Robust generation of hemangioblastic progenitors from human embryonic stem cells

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Background: Human embryonic stem cells (hESCs) are a potentially inexhaustible source of cells for replacement therapy. However, successful preclinical and clinical progress requires efficient and controlled differentiation towards the specific differentiated cell fate. **Methods:** We previously developed a strategy to generate blast cells (BCs) from hESCs that were capable of differentiating into vascular structures as well as into all hematopoietic cell lineages. Although the BCs were shown to repair damaged vasculature in multiple animal models, the large-scale generation of cells under these conditions was challenging. Here we report a simpler and more efficient method for robust generation of hemangioblastic progenitors. **Results:** In addition to eliminating several expensive factors that are unnecessary, we demonstrate that bone morphogenetic protein (BMP)-4 and VEGF are necessary and sufficient to induce hemangioblastic commitment and development from hESCs during early stages of differentiation. BMP-4 and VEGF significantly upregulate T-brachyury, *KDR*, *CD31* and *Lmo2* gene expression, while dramatically downregulating *Oct-4* expression. The addition of basic FGF during growth and expansion was found to further enhance BC development, consistently generating approximately 1×10^8 BCs from one six well plate of hESCs. **Conclusion:** This new method represents a significantly improved system for generating hemangioblasts from hESCs, and although simplified, results in an eightfold increase in cell yield.

Hemangioblasts are bipotential progenitors that have the capacity to differentiate into both hematopoietic and endothelial cells [1]. The ability to generate an unlimited supply of these cells from human embryonic stem cells (hESCs) could have important clinical ramifications. We previously showed that hESC-derived hemangioblasts homed to the site of injury and showed robust reparative function of damaged vasculature when injected into animals with diabetes or ischemia/reperfusion injury of the retina [2]. The cells also showed a similar regenerative capacity in animal models of both myocardial infarction (50% reduction in mortality rate) and hind-limb ischemia, with restoration of blood flow to near normal levels within a month. However, to realize their clinical potential, whether for vascular repair or various hematopoietic applications, it will be necessary to develop economical conditions for the robust and efficient differentiation of hemangioblastic progenitors from hESCs. For clinical application, the cells will also need to be animal and human pathogen free, generated in the absence of serum and stromal cells.

Several groups have generated hemangioblasts using hESC differentiation systems. Wang *et al.* demonstrated that $CD45^{neg}$ PECAM-1^+, FLK-1^+ and VE-cadherin$^+$ ($CD45^{neg}$ PFV) cells, derived from embryoid bodies (EBs) cultured in fetal bovine serum (FBS) and a mixture of growth factors and cytokines, possessed hemangioblast properties [3]. However, the rarity of the cells, less than a fraction of a percent of the cells, and the presence of FBS in the differentiation system precludes the possibility of its clinical use. Similarly, Zambidis *et al.* demonstrated the formation of multipotential mesodermal–hemato-endothelial colonies using an FBS-containing culture system, although it is unclear whether these colonies can be expanded and/or whether they possess any functional activity *in vivo* [4]. Recently Kennedy *et al.* identified a human hemangioblast cell population derived from hESCs using a procedure that consisted of serum-free differentiation in a mixture of cytokines followed by expansion in serum-containing media [5]. However, all these studies utilize hESCs cultured on feeder cells and differentiated in FBS-containing medium. Importantly, to date, no other system has been described for the large-scale generation or functional assessment of hESC-derived hemangioblasts.

We previously reported a two-step approach to generate blast cells (BC) from hESCs without the use of serum [2]. These cells expressed gene signatures characteristic of hemangioblasts and

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clonally differentiated into multiple (erythroid, myeloid and multi-lineage) hematopoietic cell lineages as well as endothelial cells that expressed von Willebrand factor, CD31/PECAM-1 and VE-cadherin. When the cells were injected into animals with vascular injury, they homed to the site of injury and showed robust reparative function of the damaged vasculatures [2]. In the present study, we investigated the roles of several factors in promoting differentiation of hESCs toward hemangioblasts. We found that bone morphogenetic protein (BMP)-4, a ventral mesoderm inducer, is critical in hemangioblastic specification of hESCs, but not in the growth of BCs. VEGF, a multifunctional cytokine that plays a prominent role in normal vascular biology, is essential for efficient hemangioblastic specification of hESCs as well as the growth and expansion of BCs in this serum-free system. The addition of basic FGF (bFGF) throughout the BC growth and expansion stages, but not during EB formation (hemangioblastic specification of hESCs), also enhanced the generation of BCs significantly. Despite eliminating several expensive factors, including stem cell factor (SCF), Flt3-ligand (FL) and Tpo, these new improvements significantly optimize the generation of hemangioblasts from hESCs. In addition, we showed that feeder-free hESCs robustly generated hemangioblasts under these conditions; the development of this serum- and stromal-free system is important for clinical applications.

Materials & methods

Culture of hESCs

hESC lines WA01(H1), HUES3 and MA01 were used and maintained as previously described [6]. Briefly, hESCs were grown on mitomycin C-treated mouse embryonic fibroblast (MEF) in complete hESC media. The hESCs were passaged every 3–5 days before reaching confluence using 0.05% trypsin–0.53 mM EDTA. For feeder-free culture, the cells were then grown on hESC-qualified Matrigel™ matrix (BD Biosciences, Bedford, MA, USA) in complete modified TeSR™1 (mTeSR™1) medium (Stem Cell Technologies, Inc, Vancouver, BC, Canada), which is based on the formulation of Ludwig *et al.* [7,8]. Cells were maintained according to manufacture's suggested instructions. Briefly, cells were passaged when they reached approximately 90% confluence, usually every 5–7 days with split ratios ranging from 1:3 to 1:6. Cells were treated with dispase (1 mg/ml, BD Biosciences) and incubated for 3–5 min at 37°C to begin dislodging the colonies. Colonies were washed with DMEM/F12 (Mediatech, Manassas, VA, USA) to remove dispase solution. To extricate the colonies from the tissue culture plastic, the wells were coated with DMEM/F12 and gently scraped until all of the colonies had been displaced. The colonies were transferred to conical tubes, the wells were washed with DMEM/F12 and the cells pooled to collect any remaining in the wells. They were centrifuged for 5 min at 1000 rpm. The cell pellets were resuspended in mTeSR™1 media and transferred to Matrigel-coated six-well plates, in 2 ml of mTeSR™1 media per well. Cells were maintained at 37°C under 5% CO_2 and the mTeSR™1 medium was replenished daily.

