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Identification of the Hemogenic Endothelial Progenitor and Its Direct Precursor in Human Pluripotent Stem Cell Differentiation Cultures

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SUMMARY

Hemogenic endothelium (HE) has been recognized as a source of hematopoietic stem cells (HSCs) in the embryo. Access to human HE progenitors (HEPs) is essential for enabling the investigation of the molecular determinants of HSC specification. Here, we show that HEPs capable of generating definitive hematopoietic cells can be obtained from human pluripotent stem cells (hPSCs) and identified precisely by a VE-cadherin$^+$CD73$^-$CD235a/CD43$^-$ phenotype. This phenotype discriminates true HEPs from VE-cadherin$^+$CD73$^+$ non-HEPs and VE-cadherin$^+$CD235a$^+$CD41a$^-$ early hematopoietic cells with endothelial and FGF2-dependent hematopoietic colony-forming potential. We found that HEPs arise at the post-primitive-streak stage of differentiation directly from VE-cadherin-negative KDR^{bright}APLNR$^+$PDGFR$\alpha^{low/-}$ hematovascular mesodermal precursors (HVMPs). In contrast, hemangioblasts, which are capable of forming endothelium and primitive blood cells, originate from more immature APLNR$^+$PDGFRα^+ mesoderm. The demarcation of HEPs and HVMPs provides a platform for modeling blood development from endothelium with a goal of facilitating the generation of HSCs from hPSCs.

INTRODUCTION

Establishing a system for de novo generation of hematopoietic stem cells (HSCs) from human pluripotent stem cells (hPSCs) would provide a unique opportunity to study human HSC development and provide a novel source of therapeutic cells for blood disease. Achieving this goal requires a detailed understanding of the cellular and molecular pathways that lead to blood formation

from hPSCs, and identification of the immediate precursors of multipotential hematopoietic cells.

Avian, mouse, and human embryonic studies demonstrated that definitive HSCs that give rise to all lineages of an adult hematopoietic system are generated in the aorta-gonad-mesonephros (AGM) region and are located at the ventral aspect of the dorsal aorta (de Bruijn et al., 2002; Ivanovs et al., 2011; Pardanaud et al., 1996; Taoudi and Medvinsky, 2007). In this area, hematopoietic cells arise from a unique population of endothelial cells known as hemogenic endothelium (HE) through an endothelial-hematopoietic transition (EHT) (Boisset et al., 2010; Jaffredo et al., 2000; Zovein et al., 2008). Dynamic tracing and imaging studies conducted in vivo demonstrated that EHT represents a continuous process in which cells with endothelial characteristics gradually acquire hematopoietic morphology and phenotype (Bertrand et al., 2010; Boisset et al., 2010; Kissa and Herbomel, 2010). Definitive hematopoiesis in the AGM region is preceded by primitive hematopoiesis in the yolk sac, which initially generates primitive erythrocytes, megakaryocytes, and macrophages (Palis et al., 1999; Xu et al., 2001). The second wave of yolk sac hematopoiesis, termed erythromyeloid hematopoiesis, is associated with an expansion of erythroid precursors expressing adult β-globins and uni- and multilineage myeloid precursors (Palis et al., 1999). Although the concept of HE was developed based on observations of blood formation within the aorta, it is also known that endothelium lining nascent capillaries in the yolk sac (Ferkowicz et al., 2003), and possibly vitelline and umbilical arteries (Yokomizo and Dzierzak, 2010), has the capacity to generate blood as well.

The demonstrations of HSC formation from endothelium emphasized the need to gain access to well-defined populations of HE cells in hPSC cultures in order to develop technologies for de novo generation of HSCs from human induced pluripotent (hiPSCs) or embryonic stem cells (hESCs). In the embryo, definitive HE can be identified based on anatomical location, morphology, and expression of Runx1 (Jaffredo et al., 2010; North et al., 1999, 2002). Because these criteria cannot be entirely applied to cells differentiated in vitro, the precise identification of HE in hPSC cultures has remained a significant

challenge. Although the VE-cadherin$^+$CD41a$^-$ or CD45$^-$ phenotype is commonly used for detection and isolation of HE, it has very limited utility in human PSC cultures because it covers the entire population of endothelial cells, does not fully exclude hematopoietic cells, and does not discriminate between endothelial lineages with primitive and definitive hematopoietic potentials. In addition, the direct mesodermal precursor of HE with definitive hematopoietic potential remains largely unknown.

In this study, we show that HE progenitors (HEPs) can be generated from hPSCs and identified precisely based on VE-cadherin (CD144) expression and the lack of CD73 and CD235a/CD43 expression. We demonstrate that HEPs represent a transient population of cells with the stroma-dependent capacity to generate the entire spectrum of myeloid progenitors, including β-hemoglobin-producing erythroid cells and pan-myeloid colony-forming cells–granulocyte, erythrocyte, macrophage, megakaryocyte (CFC-GEMM). In addition, we found that the earliest VE-cadherin$^+$CD73$^-$CD43^{low}CD235a$^+$CD41a$^-$ blood cells retain endothelial potential and possess a unique FGF2-dependent hematopoietic colony-forming activity. A population of endothelial progenitors lacking hematopoietic potential (non-HEPs) was distinctively recognized by the expression of CD73 and a high level of CD117, i.e., a VE-cadherin$^+$CD73$^+$CD235a/CD43$^-$CD117^{high} phenotype. VE-cadherin$^+$CD73$^-$CD235a/CD43$^-$ HE cells originated from VE-cadherin-negative KDR^{bright}APLNR$^+$PDGFRα$^{low/-}$ hematovascular mesodermal precursors (HVMPs), which were highly enriched in cells forming hematoendothelial clusters on OP9 stromal cells. These progenitors were distinct from the more primitive APLNR$^+$PDGFRα$^+$ mesoderm, which contains a population of hemangioblasts (HBs) that have the capacity to form colonies composed of primitive-type blood cells through endothelial intermediates in serum-free semisolid medium.

RESULTS

Identification of Functionally Distinct Progenitors with Hematopoietic and/or Endothelial Potential within Emerging Embryonic VE-Cadherin$^+$ Cells Based on Expression of CD73 and CD235a

To characterize the development of various mesodermal lineages, we employed an hPSC differentiation system in coculture with OP9 (Choi et al., 2009a; Vodyanik et al., 2006, 2010). In this culture, we previously identified CD43 as a marker for hPSC-derived progenitors that have the potential to form hematopoietic cytokine-dependent colonies in semisolid medium, and demonstrated that CD43 expression separates hematopoietic cells from endothelial cells (Choi et al., 2009a; Vodyanik et al., 2006). To investigate the developmental steps immediately preceding the formation of CD43$^+$ blood cells, and map the point of divergence of hematopoietic and endothelial cell lineages, we analyzed the kinetic expression of various endothelial markers following H1 hESC differentiation in OP9 coculture. The first cells that expressed the VE-cadherin endothelial marker (Breier et al., 1996) were detected by day 4 of differentiation (Figures 1A and S1A). Upregulation of VE-cadherin expression on differentiated hESCs in OP9 coculture coincided with the expression of another endothelial marker, CD31 (PECAM) (Figure 1A). Of

interest, cells expressing CD235a (Glycophorin A), a hematopoietic marker of erythroid lineage, could be detected within the first emerging VE-cadherin$^+$ cells (Figure S1B). On the next day of differentiation (day 5), the number of VE-cadherin$^+$ cells and the proportion of CD235a$^+$ cells within this population substantially increased. All of the VE-cadherin$^+$CD235a$^+$ cells were negative for CD41a (abbreviated as V$^+$235$^+$41$^-$ cells) on day 4 of differentiation. However, on day 5 of differentiation, a small proportion of CD235a$^+$ cells coexpressing CD41a (V$^+$235$^+$41$^+$ cells) could be detected (Figures 1B and S1B). Although V$^+$235$^+$41$^+$ cells expressed a high level of CD43, which indicates hematopoietic commitment (Vodyanik et al., 2006), expression of CD43 in V$^+$235$^+$41$^-$ cells was relatively low and was best detectable with antibodies conjugated with APC or PE (Figure S1C). Thus, we combined CD235a and CD43 antibodies in our studies to achieve optimal pan-hematopoietic detection at all stages of differentiation.

A phenotypic analysis of day 5 VE-cadherin$^+$ cells revealed almost uniform expression of CD31, KDR, CD34, CD201, ESAM, and CD146 endothelial markers by these cells. However, we noticed that another typical endothelial marker, CD73 (or 5′-nucleotidase; Thomson et al., 1990), was expressed only in 20%–60% of total VE-cadherin$^+$ cells almost exclusively within the 235a/CD43$^-$ population (Figures 1B, S1B, and S1D). This observation led us to identify three distinct major subsets within emerging VE-cadherin$^+$ cells: V$^+$235$^+$41$^-$, V$^+$73$^+$, and V$^+$73$^-$235$^-$ (Figure 1B; Table 1). A kinetic analysis revealed that V$^+$73$^-$235$^-$cells represent a transient population that develops during the earliest stages of endothelial commitment but is mostly lost within the next 3 days of differentiation. The V$^+$73$^+$ population was minor at the onset of endotheliogenesis but gradually increased with advanced differentiation. The proportion of VE-cadherin$^+$ cells expressing 235a and/or CD43 hematopoietic markers peaked on day 5 of differentiation and then decreased (Figure S1B).

