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Definitive Hematopoietic Multipotent Progenitor Cells Are Transiently Generated From Hemogenic Endothelial Cells in Human Pluripotent Stem Cells

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Abstract

Generation of fully functional hematopoietic multipotent progenitor (MPP) cells from human pluripotent stem cells (hPSCs) has a great therapeutic potential to provide an unlimited cell source for treatment of hematological disorders. We previously demonstrated that CD34⁺CD31⁺CD144⁺ population derived from hPSCs contain hemato-endothelial progenitors (HEPs) that give rise to hematopoietic and endothelial cells. Here, we report a differentiation system to generate definitive hematopoietic MPP cells from HEPs via endothelial monolayer. In the presence of angiogenic factors, HEPs formed an endothelial monolayer, from which hematopoietic clusters emerged through the process of endothelial-to-hematopoietic transition (EHT). EHT was significantly enhanced by hematopoietic growth factors. The definitive MPP cells generated from endothelial monolayer were capable of forming multilineage hematopoietic colonies, giving rise to T lymphoid cells, and differentiating into enucleated erythrocytes. Emergence of hematopoietic cells from endothelial monolayer occurred transiently. Hematopoietic potential was lost during prolonged culture of HEPs in endothelial growth conditions. Our study demonstrated that CD34⁺CD31⁺CD144⁺ HEPs gave rise to hematopoietic MPP cells via hemogenic endothelial cells that exist transiently. The established differentiation system provides a platform for future investigation of regulatory factors involved in de novo generation of hematopoietic MPP cells and their applications in transplantation.

The transplantation of autologous or HLA-compatible allogeneic hematopoietic multipotent progenitor (MPP) cells allows for the cure of patients with bone marrow failure and the

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Authors' Contribution

H.B: designed research and performed research; Y.L, Y.X, D.L. H: performed research; R.A.B, L.C: wrote the paper and contributed vital new reagents; T.C and Z.Z.W: designed research, analyzed data and wrote the paper.

Supporting Information

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restoration of hematopoiesis in cancer patients treated with high-dose chemoradiotherapy. Because of a shortage in donors for bone marrow transplantation, derivation of MPP cells from human pluripotent stem cells (hPSCs) provides alternative sources and should have a direct benefit on future stem cell therapy (Kaufman, 2009). Investigation of hematopoietic differentiation of hPSCs has led to remarkable advances in understanding of the mechanisms that underline hematopoietic specification. However, generation of functional hPSC-derived hematopoietic MPP cells, which are capable of multilineage hematopoietic differentiation and long-term engraftment in vivo, remain a significant challenge. Further discovery of critical factors and development of technology for de novo MPP generation from hiPSCs should greatly facilitate a realization of therapeutic applications of personalized hiPSCs.

During embryogenesis, hemogenic endothelial cells (ECs), a specified subset of endothelial cells in the vascular endothelium, give rise to multipotent and self-renewable hematopoietic stem cells (HSCs) via endothelial-to-hematopoietic transition (EHT) (Bertrand et al., 2010; Boisset et al., 2010; Kissa and Herbomel, 2010). The bona fide HSCs emerge primarily from endothelium in the aortic-gonad-mesonephros (AGM) region (Zovein et al., 2008; Tavian et al., 2010; Rafii et al., 2013; Ivanovs et al., 2014), and are the origin of a full spectrum of blood cells sustained through the lifespan of an organism. Given the pivotal role of the hemogenic ECs in de novo generation of definitive HSCs, it is important to understand how definitive hematopoietic MPP cells generated from hemogenic ECs in the hPSC differentiation system. Several recent reports have focused on defining and characterization of hemogenic progenitors and definitive hematopoietic progenitors from various hPSC differentiation systems (Choi et al., 2012; Kennedy et al., 2012; Rafii et al., 2013), revealing the phenotypes and functionality of putative hemogenic progenitors in a specified context. Most recently, the first human HSCs are shown to emerge from the ventral domain of the dorsal aorta in the AGM region with an extensive defined phenotype including the expression of CD34, CD45, CD144 (VE-Cadherin), and CD117 (c-kit). Definitive hematopoietic MPP cells derived from hemogenic ECs of hPSCs have been reported (Lancrin et al., 2009; Choi et al., 2012; Kennedy et al., 2012; Rafii et al., 2013; Sturgeon et al., 2014; Uenishi et al., 2014; Ayllon et al., 2015), however, engraftment activity from these hematopoietic cells have not been demonstrated. A recent study demonstrated that vascular niche promotes engraftable human MPP production from hPSCs (Gori et al., 2015). The identity of hPSC-derived hematopoietic cells that possess long-term engraftment potential remains elusive. One of the possible factors contributing to the difficulty in de novo generation of engraftable hematopoietic cells from hPSCs is that definitive hemogenic ECs exist only briefly, thus definitive MPP generation via EHT must occur in a restricted developmental time window.

