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Endothelial and Hematopoietic Cell Fate of Human Embryonic Stem Cells Originates from Primitive Endothelium with Hemangioblastic Properties

Lisheng Wang,[1] Li Li,[1] Farbod Shojaei,[1,2] Krysta Levac,[1] Chantal Cerdan,[1] Pablo Menendez,[1] Tanya Martin,[1] Anne Rouleau,[1] and Mickie Bhatia[1,2,*]

[1]Robarts Research Institute
Krembil Centre for Stem Cell Biology
and Regenerative Medicine
100 Perth Drive
London, Ontario N6A 5K8
Canada

[2]Department of Microbiology and Immunology
The University of Western Ontario
London, Ontario
Canada

Summary

The cellular organization and relationships among precursors that initiate embryonic angiogenesis and hematopoiesis in the human have yet to be characterized. Here, we identify a subpopulation of primitive endothelial-like cells derived from human embryonic stem cells (hESCs) that express PECAM-1, Flk-1, and VE-cadherin, but not CD45 (CD45^{neg}PFV cells), and that are uniquely responsible for endothelial and hematopoietic development. Molecular profiling of CD45^{neg}PFV cells is consistent with endothelial and hematopoietic competency. Clonal isolation demonstrates that the CD45^{neg}PFV population includes bipotent cells with endothelial and hematopoietic capacity. We suggest that human hematopoiesis and endothelial maturation originate exclusively from a subset of embryonic endothelium that possesses hemangioblastic properties and offers a model system to study these lineage relationships in the human.

Introduction

Among vertebrates analyzed to date, initiation of embryonic hematopoiesis has indicated a close association with the major vasculature (Shalaby et al., 1997; Wood et al., 1997). In the human, similar studies of hematopoietic and endothelial development have been limited by difficulties associated with obtaining early human embryos, and lack of conditions capable of reproducing human embryonic developmental processes in vitro. A few lines of evidence indicate that human hematopoietic cells first arise in the third week of human ontogeny within the developing blood vessels of the yolk sac and subsequently from independent origins within the wall of the embryonic aorta and vitelline artery (Oberlin et al., 2002; Tavian et al., 2001). However, the signals that govern the complex relationship between hematopoietic and endothelial cell fate remain elusive, and further investigation is dependent on the ability to identify human precursors with these developmental potentials.

*Correspondence: mbhatia@robarts.ca

Establishment of pluripotent human embryonic stem cell lines (hESC) (Thomson et al., 1998) provides a unique opportunity to investigate the early developmental processes of cell fate specification. Removal of hESCs from culture conditions containing basic fibroblast growth factor (bFGF) (Xu et al., 2001) results in the aggregation of hESCs into clusters of cells termed human embryoid bodies (hEB) capable of differentiation into all primary germ layers (Schuldiner et al., 2000). However, it is unclear whether the use of hESCs will recapitulate hematopoietic and endothelial development as described in the mouse or model the human embryo.

Here, we identify a population of primitive endothelial-like cells derived from human embryonic stem cells (hESCs) that express PECAM-1, Flk-1, and VE-cadherin, but not CD45 (CD45^{neg}PFV cells), that are uniquely responsible for endothelial and hematopoietic development, offering a model system to study mechanisms of endothelial and hematopoietic lineage relationships in the human.

Results

Characterization of Human EB Development Prior to Hematopoietic Commitment

Using conditions previously optimized to promote hematopoietic differentiation (Chadwick et al., 2003), we profiled hEB development to identify the onset of hematopoietic commitment by detection of cells expressing the panleukocyte marker CD45 and functional measurement of hematopoietic progenitor capacity as determined by the colony-forming unit (CFU) assay (Eaves et al., 1999).

Evidence of hematopoietic commitment was observed on day 11 of hEB development in 18 independent experiments. CD45-expressing hematopoietic cells emerged only after 10 days of hEB development (Figures 1A and 1B), and functional hematopoietic progenitors were detected at day 15 (Figure 1C). Individual hEBs were sectioned and analyzed by immunohistochemistry, and no CD45 expression was detectable until day 11 (Figure 1D). The number of CD45+ hematopoietic cells increased beyond day 11 until large clusters of hematopoietic cells were detected at day 15 (Figure 1D), suggesting that hematopoietic precursors must emerge prior to the onset of hematopoietic cell fate at day 11 of hEB development.

To examine the hematopoietic potential of cells comprising hEBs prior to the onset of hematopoietic commitment at day 11 of hEB development, cells were harvested at days 3, 7, and 10 from hEBs, verified to be devoid of hematopoietic cells (Figures 1E and 1F), and then cultured for 7 days in hematopoietic conducive conditions (Hem-Culture) (Bhatia et al., 1997). CD45+ hematopoietic cells and hematopoietic progenitors (CFU) (Figures 1E and 1F) could be induced only between days 7 and 10 of hEB development, but not at day 3 (Figure 1F). Based on these results, we define a stage of hEB development where hematopoietically competent precursors emerge within hEBs.