As demonstrated in Figure 1B, all three major VE-cadherin$^+$ cell subsets had very similar endothelial phenotypes and were capable of acetylated low-density lipoprotein (AcLDL) uptake, indicative of endothelial function. However, we noticed that expression of CD117 (c-Kit), a marker for early-stage angiohematopoietic progenitors, was highest in V$^+$73$^+$ cells, and its expression was almost undetectable in V$^+$235$^+$41$^-$ cells. V$^+$73$^-$235$^-$ cells expressed an intermediate level of CD117 (Figure 1B). We also found that, in contrast to other day 5 VE-cadherin$^+$ subsets, V$^+$73$^+$ cells lacked the expression of CD226 (DNAM-1), a cell surface marker typically found on hematopoietic cells (Kojima et al., 2003; Shibuya et al., 1996). Morphologically, the V$^+$235$^+$41$^-$ population consisted predominantly of cells with a high nuclear/cytoplasmic ratio, which is typical for immature hematopoietic cells. In contrast, almost all V$^+$73$^+$ cells had a characteristic endothelial morphology. V$^+$73$^-$235$^-$ cells had an intermediate morphology resembling those of both V$^+$235$^+$41$^-$ and V$^+$73$^+$ cells, i.e., pale blue cytoplasm similar to endothelial cells, and a higher nuclear/cytoplasmic ratio similar to immature hematopoietic cells (Figure 1B).

To fully analyze the differentiation potential of the newly discovered VE-cadherin$^+$ cell subsets, we isolated each one by fluorescence-activated cell sorting (FACS), cultured it in
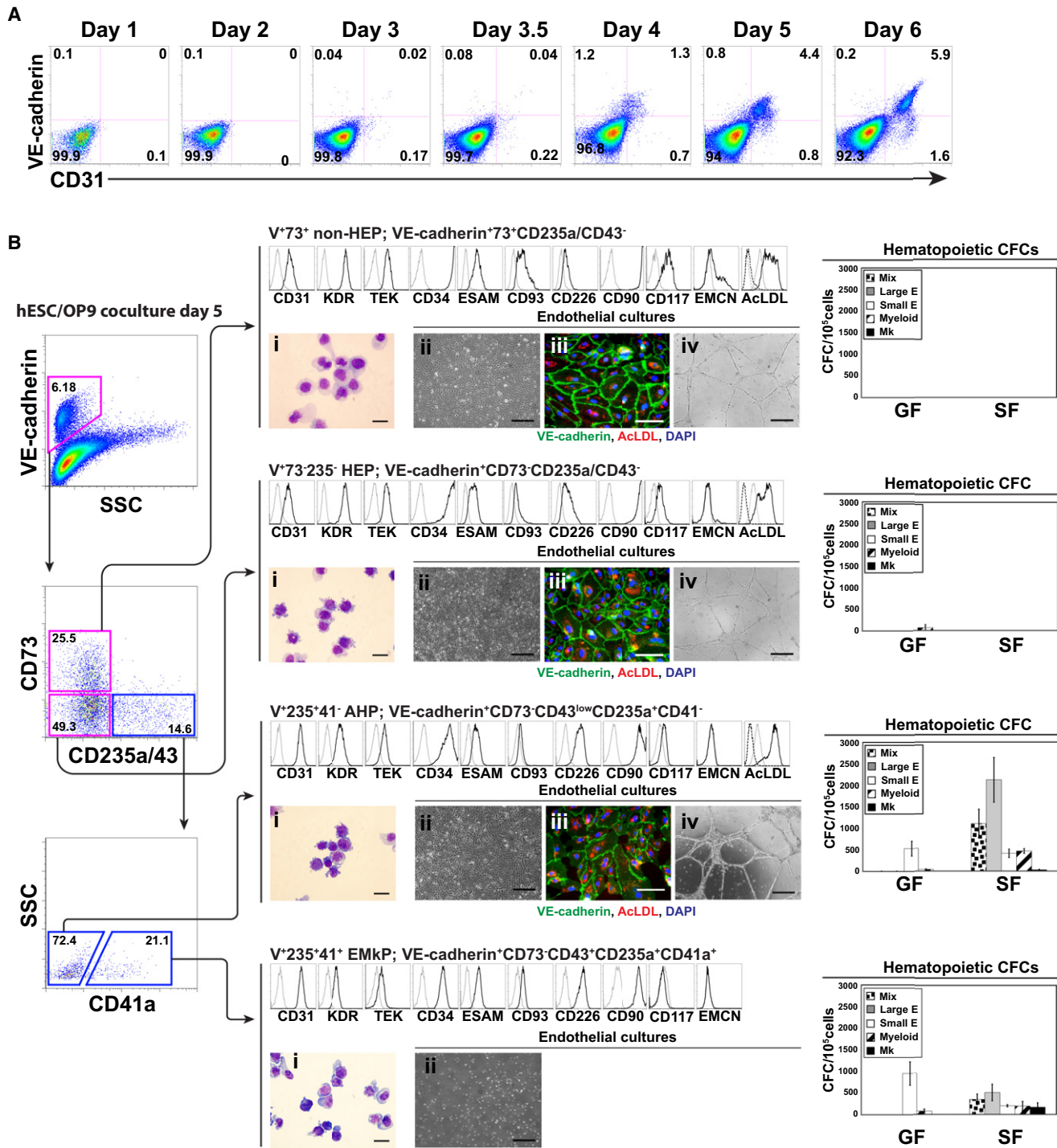
Figure 1. Characterization of Major Subsets of VE-Cadherin+ Cells Generated from hESCs after 5 Days of Coculture on OP9

(A) Kinetics of VE-cadherin and CD31 expression in differentiated H1 hESCs.

(B) Characterization of endothelial and hematopoietic CFC potentials of freshly isolated day 5 VE-cadherin+ subsets. Histograms represent the expression of typical endothelial molecules by indicated cell subsets. AcLDL histograms show flow cytometric profiles of cells incubated with AcLDL at 37°C (AcLDL uptake; black histogram) versus 4°C (AcLDL binding control; gray histogram). (i) Wright-stained cytospins demonstrate the morphology of isolated cells (bar = 20 μm). Endothelial culture panels show (ii) phase-contrast images (bar = 400 μm), (iii) immunofluorescent analysis (bar = 100 μm), and (iv) tube formation (bar = 400 μm). The hematopoietic CFC potential of sorted day 5 VE-cadherin+ subsets was evaluated in serum-free MethoCult (SF) supplemented with FGF2, SCF, IL6, IL3, and EPO, and in standard serum-containing GF+ H4435 MethoCult. Error bars are means ± SE of three experiments.

See also Figures S1 and S3.

Table 1. Phenotypic Features and Definition of Subsets with Endothelial and/or Hematopoietic Potential from hPSCs Analyzed and Characterized in This Study

Abbreviation	Phenotype	Day of Isolation[a]	Definition
V^{+}73^{−}235^{−} HEP	VE-cadherin^{+}CD73^{−}CD235a/CD43^{−}CD117[intermediate]	5	Hemogenic endothelial progenitors that have primary endothelial characteristics and lack hematopoietic CFC potential and surface markers, but are capable of generating blood and endothelial cells upon coculture with stromal cells
V^{+}73^{+} Non-HEP	VE-cadherin^{+}CD73^{+} CD235a/CD43^{−}CD117^{high}	5	Nonhemogenic endothelial progenitors that have all of the functional and molecular features of endothelial cells, and form endothelial colonies on OP9
V^{+}235^{+}41^{−} AHP	VE-cadherin^{+}CD73^{−}CD43^{low} CD235a^{+}CD41a^{−}CD117^{−}	5	Angiogenic hematopoietic progenitors that possess primary hematopoietic characteristics and FGF2 and hematopoietic cytokine-dependent colony-forming potential in serum-free medium, but are capable of generating endothelial cells
V^{+}235^{+}41^{+} EMkP	VE-cadherin^{+}CD73^{−}CD43^{+} CD235a^{+}CD41a^{+}	5	Hematopoietic cells that are enriched in erythromegakaryocytic progenitors
MHP	lin^{−}CD34^{+}CD43^{+}CD45^{+}CD38^{−}	8	Multipotential hematopoietic progenitors that lack expression of lineage-specific hematopoietic markers (lin^{−}) and form a full spectrum of myeloid colonies in serum-containing semisolid medium supplemented with hematopoietic cytokines
K[br]A[+]P[−] HVMP	[EMH]lin[−]KDR[bright]APLNR[+] PDGFRα[low/− b]	4	Hematovascular mesodermal precursors that express genes associated with lateral plate/extraembryonic mesoderm and angiohematopoietic commitment, but lack the expression of primitive streak genes. These cells are highly enriched in bipotential cells that form hematoendothelial clusters on OP9.
A[+]P[+] PM	[EMH]lin[−]APLNR[+]PDGFRα[+ b]	3	Primitive posterior mesoderm enriched in cells that express a typical primitive streak and lateral plate/extraembryonic mesoderm genes. These cells have potential to form FGF2-dependent blast (hemangioblast) colonies in serum-free medium.