We and others have identified hematopoietic and endothelial progenitors in differentiated hPSCs, based on markers expressed in endothelial and hematopoietic progenitor cells, including CD34, KDR (VEGFR2 or FLK1), CD31 (PECAM1), and CD144 (Kennedy et al., 2007; Choi et al., 2012; Kennedy et al., 2012; Wang et al., 2012; Bai et al., 2013; Rafii et al., 2013; Xie et al., 2015). We previously demonstrated that CD34⁺CD31⁺CD144⁺ population from hPSCs contains hemato-endothelial progenitors (HEPs) that give rise to hematopoietic cells and endothelial cells (Bai et al., 2010; Bai et al., 2013; Xie et al., 2015). The key transcription factors required for definitive hematopoietic cell generation from hemogenic

ECs, including SCL and RUNX1 (Lacaud et al., 2002; Patterson et al., 2005; Nottingham et al., 2007; Dzierzak and Speck, 2008; Chen et al., 2009; Lam et al., 2010; Ren and Gomez, 2010; Van et al., 2012; Ran et al., 2013; Zhen et al., 2013), are expressed in CD34⁺CD31⁺CD144⁺ cells (Bai et al., 2013). In the present study, we established a stepwise differentiation system to interrogate hematopoietic cells from the endothelial monolayer derived from HEPs. Our data indicated that the CD34⁺CD31⁺CD144⁺ population is endowed with hemogenic activity to generate definitive MPP cells that gave rise to multilineage hematopoietic cells, including definitive erythroid cells and T lymphocytes. Our study provides insight into the feature of hemogenic endothelial cells, and provides a unique system for future investigation of the mechanisms underlying the development and specification of human MPP cells through hemogenic ECs.

Materials and Methods

Cell lines

The H1 and H9 hESCs were obtained from the WiCell research Institute (Madison, WI), and used between passages 40 and 60. The BC1 hiPSC line was established by Dr. Linzhao Cheng at Johns Hopkins University from human bone marrow CD34⁺ cells by transient expression of a non-integrating plasmid (Chou et al., 2011). The hPSCs were cultured on Vitronectin-coated plates in E8 medium (Life Technologies, Carlsbad, CA) and growth media was changed daily (Wang et al., 2013). The hPSCs were subcultured every 4–5 days with a treatment of 0.5 mM EDTA for passaging.

Differentiation of embryoid bodies

The hPSC differentiation was induced, as previously described (Bai et al., 2010, 2013; Xie et al., 2015). To generate hanging-drop embryoid bodies (EBs), 1,000 cells per drop in 25 μ l was cultured on petri dishes to form EBs in the presence of 10 μ M ROCK inhibitor (Y27632), 2 ng/ml TGF- β 1, and 0.05% polyvinyl alcohol (PVA) in a serum-free differentiation medium (SFM) containing IMDM/F12 (1:1), 0.5% BSA (Sigma–Aldrich), 1% ITS-X (Life Technologies), 1% chemical defined lipid (Life Technologies), 2 mM Glutamax (Life Technologies), 50 μ g/ml L-ascorbic acid (Sigma–Aldrich), and 450 μ M 1-Thioglycerol (Sigma–Aldrich, St, Louis, MO). After 2 days of hanging-drop EB formation, EBs were collected, and transferred to ultra-low attachment dishes in SFM. The differentiation media was changed every 2 days with a fresh supplement of growth factors, BMP4, FGF2, VEGF and TGF- β inhibitor (SB431542), as indicated in Figure 1A. Similar results were obtained by forming EBs individually in round bottom 96-well plates by aggregating 3,000–4,000 hPSCs per well after a centrifugal spin (Spin-EBs) as previously described (Wang et al., 2013).

Hemogenic endothelial cell culture and hematopoietic differentiation

After 8–10 days of EB differentiation, EBs were treated by TrypLE (Life Technologies) at room temperature for 10 min to obtain a single-cell suspension. The CD34⁺CD31⁺CD144⁺ cells were isolated by fluorescence-activated cell sorting (FACS) with FACS Aria II (BD Biosciences, Franklin Lakes, NJ). In some experiments, the CD34⁺CD31⁺ cells were

positively selected by using the MultiSort immunomagnetic separation system (Miltenyi Biotec, San Diego, CA) according to the manufacturer's instruction.

For hematopoietic differentiation from hemogenic ECs, the isolated CD34⁺CD31⁺CD144⁺ cells were cultured on collagen-I coated plates (5×10⁴ cells per well in 12-well plate) in serum-free endothelial cell growth medium (ECGM) (Bai et al., 2013; Xie et al., 2015). After 24 h, unattached cells were removed and adherent cells were washed with PBS and continually cultured for three additional days in ECGM or in hematopoietic cell growth medium (HCGM) containing 50 ng/ml stem cell factor (SCF), 50 ng/ml FLT3-ligand (F3L), and 50 ng/ml thrombopoietin (TPO). The semi-attached hematopoietic colonies were counted after 3 days. The hematopoietic cells (including suspension cells and semi-attached cells) were collected and analyzed by flow cytometry. A photograph was taken every 60 min from 24 h to 96 h for cells cultured in ECGM by using a time-lapse microscopy (Leica AF6000 LX System). The video was assembled with 0.2 sec frame images (Supplemental video).

For T cell differentiation, hematopoietic cells in suspension were harvested after 3 days of hemogenic EC culture, and then cultured on OP9-DLL1 (Holmes and Zuniga-Pflucker 2009) or on collagen-I coated dishes in HCGM containing SCF, F3L and IL-7 for 14 days. For erythroid differentiation, CD34⁺CD31⁺CD144⁺ cells were subjected for erythroid differentiation culture in HCGM with 3 units/ml erythropoietin (EPO) for 6–18 days, as previously described (Xie et al., 2015).

Dil-Ac-LDL uptake assay

CD34⁺CD31⁺CD144⁺ cells (2.5 × 10⁵ cells per well in 12-well plate) were cultured on collagen-I coated plates in ECGM for 24 h. After removing suspension cells, 10 µg/ml Dil-Ac-LDL (Biomedical Technologies Inc.) was added in ECGM then adherent cells were cultured for 6 h. The anti-human-CD144-FITC antibody was used to identify endothelial cells. Photos were taken by a fluorescence microscope (Olympus).

