Hemangioblasts from human embryonic stem cells generate multilayered blood vessels with functional smooth muscle cells

Background: The formation and regeneration of functional vasculatures require both endothelial cells (ECs) and vascular smooth muscle cells (SMCs). Identification and isolation of progenitors with potential for both EC and SMC lineage differentiation from an inexhaustible source, such as human embryonic stem (hES) or induced pluripotent stem cells, will be desirable for cell replacement therapy. Method: Recently, we have developed a serum-free and animal feeder-free differentiation system to generate blast cells (BCs) from hESCs. These cells possess the characteristics of hemangioblasts in vitro and are capable of repairing damaged retinal vasculatures, restoring blood flow in hind-limb ischemia and reducing the mortality rate after myocardial infarction in vivo. We demonstrate here that BCs express markers of SMCs and differentiate into smooth muscle-like cells (SMLCs), in addition to ECs and hematopoietic cells. Results: When BCs from individual blast colonies were cultured in SMC medium, they differentiated into both ECs and SMLCs, which formed capillary-vascular-like structures after replating on Matrigel™. The SMLCs expressed SMC-specific markers (α-SM actin and calponin) and contracted upon treatment with carbachol. When implanted in nude mice, these cells formed microvasculature with ECs in Matrigel plaques. The BCs differentiated into both ECs and SMLCs, and incorporated into blood vessels after injection into ischemic tissue. Conclusion: These results demonstrate that hemangioblasts (BCs) generated from hESCs are tripotential and can provide a potentially inexhaustible source of cells for the treatment of human blood and vascular diseases.

KEYWORDS: blast cell, differentiation, endothelial cell, hemangioblast, hematopoietic cell, human embryonic stem cell, smooth muscle cell

Human embryonic stem cells (hESCs) have previously been shown to differentiate into a variety of important hemangiogenic-lineage cell types, including hemangioblasts themselves [1–11]. The latter have been traditionally defined as the bipotential precursor of hematopoietic and endothelial cells (ECs) [12], and can be generated from mouse, nonhuman primate and hESCs using established in vitro differentiation systems [13–19]. We recently developed a simple strategy to efficiently and reproducibly generate hemangioblasts/blast cells (BCs) from multiple hESC lines [19]. These BCs express gene signatures characteristic of hemangioblasts and can differentiate into ECs as well as multiple hematopoietic cell lineages [19]. When the BCs were injected into animals with spontaneous Type II diabetes or ischemia/reperfusion (I/R) injury of the retina, they homed to the site of injury and showed robust reparative function of the damaged vasculature. The cells also showed a similar regenerative capacity in nonobese diabetic/severe combined immunodeficiency β2 / mouse models of both myocardial infarction (50% reduction in mortality rate) and hind-limb ischemia, with restoration of blood flow in the latter model to near normal levels [19]. Fluorescent immunocytochemistry showed that the vascular lumens were surrounded by human ECs in both diabetic and I/R damaged retinas. Similarly, confocal microscopy confirmed the incorporation of human ECs into the lumens of microvessels in the infarcted heart and ischemic limb tissues, and the presence of vascular organization in intramuscular areas with human cells [19].

However, formation of mature and functional vasculature, except for capillary vessels, requires the interaction of ECs and smooth muscle cells (SMCs), the later playing a critical role in the structural and functional support of the vascular network [20–22]. Previous studies have shown that single cell-derived blast colony forming cells (BL-CFCs) from mouse ESCs (the mouse equivalent to human BCs) can produce hematopoietic cells as well as ECs and SMCs when cultured under proper conditions [23,24]. This suggests that, at least in some species, hemangioblasts might be a tripotential progenitor for hematopoietic cells, ECs and SMCs.

To date, nonhuman primate and human BCs (hemangioblasts) have not been shown to possess the ability to differentiate into SMCs. Here we report that single cell-derived BCs...
can differentiate into hematopoietic cells, ECs, as well as SMCs under appropriate conditions. When BCs were cultured in SMC medium, they differentiated into both ECs and SMCs, which formed vascular-like structures after replating on Matrigel™. SMCs derived from BCs were capable of contraction when treated with the acetylcholine receptor agonist carbachol and incorporated in blood vessels upon administration into ischemic tissues. These results, combined with our previous studies [19], clearly demonstrate that BCs are capable of differentiating into ECs and SMCs both in vitro and in vivo. The cells participated in the formation of new blood vessels and the repair of injured vessels in multiple vasculatures, suggesting a potential future therapeutic role using these early cellular developmental components to restore vascularization and function in patients with vascular disease.

