE-cadherin	6608.01	8902.53
SMC Specific Genes		
calponin	0.01	0.12
alpha-SMA	0.18	0.33
SM22	0.03	0.06
SM1	0.02	0.06

B		
Gene	HUVECs in EC Culture	HUVECs in SMC Culture
myocardin	0.04	0.22