Vascular-like network formation assay

CD34⁺CD31⁺CD144⁺ cells were cultured on collagen-I coated plates in ECGM for 24 h. After removing suspension cells, 5 × 10⁴ adherent cells were resuspended in 0.5 ml ECGM, and then cultured on a Matrigel-coated 24-well plate. The results were examined after 16 h of culture.

Immunocytochemistry

The cells were fixed with 4% paraformaldehyde (PFA) in PBS at room temperature for 10 min, permeabilized with 0.1% Triton X-100 in PBS at room temperature for 10 min, and then incubated with 1% BSA in PBS for 30 min to block nonspecific binding. The treated cells were then incubated with the following antibodies as shown in figures: CD144-Alexa Fluor 488 (eBioscience), CD45-PE (BD Biosciences), CD71-FITC (eBioscience), and CD235a-PE (BioLegend) for one hour at room temperature. The nuclei were stained by 0.1 mg/ml DAPI for 3 min. The results were examined by a fluorescence microscope.

Real-time RT-PCR analyses

Total RNAs from undifferentiated hPSCs and EBs at different time points were isolated by using Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA). To eliminate DNA contamination, the RNA samples were treated with DNase I (RNase free) on the column, following manufacturer's instruction. Total RNA (1 µg) was used for each reverse transcription reaction with SuperScript III. Real-time qPCR was performed on an iQ5 thermal cycler (Bio-Rad Laboratories), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal standard for normalization. The specific primers for each of the globin mRNAs were as following: ϵ -globin (*HBE*) forward (GCCTGTGGAGCAAGATGAAT) and reverse (GCGGGCTTGAGGTTGT); γ -globin (*HBG*) forward (CTTCAAGCTCCTGGGAAATGT) and reverse (GCAGAATAAAGCCTACCTTGAAAG); β -globin (*HBB*) forward (GGCACCTTTGCCACACTG) and reverse (CACTGGTGGGGTGAATTCTT).

Flow cytometric analysis

Adherent cells or EBs were dissociated to form a single-cell suspension by TrypLE (Life Technologies) treatment, and washed with FACS buffer (1% FBS and 1 mM EDTA in PBS). The dissociated cells were resuspended in FACS buffer, and labeled with fluorochrome-conjugated anti-human CD34-APC (clone# AC136, Miltenyi Biotec), CD31-PE (clone# AC114.5, Miltenyi Biotec), CD144-Alexa Fluor 700 (clone# 16B1, eBioscience), CD45-BV605 (clone# HI30, BioLegend), CD41-APC/Cy7 (clone# HIP8, BioLegend), CD235a-PE (clone# HIR2, eBioscience), CD43-BV421 (clone# 1G10, BD Biosciences), CD73-PE/Cy7 (clone# AD2, BioLegend), CD117-PE/Cy7 (clone# 104D2, BioLegend), CD127-FITC (clone# eBioRDR5, eBioscience), CD14-FITC (clone# 61D3, eBioscience), or KDR-PerCP (clone# HKDR-1, BioLegend). Flow cytometry was performed on LSR II analyzer (BD Biosciences). Data analysis was performed using FlowJo software or FCS Express software.

Cytospins and Wright-Giemsa staining

Cells were collected from liquid cultures, washed with PBS, and spun onto glass slides using a cytospin apparatus. Slides were fixed in methanol: acetone (80:20 volume), and then stained with Wright-Giemsa solution (Sigma-Aldrich).

CFC assay (colony-forming cell assay)

Hematopoietic colony-forming cell (CFC) assay was performed as described in our previous studies (Chen et al., 2007; Wang et al., 2007) with minor modification. The cells (1×10^4) were mixed with MethoCult[®] methylcellulose-based media (StemCell Tech, H4435), following manufacturer's instruction. The hematopoietic colonies were photographed and counted after 2 weeks of culture. Each type of colony was classified according to morphology. Each assay was performed in triplicate.

Statistical analysis

The data were subjected to statistical analysis by the Student's t-test. Results with a value of $P < 0.05$ were considered statistically significant.

Results

Establishment of a stepwise differentiation system for hemogenic EC culture

To generate multilineage hematopoietic cells from hPSCs (hESCs or hiPSCs), we induced hPSC differentiation in a stepwise manner (Fig. 1A). The hematopoietic differentiation of hPSCs was initiated through embryoid body (EB) formation in a serum-free medium with an optimized stage-specific combination of BMP4, FGF2, and VEGF-A, as we previously described (Bai et al., 2013; Xie et al., 2015). We have previously shown that CD34⁺CD31⁺CD144⁺ cells express *SCL* and *RUNX1*, the two key transcription factor genes required for definitive hematopoietic cell development, and contain HEPs that give rise to hematopoietic cells and endothelial cells. To investigate the developmental stage at which these cells are derived, we analyzed the kinetics of CD34, CD31, and CD144 expression and the emergence of CD34⁺CD31⁺CD144⁺ cells. During hPSC differentiation, EBs were collected at different time points and assessed by flow cytometry. The earliest detection of CD34⁺ cells, CD31⁺ cells, and CD144⁺ cells was around day 4, and the numbers of positive cells steadily increased in a similar kinetics (Fig. 1B left panel). The CD34⁺CD31⁺CD144⁺ cells also appeared as early as day 4 and peaked around day 8 (Fig. 1B right panel). Further analysis of CD34⁺CD31⁺CD144⁺ population at EB day 8 (Fig. 1C) indicated that the majority of the CD34⁺CD31⁺CD144⁺ cells (more than 95%) co-expressed KDR (VEGFR2/FLK1). Whereas they were negative for CD45 and CD117 (c-Kit), markers of human HSPCs from AGM (Ivanovs et al., 2014), a small subset of CD34⁺CD31⁺CD144⁺ cells expressed CD43 and CD235a, the markers of primitive hematopoietic cells at early hematopoietic developmental stage (Kennedy et al., 2012; Sturgeon et al., 2014).