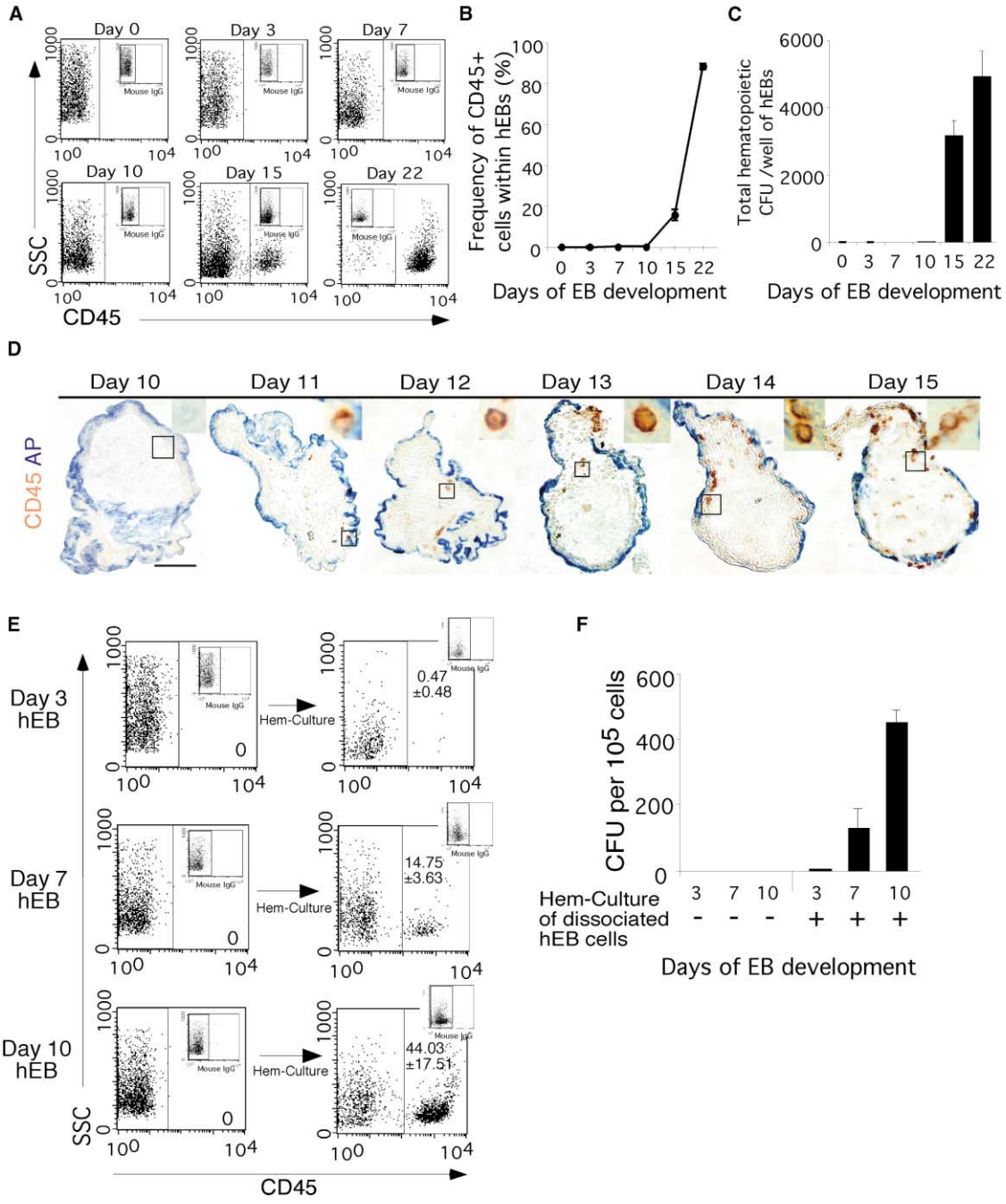

Figure 1. Characterization of Hematopoietic Cell Fate Emergence within Developing hEBs

(A) Single cells were prepared from hEBs that had been cultured in the presence of BMP-4 and cytokines, stained with fluorochrome-conjugated monoclonal antibody CD45, and analyzed by flow cytometry at time points indicated. No CD45+ cells were detected prior to 10 days of hEB development as shown in this representative example.

(B) Summary of CD45 expression of cells comprising treated hEBs.

(C) Hematopoietic progenitors detected by the CFU assay were analyzed at various periods of hEB development as indicated.

(D) hEBs were sectioned and analyzed for CD45 (red) and alkaline phosphatase (blue) every day between day 10 and 15 of development. CD45+ (red) cells only emerged after 11 days (scale bar, 100 μm).

(E) Single cells were prepared from hEBs at days 3, 7, and 10 prior to CD45+ emergence and analyzed by flow cytometry (left). Single cells were cultured in Hem-cultures for 7 days and then analyzed for CD45 (right) and CFU.

(F) CD45 expression and functional CFU capacity was detected only after Hem-culture from cells derived from days 7 and 10 hEBs (n = 5).

Characterization of Candidate Hematopoietic Precursors Arising from hESCs

To identify cells contributing to hematopoiesis during hEB development, we compared cell surface markers in undifferentiated hESCs and cells comprising day 3 hEBs when hemogenic potential was absent, to hematopoietically competent cells present at days 7 and 10 of hEB development (Figures 1E and 1F). Of a series of

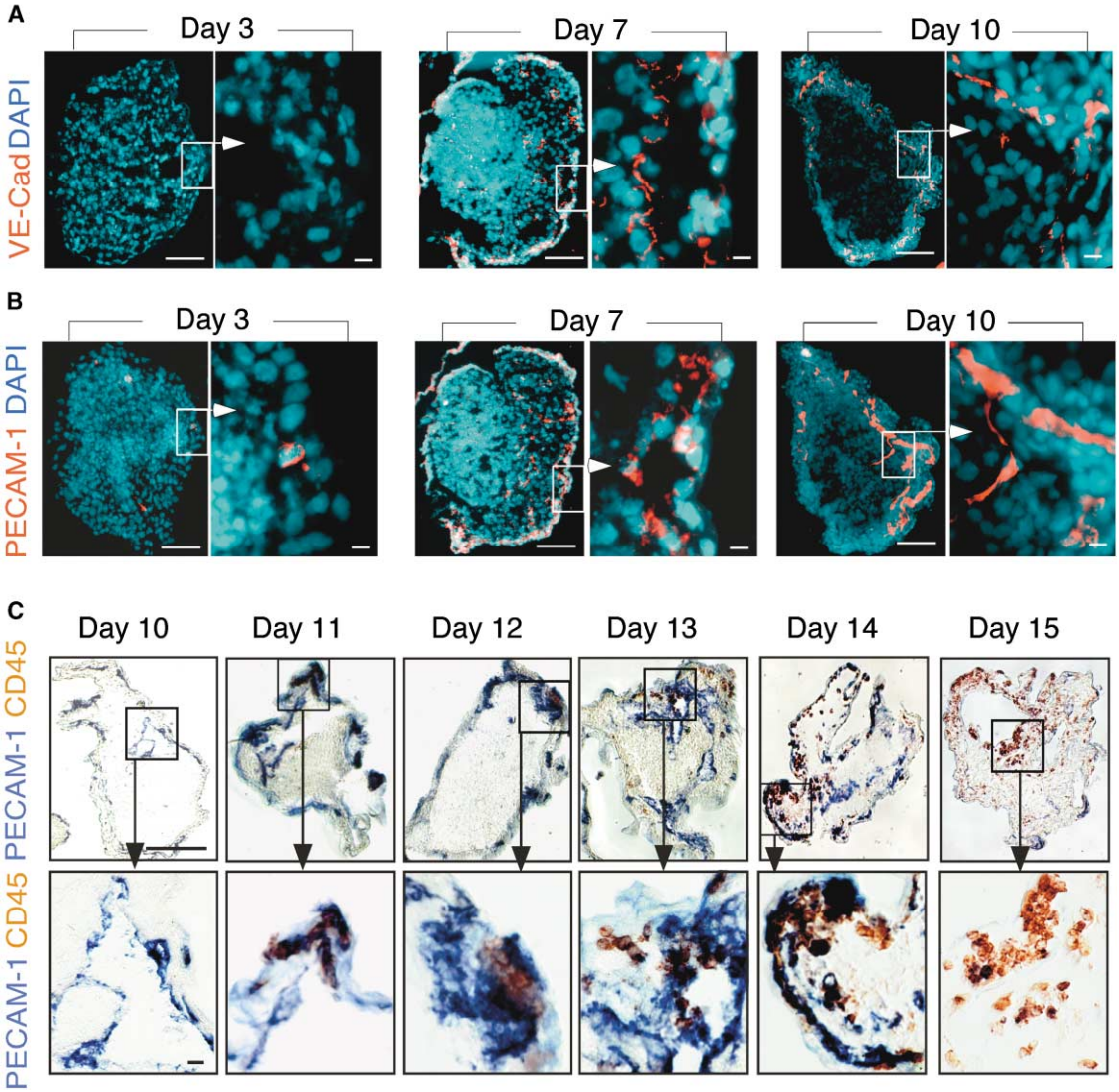

Figure 2. In Vivo Emergence of VE-Cadherin and PECAM-1-Expressing Cells within Developing hEBs

(A) Staining of VE-cadherin (red) and counterstaining with DAPI (blue) of hEBs. Positive VE-cadherin staining was observed at 7 and 10 days. (B) Staining of PECAM-1 (red) and DAPI (blue) of sectioned hEBs. Cells with endothelial-like morphology (red) could be visualized at day 3 and at a higher frequency by days 7 and 10. (C) hEBs were harvested every day between days 10 and 15 and stained with CD45 (brown) and PECAM-1 (blue). The majority of CD45+ cells coexpressed PECAM-1 (purple, double-positive PECAM-1+CD45+ cells). By day 15, cells expressing CD45+ but absent for PECAM-1 were detected. Long scale bar, 100 μm; short scale bar, 10 μm.

markers compared and shown in Supplemental Table S1 at http://www.immunity.com/cgi/content/full/21/1/31/DC1, VE-cadherin and PECAM-1 were the only candidate markers that were absent from hESCs but were upregulated prior to hematopoietic emergence between days 3 and 10 of hEB development.