Immunoflourescent cytochemistry analysis

Feeder-free hESC colonies were assayed for Oct-4 and Tra-1–60 expression using immunofluorescence. The cells were fixed with 4% paraformaldehyde, washed with phosphate-buffered saline (PBS), and blocked with 5% normal goat serum (Vector Labs, Burlingame, CA, USA), 1% bovine serum albumin (Sigma, St Louis, MO, USA) and 0.2% Triton-X-100 (Sigma) in PBS for 30 min at room temperature. Cells were incubated with primary antibodies against Oct-4 (Santa Cruz Biotechnology, Santa cruz, CA, USA) or Tra-1–60 (Millipore/Chemicon, Temecula, CA, USA), in blocking solution, overnight at 4°C, washed with PBS and incubated with a biotin conjugated secondary antibody (Jackson ImmunoResearch Labs, West Grove, PA, USA), in blocking solution, for 45 min at room temperature. After further washing, cells were incubated with Alexa 954 conjugated streptavidin (Invitrogen/Molecular probes, Carlsbad, CA, USA), for 15 min at room temp followed by an extended final wash in PBS. Cells were mounted in Prolong Gold with DAPI (Invitrogen/Molecular Probes).

Differentiation of hemangioblasts from hESCs

To induce hESCs cultured on MEFs into hemangioblasts, 80–90% confluent plates were dissociated by 0.05% trypsin digestion. To differentiate feeder-free hESCs into hemangioblasts, 85–90% confluent cells were dislodged from the Matrigel matrix using the protocol described earlier. Cells from both conditions were plated on ultra-low dishes (Corning, NY, USA) in Stemline II (Sigma) medium with different doses of BMP-4, VEGF and bFGF as described previously [2]. Half of the medium was

replaced after 48 h with fresh medium containing the same cytokines or the same medium plus SCF, FL and Tpo (20 ng/ml, R&D System, Inc., Minneapolis, MN, USA), which depend on different experiment conditions. After 3.5 days, EBs were collected and dissociated by 0.05% trypsin. Single-cell suspensions were obtained by passing the cells through 22-gauge needle and through a 40-µm cell strainer, collected by centrifugation and resuspended in 50–100 µl of Stemline II media. Cells (0.75×10^5 to 1×10^5) were mixed with 2.5 ml of blast colony growth medium (BGM) as previously described [2], plated in ultra-low dishes and incubated at 37°C. Blast colonies derived from both MEF and feeder-free hESCs were observed 3–4 days after plating, followed shortly thereafter by rapid expansion. BCs are defined in the current study as cells obtained from day-6 blast colonies.

Enrichment of hemangioblast precursors

Potential BC precursor surface markers CD31, CD34, KDR, CXCR-4, CD133, ACE, PCLP1, PDGFRα, Tie-2, Nrp-2, Tpo-R and bFGFR-1 were selected for cell enrichment. All antibodies are mouse monoclonal IgG isotype and they are: CD31 and CD34 (Dako Cytomation, Carpinteria, CA, USA), KDR and Tpo-R (R&D Systems, Inc.), CXCR-4 (Abcam Inc., Cambridge, MA, USA), Nrp-2, ACE, PCLP1 and PDGFRα (Santa Cruz Biotechnology), Tie-2 (Cell Signaling Technology, Inc., Danvers, MA, USA), bFGFR-1 (Zymed Laboratories, San Francisco, CA, USA) and CD133 (Miltenyi Biotech, Auburn, CA, USA). Antibody cocktail assembly was performed by EasySep 'Do-it-Yourself' Selection Kit (Stem Cell Technologies). Cell suspensions derived from EBs were centrifuged at 1200 rpm for 4 min and resuspended in PBS with 2% FBS/1 mM EDTA buffer at a concentration of $1–2 \times 10^6$ cells/100 µl. The cells were mixed with different antibody cocktails for 15 min at room temperature and then incubated with EasySep Nanoparticle at room temperature for 10 additional minutes. Positive selected cells were separated after pouring off supernatant when placing tube with cells in a Magnet holder. Antibody selected positive cells (1×10^5) were mixed with 2.5 ml of BGM and plated for blast colony development.