To test whether CD34⁺CD31⁺CD144⁺ cells give rise to hematopoietic cells from HEPs through hemogenic EC intermediate, we isolated CD34⁺CD31⁺CD144⁺ cells from EBs between day 6 and 8, and cultured them to form endothelial cell monolayer in a serum-free endothelial cell growth medium (ECGM) containing VEGF-A and FGF2 (Bai et al., 2013). The non-adherent cells were carefully washed out after 24 h. The remaining cells formed an endothelial monolayer that exhibited classic endothelial characteristics, such as the up-taking of Dil-Ac-LDL (Fig. 2A), expression of CD144 (VE-cadherin) at the cell-cell junction (Fig. 2A), and forming a vascular-like network on Matrigel (Fig. 2B). The ECs derived from CD34⁺CD31⁺CD144⁺ cells remained positive for CD34, CD31, and CD144, but negative for CD235a (Glycophorin A), CD41, CD117, and CD45 (Fig. 2C), indicating that EC monolayer contained no pre-committed hematopoietic cells.

To induce hematopoietic differentiation from ECs from HEPs, we followed the emergence of hematopoietic clusters from EC monolayer by using a time-lapse microscope to monitor endothelial-to-hematopoietic transition (EHT). By analyzing the photographs taken in a designated culture area after removal of unattached cells, we found that round-shape hematopoietic-like cells emerged from ECs as early as 8 h after removal of unattached cells, and formed semi-attached hematopoietic clusters that were recognizable as early as 24 h (Fig. 3A). To confirm that the semi-attached clusters are nascent hematopoietic cells, we performed immunocytochemistry analysis of CD45⁺ cells on the semi-attached clusters after 72 h of hemogenic EC culture (Fig. 3B). Approximately 60% of hemogenic clusters

contained round-shaped CD45⁺ cells (Fig. 3C). Interestingly, some hemogenic clusters contained both CD45⁺ and CD45⁻ cells indicating that heterogenous cell populations existed in individual hemogenic clusters. It is unclear whether hematopoietic clusters contain cells from different stages of hematopoietic development. Based on the functionality potential of the endothelial monolayer from CD34⁺CD31⁺CD144⁺ cells in giving rise to hematopoietic cluster through EHT, we referred to this endothelial cell culture as hemogenic EC culture.

Hemogenic activity from triple-negative (CD41⁻CD43⁻CD73⁻) cells

To further identify putative hemogenic progenitors from HEPs, we characterized hemogenic potential of distinctive subpopulations of CD34⁺CD31⁺CD144⁺ cells. It has been reported that hemogenic ECs are enriched based on the absence of CD43, CD41, and CD73 on cell surface (Choi et al., 2012; Kennedy et al., 2012; Rafii et al., 2013). In a representative experiment, our study of flow cytometry of day 8 EBs indicated that CD34⁺CD31⁺ population contained 17% of CD144⁺CD73⁻ cells in which 86% of cells were CD41⁻CD43⁻ cells (Fig. 4A). The CD41⁺ cells, CD43⁺ cells, CD73⁺ cells, and their negative counterparts were isolated from CD34⁺CD31⁺CD144⁺ population by FACS cell sorting, and assessed for their ability to generate hematopoietic clusters from endothelial monolayer in hemogenic EC culture. The CD43⁻ cells, CD41⁻ cells, and CD73⁻ cells generated significantly higher numbers of hemogenic colonies than their CD43⁺, CD41⁺, and CD73⁺ counterparts (Fig. 4B). To assess the development of hemogenic progenitors during hPSC differentiation, we analyzed the kinetics of CD34⁺CD31⁺CD144⁺CD73⁻ cells in EBs at different time points. As shown in Figure 4C, approximately 30% of CD34⁺CD31⁺ cells were CD144⁺CD73⁻ cells on EB day 6, and CD144⁺CD73⁻ cells significantly decreased after EB day 6.

Multilineage and definitive hematopoietic cells derived from hemogenic EC culture

To examine multilineage hematopoietic potential of HSPCs from hemogenic EC culture, we harvested hematopoietic cells in suspension after 3 days, and analyzed hematopoietic markers by flow cytometry (Fig. 5A). The vast majority of hematopoietic cells remained CD31 positive (90.3±4.8%), whereas 61.2±12.2% of them remained CD34 positive. Subsets of the hematopoietic cells expressed CD117 (c-Kit) (54.6±14.6%), CD127 (IL-7R-α) (16.6±5.7%), CD33 (21.1±5.4%), CD235a (23.5±11.6%), CD45 (26.1±14.2%), and CD14 (9.5±5.3%). To determine multilineage hematopoietic potential, CFC assays were performed from hematopoietic cells in suspension from hemogenic EC culture. As shown in Figure 5B, multilineage hematopoietic colonies were observed, including BFU-E/CFU-E, CFU-GEMM, CFU-G/M/GM. The majority colonies represented granulocyte and macrophage (Fig. 5C). These data indicated that hematopoietic cells derived from hemogenic EC culture are heterogeneous, consisting of a broad spectrum of hematopoietic progenitors and derivatives.