Materials & methods

hESC culture & differentiation

The hESC lines used in this study were previously described H1 and H7 (NIH-registered as WA01 and WA07) and were grown on mitomycin C-treated mouse embryonic fibroblasts in complete hESC media until they reached 80% confluence as described previously [25]. hESCs were dissociated by 0.05% trypsin-0.53 mM EDTA (Invitrogen, Carlsbad, CA, USA) for 2–5 min and collected by centrifugation at 1000 rpm for 5 min. To induce hemangioblast precursor (mesoderm) formation, hESCs (2–5 × 10^5 cells/ml) were plated on ultra-low dishes (Corning, NY, USA) in Stemline II medium (Sigma, St Louis, MO, USA) with the addition of bone morphogenic protein (BMP)-4 and VEGF165 (50 ng/ml, R&D Systems) and cultured in 5% CO2 as described previously [19]. After 48 h, half the media was removed and fresh media was added with the same final concentrations of BMP-4 and VEGF, plus stem cell factor, thrombopoietin and Flt3 ligand (20 ng/ml, R&D Systems, Minneapolis, MN, USA). After 3.5 days, embryoid bodies (EBs) were collected and dissociated by 0.05% trypsin-0.53 mM EDTA (Invitrogen). A single cell suspension was prepared by passing the cells through a 22G needle 3–5 times followed by centrifugation at 1000 rpm for 5 min, resuspended in 50–100 µl of Stemline II medium and plated in blast-colony expansion medium as previously reported [19]. BCs were collected 6–7 days after replating.

Hematopoietic & endothelial differentiation

For hematopoietic colony-forming unit (CFU) assay, BC single cell suspensions were mixed with 1 ml of serum-free hematopoietic CFU medium (Stem Cell Technologies, Vancouver, BC, Canada) plus 0.5% EX-CYTE (Seralogicals/Celliance, Kankakee, IL, USA). Hematopoietic CFUs were examined under microscope 10–14 days after initial plating.

For EC differentiation, BCs were obtained from 6- to 7-day-old blast colonies as before [19], and plated on fibronectin-coated culture slides (BD Biosciences, San Diego, CA, USA) in EGM2 medium (Lonza, Walkersville, MD, USA) for 3–5 days. For acetylated low-density-lipoprotein (Ac-LDL) uptake, 10 µg/ml of Alexa Fluor 594-labeled Ac-LDL (Invitrogen) was added in the culture and incubated for 4–6 h. Then cells were washed three times with 1X phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 30 min. The uptake of Ac-LDL was visualized under a fluorescent microscope. For network formation, 0.5–1 × 10^5 cells in 0.3 ml of EGM2 medium were plated on top of Matrigel (BD Bioscience) in four well-plates and the formation of capillary-like (tube) structures was verified after 16–24 h of incubation.

SMC differentiation

To examine the potential of BCs to differentiate toward SMCs, BCs obtained from 6- to 7-day-old blast colonies were plated on fibronectin-coated culture slides (BD Bioscience) in SMC medium (Lonza) for 2–10 days. Cells were fixed at day 2, 5, 7 and 10 with 4% paraformaldehyde (PFA) for 15 min, washed with PBS and permeabilized with 0.4% Triton X-100. The labeled Ac-LDL (Invitrogen) was added in the culture and incubated for 4–6 h. Then cells were washed three times with 1X PBS and fixed with 4% paraformaldehyde for 30 min. The uptake of Ac-LDL was visualized under a fluorescent microscope. For network formation, 0.5–1 × 10^5 cells in 0.3 ml of EGM2 medium were plated on top of Matrigel (BD Bioscience) in four well-plates and the formation of capillary-like (tube) structures was verified after 16–24 h of incubation.