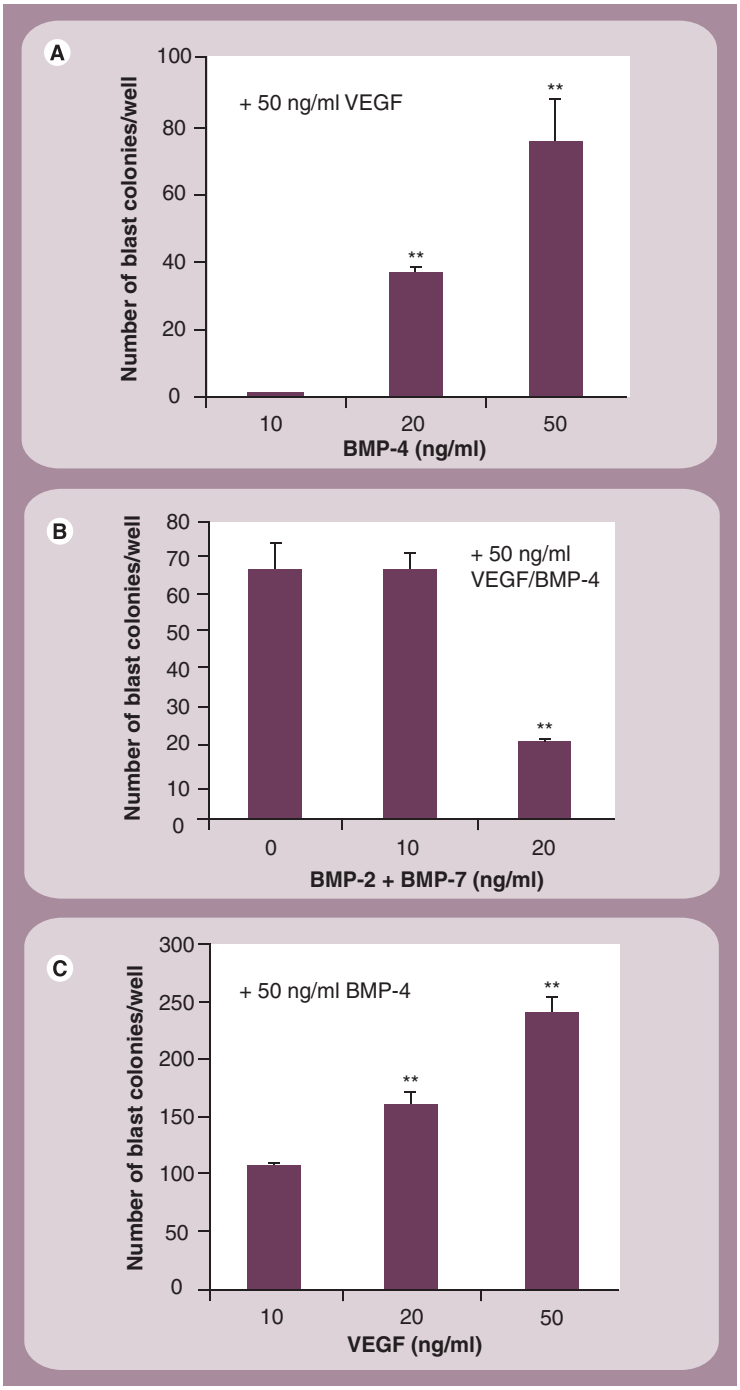
Real-time RT-PCR & data analysis

Total RNA was extracted from EBs or undifferentiated hESCs using RNeasy Micro Kits (Qiagen, Valencia, CA, USA) according to manufacturer's protocol. cDNAs were synthesized using BD SMART PCR cDNA Synthesis Kit (BD Biosciences) per manual instructions. Real-time RT-PCR (qRT-PCR) was performed using FullVelocity SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA, USA). The reactions were set up in triplicate with the following components per reaction: 50 ng of template, 0.2 µm of each primer and 1X Master mix. Gene-specific sequences of the primers used are listed in Table 1, and annealing temperature for all primers is 55°C. Amplification and real-time data acquisition were performed in a Stratagene Mx3005P with MxPro version 3.0 software. The following cycle conditions were used: one cycle of 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 55°C for 1 min, 72°C for 30 s followed by a final cycle of 95°C for 1 min, 55°C for 30 s and 95°C for 30 s. Relative quantification of each target gene was performed based on cycle threshold (C_T) normalization to β-actin (ΔC_T) using the $\Delta\Delta C_T$ method [9]. Analysis of relative gene-expression data using real-time quantitative PCR and the $2(-\Delta\Delta(T))$ method [9], where the ΔC_T of each examined gene in the experimental samples was compared with average ΔC_T of each gene in an undifferentiated hESC control

Table 1. Sequences of gene-specific primers used in real-time RT-PCR.

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)	Ref.
OCT-4	GAAGGTATTCAGCCAAACGC	GTTACAGAACCACACTCGGA	
BRACH	TGCTTCCCTGAGACCCAGTT	GATCACTTCTTTCCTTTGCATCAAG	[33]
MixL1	CCGAGTCCAGGATCCAGGTA	CTCTGACGCCGAGACTTGG	[33]
KDR/Flk1	CCAGCCAAGCTGTCTCAGT	CTGCATGTCAGGTTGCAAAG	[4]
CD31	GAGTCCTGCTGACCCTTCTG	ATTTTGCACCGTCCAGTCC	[4]
Scl/TAL1	ATGAGATGGAGATTACTGATG	GCCCCGTTCACATTCTGCT	[4]
LMO2	AACTGGGCCGGAAGCTCT	CTTGAAACATTCCAGGTGATACA	[4]
GAPDH	CGATGCTGGCGCTGAGTAC	CCACCACTGACACGTTGGC	
β-actin	GCGGGAAATCGTGCGTGACA	GATGGAGTTGAAGGTAGTTTCG	

Figure 1. Effects of bone morphogenetic proteins and $VEGF_{165}$ on the development of blast colonies.



(A) Different doses of BMP-4 were added in embryoid body (EB) medium containing 50 ng/ml of $VEGF_{165}$, and a dose-dependent development of blast colonies was observed for BMP-4. **(B)** EB medium containing 50 ng/ml of BMP-4 and $VEGF_{165}$ were supplemented with different doses (0, 10 and 20 ng/ml) of BMP-2 and -7. BMP-2 and -7 failed in promoting blast colony development. **(C)** Different doses of $VEGF_{165}$ were added in EB medium containing 50 ng/ml of BMP-4. The development of blast colonies is $VEGF_{165}$ dose dependent.
**p < 0.01, n = 3. 1×10^5 cells from day 3.5 EBs were plated per well.
BMP: Bone morphogenetic protein.

sample ($\Delta\Delta C_T$). Then the fold change in expression was calculated as $2\text{-h}\Delta\Delta^{CT}$. The negative fold difference data was convert to a linear 'fold change in expression' value using the following formula: linear fold change in expression = -(1/fold change in expression).

Statistical analysis

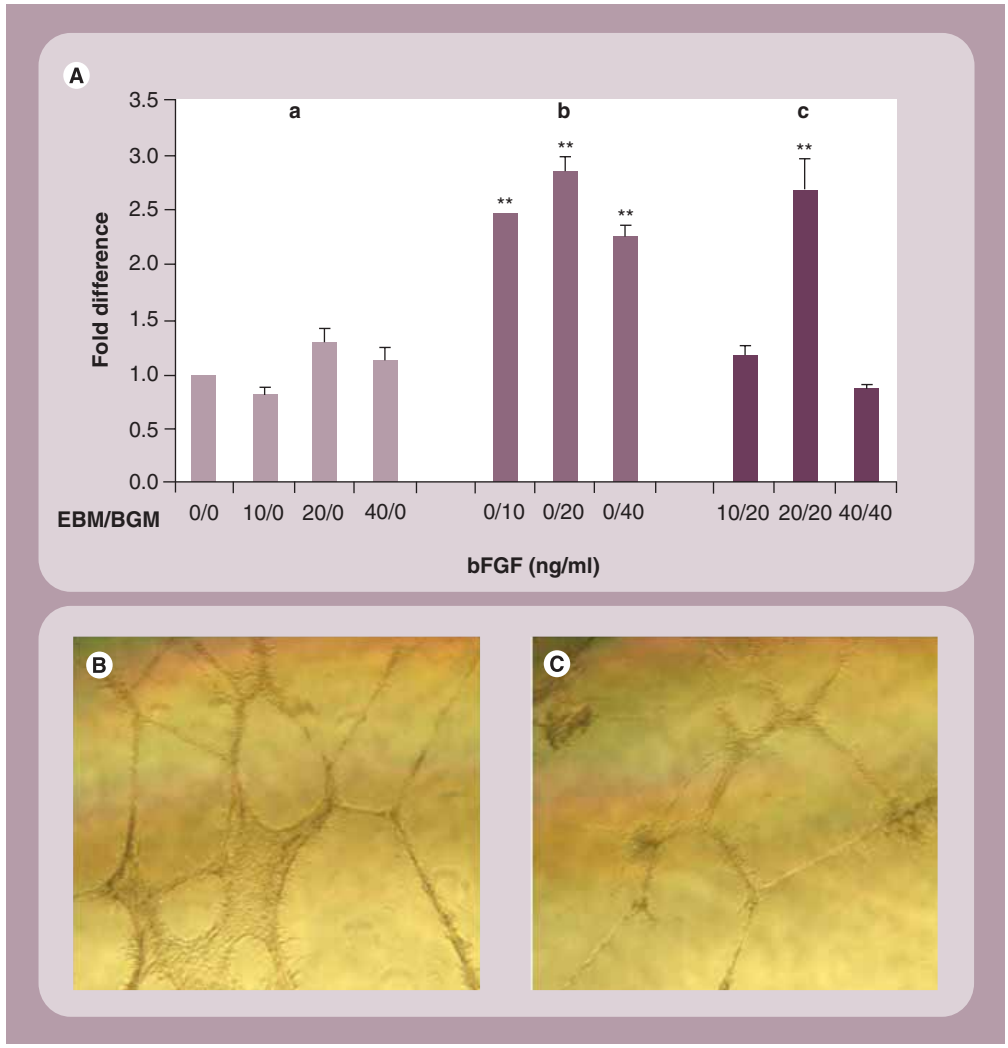
All data were presented as mean ± SEM. Intergroup comparisons were performed by unpaired Student's t-test using GraphPad Prism, version 4, software (GraphPad Software, Inc., San Diego, CA, USA). $p < 0.05$ was interpreted as statistically significant.