[a] Day of isolation indicates the day of hPSC differentiation in coculture with OP9 on which the indicated cell subsets were optimally detected and isolated from cultures.
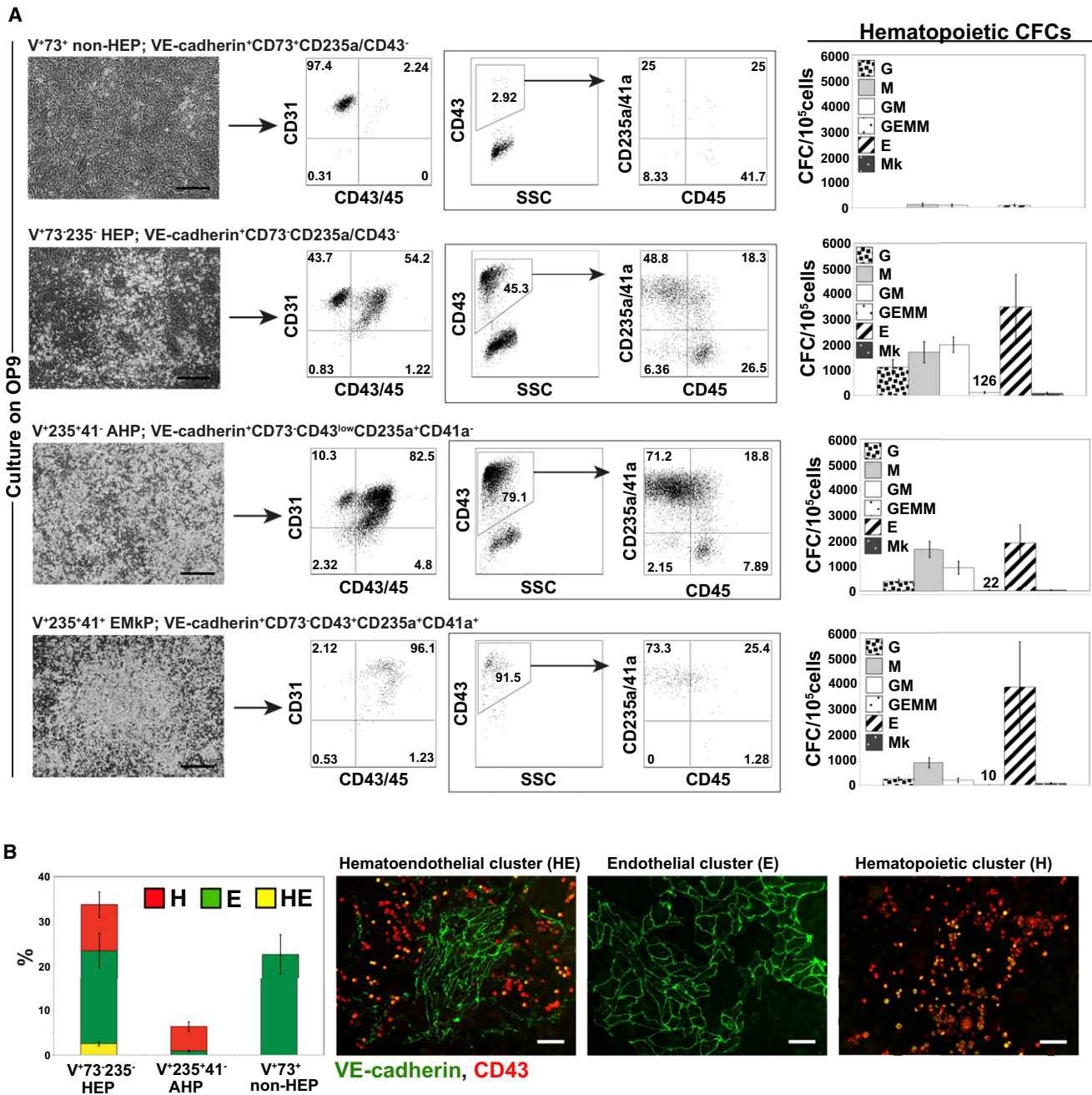[b] [EMH]lin[−] denotes lack of expression of CD31, VE-cadherin endothelial, CD73 and CD105 mesenchymal/endothelial cell markers, and CD43 and CD45 hematopoietic cell markers. See also Figure S2.

endothelial conditions, and assayed it for hematopoietic colony-forming activity (Figure S2). As shown in Figure 1B, the three major day 5 VE-cadherin^{+} subsets (V^{+}73^{−}235^{−}, V^{+}235^{+}41^{−}, and V^{+}73^{+} cells), but not the minor V^{+}235^{+}CD41^{+} subset, formed a monolayer of adherent cells with endothelial morphology when cultured on fibronectin in endothelial growth medium. Consistent with their endothelial nature, these cells expressed VE-cadherin, took up AcLDL, and formed vascular tubes in Matrigel matrix. In contrast, hematopoietic CFC potential was detected almost exclusively within V[+]235[+]41[−] and V[+]235[+]41^{+} cells. Although the hematopoietic CFC potential of V[+]235[+]41^{−} cells in standard serum-based CFC medium was low and mostly restricted to small CFC-E, we found that the number and spectrum of hematopoietic CFCs were markedly increased in serum-free medium containing FGF2, SCF, EPO, IL-3, and IL-6. In the serum-free conditions, day 5 V[+]235[+]41^{−} cells formed large erythroid, megakaryocyte, myeloid, and mixed colonies composed of erythroid cells, macrophages, and megakaryocytes, indicating that emerging blood cells expressing the CD235a erythroid marker

had multilineage potential (Figures S3A and S3B). To define which growth factors are required for V[+]235[+]41^{−} cells to form hematopoietic colonies, we eliminated each cytokine individually from clonogenic cultures. These experiments demonstrated that both FGF2 and EPO were essential for the development of large CFC-E and CFC-Mix (Figure S3C). The removal of SCF almost entirely abrogated CFC-Mix, but had little effect on large CFC-E. Myeloid colonies required IL-3 and FGF2 for optimal development. The day 5 V[+]235[+]41^{+} cells formed predominantly CFC-E and CFC-Mix; however, they had downregulated expression of APLNR and TEK, and failed to grow into endothelial cells in endothelial conditions, indicating that the acquisition of CD41a expression was associated with the complete loss of endothelial potential (Figures 1B and S1D).

The hemogenic potential of embryonic endothelial cells can be identified in culture with bone marrow stromal cells (Nishikawa et al., 1998; Oberlin et al., 2002); thus, we cultured day 5 VE-cadherin^{+} subsets on OP9 separately. In these conditions, both V^{+}73^{−}235^{−} and V^{+}235^{+}41^{−} cells generated CD31^{+}CD43/45^{−}

A

Hematopoietic CFCs

V^{+}73^{+} non-HEP; VE-cadherin^{+}CD73^{+}CD235a/CD43^{−}

V^{+}73^{−}235^{−} HEP; VE-cadherin^{+}CD73^{−}CD235a/CD43^{−}

V^{+}235^{+}41^{−} AHP; VE-cadherin^{+}CD73^{−}CD43^{low}CD235a^{+}CD41a^{−}

V^{+}235^{+}41^{+} EMkP; VE-cadherin^{+}CD73^{−}CD43^{+}CD235a^{+}CD41a^{+}

B

VE-cadherin, CD43

Hematoendothelial cluster (HE)   Endothelial cluster (E)   Hematopoietic cluster (H)

Figure 2. Stroma-Dependent Hematopoietic and Endothelial Potential of Day 5 VE-Cadherin[+] Subsets

(A) Phase-contrast images of cultures (bar = 400 μm), flow cytometric analysis and hematopoietic CFC potential (GF+ H4435 serum-containing MethoCult) are shown. Error bars are means ± SE of three experiments. The numbers show mean counts for CFC-GEMM.

(B) A single-cell deposition assay to detect the frequency of endothelial, hematopoietic, and bipotential hematoendothelial progenitors. Immunofluorescent staining of clusters formed by single cells after 10–12 days of culture on OP9 using CD43 and VE-cadherin antibodies is shown (bar = 100 μm). Graph shows the frequency of each type of progenitor as a percentage of cluster-containing wells versus total cell-deposited wells. Error bars are means ± SE of three experiments.

endothelial cells and a significant amount of CD43^{+} blood cells (Figure 2A). The CD43^{+} cells consisted of CD235a/CD41a^{+} erythromegakaryocytic cells and CD235a/CD41a^{−}CD45^{+/−} multipotent hematopoietic progenitors (MHPs), which we typically observe from hESCs differentiated on OP9 for 8–9 days (Vodya-nik et al., 2006). These day 8–9 CD235a/CD41a^{−}CD45^{+/−} MHPs express CD34 but lack CD38 and other hematopoietic lineage markers, i.e., they have a lin^{−}CD34^{+}CD43^{+}CD45^{+/−}CD38^{−} phenotype (Vodyanik et al., 2006). Although both V^{+}73^{−}235^{−} and V^{+}235^{+}41^{−} cells generated a broad range of hematopoietic

colonies in standard serum-containing CFC medium after culture on OP9, the $V^+73^-235^-$ cells formed a higher number of myeloid colonies, including large multicentric pan-myeloid GEMM colonies. In contrast, V^+73^+ cells formed mostly $CD31^+CD43/45^-$ endothelial cells with very few hematopoietic cells (Figure 2A).

To determine the frequency of progenitors with hematopoietic and endothelial potential in each day 5 VE-cadherin$^+$ subset, we performed a single-cell deposition assay. As shown in Figure 2B, V^+73^+ single cells generated only endothelial clusters on OP9 with a frequency of approximately one in five, and $V^+235^+41^-$ cells formed predominantly hematopoietic cell clusters. Although the majority of $V^+73^-235^-$ cells gave rise to either hematopoietic or endothelial clusters, 2.5% of them had the potential to form hematoendothelial clusters, indicating the presence of bipotential progenitors within this population.