VE-cadherin-expressing cells were observed in discrete clusters in days 7 and 10 hEBs but were not detected at day 3 (Figure 2A), whereas PECAM-1, a marker associated with cells capable of early hematopoietic potential in the human embryo (Oberlin et al., 2002), was first detected at day 3 by immunostaining (Figure 2B) and significantly increased at day 7 through day 10. At day 10, hEBs containing PECAM-1+ cells were devoid of committed hematopoietic cells (CD45+), whereas, by day 11, CD45+ cells emerged and were exclusively found adjacent to PECAM-1+ cells. Most CD45+ cells costained with PECAM-1 after day 11 to day 15 of hEB development (Figure 2C) were consistent with the phenotype of the first emerging hematopoietic cells in the human embryo (Oberlin et al., 2002) and were never observed in hEBs independent of adjacent PECAM-1+ cells (Figure 2C). We suggest that a subpopulation of VE-cadherin and PECAM-1-expressing cells within hEBs is associated with subsequent hematopoietic commitment.

Isolation of Hematopoietic Precursors from Human EBs

VE-cadherin/PECAM-1+ cells were directly isolated from day 10 hEBs by FACS and compared to all remaining cells within the hEB (remaining day 10 hEB cells). Single viable cells excluding 7-AAD (gated R1) were isolated based on the absence or presence of

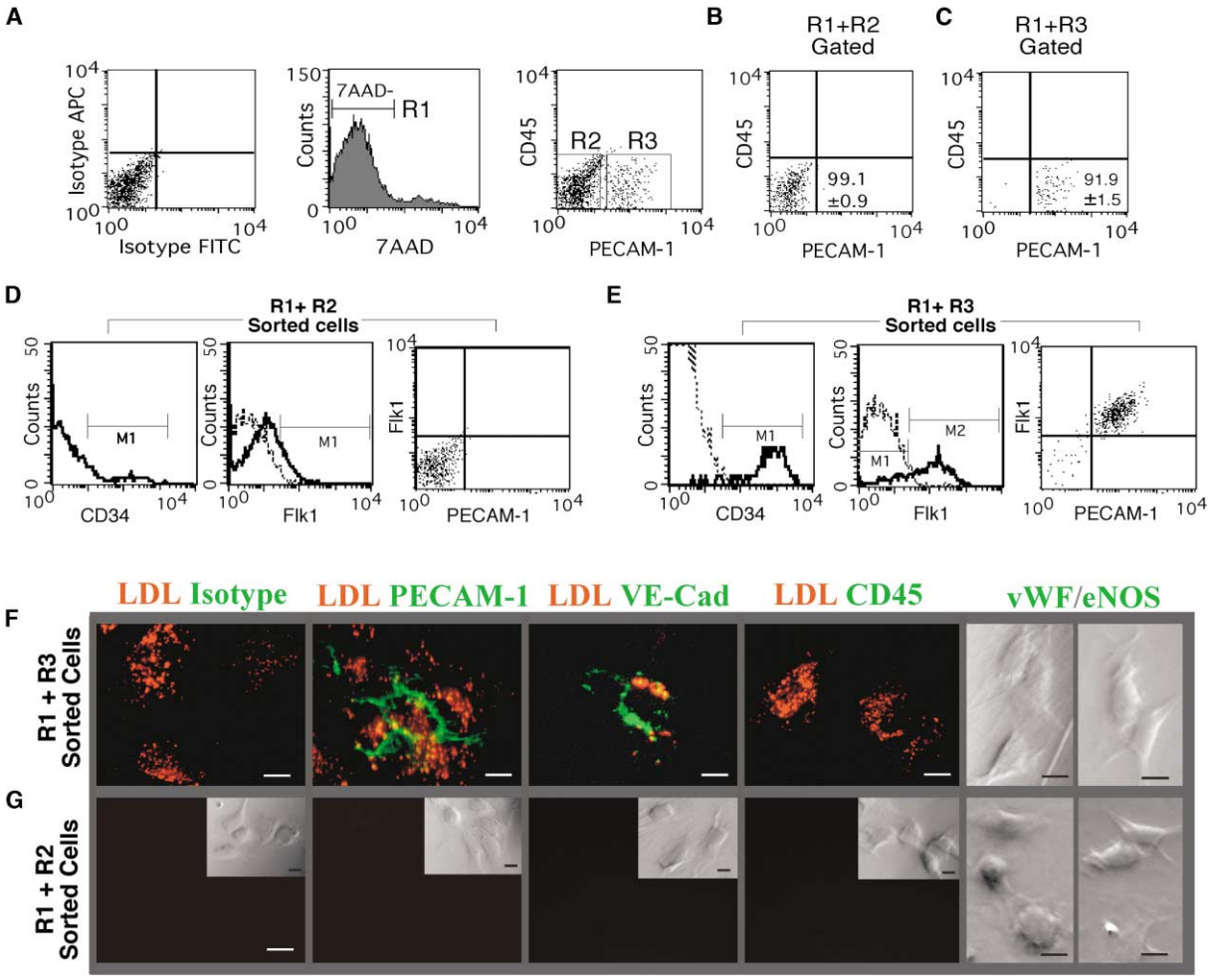

Figure 3. De Novo-Isolated Subsets Derived from Day 10 hEBs Possess Endothelial Properties

Subsets were isolated from day 10 hEBs (excluding 7AAD+ cells) and reanalyzed ([A]–[C]; n = 8). In contrast to the R1 + R2 sorted subset that was negative for CD34 and Flk-1 cell surface expression (D), up to 97% of R1 and R3 sorted cells (E) expressed CD34 (92.11% ± 4.84%; n = 6) and Flk-1 (84.34% ± 13.14%); however, all cells within the R1 and R3 sorted population expressing PECAM-1 expressed Flk-1 and vice versa. (F) Determined by immunohistochemistry, R1 + R2 isolated cells possessed metabolic function for DiI-Ac-LDL uptake (red) and coexpressed the endothelial marker VE-cadherin (green) but lacked expression of CD45, vWF, and eNOS. (G) Remaining cells within the day 10 hEB gated R1 and R3 were negative for markers indicated. Staining of cells with vWF and eNOS is overlayed onto Hoffman images. Scale bar, 10 μm.

PECAM-1 (R2 and R3, respectively; Figure 3A). Upon reanalysis, the purity of viable R1 + R2 gated cells was 99.1% ± 0.9%, and R1 + R3 gated cells was 91.9% ± 1.5% for PECAM-1 (Figures 3B and 3C). In contrast to R1 and R2 sorted cells (Figure 3D), an average of 92.1% ± 4.8% of R1 + R3 gated cells coexpressed CD34 (Figure 3E) and 84.3% ± 13.1% coexpressed Flk-1 (Figure 3E). R1 + R2 sorted remaining day 10 hEB cells were almost exclusively devoid of either PECAM or Flk-1 expression (Figure 3D, right panel). The R1 and R3 sorted fraction contained a low frequency of Flk-1− cells (Figure 3E, M1). However, these cells were also devoid of PECAM-1 expression (Figure 3E, right panel), indicating that they most likely originate from the abundant PECAM-1−Flk-1− cells comprising day 10 hEBs during purification of the PECAM-1+Flk-1+ cells. The presence of rare cells with either a PECAM-1+Flk-1− or PECAM-1−Flk-1+ phenotype within the R1 + R3 sorted fraction was ambiguous but could not be formally excluded based on quadrant analysis using flow cytometry (Figure 3E, right panel). However, based on the proximity of cells with either a PECAM-1+Flk-1− or PECAM-1−Flk-1+ phenotype to the major PECAM-1+Flk-1+ subset, these few events most likely represent cells with slightly less detectable PECAM-1 or Flk-1 fluorescence that upon reanalysis fall just outside the PECAM-1+Flk-1+-defined quadrant (Figure 3E, right panel), thereby precluding any meaningful separation for subsequent functional analysis. Direct isolation of CD45−, Flk-1+, or double positive Flk-1+/PECAM-1+ cells from day 10 hEB demonstrated that VE-cadherin segregated with either of these populations, indicating that any of these sorting strategies isolate a functionally and phenotypically similar population with a CD45−, VE-cadherin+/PECAM-1+/Flk-1+ phenotype (Supplemental Figure S1 on *Immunity*'s website).