Results

Both BMP-4 & VEGFs are required for hemangioblast development

We previously described a serum-free system to induce hESC differentiation toward the hemangioblastic and hematopoietic lineages [2,10]. Although BMP-4, VEGF and a cocktail of early hematopoietic cytokines were used, the absolute requirement and optimal concentrations of the individual factors were not examined. In order to reduce the expense and effort necessary to generate hemangioblasts for future research and clinical applications, we specifically examined the minimal requirements and effects of VEGFs, BMPs and three early hematopoietic cytokines (TPO, FL and SCF) on the efficient development of blast colonies from hESCs. We found that BMP-4 is absolutely required for the development of blast colonies under serum-free conditions. No blast colonies were obtained without the supplement of BPM-4 in the medium during EB formation and a clear dose-response effect of BMP-4 was observed for the formation of blast colonies from hESCs (Figure 1A). Furthermore, we found that BMP-4 could not be substituted by other members of the BMP family. BMP-2 and -7 alone, or a combination of the two, failed to promote BC development. Furthermore, supplementation of BMP-2 and -7 in EB medium containing BMP-4, either showed no effect (10 ng/ml) or inhibited (20 ng/ml) blast colony development (Figure 1B). However, addition of BMP-4, and BMP-2 and/or BMP-7 in BGM did not have any effect on the development of blast colonies, suggesting that BMP-4 only promotes the mesoderm/hemangioblastic specification stage, but not the growth and expansion of BCs. Similarly, no blast colonies developed when $VEGF_{165}$ was eliminated from the EB formation medium. $VEGF_{165}$ was found

Figure 2. The effect of basic FGF on the development of blast colonies added during different stages.

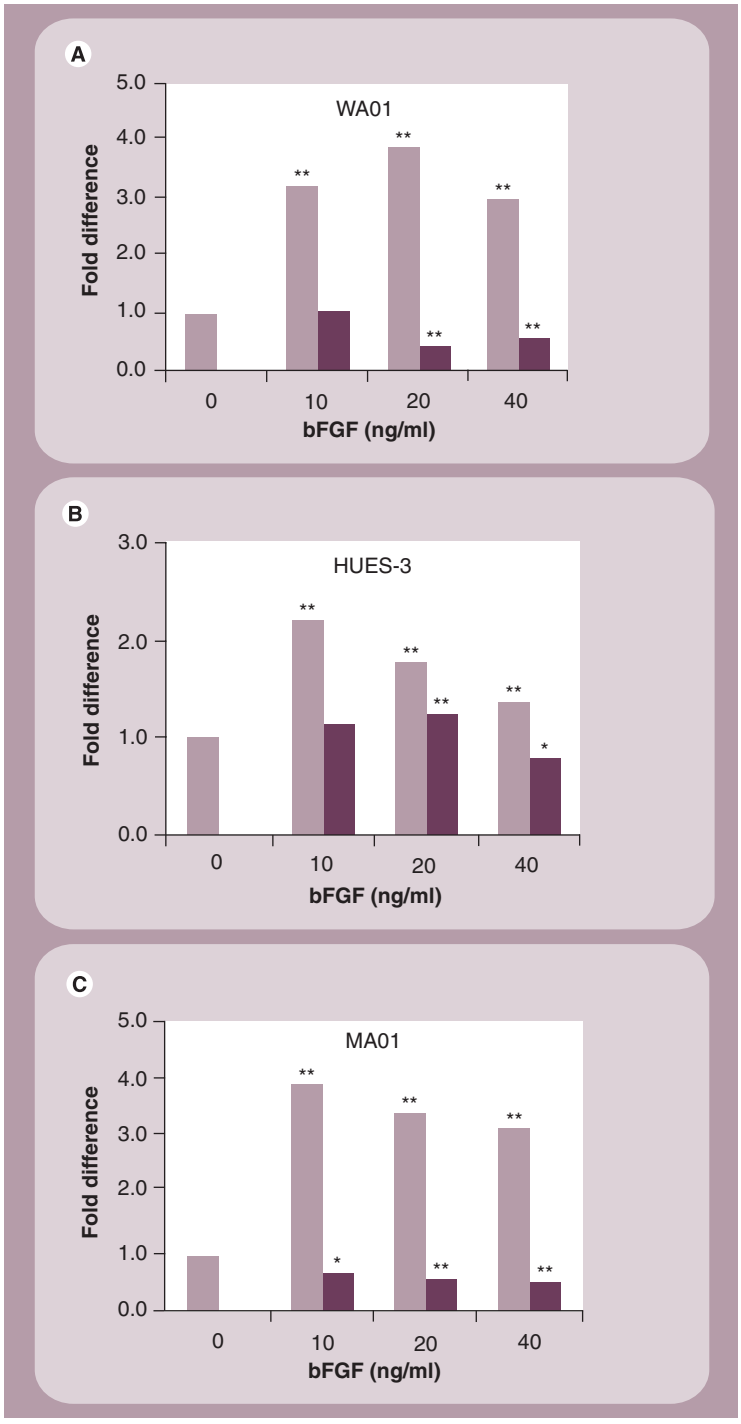


(A-a) Different doses of bFGF were added in EBM; **(A-b)** Different doses of bFGF were supplemented in BGM; **(A-c)** Different doses of bFGF were added in both EBM and BGM. **p < 0.01, n = 3. **(B & C)** Network-like structure formation of endothelial cells derived from blast cells developed in BGM with **(B)** and without **(C)** bFGF**.** Endothelial cells from both sources formed network-like structures with no obvious difference.
bFGF: Basic FGF; BGM: Blast colony growth medium; EBM: Embryoid body medium.