Based on the functional and phenotypical properties of each VE-cadherin$^+$ subset, we defined them as follows: (1) $V^+73^-235^-$ are HEPs that have primary endothelial characteristics but lack hematopoietic CFC potential and surface markers, express an intermediate level of CD117, and are capable of generating blood and endothelial cells upon coculture with stromal cells; (2) $V^+235^+41^-$ are angiogenic hematopoietic progenitors (AHPs) that possess primary hematopoietic characteristics but are capable of generating endothelial cells; and (3) V^+73^+ are non-HEPs that have all the functional and molecular features of endothelial cells, form endothelial colonies on OP9, and express a high level of the early progenitor marker CD117 (see also Table 1).

Molecular profiling studies revealed a high similarity between the day 5 VE-cadherin$^+$ population subsets. All subpopulations of these cells expressed the typical endothelial genes (*TFP*, *HIF1A*, *AAMP*, *F2R*, *EDF1*, and *PROCR*) and the genes associated with angiohematopoietic and HSC development (*FLI1*, *TEK*, *LMO2*, *TAL1*, *RUNX1*, *CBFB*, *PBX1*, *PTEN*, and *TCEA1*). However, $V^+235^+41^-$ AHPs expressed higher levels of hematopoietic-specific genes and lower levels of the typical endothelial (*CAV1*, *CTGF*, *APOLD1*, and *AMOT*) and endothelial junction (*CDH5*, *CDH2*, and *CLDN5*) genes (Figure 3A). In contrast, $V^+73^-235^-$ HEPs expressed higher levels of the endothelial genes *CLDN5*, *CAV1*, and *MMRN1N*, and lacked the expression of hematopoietic genes. In comparison with the HEPs, the V^+73^+ non-HEPs expressed higher levels of the endothelial genes *EMCN*, *CAV1*, *CXCR4*, *CLDN5*, and *COL15A1* (Figure S4A). Genes that were found to be more highly expressed in HEPs versus non-HEPs included *NTS* neurotensin; *BMPER*, an endothelial regulator that controls BMP4-dependent angiogenesis (Heinke et al., 2008); and *SMAD6*, a negative regulator of BMP signaling (Ishida et al., 2000) and RUNX1 activity (Knezevic et al., 2011).

### Hemogenic Endothelial Cells Originate from a Unique ^{EMH}lin-KDR^{bright}APLNR$^+$PDGFR$\alpha^{low/-}$ Population of Mesodermal Cells with Hematovascular Potential

To identify the direct mesodermal precursor of HE cells, we analyzed the expression of the mesodermal markers APLNR (D'Aniello et al., 2009; Vodyanik et al., 2010), KDR (Shalaby et al., 1997), and PDGFRα (CD140a) (Kataoka et al., 1997) in differentiated hESCs before the first VE-cadherin$^+$ cells could

be detected. This analysis revealed the population of KDR^{bright} APLNR$^+$ cells that was initially detected on day 3.5 of differentiation (Figure 4A) immediately preceding the formation of the first VE-cadherin$^+$ cells in hESC/OP9 coculture (Figure 1A). Emerging day 3.5 KDR^{bright}APLNR$^+$ cells essentially lacked the typical CD31, VE-cadherin endothelial, CD73, CD105 mesenchymal/endothelial, and CD43, CD45 hematopoietic cell markers (hereafter referred to as ^{EMH}lin$^-$ cells); however, the early VE-cadherin$^+$ cells became clearly detectable within this population from day 4 of differentiation (Figure 4B). A flow cytometric analysis of day 4 VE-cadherin$^-$KDR^{bright}APLNR$^+$ cells revealed that they maintained the ^{EMH}lin$^-$ phenotype (^{EMH}lin$^-$KDR^{bright} APLNR$^+$). However, in contrast to the more-primitive day 2 and day 3 APLNR$^+$ (Vodyanik et al., 2010) and day 4 KDR^{dim} mesodermal cells, day 4 ^{EMH}lin$^-$KDR^{bright}APLNR$^+$ cells had downregulated expression of PDGFRα (Figure 4B). Although these day 4 ^{EMH}lin$^-$KDR^{bright}APLNR$^+$PDGFRα$^{low/-}$ (K^{br}A$^+$P$^-$) cells lacked the most specific endothelial markers, VE-cadherin and CD31, they expressed other markers typically found on endothelial cells, including TEK, CD34, CD201, and CD146 (Figures 4B and S5A), suggesting that these mesodermal cells could be direct precursors of endothelial progenitors in hESC cultures. To confirm our hypothesis, we isolated day 4 K^{br}A$^+$P$^-$, KDR^{dim}, and KDR$^-$ cells using flow cytometry (Figure 4C) and cultured them on OP9.

As shown in Figure 4C, after 5–6 days of culture on OP9, only K^{br}A$^+$P$^-$ cells generated both $CD31^+CD43/45^-$ endothelial cells and $CD43/CD45^+$ hematopoietic cells, whereas KDR^{dim} cells predominantly generated $CD146^+CD31^-$ mesenchymal cells, few endothelial cells, and almost no blood cells. KDR$^-$ cells lacked hematovascular potential completely. Of importance, day 4 K^{br}A$^+$P$^-$ cells generated the $V^+73^-235^-$, $V^+235^+41^-$, and V^+73^+ subsets we observed on day 5 of primary hESC/OP9 coculture (Figure 4C). It should be also noted that K^{br}A$^+$P$^-$ cells were multipotential and capable of differentiating into $CD146^+$ $CD31^-$ mesenchymal cells in addition to blood and endothelial cells (Figure 4C). Double staining of K^{br}A$^+$P$^-$ cells grown on OP9 with VE-cadherin and CD43 antibodies revealed that they formed HE clusters, i.e., sheets of endothelial cells generating nonadherent blood cells (Figure 4D). Morphological examination of HE clusters at different stages of development revealed that endothelial cells within these clusters gradually transitioned into hematopoietic cells. During the early stages of transition, VE-cadherin$^+$ cells had upregulated CD43 expression and transformed from a cuboidal to a round cell morphology (Figure 4D; Movie S1). Single-cell deposition experiments demonstrated that K^{br}A$^+$P$^-$ cells formed HE clusters at a high frequency (approximately one in 10 cells), strongly indicating that these cells represent the direct precursors of HE (Figure 4E).

To confirm that K^{br}A$^+$P$^-$ cells are direct precursors of the HEPs, we isolated these cells from day 3.5 of hESC/OP9 cocultures, before VE-cadherin$^+$ cells became detectable (see Figure 1A), and recultured them on OP9 for 2 days. A flow cytometric analysis of these K^{br}A$^+$P$^-$ secondary cultures revealed that they had upregulated VE-cadherin expression and differentiated into the $V^+73^-235^-$, $V^+235^+41^-$, and V^+73^+ subsets we observed on day 5 of primary hESC/OP9 coculture (Figure S5B). When we isolated these subsets from the secondary cocultures
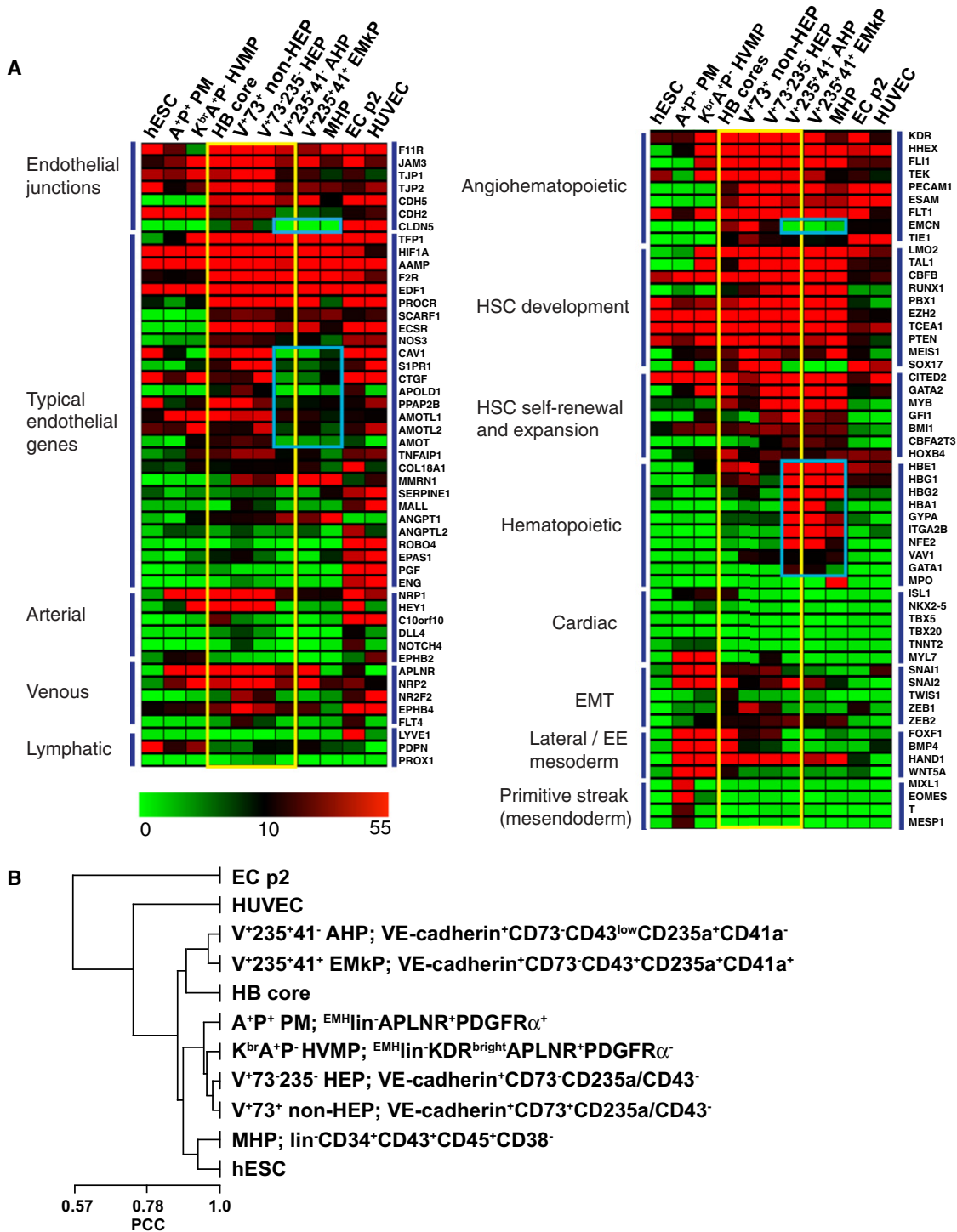
Figure 3. Gene Expression Profiling of Undifferentiated H1 hESCs, Differentiated H1 hESC Populations, and Human Umbilical Endothelial Cells