Immunocytochemistry

To examine the expression of SMC genes, the permeabilized cells were incubated with primary antibodies against α-smooth muscle actin (ASMA) and calponin (Dako North America, Carpinteria, CA, USA) at 4°C overnight followed by secondary antibodies conjugated with Rhodamine (Jackson ImmunoResearch, West Grove, PA, USA) for 60 min and examined under fluorescent microscope. For the expression of von
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Willebrand factor (vWF), PECAM-1 (CD31), VE-cadherin and KDR, cells were permeabilized and then incubated with primary antihuman vWF (Dako), PECAM-1 (Dako), KDR (Cell Signaling Technology, Danvers, MA, USA) and VE-cadherin (R&D Systems) antibodies, respectively, overnight at 4°C, then incubated with corresponding secondary antibodies conjugated with Rhodamine or fluorescein isothiocyanate (FITC) for 60 min. Human aorta smooth muscle cells (AoSM) and human umbilical vein ECs (Lonza) were used as positive controls and neonatal human dermal fibroblasts (Lonza) were used as a negative control.

Reverse transcriptase-PCR analysis
Between 20 and 30 individual blast colonies were handpicked on day 6 and smooth muscle gene expression was analyzed by reverse transcriptase-PCR. In brief, total RNA was isolated using an RNeasy Micro Kit (Qiagen, Valencia, CA, USA). cDNA pools were constructed using the SMART cDNA synthesis kit (Clontech, Mountain View, CA, USA), as previously reported [2]. Primers (Supplementary Table 1) (see online www.futuremedicine.com/toc/rme/4/1) specific to smooth muscle calponin, SM22, ASMA and GAPDH (quality control), as reported previously [26], were used to amplify corresponding messages by PCR. Human AoSM cell total RNA was used as a positive control and mouse fibroblast 3T3 total RNA was used as a negative control. PCR products were separated on a 1% agarose gel and visualized by ethidium bromide fluorescence.

Contraction assay
BCs were cultured in SMC medium for three passages. Contraction was induced by incubating the cells with the addition of 10⁻⁵ M carbachol for 45 min. Photographs were taken before the addition of carbachol (t0) and 45 min after adding carbachol (t45). Real-time video was captured by using a Hamamatsu C4742–95 camera (Hamamatsu Photonics, Birdgewater, NJ, USA). Human fetal fibroblasts and neonatal dermal fibroblasts (Lonza) were used as negative controls and human AoSM cells (Lonza) were used as positive controls.

Figure 1. Characterization of smooth muscle-like cells derived from purified blast cells. (A) Immunostaining of calponin in smooth muscle cells (SMCs) derived from WA01 cells. (B) Immunostaining of calponin in SMCs derived from WA07 cells. (C) Immunostaining of smooth muscle actin (SMA) in SMCs derived from WA01 cells. (D) Immunostaining of SMA in SMCs derived from WA07 cells. (A–D) magnification x200.
Network formation by SMCs & ECs from BCs

BCs were differentiated on fibronectin-coated culture slides (BD Bioscience) in SMC medium for 2 weeks and then transferred onto Matrigel (BD Biosciences) matrices. Matrigel was added to each well of a four-well plate (0.2 ml) and was subsequently aspirated, leaving a thin layer remaining on the plate to solidify at 37°C for 45 min. The above differentiated cells (0.5 x 10⁵) in 0.3 ml of SMC medium were plated on top of the Matrigel layer. After the formation of capillary-like structures was observed (~after 16 h incubation) the cells were fixed with 4% PFA, washed with 1X PBS and permeabilized with 0.4% Triton X-100. To examine the expression of SMC and EC markers, the permeabilized cells were incubated with primary antibodies against vWF and calponin or vWF and ASMA (Dako) at 4°C overnight, followed by Rhodamine and FITC conjugated secondary antibodies and examined using fluorescent microscopy.