to promote the development of blast colonies in a dose-dependent manner (Figure 1C). $VEGF_{121}$, an isoform of VEGF members that can only bind to KDR and FLT1 receptors [11], can be used as a substitute of $VEGF_{165}$ in promoting the development of blast colonies from hESCs; almost identical numbers of blast colonies (68 ± 5 vs 67 ± 12) were developed when 50 ng/ml of either $VEGF_{165}$ or $VEGF_{121}$, which is the optimal dose under serum-free condition, was added in EB medium. However, in contrast to BMP-4, no blast colonies were obtained if VEGF was absent in BGM, demonstrating that VEGF plays a critical role both in early stage of mesoderm/hemangioblastic specification and in the growth and expansion of BCs.

In our original report [2], TPO, FL and SCF were added 48 h after plating hESCs in EB medium in an effort to further promote early hematopoeitic progenitor growth and expansion. Here we examined whether TPO, FL and SCF played any role in the specification of hESCs toward the mesoderm/hemangioblast lineage. EBs were formed by plating hESCs in Stemline II

Figure 3. The effect of basic FGF on the development of blast colonies from three human embryonic stem cell lines.



Light gray: Different doses of bFGF were added in blast colony growth medium.
Dark gray: Various doses of bFGF were added in embryoid body medium.
*p < 0.05; **p < 0.01; n = 3.
bFGF: Basic FGF.

medium with 50 ng/ml of BMP-4 and VEGF, and divided into two wells after 48 h: to one well, 20 ng/ml of TPO, FL and SCF was added, to the other well, no additional factor was added, and the EBs were incubated for another 36 h. EBs were then collected and single cell suspension was obtained and plated for blast colony formation. Our results show that supplement of TPO, FL and SCF during EB formation has no effect on the development of blast colonies, 242 ± 16 versus 287 ± 33 blast colonies developed per 1×10^5 cells derived from EBs treated with and without TPO, FL and SCF, respectively. However, both TPO and FL are required for blast colony growth and expansion.

bFGF promotes the growth, but not commitment, of hemangioblasts from hESCs

Previous studies have shown that supplement of bFGF during early differentiation promotes murine and human ESC hematopoietic development [5,12–14]. Thus, we investigated whether the addition of bFGF during the EB differentiation stage would enhance blast colony formation from hESCs. Addition of bFGF during EB formation had no effect on the development of blast colonies, and, in fact, at a higher dose (40 ng/ml) inhibited the formation of blast colonies from multiple hESC lines (Figures 2A & 3). By contrast, the addition of bFGF in BGM significantly enhanced the development of blast colonies (Figures 2A & 3). Both the number of blast colonies ($p < 0.001$) and total number of BCs increased significantly compared with BGM without bFGF supplementation. With bFGF at optimal dose (20 ng/ml) in BGM, the blast colonies are larger and healthier, and we consistently harvest approximately 1×10^8 BCs from one six-well plate of high-quality WA01 hESCs (approximately 1.2×10^7 cells) after 6 days growth, which is 8 ± 1-fold higher than that obtained from BGM without the supplement of bFGF.

To investigate the lineage differentiation potentials of BCs generated with and without supplementation of bFGF, equal numbers of pooled BCs were plated for hematopoietic and endothelial lineage differentiation as previously described [2]. For hematopoietic CFU formation, 129 ± 9 and 86 ± 22 CFUs/10^4 BCs were formed from BCs derived from BGMs supplemented with and without bFGF (20 ng/ml), respectively. Furthermore, no difference was observed for the development of different CFUs (CFU-mix, CFU-G, CFU-M and CFU-E) between the two groups (data not shown). For endothelial lineage differentiation, more BCs (62 ± 3%) from BGM with bFGF (20 ng/ml) differentiated into endothelial

cells than BCs (55 ± 3%) derived from BGM without bFGF supplement. Endothelial cells from both sources formed capillary vascular-like structures efficiently after plating on Matrigel (Figure 2B & C). These results suggest that bFGF promotes the growth of BCs, but does not cause preferential lineage differentiation.

Robust generation of hemangioblasts from hESCs maintained without feeder cells

It has been reported that hESCs maintained on MEF feeders contain the nonhuman sialic acid *N*-glycolylneuraminic acid (Neu5Gc) [7,8,15], and that animal sources of Neu5Gc can cause a potential immunogenic reaction with human complement. The culturing of hESCs on MEF feeder layers prevents complete elimination of animal Neu5Gc, and raises concerns for the potential clinical applications of hemangioblasts generated from hESC lines maintained under these conditions. Therefore, we have taken steps to determine whether hemangioblasts can be generated from hESCs maintained without MEF feeders. Three hESC lines were passaged with dispase onto plates coated with hESC-qualified Matrigel matrix, and maintained in mTeSR medium as described in Materials & methods. Their undifferentiated state was confirmed with immunoflourescence staining for the expression of Oct-4 and Tra-1–60 antigens and colony morphology (Figure 4A–H). These cells were collected and utilized for the development of BCs using the optimized conditions described earlier. Interestingly, a significantly higher number of BCs were observed with feeder-free hESCs as compared with hESCs cultured on MEF feeders when identical numbers of EB cells were plated (Figure 4I; $p < 0.05$). These results were observed for all three tested hESC lines WA01, MA01 and HUES-3 (data not shown).