(A) Heat maps of selected genes associated with endothelial and hematopoietic cells, EMT, lateral plate/EE mesoderm, and primitive streak. HB cores are endothelial intermediates formed by HBs in serum-free clonogenic medium. The gene expression levels are estimated in transcripts per million (tpm). EC p2, second passage of endothelial cells obtained from day 8 CD31⁺CD43⁻ differentiated H1 cells; EE, extraembryonic; EMT, epithelial-mesenchymal transition; HUVEC, human umbilical vein endothelial cells. See Table 1 for other abbreviations.

(B) Pearson correlation analysis of global gene expression.

See also Figure S4.
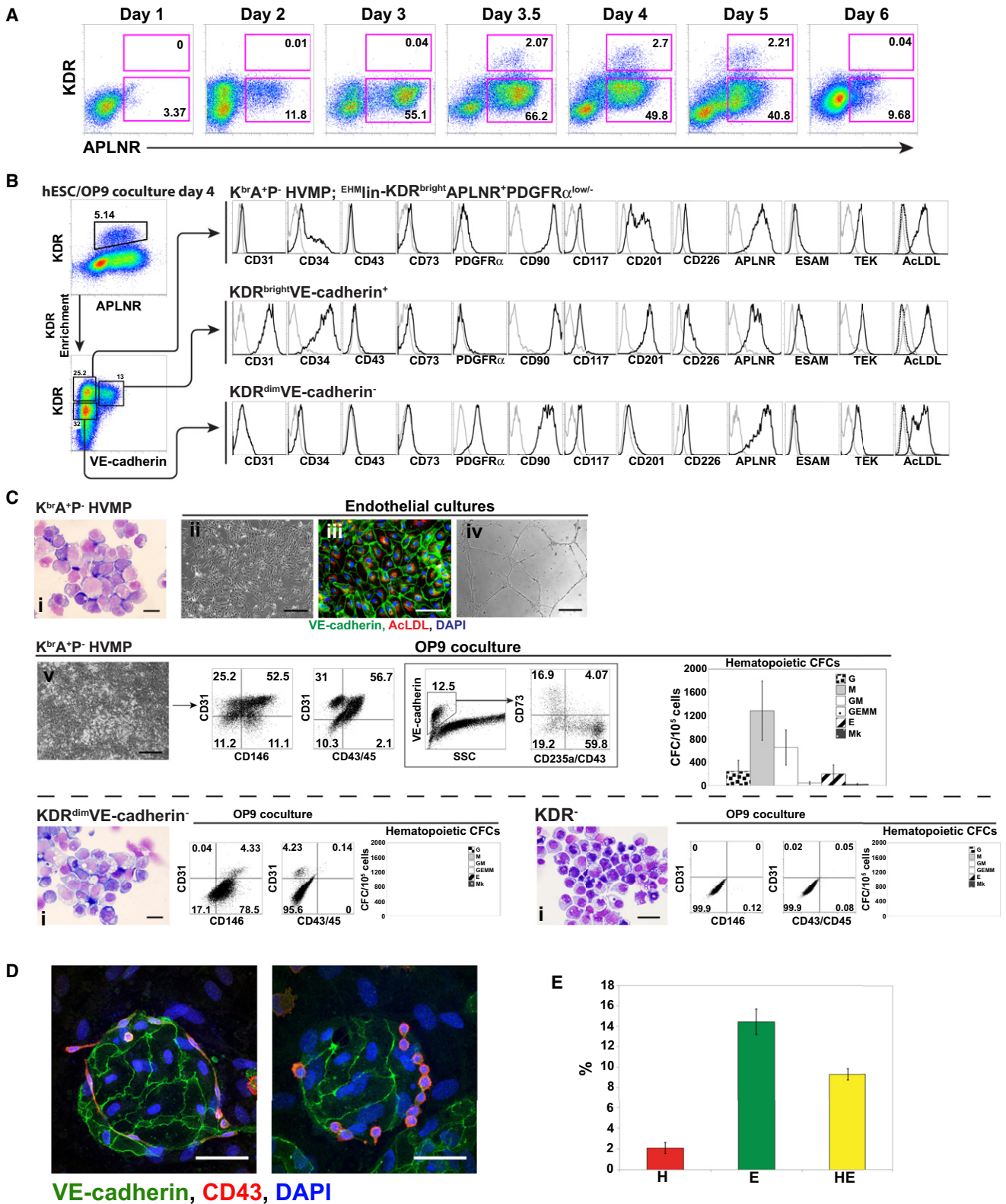
Figure 4. Phenotypic and Functional Characterization of Major Subsets of Day 4 hESC-Derived Mesodermal Cells

(A) Kinetics of expression of APLNR and KDR in differentiated H1 hESCs.

(B) Flow cytometric analysis of day 4 hESC-derived mesodermal cells that were first magnetically sorted for the KDR+ population.

by FACS and analyzed them for endothelial and hematopoietic potentials, we found that the $V^+73^-235^-$, $V^+235^+41^-$, and V^+73^+ cells generated from isolated day 3.5 $K^{br}A^+P^-$ cells had the same hematopoietic and endothelial differentiation potentials as the primary day 5 HEP, AHP, and non-HEP subsets, respectively (Figure S5C).

A morphologic analysis revealed that $K^{br}A^+P^-$ cells were large, blast-like cells that were noticeably different from KDR^{dim} and KDR^- cells, which had a more abundant and vacuolated cytoplasm (Figure 4C). Molecular profiling studies revealed that in $K^{br}A^+P^-$ cells, expression of transcriptional regulators of hematopoietic and endothelial development (LMO2, TAL1, CBFB, GATA2, and FLI1) was upregulated, whereas expression of the primitive streak genes MIXL1, EOMES, T, and MESP1 was downregulated (Figure 3A). However, these cells retained high expression levels of genes representing lateral plate/extraembryonic mesoderm (FOXF1, BMP4, and WNT5A). Based on their phenotypic features, gene expression profile, morphology, and functional properties, we designated $K^{br}A^+P^-$ mesodermal cells as HVMPs. These precursors may resemble embryonic angioblasts, which are defined as cells that have not yet formed a lumen and express certain (but not all) endothelial markers. They are committed to differentiate into endothelial cells and give rise to vascular primordia (Risau and Flamme, 1995).

BL-CFCs Represent Angiohematopoietic Progenitors with Primitive Hematopoietic Potential Originating from $^{EMH}lin^-APLNR^+PDGFR\alpha^+$ Mesoderm

Blast CFCs (BL-CFCs) were identified by the Keller group as progenitors that generate blast colonies composed of cells with hematopoietic and endothelial potential (Choi et al., 1998). Widely referred to as HBs, BL-CFCs represent the earliest cells with detectable hematopoietic potential in mouse and human ESC differentiation systems (Choi et al., 1998; Kennedy et al., 2007). However, the exact position of HBs (BL-CFCs) within the hierarchy of human angiohematopoietic cells and their developmental potential remains unclear. Various studies have described BL colonies from differentiated hESCs at early stages of mesodermal development (Davis et al., 2008; Kennedy et al., 2007), as well as from cells at more advanced stages of differentiation, including cells already expressing endothelial markers (Lu et al., 2008; Zambidis et al., 2008). Moreover, two types of HB colonies (one with and one without myeloid potential) were recently described (Kennedy et al., 2007).