Since some markers are shared between endothelial and hematopoietic cells, definitive evidence to distinguish these lineages requires a combination of phenotypic and functional indices (Ema et al., 2003; Nishikawa et al., 1998). Accordingly, we have used the absence of CD45 and hematopoietic progenitor function, in addition to the presence of PECAM-1, CD34, Flk-1, VE-cadherin, uptake of LDL, wWF, and eNOS, as our criteria for defining endothelial cells derived from hEBs (Rafii and Lyden, 2003). To avoid false negatives or false positives, human umbilical vein endothelial cells (HUVEC) and mouse embryonic fibroblast cells (MEF) served as respective controls (Figure 5).

Selected subsets shown in Figure 3D (R1 + R2) and Figure 3E (R1 + R3) were analyzed by immunohistochemistry after de novo isolation (Figure 3G). The subset of CD45− cells from day 10 hEBs that were isolated based on PECAM-1 or Flk-1 all demonstrated the functional ability to uptake Dil-Ac-LDL and also expressed PECAM-1 and VE-cadherin (Figure 3F). Cells capable of LDL uptake were confirmed to be nonhematopoietic by the absence of CD45 expression (Figure 3F). However, more mature features of functional endothelium (Rafii and Lyden, 2003), such as expression of vWF and eNOS, were not detected, suggesting this subset does not contain fully mature endothelial cells (Figure 3F). In contrast, the remaining day 10 hEB cells (R1 and R2) did not possess any of these endothelial characteristics (Figure 3G). Based on these multiple parameter analyses, we define this unique subset of cells at day 10 of hEB development that lack CD45 expression; express PECAM-1, Flk-1, and VE-cadherin; and possess phenotypic and functional endothelial-like properties, as CD45^{neg}PFV cells.

Hematopoietic Cells Are Derived from Endothelial-like CD45^{neg}PFV Precursors Isolated from hEBs

CD45^{neg}PFV and remaining day 10 hEB cells were cultured in Hem-culture conditions for 7 days and then assessed for hematopoietic commitment by CD45 expression and the ability to give rise to hematopoietic progenitors (CFU) compared to unsorted cells derived from day 10 hEBs. Total number of cells from CD45^{neg}PFV cultures increased (1.36- ± 0.47-fold) over 7 days and appeared as single round cells (Figure 4A), where up to 98.5% of these cells were CD45+ (Figure 4A), with 8.3% ± 0.7% coexpressing CD34 (Figure 4A). In contrast to CD45^{neg}PFV cells, the remaining day 10 hEB cells or unsorted cells demonstrated a decrease in cell number by 5.48- ± 1.87-fold after 7 days in hematopoietic culture conditions. Overall, the number of CD45+ hematopoietic cells generated from CD45^{neg}PFV cells was 28.7- ± 8.2- and 146.1- ± 89.5-fold higher than from unsorted or remaining day 10 hEB cells, respectively (Figures 4B and 4C). In addition, cultured CD45^{neg}PFV cells generated a 124-fold higher number of colonies compared to remaining day 10 hEB cells or unsorted cells (Figure 4D). As shown, morphology and phenotype of CFUs derived from CD45^{neg}PFV cells were similar to adult hematopoietic sources (Figures 4E–4M).

CD45+CD34+ and remaining CD45+CD34− subpopulations were isolated from 7 day Hem-cultures of CD45^{neg}PFV cells (Figures 4N and 4O) and assayed for CFU capacity. A low frequency of CFU was obtained from the CD45+CD34− population (1 in 1069), whereas CD45+CD34+ cells were highly enriched (1 in 50) for CFU (Figure 4Q). Thus, hESC-derived hematopoietic progenitor capacity was enriched in the CD45+CD34+ subfraction. Our results indicate that CD45^{neg}PFV cells represent a unique subpopulation of endothelial-like cells that exclusively account for hematopoietic development from hESCs, giving rise to CD45+CD34+ cells with multilineage progenitor capacity.

Endothelial-like CD45^{neg}PFV Precursors Isolated from hEBs Are Capable of Endothelial Maturation

CD45^{neg}PFV cells and remaining day 10 hEB cells were cultured for 7 days in conditions conducive to endothelial maturation (endo-culture) containing pituitary extracts and VEGF (Murohara et al., 2000). After 7 days in endo-culture conditions, functional uptake of Dil-Ac-LDL and expression of PECAM-1, VE-cadherin, CD45, vWF, eNOS, and hematopoietic progenitor capacity were examined. Endo-cultured CD45^{neg}PFV cells became attached and spindle shaped (Figure 5), strongly expressed PECAM-1 and VE-cadherin, and possessed higher LDL uptake capacity (Figure 5), as compared to de novo-isolated cells (Figure 3F). In contrast to properties of de novo-isolated CD45^{neg}PFV cells (Figure 3F), endo-cultured CD45^{neg}PFV cells expressed vWF and eNOS (Figure 5) indicative of endothelial maturation. No CD45 expression could be detected after culture under these conditions (Figure 5), consistent with the complete absence of hematopoietic CFU capacity (data not shown). Remaining day 10 hEBs were negative for all endothelial markers tested after 7 days of endo-culture (Figure 5). Our combined results indicate that CD45^{neg}PFV cells represent a unique population of endothelial-like cells with hemogenic properties and retain the functional ability to differentiate into mature endothelium.

CD45^{neg}PFV Cells Possess a Distinct Molecular Profile Consistent with Both Hematopoietic and Endothelial Potential

The unique ability of CD45^{neg}PFV cells to differentiate into both hematopoietic and endothelial cells (Figures 4 and 5) prompted us to investigate their gene expression signature. Classification of differentially regulated genes in CD45^{neg}PFV versus hESCs and day 10 hEBs divided them into three groups, including genes representing endothelial (Figure 6A), hemogenic (Figure 6B), and hemogenic-endothelial (Figure 6C) potentials. See Supplemental Table S2 on Immunity's website for the complete differential gene expression profile of upregulated genes in CD45^{neg}PFV cells. Endothelial factors *vWF* (Guo et al., 2003a) and *eNOS* (Venugopal et al., 2002) transcripts were highly expressed, consistent with the upregulation of protein expression upon endo-culture (Figure 5). In addition, expression of several adhesion molecules, such as *CD34*, *VE-cadherin*, *PECAM-1*, *Endothelin*, *LFA-3*, and *E-Selectin*, was also consistent with the cellular characteristics of embryonic endothelium (Neumuller and Menzel, 1997). Consistent with the de novo endothelial-like properties of CD45^{neg}PFV cells, several signal transduction genes known to play a role in endothelium, including *TIE-2*, *Neuropilin*, *EDG-1*, and