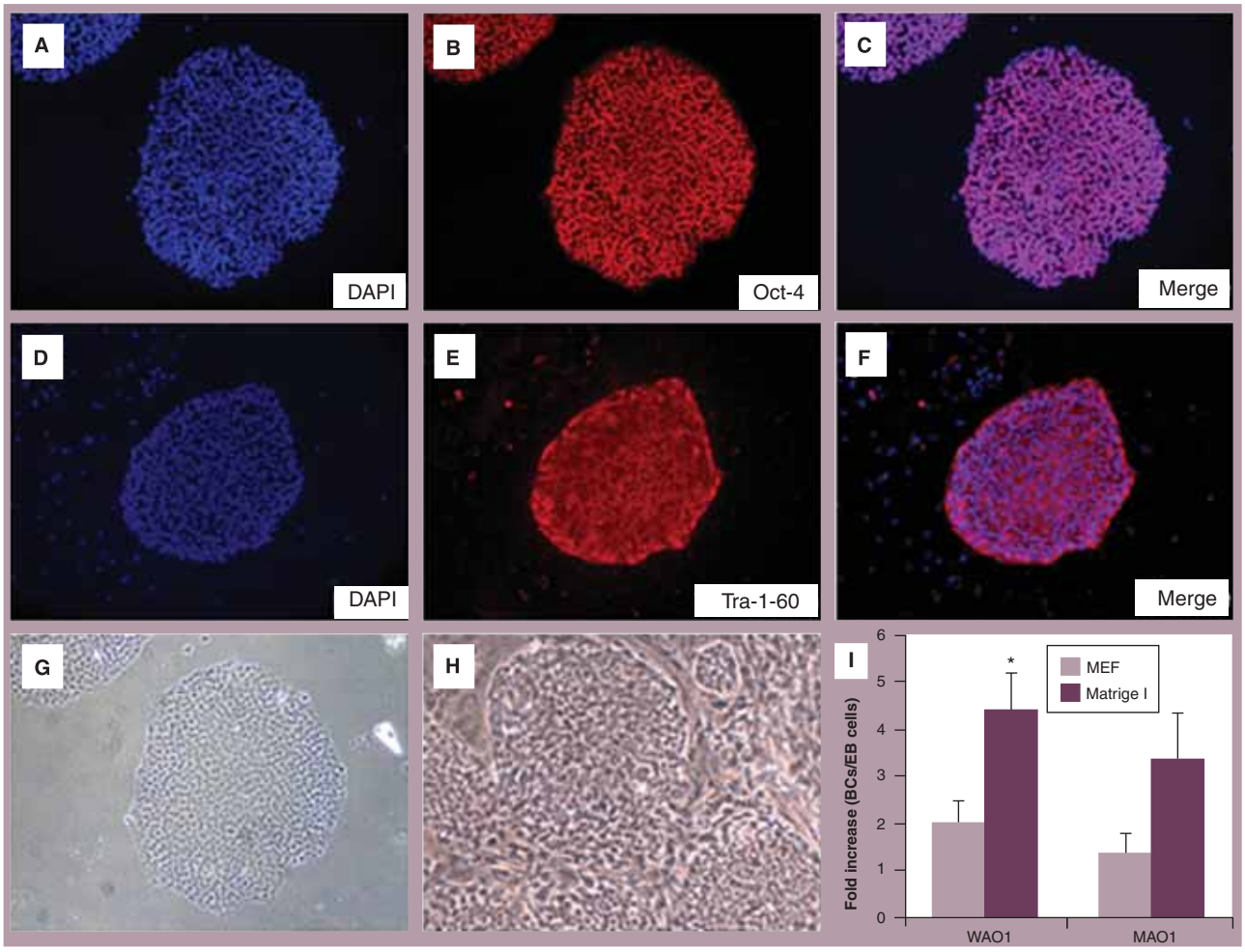
Mechanism underlying the effects of BMP-4 & VEGF on hemangioblast development

In order to dissect the molecular mechanism underlying the effects of BMP-4 and VEGF on hemangioblast development from hESCs, we compared the expression of genes associated with the development of hemangioblasts in 3.5-day-old EBs that were formed in Stemline II medium both with and without each factor, as well as with a combination of BMP-4 and VEGF. Gene expression was analyzed by qRT-PCR and compared with their levels in undifferentiated hESCs. EBs formed without any factor expressed higher levels of *OCT-4*, a marker for hESCs, than undifferentiated hESCs. Supplementation of VEGF in EB medium led to a moderate downregulation of *OCT-4* expression, whereas the addition of BMP-4 or BMP-4 plus VEGF resulted in a significant decrease in *OCT-4* expression ($p < 0.0005$; Figure 5). There was no additive effect of BMP-4 and VEGF on *OCT-4* expression. The expression of T-brachyury gene, the earliest marker expressed in mesoderm cells, was downregulated in all samples except EBs derived from cultures containing both BMP-4 and VEGF (the latter showing a significant increase in its expression; $p < 0.0005$). Similar expression patterns were observed for *CD31* and *Lmo2*; significantly increased levels of expression were only detected in EBs exposed to a combination of BMP-4 and VEGF ($p < 0.0005$). *KDR*, one of the most studied VEGF receptor, has been shown to be expressed in all hESC lines [4,5]; its expression was dramatically downregulated in EBs derived from media with no addition of exogenous factor, and with supplement of BMP-4 or VEGF alone. However, a moderate but significant increase in *KDR* expression was observed in EBs formed in the presence of BMP-4 and VEGF ($p < 0.002$), a condition that promoted efficient development of hemangioblasts from hESCs. Surprisingly, in contrast to a recent report [14], substantial decreases in the expression of *MixL-1* and *SCL/TAL-1* genes were detected in EBs formed in all conditions. One possible explanation is that growth in different serum-free media caused a different expression pattern in these genes. Nevertheless, these results suggest that the commitment and development of mesoderm/hemangioblast from hESCs requires both BMP-4 and VEGF, consistent with the results of blast colony development (Figure 1).

Identification of surface markers for progenitors of blast cells

In our original method [2], BCs were generated by replating day 3.5 EBs cells in 1% methylcellulose supplemented with defined factors. This strategy is important when identifying BCs that possess the potential to form hematopoietic and endothelial cells, and it is also reproducible when generating BCs from hESCs. However, this approach utilizes dishes in standard tissue culture incubators, and thus cannot be adapted to rotary bioreactors for scale-up. This limitation is mainly due to the fact that cells from day 3.5 EBs are heterogeneous and include undifferentiated hESCs (only a portion of the cells are BC

Figure 4. Human embryonic stem cells grown under feeder-free conditions retain pluripotency markers and are capable of robust hemangioblast differentiation.