Transplantation in nude mice

BCs were differentiated in SMC medium for 2 weeks, then mixed with 750 µl Matrigel (BD Biosciences), 50 ng/ml VEGF and 20 ng/ml bFGF. The cell–Matrigel suspension was injected subcutaneously into the dorsal region of 1-month-old female nude mice. After 28 days the implants were removed, embedded, frozen in Tissue-Tek OCT embedding medium (Sakura Finetek USA Inc., Torrance, CA, USA) and sectioned for histological examination. Briefly, 10 µm Matrigel sections were fixed with 4% PFA, permeabilized with 0.5% Triton X-100 and incubated with rabbit primary antibodies against ASMA (Abcam, Cambridge, MA, USA. Cross-reactive with both mouse and human antigens) and PECAM-1 (Proteintech Group, Chicago, IL, USA. Cross-reactive with both mouse and human antigens) at 4°C overnight, followed by FITC-conjugated secondary antibodies (Jackson ImmunoResearch) and examined using fluorescent microscopy. As a control, SMC medium mixed with Matrigel
plus VEGF and basic FGF was injected and processed as described earlier.

- Transplantation into mice with hind-limb ischemia

Hind-limb ischemia was induced by femoral artery ligation surgery as described previously [19] and 6 x 10^7 BCs or cell-free medium (100 µl) were injected into the area of peri-ischemic muscle. A total of 4 weeks after the injection, the mice were sacrificed and the limbs were embedded in OCT, sectioned, fixed with 4% PFA, permeabilized with 0.5% TritonX and incubated with antibodies against ASMA (Abcam, cross-reactive with both mouse and human antigens) and human specific mitochondrial antigen (Millipore/Chemicon, Temecula, CA, USA) at 4°C overnight, followed by Rhodamine and FITC-conjugated secondary antibodies (Jackson ImmunoResearch) and examined using fluorescent microscopy.

Results

- BCs possess the potential to differentiate into SMCs in addition to ECs and hematopoietic cells in vitro

We have recently demonstrated the generation of functional BCs from hESCs with both hematopoietic and endothelial potential. These cells rapidly repaired damaged blood vasculatures and restored blood flow in ischemic tissues, suggesting that they may be capable of forming functional vessels composed of ECs and SMCs. BCs from mouse ESCs have previously been shown capable of generating hematopoietic cells, ECs and SMCs. However, until now, there was no evidence that BCs or their equivalents generated from hESCs were able to differentiate into all three types of cells. Therefore, we examined whether BCs could differentiate into these three cell lineages in vitro by plating early stage (day 3–4) EBs derived from WA01 and WA07 cells and expanded in blast colony growth medium for 6–7 days as described [19]. BCs were then isolated and subjected to three different differentiation conditions.

To induce SMC differentiation, BCs were cultured on fibronectin-coated plates in SMC growth medium for up to 10 days and then stained with calponin and ASMA antibodies. Calponin-positive cells appeared 1–2 days after plating and the numbers of positive cells remained constant throughout the 10-day differentiation period (Figure 1A & B). For cells derived from WA01 cells, 10–15% of adherent cells were positive for calponin, while approximately 20–25% of adherent cells derived from WA07 cells expressed calponin (Figure 2), suggesting that

SMC differentiation efficiency is cell line specific. ASMA-positive cells were observed after culturing the cells in SMC medium for 5–7 days (Figure 1C & D), and the percentage of ASMA-positive cells was dependent on the initial plating density and the length of culture time. More ASMA-positive cells were observed when the cells were cultured for 10 days or longer at a high cell density, suggesting that SMLCs derived from BCs need to be cultured for a longer period of time to express more mature contractile SMC markers.

As reported previously [19], single BC suspensions plated in serum-free methylcellulose medium with a spectrum of hematopoietic cytokines for 10–14 days, colonies of erythroid, myeloid, macrophage and multilineage hematopoietic cells were observed (Supplementary Figure 1). To determine the endothelial potential, BCs were cultured on fibronectin-coated plates with endothelial growth medium and analyzed for expression of EC markers, Ac-LDL uptake and capillary-vascular structure formation. These adherent cells were positive for EC markers (CD31/PECAM-1, vWF, KDR and VE-cadherin) and took up Ac-LDL (Supplementary Figure 2A & B). After replating on Matrigel matrix for less than 24 h, these cells formed capillary

![Figure 3](image-url)

**Figure 3.** Smooth muscle gene-expression analysis of blast cells derived from 3-day-old and 4-day-old embryoid bodies before plating under smooth muscle cell conditions. Expression of various smooth muscle genes associated with development of smooth muscle cells in blast cells derived from 3-day-old and 4-day-old embryoid bodies. GAPDH was used as an internal control to normalize gene expression.