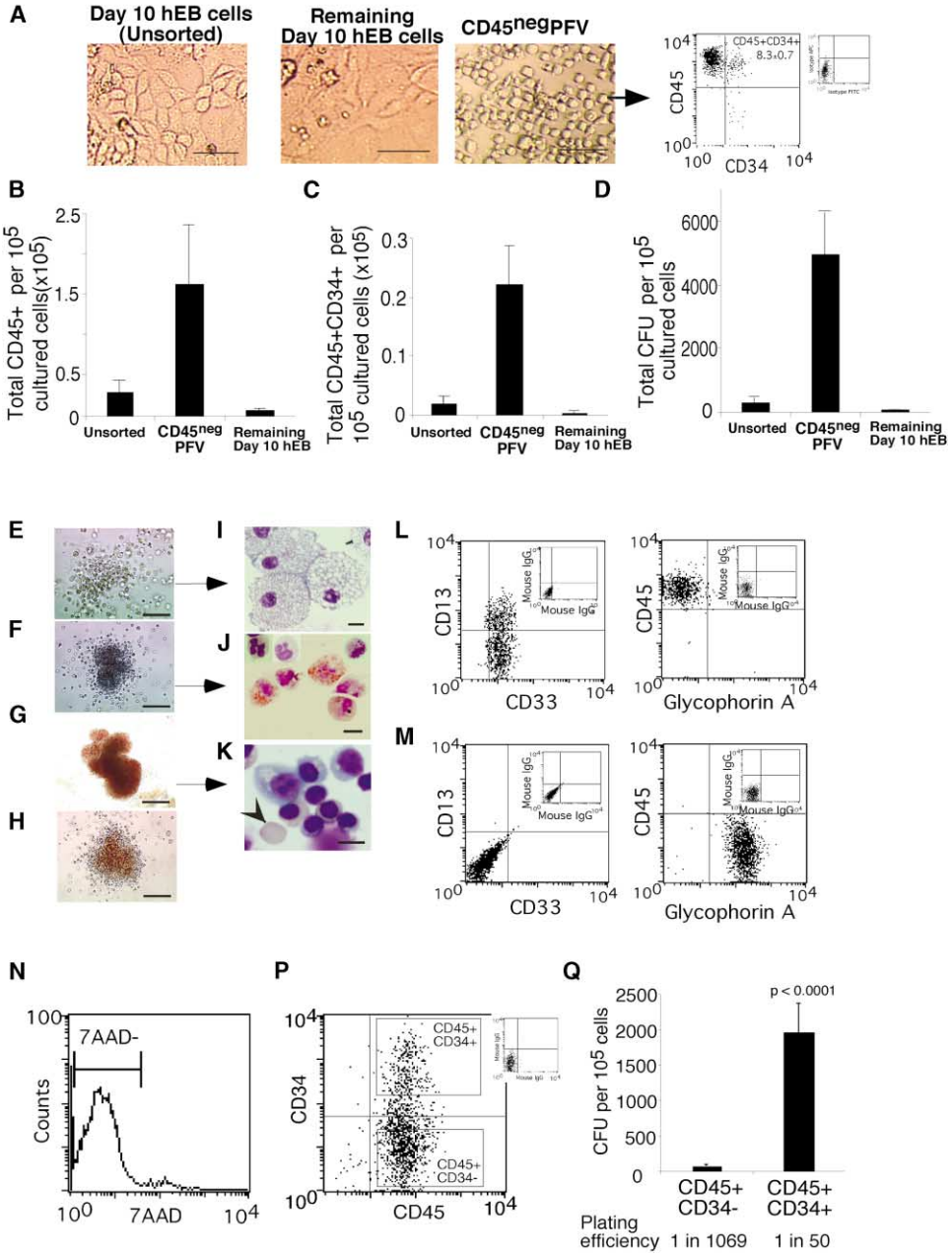

Figure 4. CD45^{neg}PFV Cells Derived from Day 10 hEBs Possess Hemogenic Potential

(A) Total cell numbers after Hem-culture increased in cultures seeded with CD45^{neg}PFV cells (1.36- ± 0.47-fold increase; n = 6), and the majority of cells appeared as single, round cells (scale bar, 50 μm). CD45^{neg}PFV subset gave rise to up to 98.5% of CD45+ hematopoietic cells, and 8.3% ± 0.7% of those CD45+ cells were also CD34+. In contrast, remaining day 10 hEB subset (appeared spindle shaped) demonstrated no growth in Hem-culture, and total cell numbers decreased significantly (5.48- ± 1.87-fold decrease; n = 6). Total CD45+ cells (B) or CD45+CD34+ cells (C) and CFUs (D) derived from unsorted or sorted CD45^{neg}PFV and remaining day 10 hEB cells. CD45^{neg}PFV subset showed 124.20 (±66.70; n = 3) -fold higher numbers of CFU than remaining day 10 hEB subset. Representative colonies from hEB-derived CD45+ cells are depicted, including macrophage (E), granulocyte (F), erythroid (G), and multipotent (H) colonies. Cells from the macrophage, granulocyte, or erythroid colonies, identified by light microscopy, contained monocytes (I), granulocytic (J), or erythroid cells (K), by Wright-Giemsa staining. The granulocytic cells were characterized by segmented nuclei and pink or dark blue cytoplasmic granules ([J] and insets). Cells comprising myeloid colonies expressed CD45+, myelomonocytic markers CD33 and CD13, and lacked erythroid glycophorin A marker (L). Erythroid colonies contained mature enucleated erythrocytes ([K], arrow), expressed glycophorin A, and CD45− (M) (scale bar, 100 μm in [E]–[H]; scale bar, 10 μm in [I]–[K]). CD45+ cells were separated into CD45+CD34+ and CD45+CD34− subfractions (N and O), and CFU efficiency of CD45+CD34− and CD45+CD34+ cells was compared (P).

TEK (Breier, 2000), were highly expressed. As would be expected in cells with endothelial-like characteristics, hematopoietic cytokine signaling pathways were not prominent (data not shown); however, CD45^{neg}PFV cells expressed transcription factors that would be required for hemogenic potential and priming of the hematopoi-