Because T cell differentiation occurs in definitive hematopoiesis, it has been used as an indicator to outline definitive hematopoietic progenitors in hPSC differentiation systems (Kennedy et al., 2012; Sturgeon et al., 2014; Uenishi et al., 2014; Ditadi et al., 2015). To assess lymphoid potential of hematopoietic cells from hemogenic ECs, we harvested hematopoietic cells in suspension on day 3, and sub-cultured them for 2 weeks on OP9-

DLL1 feeder cells, in which Notch ligand, delta-like 1 (DLL1), is ectopically expressed (Holmes and Zuniga-Pflucker, 2009). After 2 weeks of coculturing with OP9-DLL1 in the presence of IL-7, a cell population within lymphoid gate appeared (Fig. 5D, left). Flow cytometry analyses revealed several T cell markers, including CD4, CD8, CD3, and T cell receptor $\gamma\delta$ (TCR $\gamma\delta$), were highly expressed, indicating that T lymphoid differentiation from HSPCs occurs (Fig. 5D right).

To further assess definitive hematopoietic potential, we induced hematopoietic differentiation to erythroid cells by adding EPO during hemogenic EC culture. After 15 days of erythroid differentiation, the majority of hematopoietic cells in suspension expressed erythroid markers, CD235a and CD71 ($72.0\pm9.5\%$) (Fig. 6A). Morphologic analysis by Wright-Giemsa staining revealed that erythroid cells on day 7 contained blue-gray cytoplasm and a nucleus with clumped chromatin, a feature of basophilic erythroblasts (Fig. 6B). Progressive erythroid maturation continued, and by day 15 erythroid cells were small in size with bright pink cytoplasm, low nuclear cytoplasmic ratio, and a condensed nucleus (Fig. 6B). By day 18, approximately 1–5% of CD235a⁺CD71⁺ erythrocytes failed DAPI staining for nuclei, observed by confocal fluorescent microscopy, suggesting erythrocyte enucleation (Fig. 6C). Gene expression patterns of hemoglobin variants represent the progress of erythropoiesis during the development. Using hemoglobin as an indicator, we tested whether hematopoietic cells in suspension from hemogenic EC culture differentiate to definitive erythrocytes. The expression of *HBB* gene (coding the β -subunit of adult hemoglobin) increased from day 6 to day 15 significantly (Fig. 6D). Because CD235a⁺ cells and CD45⁺ cells were observed in hematopoietic cells on day 3 of hemogenic EC culture, we examined if CD45⁺ cells and CD235a⁺ cells have different definitive potential. CD235a⁺ cells and CD45⁺ cells were isolated from hematopoietic cells after 3 days of hemogenic EC culture, and induced for erythroid differentiation for 7 days and were then analyzed for β -globin expression. As shown in Figure 6E, *HBB* gene expression level was significantly higher in erythrocytes differentiated from CD45⁺ cells, compared to that from CD235a⁺ cells, suggesting that CD45⁺ cells from hemogenic EC culture contain enriched definitive MPP cells. Taken together, the data of T cell differentiation and erythroid differentiation indicated that hematopoietic cells derived from HE culture contain MPP cells that possess a definitive hematopoietic potential.

Generation of hematopoietic cells from transient hemogenic ECs is promoted by hematopoietic growth factors

Although the development of hematopoietic clusters occurred in endothelial cell growth conditions, it is of great interest to know whether hematopoietic growth factors instruct de novo MPP generation from hemogenic ECs. Therefore, after establishing endothelial monolayer from CD34⁺CD31⁺CD144⁺ cells, we switched the culture medium from ECGM to hematopoietic cell growth medium (HCGM) containing SCF, TPO, and F3L for 3 days, and then scored hematopoietic clusters. Compared to the cells continually grown in EC growth condition, the cells cultured in hematopoietic growth condition gave rise to a significantly increased number of hematopoietic clusters (Fig. 7A), indicating that hematopoietic growth factors promote hematopoietic cell generation from hemogenic ECs.

By assessment of hematopoietic clusters from total input cells, we found that approximately 0.5–2.5% of CD34⁺CD31⁺CD144⁺ cells had hemogenic potential.

To understand the developmental progression of hemogenic activity, we cultured CD34⁺CD31⁺CD144⁺ cells in endothelial cell growth condition for varying periods of time (0, 24, 48, and 72 h) before switching to hematopoietic growth conditions. The frequency of hematopoietic clusters was scored after an additional 3 days of culturing in HCGM.

Interestingly, the hemogenic potential of ECs gradually decreased after culturing in endothelial growth condition, resulting in less hematopoietic clusters. As shown in Figure 7B, compared to immediate culture in HCGM after removal of unattached cells (0 hours), the number of hematopoietic clusters decreased 30% after 24 h and 70% after 48 h in the endothelial growth condition. Furthermore, one passage (P1) of ECs derived from CD34⁺CD31⁺CD144⁺ cells in endothelial growth condition resulted in complete loss of ability to generate hematopoietic clusters (Fig. 7C). Gene expression analysis indicated that, concomitant with the gradual loss of hemogenic potential in endothelial growth condition, the expression of functional EC-specific genes, *von Willebrand factor* (vWF) and *CD106* (*VCAM-1*), were gradually increased, whereas the expression of key regulators of hemogenic ECs, *RUNX1*, and *SCL*, were decreased (Fig. 7D). Our findings demonstrate that hemogenic activity is possessed by a transient cell population that functions as an in vitro equivalent of hemogenic ECs, and these cells lose their hemogenic potential if propagated in endothelial growth condition.