AoSM: Aorta smooth muscle cell; bp: Base pair.
vascular-like structures that also took up Ac-LDL (Supplementary Figure 2C), confirming their endothelial lineage. These experiments demonstrated that BCs from hESCs can differentiate into hematopoietic cells, ECs and SMLCs in vitro under appropriate conditions.

Differentiation of SMCs from individual BCs
To determine SMC differentiation potential of a single blast colony, we handpicked 20 individual blast colonies, isolated their RNA and analyzed the expression of calponin, SM22 and ASMA genes. As shown in Figure 3, all blast colonies derived from day 3 and day 4 EBs expressed calponin, SM22 and ASMA. To further demonstrate their SMC differentiation potential, individual blast colonies were handpicked, cultured in SMC medium for 10–14 days and stained with the SMC marker calponin; 95% (83 out of 88) and 91% (71 out of 78) of the colonies generated calponin-positive cells for WA07 and WA01 cell lines, respectively. The percentage of calponin-positive cells varied substantially among individual colonies. In some colonies, there were only a few calponin-positive cells (Supplementary Figure 3A), but in other cases the majority of the cells in the colony expressed calponin (Supplementary Figure 3B & C). Overall, the majority of colonies observed contained 10–20% calponin-positive cells. We have shown previously that blast colonies are clonogenic and single individual blast colonies differentiate into both hematopoietic progenitors and ECs [19]. The above results demonstrate that BCs are tripotential and capable of differentiating into hematopoietic, endothelial and smooth muscle lineages, which is consistent with our previously reported in vivo functional studies [19].

SMCs derived from BCs are functional
One functional characteristic of SMCs is their ability to contract in response to stimuli. To evaluate whether SMLCs derived from BCs are functional, the cells were cultured in SMC growth medium for three passages and exposed to carbachol (an acetylcholine receptor agonist) for 45 min. As shown in Figure 4A & B and real-time captured videos (Supplementary Video 1), upon exposure to carbachol, these cells shortened and narrowed morphologically within 30 min, resembling the contractile activity of SMCs, which is similar to that of HV-SMCs (Supplementary Figure 4A & B). Human fetal fibroblasts treated with carbachol under the same conditions exhibited no contraction over the course of 45 min (Figure 4C & D; Supplementary Video 2).

Another characteristic of SMCs is their interaction with ECs and participation in the formation of blood vessels. We next examined whether SMLCs derived from BCs can form vascular structure by interacting with ECs. BCs were differentiated on fibronectin-coated dishes in SMC medium for 1–2 weeks then transferred onto Matrigel plates. The formation of vascular-like structures was observed within 12 h. As shown in Figure 5, double immunostaining showed that these structures consisted of vWF-positive endothelial tubes associated with calponin- (Figure 5A & B) and ASMA- (Figure 5C) positive cells, a feature compatible with that of pericytes in capillary vessels. We also observed that some cells in these structures were positive for both calponin and vWF (Figure 5). These results demonstrate that SMLCs derived from BCs coordinate in concert with ECs to form vascular-like network in vitro.

To further demonstrate the participation of SMLCs and ECs in the formation of vascular structures, BCs were cultured on fibronectin-coated dishes in SMC medium for 2 weeks, mixed with Matrigel and injected subcutaneously into the dorsal region of nude mice. The implant was removed, sectioned and stained with antibodies...
against PECAM1 (CD31) and ASMA 4 weeks after the injection. Staining with both CD31 and ASMA antibodies showed individual positive cells (Figure 6A & B) and tube-like structures (microvessels) (Figure 6A & B) inside the Matrigel, confirming the differentiation of these cells into ECs and SMLCs and their involvement in vascular structure formation. Medium control showed no positive staining for either CD31 or ASMA (Figure 6C & D). One of the specific limitations of this study is that double staining could not be performed. Most mouse monoclonal antibodies tested did not work well on samples embedded in Matrigel, and both antibodies against CD31 and ASMA that work on these samples are rabbit polyclonal antibodies, precluding double staining.