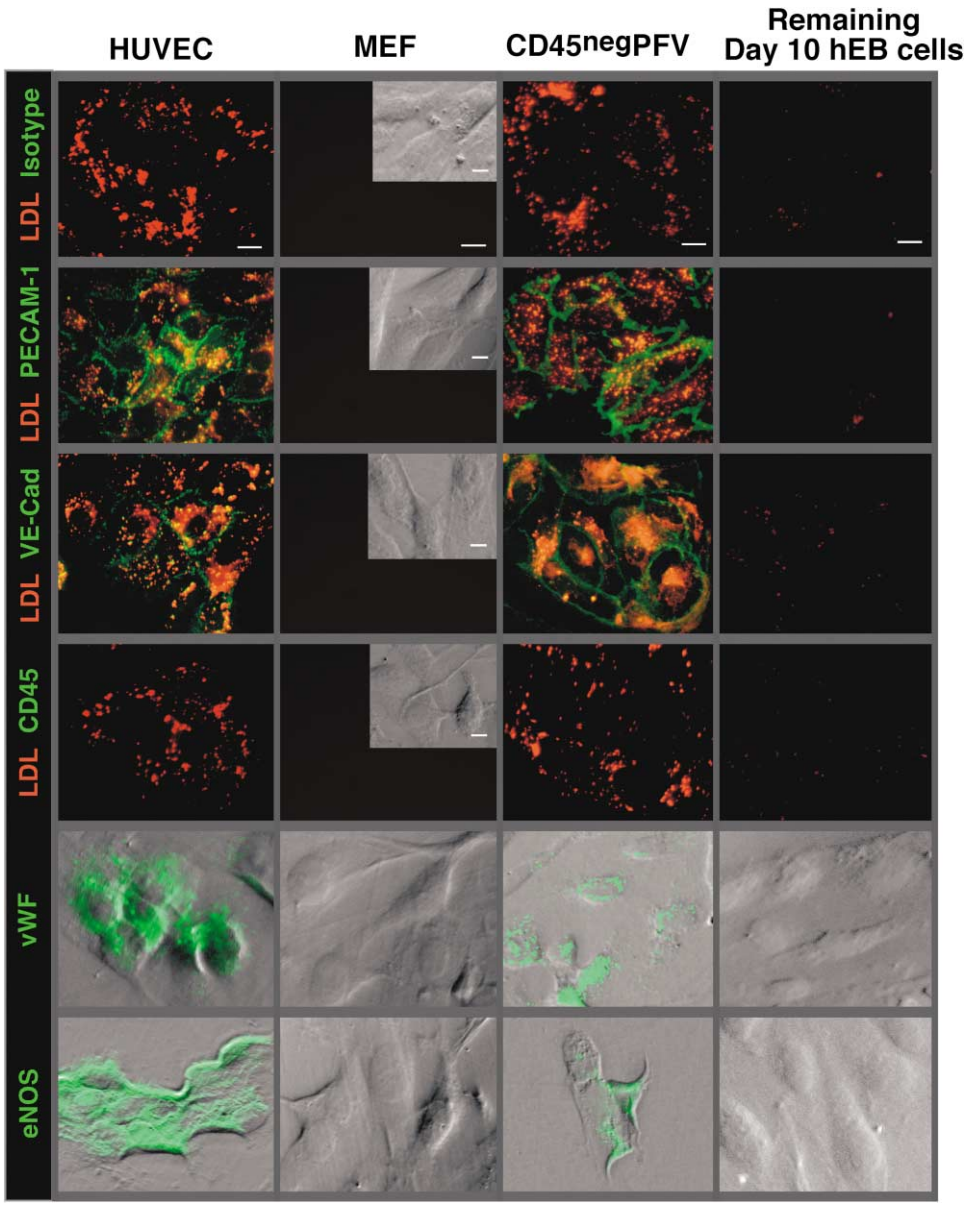

Figure 5. Human EB-Derived CD45^{neg}PFV Are Capable of Endothelial Maturation

Using primary HUVEC cells (column 1) as a positive control and mouse embryonic fibroblasts (MEF, column 2) as a negative control, CD45^{neg}PFV (column 3) and remaining day 10 hEB cells (column 4) were cultured in endo-culture and analyzed for endothelial cell fate. For Dil-Ac-LDL uptake (red), cells were incubated with red-fluorescent Dil-Ac-LDL prior to immunocytochemical analysis, such as PECAM-1, VE-cadherin (VE), vWF, and eNOS markers, shown in green. vWF and eNOS staining was overlaid onto Hoffman images. Cultured remaining day 10 hEB cells showed little Dil-Ac-LDL uptake and were negative for endothelial markers shown (column 4). No positive stain was observed for CD45 (n = 4). Scale bar, 10 μm. Negative staining of MEFs was overlaid onto Hoffman images in insets.

etic cell fate programs. These hemogenic factors included *FLI-1*, *MPL*, *Lymphocytic leukemia derived sequence-1 (LLD-1)*, *MYB*, and *ERG* (Orkin and Zon, 1997). Genes associated with primitive cells with both endothelial and hemogenic potential were also highly expressed in CD45^{neg}PFV cells, supportive of human hemangioblast function of CD45^{neg}PFV cells. These include *FLT-1* (Casella et al., 2003), *FLK-1* (Ema et al., 2003), *TAL-1* (Ema et al., 2003), *FLT-4* (Fielder et al., 1997), *FOG-1* (Katz et al., 2002, 2003), *RUNX-1* (Bailey and Fleming, 2003), and HEX (Guo et al., 2003b). Therefore, the molecular profile of human CD45^{neg}PFV is consistent with the dual hemogenic and endothelial potential of these cells.

The CD45^{neg}PFV Subpopulation Contains Single Cells with Both Hematopoietic and Endothelial Capacity

Single CD45^{neg}PFV cells isolated from day 10 hEBs were deposited into individual wells of a 96-well plate and inspected daily. A total of 1169 individual wells targeted for single CD45^{neg}PFV cell deposition were visually inspected at 2 hr and again at 16 hr post-clonal isolation. This inspection was performed by two independent ob-

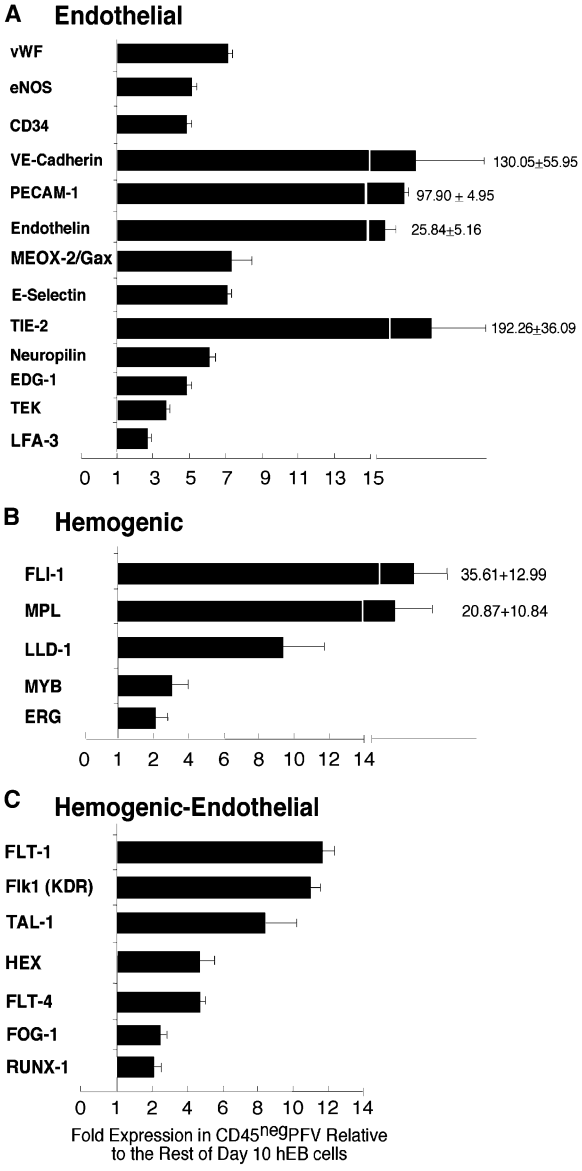

Figure 6. Molecular Profiling of CD45^{neg}PFV Cells

Total RNA of hESC cells, CD45^{neg}PFV, and remaining day 10 hEB cells were extracted, and aRNA was generated to hybridize human HG-U133AB Affymetrix chips. Expression level of CD45^{neg}PFV and remaining cells within day 10 hEBs was calculated relative to hESC cells. Differentially regulated genes were defined as those being upregulated more than 2-fold in CD45^{neg}PFV versus remaining day 10 hEB cells and being statistically significant ($p < 0.05$) using three biological replicates. Markers representing endothelial potential of CD45^{neg}PFV (A), defining hemogenic potential (B), and describing both hemogenic and endothelial potentials (C) are shown.

servations blinded to the others' results. Both observers demonstrated 100% concordance of wells that contained more than one cell, at either time point after deposition, and these wells were then excluded from subsequent analysis.