Discussion

Decades-long investigations of hematopoietic differentiation from hPSCs have led to a remarkable advance in our understanding of human hematopoiesis. However, identification and derivation of hPSCs-derived MPP cells that are capable of long-term engraftment and differentiation to multilineage hematopoietic cells remain major challenges, which present an impediment to the utilization of hPSC-derived MPP cells as a promising source for stem cell therapy. Here, we described a stepwise serum-free and feeder-free differentiation system to generate multilineage definitive hematopoietic cells from HE cells derived from hPSCs. The approach possesses relatively simple differentiation procedure and cytokine usage, as well as adequate efficiency in terms of time and progenitor cell percentage. By controlling exact cell number in EB formation, it allowed to generate reproducible experimental results. Given the avoidance of utilizing exogenous animal products, the approach provides a promising tool of potential clinical application. With this defined system, we have provided evidences that hematopoietic cells emerged from hPSC-derived endothelial cells are capable of giving rise to definitive hematopoietic cells of different fates.

We previously demonstrated that hPSC-derived CD34⁺CD31⁺CD144⁺ cells have hematopoietic and endothelial potential (Bai et al., 2010; Bai et al., 2013), thereby are referred to as hemato-endothelial progenitors (HEPs). The majority of the CD34⁺CD31⁺CD144⁺ cells co-expressed KDR (VEGFR2/FLK1), a mesodermal marker expressed in early precursors of hematopoiesis in the hPSC differentiation system (Kennedy et al., 2007), whereas they were negative for CD45 and CD117 (c-Kit), markers expressed in

human HSPCs from AGM (Ivanovs et al., 2014). CD41, CD43, and CD235a cells in the early hematopoietic compartment define their primitive hematopoietic nature (Kennedy et al., 2012; Sturgeon et al., 2014). We found that small subsets of CD34⁺CD31⁺CD144⁺ cells expressed CD41, CD43, and CD235a (Fig., 4 1), suggesting that the CD34⁺CD31⁺CD144⁺ population contains primitive hematopoietic cells. We found that the frequency of CD34⁺CD31⁺CD144⁺ cells from H9 hESCs was slightly lower than that from H1 hESCs and BC1 hiPSCs. However, once those cells were selected for further experiment, the hemogenic potential showed no significant difference among H1, H9, and BC1 hPSCs.

Although hematopoietic developmental hierarchy remains to be clarified and refined, numerous evidences indicated that MPP cells originate from endothelial intermediates, HE cells, in early development and subsequently give rise to the full spectrum of blood cells (Slukvin, 2013). In this study, we intended to characterize hemogenic potential in the endothelial monolayer established from CD34⁺CD31⁺CD144⁺ HEPs. To initiate from an endothelial stage, we eliminated pre-existing hematopoietic-committed cells from the CD34⁺CD31⁺CD144⁺ population by culturing them in endothelial growth condition, and then washing out unattached hematopoietic-committed cells in suspension. The remaining adherent cells formed an endothelial monolayer with endothelial characteristics (Fig. 2). We observed emergence of hematopoietic-like cells from endothelial monolayer as early as 8 h after removal of unattached hematopoietic cells, and formation of recognizable semi-attached hematopoietic clusters after 24 h (Fig. 3). The timeline of hemogenic cluster development is consistent with a previously reported observation (Rafii et al., 2013). A recent study has demonstrated that definitive erythroid/myeloid progenitors and HSCs originate from distinct hemogenic ECs during mouse embryogenesis (Chen et al., 2011). We found that approximately 60% of hematopoietic clusters contained CD45⁺ cells (Fig. 3B). CD45⁻ cells were observed within CD45⁺ hematopoietic clusters, suggesting that hematopoietic cells derived from hemogenic ECs are at different developmental stages and/or different multilineage cell fates. Alternatively, discrete hematopoietic clusters (CD45⁺ and CD45⁻) may account for derivatives from distinct hemogenic EC populations (Chen et al., 2011).

Several recent studies provided important insights into identification and characterization of hemogenic ECs in hPSC differentiation systems and highlighted the utilization of surface markers CD73, CD34/CD43, and CD41a in different contexts to distinguish HE and non-HE cells (Choi et al., 2012; Kennedy et al., 2012; Rafii et al., 2013; Ditadi et al., 2015). In this study we identified enriched hemogenic potential within CD73⁻, CD43⁻, and CD41⁻ subpopulations respectively, in agreement with the previous reports (Fig. 4B). These findings enabled us to further specify hemogenic potential as CD34⁺CD31⁺CD144⁺CD41⁻CD43⁻CD73⁻ cells.