**Differentiation of BCs into SMCs in vivo**

In previous studies, we demonstrate that BCs significantly enhanced the restoration of blood flow rate in ischemic limbs as compared with control. This improvement is correlated with the incorporation of human ECs into the lumen of microvessels [19]. However, whether or not BCs differentiated into SMCs in this model was not examined. To investigate the in vivo SMC differentiation potential of BCs, we re-examined the BCs injected ischemic hind-limb samples obtained from our previous studies [19] by double immunofluorescent staining with human-specific mitochondrial antigen (Hu-Mito, red) and SMC markers ASMA (green). As shown in Figure 7A & B, double immunohistostainings of ischemic sections demonstrated the incorporation of human SMLCs in blood vessel structure (yellow, arrow), whereas no double-positive cells were observed in tissues from mice injected with cell-free medium (Figure 7C & D). This suggests that BCs differentiate into both ECs and SMCs in vivo and contribute to the repair of damaged vasculatures.

**Discussion**

Hemangioblasts have traditionally been defined as progenitors with the bipotential capacity to differentiate into hematopoietic and ECs [32]. The
existence of these common progenitors was first demonstrated using in vitro differentiation systems in mouse ESCs. Replating of mouse ESC-derived EBs resulted in the formation of BL-CFC that possessed the capability to differentiate into both hematopoietic and ECs when cultured under appropriate conditions [13,14]. Cells with bipotential characteristics have subsequently been identified in zebrafish, avian, mouse and human adult tissues [27–34], as well as during nonhuman primate and human ESC differentiation in vitro [15–19]. Recent studies have shown that BL-CFCs derived from mouse ESCs were further capable of generating SMCs in addition to hematopoietic and endothelial progeny [23,24], suggesting that BL-CFC is tripotential. However, to date, no studies have demonstrated this in the primate or human system. We recently showed that when transplanted into ischemic tissues, BCs derived from hESCs rapidly incorporated into and repaired the damaged vasculature, and restored blood flow to normal levels [19]. This suggests that some of the injected BCs may have differentiated into SMCs, a critical component of mature and functional vasculature. Here we clearly demonstrate for the first time that BCs derived from hESCs gave rise to SMLCs, as well as ECs and hematopoietic cells in vitro, confirming the tripotential nature of these cells. SMLCs derived from BCs contracted in response to carbachol stimulation, formed vasculature-like networks in alignment with ECs on Matrigel and incorporated into blood vessels in ischemic tissues, indicating that these cells are functional both in vitro and in vivo.
Blood vessels are typically composed of two major cell types: the inner endothelium, a thin layer of ECs that separates the blood from tissues; and an outer layer of mural cells (pericytes and vascular SMCs) that protect the fragile channels from rupture and help control blood flow [21]. Fibroblasts are also part of vascular structures as they secrete elastin and collagen in the media and adventitia. Although ECs play an essential role in vasculogenesis and angiogenesis and form capillary vasculatures, they alone cannot complete the process of vessel growth and development. The formation of a mature and functional vascular network requires communication between ECs and SMCs [21]. Vascular SMCs play a critical role in structural and functional support of the vascular network by stabilizing nascent endothelial vessels during vascular development and blood vessel growth. Therefore, a progenitor cell population that can differentiate into both ECs and SMCs, and that can be propagated and expanded indefinitely, could be of potential clinical value in the treatment of human diseases caused by deficient vessel growth.

Here we show that functional BCs can be efficiently generated from multiple hESC lines under well defined and serum-free conditions. Using our recently optimized system [35], approximately 10-times more BCs can be obtained as compared with our previous report [19]. The elimination of serum and other animal components from the system, as well as the ability to generate an unlimited supply of BCs, will be important for future clinical applications. The use and concentration

Figure 7. Incorporation of blast cells into the blood vessel structure of ischemic hind-limb muscle. A total of 4 weeks after injection of blast cells into ischemic hind-limb muscle, the animals were killed and the tissue was sectioned, fixed and stained for (A) SMA (green) and human mitochondrial antigen (red), ×100; (B) SMA (green) and human mitochondrial antigen (red), ×400; DAPI (blue) stains cell nuclei. (C & D) Control tissue from muscle injected with cell-free medium. (C) ×100; (D) ×400.