We previously demonstrated that BL-CFCs arise from a day 2–3 $^{EMH}lin^-APLNR^+PDGFR\alpha^+$ (A^+P^+) mesodermal population that expresses genes associated with primitive streak and lateral plate/extraembryonic mesoderm development reminis-

cent of primitive posterior mesoderm (PM) in the embryo (Vodyanik et al., 2010). We showed that BL-CFCs could be detected using serum-free FGF2-containing clonogenic medium (Vodyanik et al., 2010). Here, we also found that we could increase the number of BL-CFCs by adding the APLNR ligand apelin-12 to the clonogenic medium (Figure 5B). Although hematopoietic cytokines are commonly added to BL-CFC clonogenic medium, we avoided using them in our assay so that we could increase its specificity by eliminating false-positive results due to the detection of hematopoietic progenitors. Using an optimized BL-CFC-specific assay with FGF2 and apelin-12, we detected BL-CFC activity almost exclusively in day 3 A^+P^+ cells (Figure 5F), indicating that HBs (BL-CFCs) are distinct from day 4 HVMPs and day 5 HEPs and AHPs.

To characterize the developmental potential of BL-CFCs, we analyzed the mature BL colonies using flow cytometry and hematopoietic CFC assay. As shown in Figure 5A, HB (BL) colonies collected on day 12 of clonogenic culture consisted almost entirely of CD235a and/or CD41a expressing cells with erythroblast morphology. In contrast to erythroid colonies generated from $V^+73^-235^-$ HEPs, BL-CFCs expressed no adult β hemoglobin (Figure 5C). The replating of 20 individual blast colonies into serum-free hematopoietic clonogenic medium demonstrated that they could give rise to erythroid, megakaryocyte, and macrophage colonies, and mixed colonies composed of all three cell types (Figures 5D and 5E). When a pool of 200 blast colonies was collected, we were able to detect the same spectrum of hematopoietic CFCs. The spectra of hematopoietic CFCs were similar when BL-CFCs were replated into standard serum-containing hematopoietic CFC medium, although we observed a reduction in the number of erythroid colonies and a slight increase in macrophage colonies (not shown). These results indicate that BL-CFC hematopoietic potential is mostly restricted to primitive cells of erythromegakaryocytic and macrophage lineages.

As previously demonstrated, BL-CFCs represent single-cell-derived clonogenic progenitors that generate hematopoietic cells through the formation of an endothelial intermediate (Lancrin et al., 2009; Vodyanik et al., 2010). This transitional intermediate appeared as a core-like structure that formed during the first 3 days of clonogenic culture (Figure 5A) and distinguished HB colonies from FGF2-dependent hematopoietic colonies formed from day 5 AHPs (Figure S6A). HB cores were formed by epithelioid cells, which stained positively for VE-cadherin. However, in contrast to the membranous VE-cadherin expression typically seen in mature endothelial cells, VE-cadherin expression in the HB core cells was predominantly cytoplasmic. When HB cores were cultured in endothelial conditions, up to

(C) Endothelial and hematopoietic differentiation potential of indicated day 4 mesodermal subsets. (i) Wright-stained cytospins of freshly isolated cells (bar = 20 μm). Endothelial culture panels show (ii) phase-contrast images (bar = 400 μm), (iii) immunofluorescent analysis (bar = 100 μm), and (iv) tube formation (bar = 400 μm) by $K^{br}A^+P^-$ cells. (v) Phase-contrast image of $K^{br}A^+P^-$ cell cultures on OP9. Flow cytometric analysis shows a developmental potential of KDR subsets after secondary OP9 coculture. Hematopoietic CFC potential of KDR subsets was evaluated after secondary coculture on OP9 using standard H4435 GF+ serum-containing MethoCult. Error bars are means ± SE of three experiments.

(D) Confocal images of hematoendothelial clusters to demonstrate the early stages of endothelial-hematopoietic transition; bar = 50 μm.

(E) A single-cell deposition assay was used to detect the frequency of endothelial (E), hematopoietic (H), and bipotential hematoendothelial (HE) progenitors within $K^{br}A^+P^-$ HVMPs. The graph shows the frequency of each type of progenitor as a percentage of cluster-containing wells versus total cell-deposited wells. Error bars are means ± SE of three experiments.

See also Figure S5.
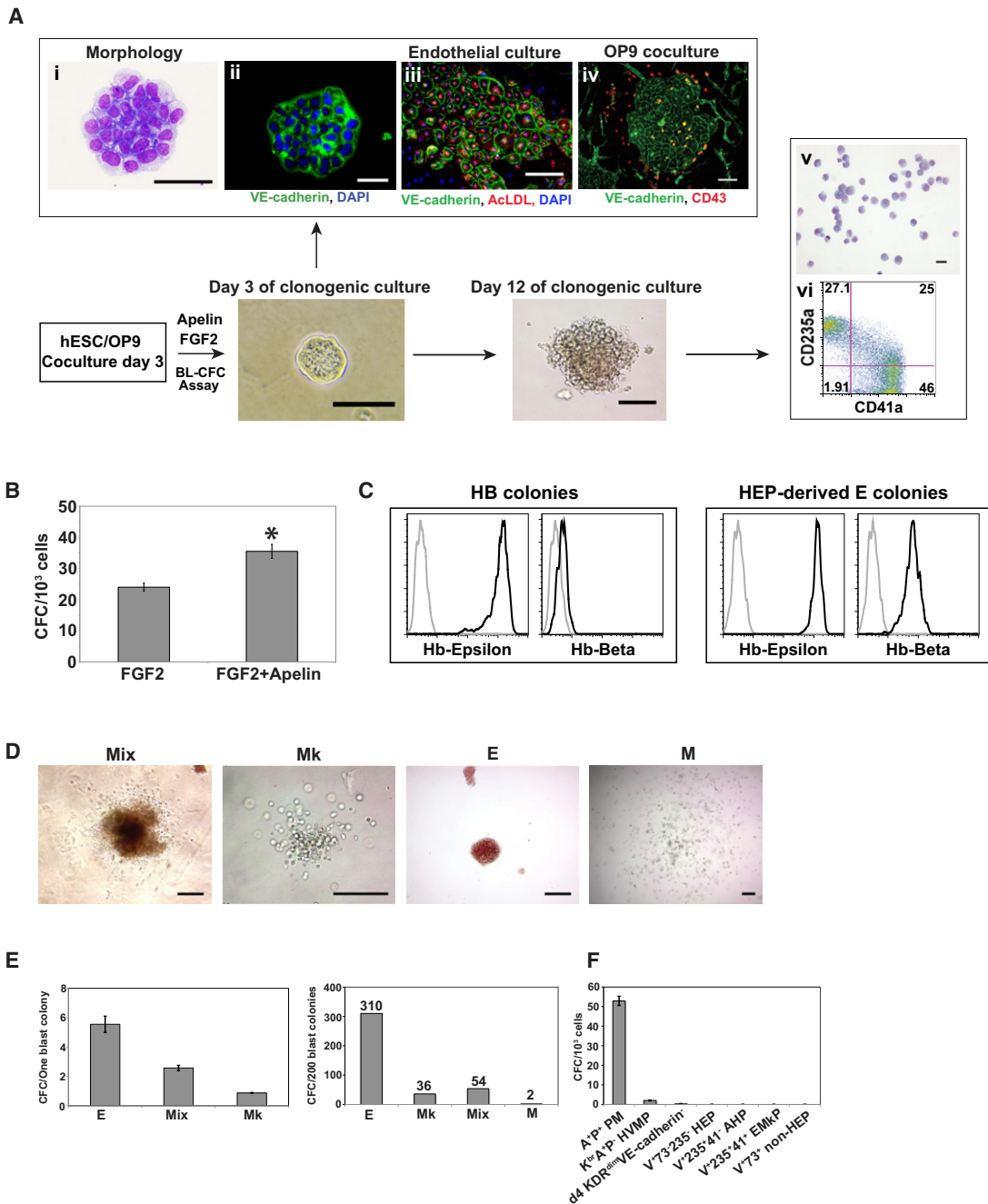
Figure 5. Characterization of BL-CFCs

(A) Hematopoietic and endothelial potential of HB colonies selected at day 3 (core stage; bar = 50 μm) and day 12 (mature blast colony; bar = 100 μm) of clonogenic culture. Top panels show (i) Wright-stained cytospins (bar = 100 μm), (ii) VE-cadherin and AcLDL staining (bar = 50 μm), (iii) endothelial culture (bar = 100 μm), and (iv) OP9 coculture (bar = 100 μm) of HB cores. Left panels show (v) cytospins (bar = 20 μm) and (vi) flow cytometric analysis of mature HB colony.

(B) Effect of adding apelin-12 on BL colony formation. Error bars are means ± SE of six experiments.

(C) Flow cytometric analysis of expression of adult and embryonic hemoglobins in HB colonies and erythroid colonies derived from $V^+73^-235^-$ HEPs after coculture on OP9.

(D) Morphology of typical hematopoietic colonies generated from day 12 BL colonies after they were replated into H4436 serum-free methylcellulose clonogenic medium containing hematopoietic cytokines; bars = 200 μm.

(E) Frequency of formation of hematopoietic colonies after HB colonies were replated into serum-free hematopoietic CFC medium. Left panel: Results of replating 20 individual colonies; error bars are means ± SE of three experiments. Right panel: Results of replating 200 blast colonies.

(F) BL-CFC potential of the indicated cell subsets detected using FGF2- and apelin 12-containing clonogenic medium. Error bars represent SEs of three experiments. See also Figure S6.

95% of them generated typical VE-cadherin⁺ endothelial clusters that were capable of incorporating AcLDL efficiently. When the HB cores were collected and cultured on OP9 with hematopoietic cytokines, they generated hematoendothelial clusters (Figure 5A).