T lymphocyte potential, along with myeloid and erythroid potential, have been used as one of the indicators for the establishment of definitive hematopoietic programs in the hPSC differentiation system (Kennedy et al., 2012; Sturgeon et al., 2014; Uenishi et al., 2014; Ditadi et al., 2015). Our data showed that the lymphocyte progenitor marker, CD127 (IL-7R), was expressed in hematopoietic cells in suspension on day 3 from hemogenic EC culture (Fig. 5A). Furthermore, after an additional 14 days of lymphocyte induction on OP9-

DLL1, a significant population of CD3⁺, CD4⁺, CD8⁺, and TCR $\gamma\delta$ cells emerged (Fig. 5D), indicating a definitive hematopoiesis from hemogenic-derived MPP cells. The detection of TCR $\alpha\beta$ was failed in FACS assay (data not shown), possibly due to the conflict of antibodies between TCR $\alpha\beta$ (clone #IP26) and CD3 (clone #OKT3), or immature T cells. During erythroid differentiation, appearance of enucleated erythrocytes after 18 days of erythroid differentiation from hematopoietic cells in suspension further demonstrated definitive hematopoietic differentiation (Fig. 6C); however, the low percentage of enucleated erythrocytes (1–5%) may result from an unfavorable condition for erythrocyte maturation. The unique hemoglobin gene expression switch from beta to alpha globin clusters at different development stages has facilitated the identification of primitive and definitive erythroid cells (Stamatoyannopoulos, 2005; Sankaran, 2010; Palis, 2014). In humans, embryonic ϵ -globin (*HBE*) gene is expressed in primitive erythroid cells, whereas fetal γ -globin (*HBG*) gene and adult β -globin (*HBB*) gene are expressed in definitive erythroid cells. Our results showed that during erythroid differentiation from hPSC-derived hemogenic ECs, the expression level of the adult β -globin gene increased steadily from day 6 to day 15 (Fig. 6D), although ϵ -globin and γ -globin genes were also highly expressed (data not shown). Erythrocytes differentiated from CD45⁺ cells expressed a higher level of β -globin than that from CD235a⁺ cells (Fig. 6E), indicating that CD45⁺ cells from hemogenic EC culture represent enriched definitive MPP cells whereas CD235a⁺ cells may represent primitive hematopoietic progenitors at this condition (Sturgeon et al., 2014).

In order to facilitate hematopoietic differentiation from hemogenic ECs, we investigated the effect of hematopoietic growth factors on hematopoietic cell generation in our established hemogenic EC culture. We found that ECs from hPSC-derived CD34⁺CD31⁺CD144⁺ cells were capable of generating multilineage hematopoietic cells in endothelial cell growth condition; and this capability is enhanced significantly in hematopoietic cell growth medium (HCGM) containing SCF, TPO, and F3L (Fig. 7A).

The hematopoietic potential of ECs was reduced after prolonged culture in endothelial growth conditions (Fig. 7B, C), suggesting that HSPCs arise from hemogenic ECs through EHT within a restricted developmental stage. It is possible that once hemogenic ECs pass a divergence point of development, they lose their ability to give rise to hematopoietic cells. Indeed, the reduced hemogenic potential of cells in hemogenic EC culture is correlated to a decreased expression of Runx1 and Scl and an increase of mature EC markers (Fig. 7D). Our observation is in agreement with a previous demonstration in the mouse differentiation system that hematopoietic generation from ECs occurs only in a specific-time window during mESC differentiation (Eilken et al., 2009). Temporal analysis of hemogenic activity and expression of Runx1 and Scl in our culture system revealed that hemogenic ECs reside within the endothelial monolayer from CD34⁺CD31⁺CD144⁺ cells as a transient population that declined quickly and diminished in 4 days of endothelial culture.

In summary, this report described a serum-free and feeder-free stepwise hPSC differentiation system to derive definitive hematopoietic MPP cells. Our findings provide new insights into the development of HSCs from hPSCs through hemogenic ECs, and laid a foundation for further investigation of regulatory factors and mechanisms involved in MPP derivation from hPSCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Differentiation of hPSCs to generate CD34⁺CD31⁺CD144⁺ HEPs. (A) Schematic outline of experimental design. CD34⁺CD31⁺CD144⁺ cells were isolated from EBs between day 6 and day 8, and cultured on collagen-I coated plates in serum-free endothelial cell growth medium (ECGM) for 24 h. Unattached hematopoietic committed cells were removed, and attached endothelial cells were cultured either in ECGM or in hematopoietic cell growth medium (HCGM) for an additional 3 days. (B) Kinetics of CD34⁺ cells, CD31⁺ cells, and CD144⁺ cells (left panel) and kinetics of CD34⁺CD31⁺CD144⁺ cells (right panel) during hPSC differentiation by flow cytometric analysis. Cell numbers were normalized by per million input of hPSCs at day 0. Data were presented as Mean $\pm$ SD from 10 independent experiments of H1, H9, and BC1 cells. (C) Subpopulation analysis of CD34⁺CD31⁺CD144⁺ cells. CD34⁺CD31⁺ cells were gated for CD144⁺ and KDR⁺ (VEGFR2/FLK1) cells. CD34⁺CD31⁺CD144⁺KDR⁺ population was further assessed for hematopoietic makers

CD43, CD235a, CD45, and CD117 (c-kit). The representative FACS data were shown from 3 independent experiments of H1 cells.

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Fig. 2.

Characterization of endothelial cells from $CD34^+CD31^+CD144^+$ cells.

$CD34^+CD31^+CD144^+$ cells derived from either H1 or BC1 cells were cultured on collagen-I coated plates in ECGM for 24 h. After removal of unattached cells, the adherent ECs formed a monolayer and were harvested and characterized by: (A) CD144 expression at cell-cell junction (green) and Dil-Ac-LDL up-taking (red), (B) Vascular-like network formation assay in Matrigel, and (C) Flow cytometry of endothelial and hematopoietic markers. The representative data were shown from five independent experiments.

Fig. 3.