After four to five passages under feeder-free conditions WA01 cells were stained for expression of the human embryonic stem cell (hESC) markers Oct-4 (**[A-C]** DAPI, Oct-4 and merged, respectively) and Tra-1–60 (**[D-F]** DAPI, TRA-1–60 and merged, respectively). **(G & H)** demonstrate differences in colony morphology when hESCs are cultured on Matrigel **(G)** versus MEFs **(H)**. Magnification ×100. **(I)** hESCs were grown either on MEFS or Matrigel and then differentiated under the optimized conditions described in Material & methods. Considerably more hemangioblast expansion was observed in Matrigel cultured cells as compared with MEF cultured hESCs. *$p < 0.03$; $n = 3$.
BC: Blast cell; EB: Embryoid body; MEF: Mouse embryonic fibroblast.

progenitors). Replating this heterogeneous population in liquid culture would therefore lead to the growth of all cells including the formation of secondary EBs from undifferentiated hESCs, excluding their possible use in clinical applications. However, if a marker(s) for the progenitor of BCs can be identified, the purified progenitor can be seeded in liquid culture adapted with a rotary bioreactor for scaled-up production of BCs. We therefore selected 12 cell surface molecules that are associated with the development of mesoderm derivatives. The corresponding antibodies were used to enrich cells from day 3.5 EBs, and the enriched cells assayed for blast colony-forming ability. As shown in Figure 6, KDR^+ cells from 3.5-day EBs generated three-times more blast colonies than the unfractioned control cells ($p < 0.01$), which is consistent with previous studies [5]. Although we also found a moderate increase in blast colonies (~1.5-fold) after plating $CD31^+$- and $CD34^+$-enriched populations, the increase did not reach statistical significance. All other enriched populations produced equal or less blast colonies as compared with unfractioned control cells, indicating that the BC progenitor does not express these molecules. The unbound (flow

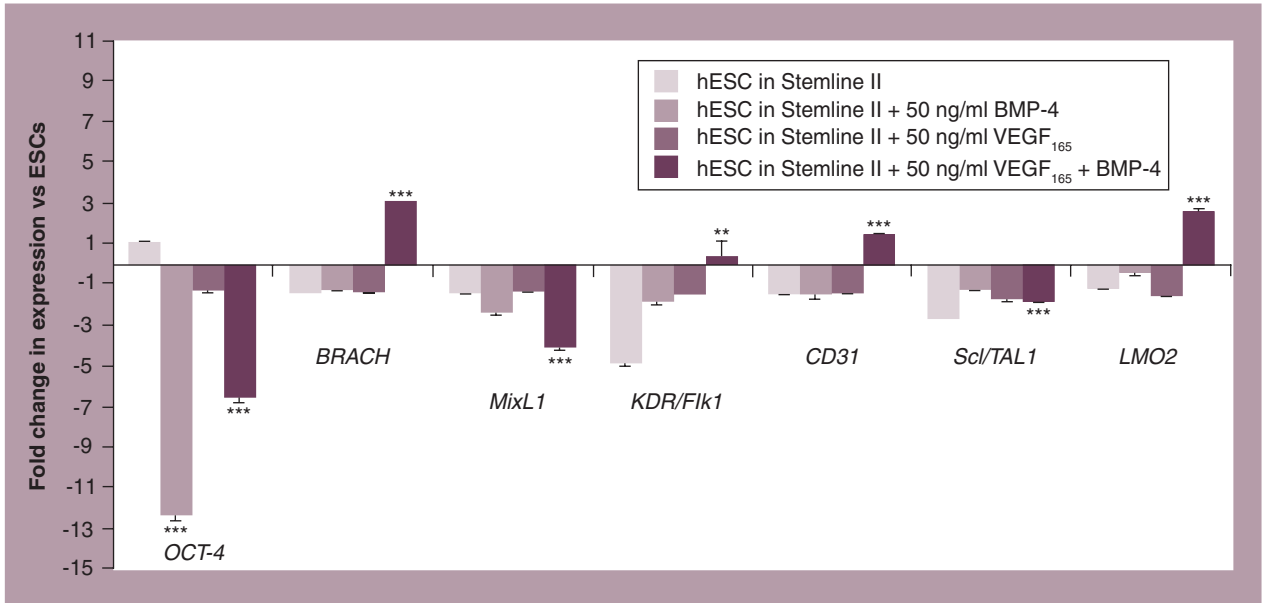
through) cells of all antibodies tested also formed similar numbers of blast colonies as the unfractioned cells, suggesting that even KDR$^+$, CD34$^+$ and CD31$^+$ cells represent a very limited portion of the cells that are capable of forming blast colonies. Owing to the limitation of the EasySep 'Do-it-Yourself' Selection Kit used in the current study, double positive cells were not examined.

Discussion

We describe a significantly improved system for generating hemangioblasts from hESCs. Although simplified, this new method results in an 8 ± 1-fold increase in BC yield. Our data also show that hESC specification and commitment to the hemangioblastic lineage is promoted very efficiently by only two factors, BMP-4 and VEGF, during EB formation in Stemline II medium. The supplement of low-dose bFGF in BGM further increases the efficiency and yield of BCs. This two-step system does not require the use of different cytokine combinations at multiple steps, nor does it require serum or conditioned medium as reported by Kennedy *et al.* [5], which could contribute to wide variations in efficiency and reproducibility. Furthermore, the elimination of several factors, including serum and animal feeder cells from our system, as well as the ability to generate significantly larger number of BCs should be desirable for future preclinical and human studies both in terms of time and cost.

BMP-4, one of the well-defined members of the BMP family, has been shown to play a pivotal role in the patterning of embryonic ventral mesoderm and the formation of precursors for hematopoietic and endothelial cells in both murine and primate ESC differentiation systems [5,14,16–23]. VEGF is a multifunctional cytokine that has also been shown to play a important role in hematopoietic differentiation of ESCs [5,13,14,20,21,24]. Our data show that both BMP-4 and VEGF are necessary and sufficient to efficiently induce hemangioblastic commitment of hESCs during the early stages of differentiation under serum-free conditions. The absence of either factor during EB formation prevents development of blast colonies. Gene-expression analyses confirmed that the upregulation of *KDR*, *CD31*, T-brachyury and *Lmo2* genes required the presence of both BMP-4 and VEGF. The effect of BMP-4 was specific, and

Figure 5. Real-time RT-PCR analysis of gene expression in embryoid bodies cultured under different conditions.