Molecular profiling studies demonstrated that HB cores had a gene expression profile very similar to that of day 5 HEPs, although the HB cores had much lower expression of *RUNX1* gene associated with definitive hematopoiesis compared with day 5 HEPs (Figures 3A and S4B). Together, these studies indicate that BL-CFCs originate from the more-primitive A⁺P⁺ PM and reflect the first wave of yolk sac hematopoiesis, which proceeds through the endothelial intermediate stage with restricted erythroid, megakaryocytic, and macrophage potential.

To find out whether the hematopoietic potential of A⁺P⁺ PM is restricted to primitive HB-derived hematopoiesis or these cells contain precursors of definitive angiohematopoietic progenitors, we isolated day 2.5 A⁺P⁺ cells and recultured them on OP9. At this stage, K^br A⁺P⁻ cells were not detected. As shown in Figure S6B, A⁺P⁺ cells rapidly expanded on OP9 and generated the entire spectrum of angiohematopoietic and hematopoietic progenitors, which we typically observed in primary hESC/OP9 coculture. These data indicate that A⁺P⁺ PM contains precursors for both primitive and definitive hematopoiesis. Although maturation of primitive angiohematopoietic progenitors was achieved in serum-free semisolid medium in the presence of FGF2, stromal factors were essential for the maturation of definitive type angiohematopoietic progenitors from A⁺P⁺ PM.

Hematoendothelial Development from iPSCs

To determine whether other hPSC lines follow patterns of hematoendothelial differentiation similar to those observed with H1 hESC, we analyzed the development of newly identified subsets of angiohematopoietic progenitors from transgene-free fibroblast-derived hiPSCs (Yu et al., 2009) and H9 hESCs. As shown in Figure S7, all examined hPSC lines formed phenotypically and functionally similar subsets of progenitors, including day 4 HVMPs, day 5 HEPs, AHPs, and non-HEPs.

DISCUSSION

During the last decade, investigators have made significant progress in identifying the major stages of hematopoietic development from hESCs/iPSCs (Kennedy et al., 2007; Vodyanik et al., 2006; Zambidis et al., 2005) and their differentiation toward particular blood lineages (Choi et al., 2009b; Lu et al., 2008; Olivier et al., 2006; Timmermans et al., 2009; Woll et al., 2005). However, the development of cells with hematopoietic reconstitution potential from ESC/iPSCs remains a challenge. Although several studies showed bone marrow engraftment of differentiated human ESCs and iPSCs, the engraftment rates were low and mostly restricted to myeloid cells (Ledran et al., 2008; Lu et al., 2009; Narayan et al., 2006; Risueño et al., 2012; Tian et al., 2006; Wang et al., 2005). The most likely explanation for these findings is that in vitro conditions do not support the formation of HSC from its direct HE precursor. Thus, it is essential to access a well-defined population of HE cells to develop an in vitro system that will enable identification of the critical factors that control the maturation of engraftable hematopoietic cells from endothelium.

Previous studies demonstrated that cells expressing endothelial molecules differentiated from mouse and human ESCs can generate blood cells (Eilken et al., 2009; Hashimoto et al., 2007; Nishikawa et al., 1998; Vodyanik et al., 2006; Wang et al., 2004). It was also shown that HE can be prospectively separated from non-HE in mouse ESC cultures based on the activity of the *Flk1* promoter/enhancer (Hirai et al., 2003). Here, we demonstrate that the CD73 phenotypic marker can be used to separate HE cells from non-HEPs prospectively. Of importance, we also found that the VE-cadherin⁺CD41a⁻CD45⁻ population in hPSC cultures includes CD235a⁺ (Glycophorin A⁺) hematopoietic progenitors, which retain angiogenic potential. On the basis of these findings, we were able to further specify the phenotype of HEPs as VE-cadherin⁺CD73⁻CD235a/CD43⁻ and demonstrate that HEPs represent a transient population of endothelial cells that emerge immediately after the beginning of endotheliogenesis in hPSC cultures and rapidly decline within next 3 days of differentiation. These HEPs have the potential to generate β-hemoglobin-producing red blood cells and the entire spectrum of myeloid progenitors, including pan-myeloid GEMM progenitors, which have been identified in human embryonic tissues but not the yolk sac (Hann et al., 1983; Huyhn et al., 1995). Although other investigators and our group previously demonstrated that CD34⁺CD43⁺ progenitors generated in hPSC/OP9 coculture have T and B lymphoid potential (Carpenter et al., 2011; Timmermans et al., 2009; Vodyanik et al., 2005, 2006), further studies will be required to prove that CD34⁺CD43⁺ cells with lymphoid potential arise directly from HEPs.

By analyzing the expression of mesodermal markers at stages preceding endotheliogenesis, we identified ᴱᴹᴴlin⁻KDR^bright APLNR⁺PDGFRα^low/⁻ HVMPs as the direct precursors of definitive-type HEPs. The HVMPs and HEPs required stromal factors for hematopoietic development and were distinct from HBs, which arise from day 3 A⁺P⁺ PM cells and can be specifically detected in serum-free semisolid medium in the presence of FGF2 and apelin-12. The hematopoietic potential of HB colonies detected using these conditions was mostly restricted to cells of erythromegakaryocytic lineage, reflecting the first wave of hematopoiesis observed in the yolk sac. These results are also consistent with mouse studies that demonstrated that Flk1-positive hemangioblastic cells are mainly primitive hematopoietic cells (Fehling et al., 2003). Hematopoietic cells within HB colonies arise through core-forming VE-cadherin⁺ cells that, in contrast to definitive angiohematopoietic progenitors, develop in serum-free medium without stromal support. HB cores have an endothelial gene expression profile and potential. However, our finding that HB cores express intracellular rather than membranous VE-cadherin indicates that they are different from definitive HEPs, and may represent a distinct type of immature cells of endothelial lineage that are more similar to angioblastic mesodermal cells than to more mature endothelial cells that line already established blood vessels. VE-cadherin⁺ cells that coexpress CD31, CD34, CD105, and TEK endothelial markers were previously identified within a subset of Flk1-positive cells in the extraembryonic mesoderm region during gastrulation

and yolk sac blood islands in embryonic day 7.0–7.5 mouse embryos (Ema et al., 2006; Yokomizo et al., 2007). These cells were able to generate endothelial and primitive blood cells. Because HB cores have similar phenotypic and functional characteristics, they could be equivalent to the VE-cadherin$^+$ cells detected within the Flk1$^+$ extraembryonic compartment.

Our study reveals a unique population of AHPs that express VE-cadherin and glycophorin A (CD235a) erythroid marker but lack CD41a. These AHPs represent multipotential hematopoietic progenitors that, similarly to BL-CFCs (HBs), require serum-free conditions and FGF2 for colony formation. However, in contrast to BL-CFCs, the development of colonies from AHPs depends on hematopoietic cytokines and does not proceed through an endothelial core stage. Another unique feature of AHPs is their angiogenic capability, which is completely lost in CD235a$^+$ CD41a$^+$ cells that arise at later stages of differentiation. FGF2- and hematopoietic cytokine-dependent colonies with and without endothelial potential have been described in the mouse yolk sac, fetal liver, and AGM (He et al., 2010; Yao et al., 2007), indicating that AHPs may have in vivo counterparts. Given the fact that AHP cells express a definitive hematopoiesis transcriptional factor, *RUNX1* (Figures 4 and S4B), and possess erythroid and uni- and multilineage myeloid differentiation potential, they may represent precursors for a transient wave of definitive erythromyeloid hematopoiesis similar to the one described in mouse yolk sac (Palis et al., 1999).

In addition, our studies provide important insight into endothelial development from hESCs. Although one commonly held view implies that all endothelial cells in PSC cultures originate from HBs, our current and prior studies (Vodyanik et al., 2010) are in agreement with other studies (Era et al., 2008) that indicate that PSCs give rise to multiple types of endothelial progenitors. Of importance, we demonstrated that emerging endothelial progenitors are multipotent and are able to differentiate into cells of other mesodermal lineages. The first progenitors with endothelial potential, the mesenchymoangioblasts, arise from PSCs on day 2 of differentiation and are capable of forming mesenchymal colonies (Vodyanik et al., 2010). HBs capable of generating primitive blood cells through endothelial intermediates in semisolid medium arise 1 day later. Endothelial intermediates that form HB colonies most likely resemble the yolk sac HE. HEPs that develop by day 5 in hPSC/OP9 coculture express *RUNX1* and have the potential to generate multipotential myeloid cells and β-globin-producing erythroid cells, and thus resemble definitive-type endothelial progenitors. Non-HEPs were distinctively recognized by the expression of CD73 (Figure 6).

CD73 (also known as 5′-ectonucleotidase) is a glycosylphosphatidylinositol (GPI)-linked 70 kDa glycoprotein that produces extracellular adenosine and is abundantly expressed by endothelial cells, mesenchymal stem cells, subsets of peripheral blood lymphocytes, and a variety of other tissues (Delorme et al., 2008; Thomson et al., 1990). CD73 is involved in the regulation of vascular permeability and maintenance of the barrier function, adaptation to hypoxia, ion and fluid transport, and regulation of inflammatory responses in the extracellular milieu (Colgan et al., 2006). Given the physiological significance of CD73, it is likely that expression of this molecule reflects differences not only in the developmental potential but also the functional properties of HE and non-HE subsets. Other distinctive features of CD73$^+$ non-HEP were the lack of CD226 hematopoietic marker expression and the strong expression of CD117 (c-KIT). CD117 is known to mark HSCs arising from the AGM, and is also found in CD45$^-$CD31$^+$ circulating endothelial progenitors and cardiac endothelial progenitors (Peichev et al., 2000; Sandstedt et al., 2010; Tallini et al., 2009). The strong expression of CD117 and the lack of hematopoietic potential in CD73$^+$ endothelial cells indicate that these cells represent a population of endothelial progenitors that are distinct from blood-forming endothelial progenitors and may resemble circulating or tissue-specific endothelial progenitors. Whether these newly identified subsets of endothelial cells possess distinct functional properties and endothelial differentiation potential remains to be explored.