Emergence of Hematopoietic cells from hemogenic ECs. (A) Development of hematopoietic clusters from EC monolayer. After culturing $CD34^+CD31^+CD144^+$ cells for 24 h to form an endothelial monolayer and removing unattached cells, the attached cells were photographed by a time-lapse microscope (Leica AF6000 LX System). The representative data were shown from 3 independent experiments of H1 cells. (B) $CD45^+$ cells in hematopoietic clusters. On day 3, the hematopoietic clusters were analyzed by staining with anti-human $CD45$ -PE. Endothelial cells were identified by anti-human $CD144$ -FITC. Nuclei were detected by DAPI. (C) The numbers of $CD45^+$ and $CD45^-$ hematopoietic clusters were counted on day 3. Data were presented as $Mean \pm SD$ from three independent experiments of H1 cells.

Fig. 4. Hemogenic potential of CD73⁻CD43⁻CD41⁻ subpopulation of CD34⁺CD31⁺CD144⁺ HEPs. (A) Representative flow cytometry of CD43 and CD41 in CD73⁻ cells in CD34⁺CD31⁺CD144⁺ cells. (B) The CD41, CD43 and CD73 subpopulations of CD34⁺CD31⁺CD144⁺ cells were isolated by FACS. Representative flow cytometric profiles showed CD41⁺ and CD41⁻ cells, CD43⁺ and CD43⁻ cells, or CD73⁺ and CD73⁻ cells within CD34⁺CD31⁺CD144⁺ cells (Top Panel). CD41⁺ and CD41⁻ cells, CD43⁺ and CD43⁻ cells, or CD73⁺ and CD73⁻ cells were cultured on collagen-I coated plates. Hemogenic clusters were counted after 3 days of culture in ECGM (Low panel). (C) CD73⁻ cells in CD34⁺CD31⁺CD144⁺ cells during hPSC differentiation. Data were presented as Mean±SD from three independent experiments of BC1 cells. * $P < 0.05$ between two groups.

Fig. 5. Definitive hematopoietic cell generation from hemogenic endothelial cells. (A) Flow cytometric analysis of hematopoietic cells in suspension from hemogenic EC culture. After culturing CD34⁺CD31⁺CD144⁺ cells for 24 h to form an endothelial monolayer and removing unattached cells, hematopoietic differentiation for HSPC generation was continued for 3 days. Hematopoietic cells in suspension were harvested and analyzed by flow cytometry on day 3. Data were presented as Mean±SD from 10 independent experiments of H1, H9, and BC1 cells. (B) Morphology of multilineage hematopoietic colonies from CFC assay of hematopoietic cells harvested in suspension on day 3 of hemogenic EC culture. The representative data were shown from 3 independent experiments of BC1 cells. (C) CFC assay of hematopoietic cells harvested in suspension. Data were presented as Mean±SD from 3 independent experiments of BC1 cells. (D) T cell

differentiation of HSPCs. On day 3 of hemogenic EC culture, hematopoietic cells in suspension were harvested, and sub-cultured on OP9-DLL1 for 2 weeks. T cells in lymphocyte gate were analyzed by flow cytometry. 7-AAD was used for detecting live cells. The representative data were shown from three independent experiments of BC1 cells.

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Fig. 6.

Erythroid differentiation from hemogenic endothelial cells. After culturing CD34⁺CD31⁺CD144⁺ cells derived from H1 cells in ECGM for 24 h, erythroid differentiation was induced in HCGM containing SCF (S), F3L (F), TPO (T), and with or without EPO (E). The representative data were shown from 3 independent experiments. (A) Flow cytometry of CD235a⁺CD71⁺ erythroid cells on day 15. (B) Wright Giemsa staining. Morphologic changes of erythroid cells were observed at different time points. Scale bar $\frac{1}{4}$ 10 μ m. (C) Immunofluorescent analysis of erythroid cells on day 12 and day 18. CD235a-PE and CD71-FITC antibodies were used to stain erythroid cells, and analyzed by a confocal microscopy. DAPI was used for nuclear staining. (D) Real-time qPCR analysis of *HBB* gene expression after 6 days and 15 days of erythroid differentiation. (E) *HBB* gene expression. CD235a⁺ and CD45⁺ cells were sorted on day 4, and then cultured in HCGM with EPO for 7 days. On day 11, cells were harvested, and RNAs were used for real-time RT-PCR analysis. The *HBB* gene expressions were normalized by its expression on day 4. GAPDH was used as an internal standard. Data were presented as Mean $\pm$ SD from three independent experiments.

Fig. 7.

Generation of hematopoietic clusters from transient hemogenic ECs. (A) The numbers of hematopoietic clusters either in ECGM containing VEGF and FGF2, or in HCGM containing SCF, F3L, and TPO in cultures at day 3. (B) Loss of hemogenic activity during endothelial cell culture. CD34⁺CD31⁺CD144⁺ cells were cultured in ECGM. After removal of unattached cells (0 h), the attached ECs either continue culture in ECGM or are switched to HCGM at different time points. The numbers of hematopoietic clusters were measured after 3 days in HCGM. (C) After one passage subculture of endothelial cells (P1) in ECGM, endothelial cells were induced for hematopoietic differentiation in HCGM for hematopoietic cell generation. The hematopoietic clusters were scored after 3 days in HCGM. (D) Real-time RT-PCR analysis of gene expression in endothelial cells. RNA samples were harvested from endothelial cells at different time points during HE culture. GAPDH was used as an internal standard. Data were represented as Mean $\pm$ SD from three independent experiments of BC1 cells. * P <0.05 between two groups.