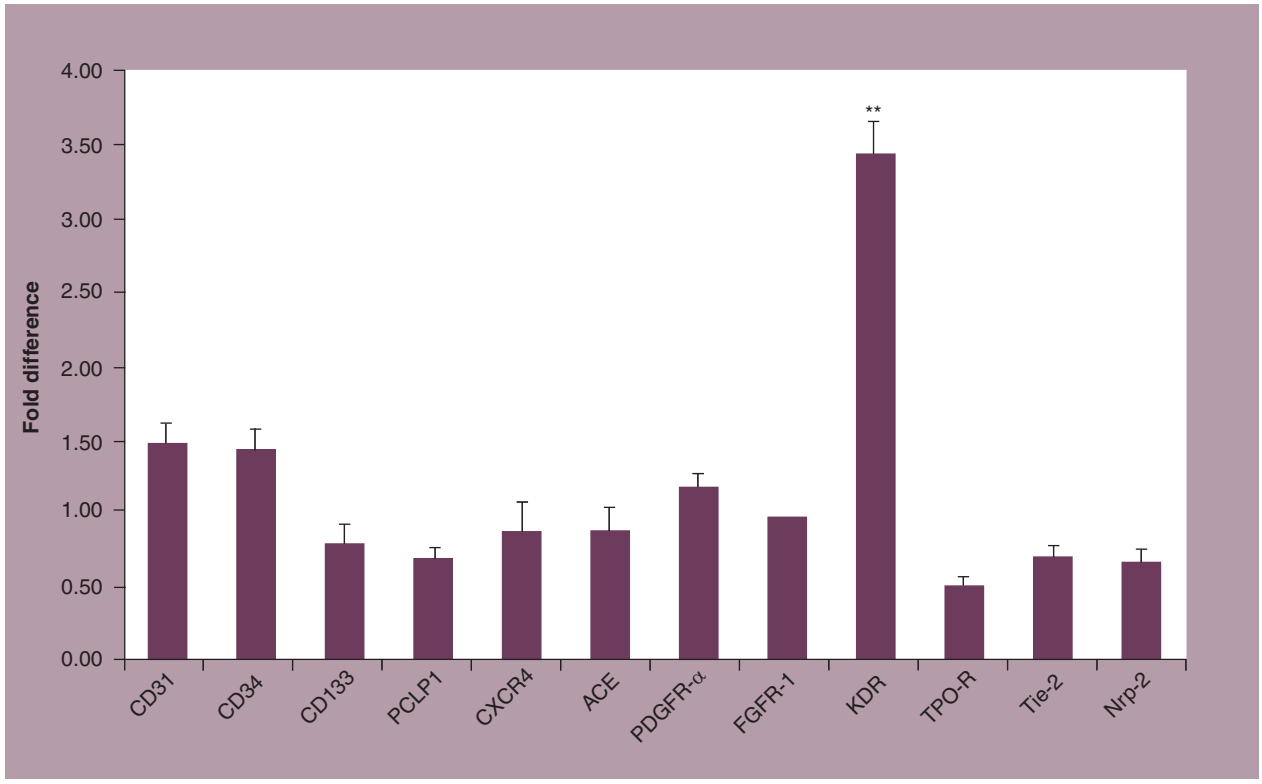


Expression levels of various genes associated with development of hemangioblasts were analyzed in embryoid bodies derived in the presence or absence of either or a combination of both bone morphogenetic protein-4 and $VEGF_{165}$. β-actin was used as an internal control to normalize gene expression. Relative gene expression is presented as a fold difference compared with average expression levels observed in undifferentiated human ESCs.

p < 0.002;*p < 0.0004; n = 3.

ESC: Embryonic stem cell.

Figure 6. Identification of surface markers for hemangioblast progenitors.



Embryoid body cells were enriched with different antibodies using EasySep Kit, then plated for the development of blast colonies.
**p < 0.01; n = 3.

other members of the BMP family showed no effects on promoting blast colony formation. However, we found that $VEGF_{165}$ could be substituted by $VEGF_{121}$. The latter can only bind to KDR and FLT1, but not NRP-1 and -2 receptors [11], consistent with observations in the mouse ESC system [25]. Our progenitor cell enrichment results confirmed these results: KDR^+ cells showed a significant increase in blast colony formation, whereas $NRP\text{-}2^+$ cells revealed a lack of enhancement of blast colony development.

bFGF has been supplemented in culture medium to sustain hESC undifferentiated status and maintain their self-renewal capacity [26–28]. FGFs have also been shown to play a role in the development of cardiomyocytes and preserve long-term repopulating hematopoietic stem cells *in vitro* [29–31]. Previous work demonstrated that bFGF induced hematopoietic and hemangioblastic specification when added to EB medium in both the mouse and human ESC differentiation systems [5,12–14]. Our results clearly demonstrated that addition of bFGF during EB formation failed to promote BC development, consistent with the fact that $FGFR^+$ cells did not increase the number of blast colonies as compared with controls. However, bFGF in BGM significantly enhanced the growth and expansion of blast colonies, which is consistent with the observation of Kennedy *et al.* [5]. There are several possible explanations for the discrepancies, and presumably reflects different properties of mouse and human ESCs [32]. In addition, in hESC studies, Kennedy *et al.* demonstrated that a multistep procedure [5], BMP-4 followed by the addition of bFGF, then by bFGF plus VEGF, resulted in the formation of hemangioblastic and hematopoietic progenitors. Pick *et al.* showed that a combination of four factors including bFGF in EB medium promoted hESC hematopoietic differentiation [14], but neither study addressed the precise role of the added factors. All of these studies, including our own, utilized different components that could have contributed to the observed discrepancies on the effects of bFGF.

In summary, we have developed a simple and efficient system for generating functional BCs from human ESCs cultured with and without

animal feeder cells. The efficient and controlled differentiation of hESCs into specific lineages in serum- and animal feeder-free systems is important for their productive use in regenerative medicine.

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Executive summary

- We describe a significantly improved system for generating hemangioblasts from human embryonic stem cells (hESCs). Although simplified, this new method results in a near-exponential (8 ± 1-fold) increase in blast cell (BC) yield.
- Our data show that hESC specification and commitment to the hemangioblastic lineage is promoted very efficiently by only two factors, bone morphogenetic protein (BMP)-4 and VEGF. The absence of either factor during embryoid body (EB) formation prevents development of blast colonies.
- Gene-expression analyses confirmed that the upregulation of *KDR*, *CD31*, T-brachyury and *Lmo2* genes required the presence of both BMP-4 and VEGF. The effect of BMP-4 was specific, and other members of the BMP family showed no effects on promoting blast colony formation.
- This two-step system does not require the use of different cytokine combinations at multiple steps, nor does it require serum or conditioned medium as previously reported, which could contribute to wide variations in efficiency and reproducibility. The ability to generate significantly larger number of BCs should be desirable for future preclinical and human studies both in terms of time and cost.
- In conclusion, we have developed a simple and efficient system for generating functional BCs from hESCs cultured with and without animal feeder cells. The efficient and controlled differentiation of hESCs into specific lineages in serum- and animal feeder-free systems is important for their productive use in regenerative medicine.

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