After 14 days in wells confirmed to contain clonal starts, individual cells were identified by DAPI staining that resulted from single CD45^{neg}PFV cell proliferation and were analyzed in situ for expression of CD45 and VE-cadherin, representing hematopoietic and endothelial cell fate, respectively. To establish appropriate in situ controls for CD45 and VE-cadherin staining, primary HUVECs, adult hematopoietic mononuclear cells (MNC), and CD45-expressing hematopoietic cells derived from hESC cells were similarly deposited (Figure 7A), stained, and compared to isotype controls (Figure 7B). Only endothelial HUVEC cells were positive for VE-cadherin (red) and negative for CD45 (green), whereas both adult and hESC-derived hematopoietic cells were positive for CD45 and negative for VE-cadherin (Figure 7B). This established our comparative criteria for identifying clones with hematopoietic, endothelial, or bipotent endothelial and hematopoietic potential.

Based on these clonal culture conditions (50:50 vol Hem:Endo Culture), a total of 1152 individual wells were analyzed using established criteria defined by positive and negative in situ controls (Figures 7A and 7B). Isolation strategy of the CD45^{neg}PFV cells and reanalysis of sort purity (98.26% ± 0.51%) for these clonal experiments is shown in Supplemental Figure S2 on *Immunity*'s website. Of the 1152 wells, 37 wells (3.2%) demonstrated clonal outgrowth, consistent with previous studies that have reported the difficulty of sustaining single cells differentiated from mammalian ES parents in culture (Choi et al., 1998; Lumelsky et al., 2001). The progeny of CD45^{neg}PFV clones detectable after cultures were subsequently examined for hematopoietic and endothelial cell differentiation by in situ analysis of individual wells.

Consistent with previous results (Figures 4 and 5), progeny of CD45^{neg}PFV clones were either exclusively hematopoietic (Figure 7C) or endothelial (Figure 7D). However, in addition to these CD45^{neg}PFV clones with unilineage differentiation capacity (Figures 7C and 7D), quantitative analysis demonstrated that 0.18% of the total wells seeded with single CD45^{neg}PFV clones were capable of giving rise to both endothelial and hematopoietic progeny (Figure 7E). These wells were interpreted to be the result of differentiation of bipotent CD45^{neg}PFV clones, suggesting that a proportion of CD45^{neg}PFV cells possess both endothelial and hematopoietic potential. Based on the clonal analysis of CD45^{neg}PFV cells derived from hESCs, we suggest that the CD45^{neg}PFV subset contains cells with human hemangioblastic properties.

Discussion

Invertebrates possess a vascular system that is open to the interstitial space and, therefore, is permissive to the entry of developing hematopoietic cells into the circulation. In the mammal, a more efficient closed circulatory system has evolved, necessitating the formation of endothelial vasculature that itself gives rise to hematopoietic cells, thereby allowing incorporation of blood cells into this closed system. Consistent with this paradigm, our study identifies CD45^{neg}PFV progenitors emerging during differentiation of hESCs and provides evidence that human hematopoietic cells arise from endothelial-like precursors that can give rise to either hematopoietic cells or committed endothelium. The ability to isolate and differentiate these precursors into either hematopoietic or endothelial lineages provides the foundation to investigate the cellular and molecular events leading to the development of these precursors in the

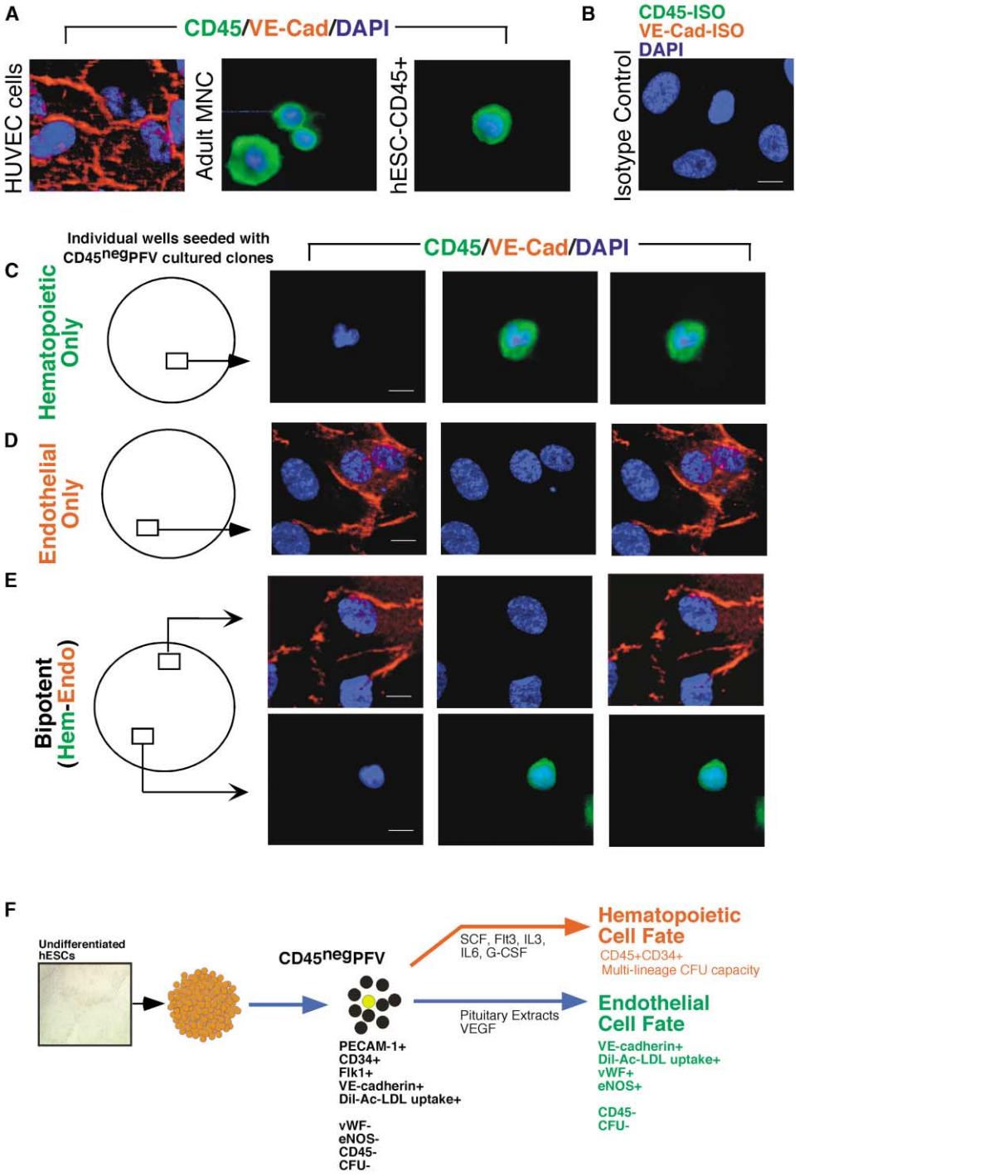

Figure 7. Clonal Analysis of the Hematopoietic and Endothelial Developmental Potential of CD45^{neg}PFV Cells

The cells resulting from single CD45^{neg}PFV cell proliferation in each well were analyzed in situ for expression of CD45 (hematopoietic cell fate) and VE-cadherin (endothelial cell fate). (A) HUVEC cells, adult hematopoietic mononuclear cells (MNC), and CD45-expressing hematopoietic cells derived from hESC cells (hESC−CD45+) as positive controls and compared to (B) isotype-staining controls (VE-cadherin+, red; CD45+, green; DAPI nucleus staining, blue). A schematic illustration representing the location of CD45^{neg}PFV progeny within individual wells containing (C) only hematopoietic progeny, (D) only endothelial progeny, or (E) individual wells containing cells of both hematopoietic and endothelial progeny originally seeded with only one CD45^{neg}PFV cell. Scale bar, 10 μm. Dispersed location of cells is due to the physical manipulations (movement) of plates necessary at 2 and 16 hr, at subsequent daily inspections, and at addition to culture media. (F) Working model of the cellular development toward endothelial and hematopoietic cell fate during hESC differentiation.

human. Furthermore, our report demonstrates that precursors of a specific tissue lineage can be prospectively isolated from hEBs, indicating the hESC differentiation progresses via discrete cell types that become more restricted, thereby describing a hierarchical organization of cell fate commitment from hESCs. We propose

a working model that outlines our current understanding of hematopoietic cell fate specification from hESCs (Figure 7F).