SMA: Smooth muscle actin.
of these cells would not be limited by availability, could be titrated to the precise clinical requirements of the individual and could be repeatedly reinfused over the lifetime of the patient if deemed necessary. Furthermore, the ability to create banks of matching or reduced-complexity HLA hESC lines and patient-specific induced pluripotent stem cells [36,37] could potentially reduce or eliminate the need for immunosuppressive drugs and/or immunomodulatory protocols altogether.

SMCs have recently been obtained from hESCs by several groups. Huang and coworkers reported that when hESCs were cultured as a monolayer and treated with all trans-retinoid acid, more than 93% cells were positive for SMC markers ASMA and SM-MHC and capable of contraction upon stimulation, although it is unclear whether these cells can be expanded and/or whether they have any functional activity in vivo [8]. Sone and coworkers found that KDR+/TRA1–60 cells derived from hESCs cultured on OP9 stromal cells differentiated into both VE-cadherin+ ECs and ASMA+- mural cells [10]. When transplanted into ischemic hind-limbs, these cells contributed to the construction of new blood vessels and improved blood flow. Ferreira and coworkers also demonstrated that CD34+ cells from day 10 EBs of hESCs cultured with fetal bovine serum (FBS) gave rise to ECs when cultured in EGM2 medium supplemented with VEGF and when grown in EGM2 medium with the addition of PDGF-BB, they differentiated into SMCs capable of contracting [9]. When implanted in nude mice, the mixture of hESC-derived ECs and SMCs contributed to the formation of functional microvessels. However, the studies by Sone and coworkers [10] and Ferreira and coworkers [9] failed to demonstrate whether ECs and SMCs were derived from a common progenitor. The results of our current and previous studies [19] demonstrate that BCs from a single clonogenic blast colony can differentiate into SMCs in addition to endothelial and hematopoietic progeny. Unfortunately, all the above hESC differentiation systems utilized FBS and/or feeder cells, containing an undefined mixture of cytokines, inducers and inhibitors that differ from batch to batch and can contribute to wide variations in efficiency and reproducibility. It has also been reported that the passage and growth status of feeder cells such as S17 and OP9 play a critical role in supporting hESC differentiation [38]. In contrast to these methods, the system described here does not require serum or feeder cells and should allow the generation of multipotential and functional BCs without the potential risk of zoonotic contamination.

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Financial & competing interests disclosure

S-J Lu, Y Ivanova, Q Feng, C Luo and R Lanza are employees of Advanced Cell Technology, a stem cell company in the field of regenerative medicine. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- We show here for the first time that blast cells (BCs; hemangioblasts) derived from human embryonic stem cells (hESCs) possess the ability to differentiate into smooth muscle cells (SMCs).
- These hemangioblasts are tripotential. In addition to expressing markers of SMCs and differentiating into smooth muscle-like cells (SMLCs), they are capable of differentiating into endothelial and hematopoietic lineages.
- When blast cells (BCs) from individual blast colonies were cultured in SMC medium, they differentiated into both endothelial cells (ECs) and SMLCs, which formed capillary-vascular like structures in vitro after replating on Matrigel™.
- SMLCs derived from BCs are functional. The cells expressed SMC-specific markers (α-smooth muscle actin and calponin) and contracted upon treatment with the acetylcholine receptor agonist, carbachol.
- When implanted in nude mice, these cells formed microvasculature with ECs in Matrigel plaques.
- The BCs differentiated into both ECs and SMLCs, and incorporated into blood vessels after injection into ischemic tissue. The cells participated in the formation of new blood vessels and the repair of injured vessels in multiple vasculatures.
- These results demonstrate that hESC-derived hemangioblasts are tripotential and can generate multilayered blood vessels with functional SMCs.
- Hemangioblasts generated from either hESCs or induced pluripotent stem cells could potentially provide an inexhaustible source of cells for the treatment of human blood and vascular diseases.
Bibliography