In conclusion, the identification of distinct subsets of cells with angiohematopoietic potential in our studies provides an hPSC-based platform for identifying molecular determinants of HSC development with the goal of facilitating the generation of HSCs from hPSCs.

EXPERIMENTAL PROCEDURES

The experimental procedures used in this study are briefly described below. The Supplemental Information contains a complete description.

Maintenance of hPSCs

H1 and H9 hESC lines were obtained from WiCell Research Institute (Madison, WI). H9-EGFP (Xia et al., 2008) was kindly provided by Su-Chun Zhang (University of Wisconsin, Madison, WI). Transgene-free DF4-3-7T and DF19-9-7T human iPSC cell lines were produced using episomal vectors (Yu et al., 2009). All hESC/iPSC lines were maintained in an undifferentiated state on irradiated mouse embryonic fibroblasts as described previously (Yu et al., 2007).

hPSC Differentiation in OP9 Coculture

hESC/iPSCs were differentiated in coculture with OP9 stromal cells provided by Dr. Toru Nakano (Osaka University, Osaka, Japan) and depleted of OP9 cells using anti-mouse CD29 antibodies (Serotec) as previously described (Vodyanik et al., 2010).
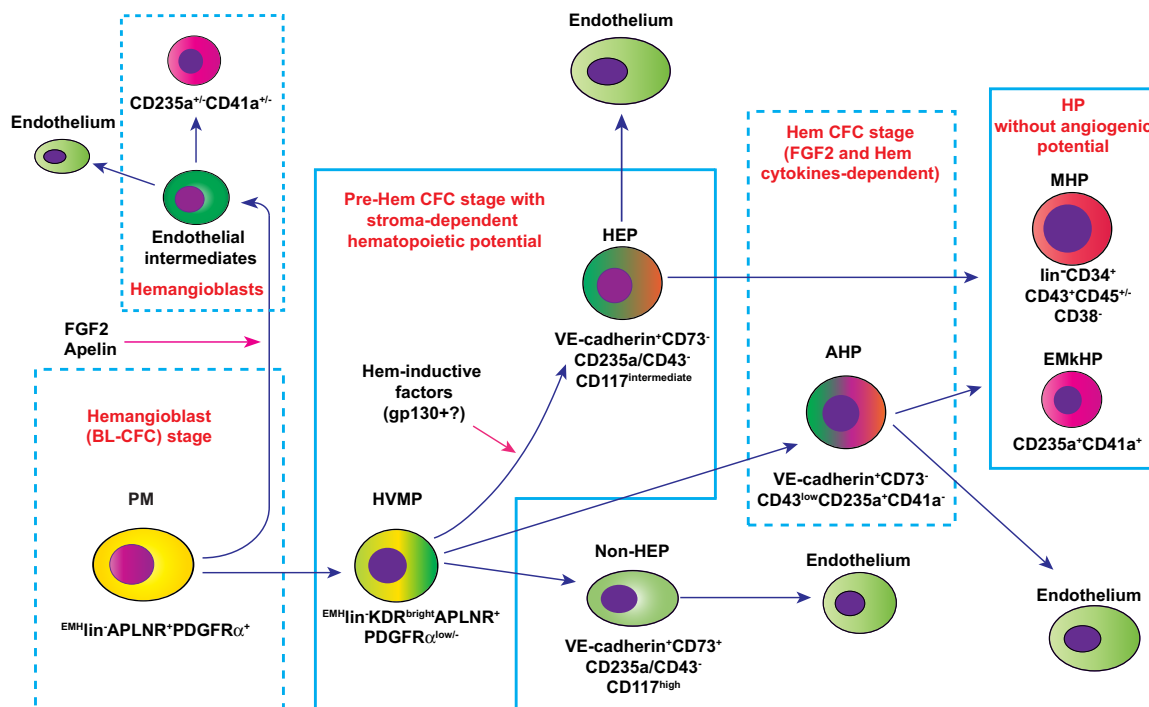
Cell Sorting and Analysis of Hematopoietic and Endothelial Potential

The approach and antibodies used to isolate distinct subsets of progenitors with angiohematopoietic potential are summarized in Figure S2 and Table S1. VE-cadherin$^+$ or CD31$^+$ cells were isolated from day 5 hESC/OP9 cocultures by positive MACS selection using the corresponding FITC-conjugated antibodies and anti-FITC magnetic beads (Miltenyi). MACS-separated cells were stained with CD73-PE, CD235a-APC, and CD43-APC, and CD41a-PECy7 antibodies and sorted using a FACSAria cell sorter (BD Biosciences) to select subsets as depicted in Figures 1B and S2. To isolate day 4 subsets from hPSC/OP9 cocultures, KDR-positive cells were selected by positive MACS selection using KDR-PE antibody (R&D Systems) and anti-PE magnetic beads (Miltenyi). After labeling with VE-cadherin-APC antibody, KDR^{bright}VE-cadherin$^-$, KDR^{dim}VE-cadherin$^-$, and KDR$^-$ cells were further separated using the FACSAria sorter. APLNR$^+$ cells were isolated from day 3 hESC/OP9 cocultures by MACS sorting with APLNR-APC antibodies and APC-magnetic beads or FACSAria sorter after depletion of OP9 with anti-mouse CD29 antibodies as previously described (Vodyanik et al., 2010). The hematopoietic and endothelial potential of isolated cells was evaluated before and after secondary coculture with OP9 via CFC assay, endothelial culture, and flow cytometry (see Supplemental Information).

Statistical Tests

The significance of differences between the mean values was determined by paired Student's t test.

Endothelium

CD235a$^{+/-}$CD41a$^{+/-}$

Endothelium

Endothelial intermediates

Hemangioblasts

Pre-Hem CFC stage with stroma-dependent hematopoietic potential

Hem CFC stage (FGF2 and Hem cytokines-dependent)

HP without angiogenic potential

HEP

MHP

FGF2 Apelin

VE-cadherin$^+$CD73$^-$ CD235a/CD43$^-$ CD117^{intermediate}

lin$^-$CD34$^+$ CD43$^+$CD45$^{+/-}$ CD38$^-$

Hem-inductive factors (gp130+?)

AHP

EMkHP

Hemangioblast (BL-CFC) stage

CD235a$^+$CD41a$^+$

PM

HVMP

VE-cadherin$^+$CD73$^-$ CD43^{low}CD235a$^+$CD41a$^-$

^{EMH}lin$^-$APLNR$^+$PDGFRα^+

^{EMH}lin$^-$KDR^{bright}APLNR$^+$ PDGFR$\alpha^{low/-}$

Non-HEP

Endothelium

Endothelium

VE-cadherin$^+$CD73$^+$ CD235a/CD43$^-$ CD117^{high}

Figure 6. Distinct Stages of Angiohematopoietic Differentiation from hPSCs in Coculture with OP9

Coculture of hESC with OP9 induces mesendodermal differentiation. The first cells with angiohematopoietic potential arise within ^{EMH}lin$^-$APLNR$^+$PDGFRα^+ mesoderm. These cells have the potential to form BL (HB) colonies, which can be specifically detected in serum-free semisolid medium containing FGF2 and apelin. Development of BL colonies proceeds through a core stage in which mesodermal cells form clusters of tightly packed endothelial intermediates (cores). Subsequently, core-forming endothelial cells give rise predominantly to primitive erythromegakaryocytic cells. Advanced mesodermal commitment of hESCs toward hematoendothelial lineage in coculture with OP9 is associated with upregulation of KDR and downregulation of PDGFRα within the APLNR$^+$ population, and the development of HVMPs. These cells are highly enriched in bipotential hematoendothelial cluster-forming cells. After gaining VE-cadherin expression, the cells gradually acquire an endothelial or hematopoietic cell morphology and gene expression profile. The earliest hematopoietic progenitors emerging within the VE-cadherin$^+$ population, AHPs, display a CD43^{low}CD235a$^+$ phenotype and possess endothelial and FGF2-dependent erythromyeloid potential. Expression of CD73 within the VE-cadherin$^+$CD235a/CD43$^-$ population discriminates non-HEPs and HEPs. HEPs do not form hematopoietic CFCs in semisolid medium, but are capable of generating the entire spectrum of definitive myeloid cells and β-globin-producing red blood cells when cultured on OP9. Progressive hematopoietic differentiation is associated with upregulation of CD43 expression, acquisition of CD41a and/or CD45 markers, and loss of endothelial potential. A similar pattern of hematoendothelial development is observed in hiPSCs. See also Figure S7.

ACCESSION NUMBERS

The RNA-Seq data have been deposited in the Gene Expression Omnibus database (accession number GSE39661).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, three tables, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.08.002.

LICENSING INFORMATION

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