Functional hemogenic capacity emerged between 7 and 10 days of hEB development. Based on the literature and available markers for human cells, our study used the presence of PECAM-1, CD34, Flk-1, VE-cadherin, uptake of LDL, and absence of CD45 or hematopoietic progenitor function as the multiparameter criteria for defining endothelial-like cells (Rafii and Lyden, 2003). A unique subset of CD45^{neg} hEB cells lacking hematopoietic progenitor capacity but possessing endothelial-like properties could be isolated from hEBs prior to the emergence of hematopoietic cell fate. Despite functional DiI-Ac-LDL uptake, expression of Flk-1, VE-cadherin, and CD34, these endothelial-like cells lacked more mature endothelial properties, such as eNOS or vWF factor expression (Rafii, 2000). Hemogenic capacity of hEBs was unique to endothelial-like CD45^{neg}PFV cells, and little hematopoiesis was detected in remaining hEB cells, suggesting that the CD45^{neg}PFV subset exclusively contained hematopoietic developmental potential (Figure 7F). When stimulated with VEGF and pituitary hormones, CD45^{neg}PFV cells were able to produce mature endothelial cells that continued to express endothelial markers, and upregulated eNOS and wWF expression (Figure 7F). Taken together, our study reveals that the CD45^{neg}PFV population is responsible for both hematopoietic and endothelial cell fate and contains CD45^{neg}PFV clones with bipotent lineage potential, thereby providing a model system to generate functional human hemangioblasts (Traver and Zon, 2002).

The use of surrogate cell surface markers to discriminate murine hematopoietic precursors during embryo development or differentiation of mouse ES cells has been reported. However, our current study indicates that many of these markers are expressed on undifferentiated hESCs and were not regulated upon differentiation of hEBs, thereby excluding their use for identification of hESC-derived hematopoietic precursors. These results illustrate differences in cellular development of mouse and human ESCs toward the hematopoietic lineage and suggest that uncovering the mechanisms of human hematopoietic cell fate decisions will depend on human systems. Due to the difficulty in studying these complex processes during the early stages of human embryo development, differentiation of hESCs provides an ideal alternative model.

Experimental Procedures

hESC Cell Culture and hEB Formation

Maintenance of hESC lines H9 or H1 (Thomson et al., 1998), formation of hEB, and preparation of single cells from hESC, hEB, and hematopoietic colonies were performed as previously described (Chadwick et al., 2003; Xu et al., 2001).

Analysis of Cell Surface Markers by Flow Cytometry

Single-cell suspensions (2–5 $\times$ 10^5 cells/mL) were stained with fluorochrome-conjugated monoclonal antibodies (mAb), including CD45, CD34, PECAM-1, c-Kit, CD33, CD13, CD41 (Becton Dickinson [BD]), AC133 (Miltenyi Biotech, Germany), Glycophorin A (Immunotech, France), CD105 (Accurate Chem & Sci Co.), VE-cadherin (Alexis Biochemicals), or their corresponding IgG isotype controls (BD), at a concentration of 5 μg/mL. Flk1 (Research Diagnostic Inc.) analysis was performed according to the methods described (Boldicke et al., 2001). Live cells identified by 7-AAD exclusion (Immunotech) were analyzed for surface marker expression using a FACSCalibur and Cell Quest software (BD).

Immunohistochemistry of hEB Sections

Cultured hEBs were snap frozen and cryosectioned as described (Chadwick et al., 2003). For each staining, three sections per specimen at an interval of 30 serial sections were used. The mAb used in this study were mouse anti-human VE-cadherin, PECAM-1, CD45, and mouse IgG1κ isotype (BD) at 5 μg/mL. Sections were incubated with the above mAbs for 2h, followed by Alex Flour 568 goat anti-mouse IgG (Molecular Probes) for 30 min, and then counterstained with DAPI. For PECAM-1/CD45 double staining, sections were first stained with anti-PECAM-1 mAb by Vector ABC-AP kit and visualized with Vector Blue. Subsequently, the sections were incubated with anti-CD45 mAb for 30 min by Vector MOM kit and visualized with Vector ABC-peroxidase using AEC. CD45/AP double staining was performed as described (Chadwick et al., 2003).

Preparation of Isolated Subpopulations and Single CD45^{neg}PFV Cells

CD45^{neg}PFV and remaining day10 hEB cells were dissociated from day 10 hEB and stained with Flk1 followed by PECAM-1-FITC (PharMingen), CD45-APC (BD), and 7-AAD. Cell subpopulations or single cells were sorted on a FACSVantage SE (BD).

Culture of hEB-Derived CD45^{neg}PFV and Remaining Day 10 hEB Cells

Isolated CD45^{neg}PFV and remaining day 10 hEB subpopulations were seeded on fibronectin-coated plates (5 $\times$ 10^4 cells/cm^2) and cultured for 7 days in conditions previously designed and shown to sustain human hematopoietic stem cells (Bhatia et al., 1997) or in Medium-199 supplemented with 20% FBS, 50 μg/mL of bovine pituitary extract as an endothelial cell growth supplement (Invitrogen), 10 IU/mL of heparin (Leo Pharna Inc.), and 5 ng/mL of hVEGF (R & D Systems). Medium was changed at days 2, 4, and 6.

Uptake of Acetylated LDL and In Situ Immunocytochemical Staining for PECAM-1, VE-Cadherin, CD45, vWF, and eNOS

HUVEC (positive control), mouse embryonic fibroblasts (MEF, negative control), de novo isolated or cultured CD45^{neg}PFV, and remaining day 10 hEB cells were incubated with 10 μg/mL of DiI-Ac-LDL (Molecular Probes) at 37°C for 4 hr. After fixation, cells were stained for 30 min with 5 μg/mL of primary anti-PECAM-1, VE-cadherin, CD45, mouse IgG1κ isotype (BD), vWF (DAKO), or eNOS mAb (BD), followed by fluorescein-conjugated goat anti-mouse IgG (IMMUNOTECH). The cells resulting from single CD45^{neg}PFV cell proliferation in each well were fixed and stained with anti-CD45 as described above and then double stained with VE-cadherin mAb by Vectors MOM kit, visualized with Vectors Texas Red-Streptavidin, and counterstained with DAPI. All immunostaining specimens were coded and analyzed blindly with computer-assisted image analysis capabilities (Image Pro-Plus).

Molecular Profiling

Total RNA was extracted using Qiagen RNAeasy kit (Qiagen) and was amplified using Message Amp aRNA kit (Ambion) (Raghavachari et al., 2002). Fifteen micrograms of fragmented antisense RNA (aRNA) was used for hybridizing human HG-U133AB arrays (Affymetrix) at the London Regional Genomic Center (Ontario, Canada). GeneSpring 6.0 was used for data analysis. Genes that were flag passed in at least one of the populations and significantly ($p < 0.05$, different by 2-fold) upregulated in CD45^{neg}PFV, in comparison to both hESC and remaining day10 hEB cells, are shown.

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