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Dysregulated Gene Expression During Hematopoietic Differentiation From Human Embryonic Stem Cells

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The generation of hematopoietic cells from human embryonic stem cells (hESC) has raised the possibility of using hESC as an alternative donor source for transplantation. However, functional defects identified in hESC-derived cells limit their use for full lymphohematopoietic reconstitution. The purpose of the present study was to define and quantitate key functional and molecular differences between CD34^{+} hematopoietic progenitor subsets derived from hESC and CD34^{+} subsets from umbilical cord blood (UCB) representing definitive hematopoiesis. Two distinct sub-populations were generated following mesodermal differentiation from hESC, a CD34^{bright} (hematoendothelial) and CD34^{dim} (hematopoietic-restricted) subset. Limiting dilution analysis revealed profound defects in clonal proliferation relative to UCB particularly in B lymphoid conditions. Transcription factors normally expressed at specific commitment stages during B lymphoid development from UCB-CD34^{+} cells were aberrantly expressed in hESC-derived CD34^{+} cells. Moreover, strong negative regulators of lymphopoiesis such as the adaptor protein *LNK* and CCAAT/enhancer-binding protein-α (*CEBPα*), were exclusively expressed in hESC-CD34^{+} subsets. Knockdown of *LNK* lead to an increase in hematopoietic progenitors generated from hESCs. The aberrant molecular profile seen in hESC-CD34^{+} cells represents persistence of transcripts first expressed in undifferentiated hESC and/or CD326-CD56^{+} mesoderm progenitors, and may contribute to the block in definitive hematopoiesis from hESC.

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INTRODUCTION

The finding that CD34^{+} cells can be generated from human embryonic stem cells (hESC) has raised the possibility of using hESC as an alternative source of hematopoietic stem cells (HSC) for transplantation.[1] The induction of hematopoietic differentiation from hESCs has also been proposed as a tool to study embryonic and fetal hematopoietic development. In order to recapitulate the full range of mammalian hematopoiesis, a system that can generate a tightly controlled sequence of events marked by mesoderm commitment, primitive hematopoiesis, and definitive hematopoiesis is required. The final stage of this process, the production of definitive hematopoietic stem cells with high proliferative capacity and full lymphohematopoietic lineage potential, is the most relevant end point for clinical transplantation of hESC-derived hematopoietic cells.

Although numerous reports have shown that hematopoietic cells can be generated from hESCs, most studies have focused on erythroid and myeloid differentiation.[1–12] Although natural killer (NK) cell[2,7] and T cell differentiation[13,14] have been reported, the few studies that have analyzed B lymphoid differentiation[2,15,16] have found this lineage potential either absent[16] or severely impaired[2,15] in hESC-derived progenitors. Studies employing microarray profiling[17] or PCR-Long SAGE library screening[18] have compared hESC-derived CD34^{+} cells with CD34^{+} cells isolated from fetal liver and umbilical cord blood (UCB), but have not identified regulatory genes expressed exclusively in hESC-derived CD34^{+} cells that can account for the functional defects in these progenitors.[6] One study suggested high expression of ID proteins as a potential block in B cell development from hESCs.[16] However, as CD34 expression encompasses a heterogeneous population of stem and progenitor cells with markedly different lineage potentials, comparisons of gene expression in hESC-derived CD34^{+} cells to total CD34^{+} cells from UCB[16] has limited usefulness in defining specific defects of B lymphoid differentiation potential.

CD34^{bright} and CD34^{dim} populations arising from hESC, are immunophenotypically and functionally consistent with hematoendothelial and hematopoietic progenitors, respectively. Although endothelial and hematopoietic-specific gene expression has been studied,[11] differential expression of genes important in B cell development has not been analyzed in these subsets. In the present study, quantitative assays showed that hESC-CD34^{+} cells had significantly reduced proliferative capacity and cloning frequency relative to UCB CD34^{+} cells, and although hESC-CD34^{+} cells readily produced erythroid, myeloid (primarily monocytic), and NK cells, B lymphoid potential was absent, consistent with studies from other groups.[2,16] Our goal was to determine whether gene expression profiles of hESC-CD34^{bright} and CD34^{dim} cells could identify critical blocks

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to normal definitive hematopoiesis, thereby identifying potential targets for therapeutic manipulation.

B lymphoid commitment and differentiation, a hallmark of definitive hematopoiesis, requires the coordinated expression of several transcription factors, notably *PU.1*, *E2A*, early B cell factor 1 (*EBF1*), and *PAX-5*, each of which act at distinct stages of B cell development.[19] B cell development is negatively regulated by ID proteins, which are known to block functions of E2A[20,21] and by CEBPα, which can reprogram B cells to monocytes by inhibiting PAX-5 function.[22] Through analysis of specific populations derived from hESC and UCB, we find that transcription factors critical for specific stages of B lymphoid development from UCB cells are aberrantly expressed in hESC-CD34$^+$ subsets. In addition, negative regulators such as *LNK* and *CEBPα*, are highly expressed in CD34^{bright} and CD34^{dim} cells, respectively, but are not detectable in relevant subsets from UCB. We find that downregulation of *LNK* transcription through short hairpin RNA (shRNA)-based targeting, significantly increases hematopoietic output at the expense of endothelial differentiation demonstrating that these aberrant transcription profiles have functional consequences for hematopoietic differentiation. Further analysis demonstrates that both B cell-"specific" positive regulators and negative regulators expressed in hESC-CD34$^+$ cells are also expressed at high levels in undifferentiated hESC and/or in CD326-CD56$^+$ embryonic mesoderm progenitor cells, a mesoderm-specific population that arises from hESC before hematopoietic commitment and CD34 expression, and which is able to generate all mesoderm derivatives.[23] Our data thus reveals a dysregulated gene expression pattern in hESC-derived progeny, which may reflect the persistence of "transcriptional memory" from the pluripotent stage, and which likely hinders normal definitive hematopoiesis.

RESULTS

Timing of hematopoietic differentiation during EB formation

A serum-free, stroma-free system of differentiation was used to study the immunophenotype and gene expression during the earliest stages of hematopoietic commitment that occur spontaneously in embryoid bodies (EBs), with minimal influence of exogenous factors (**Supplementary Figure S1**).[10] The combination of bone morphogenetic protein-4 (BMP-4) and basic fibroblast growth factor was sufficient to generate CD34$^+$ cells in serum-free, stroma-free conditions. As previously described,[8,23] c-kit and KDR (vascular endothelial growth factor receptor 2) expression was detected at low levels on undifferentiated hESC (data not shown). A distinct c-kit^{high} population was generated by day 2, whereas KDR was upregulated on a subset of cells by day 5 (**Figure 1a**). Expression of CD34 peaked at day 8; coinciding with the peak of expression of the endothelial markers CD31 and KDR. Expression of the pan-leukocyte marker CD45 remained undetectable for at least the first 12 days of EB culture.

Analysis of key transcriptional regulators involved in mesoderm and hematopoietic development is shown in **Figure 1b**. Expression of the mesoendodermal gene *T* (brachyury homolog) was detected early, by day 2. *SCL*, *GATA-1*, and *HOXB4,* transcriptional factors essential for normal hematopoietic development, were upregulated by day 5 of EB formation. Thus, the frequency of CD34$^+$ cells in hEBs peaked soon after the onset of expression of key hematopoietic transcription factors. As CD34 expression in hEBs peaked on day 8 and declined thereafter, we chose this time point for further immunophenotypic, functional, and molecular analysis of hESC-derived CD34$^+$ cells before further lineage commitment, comparing them with CD34$^+$ cells from UCB as a representative source of definitive HSC and progenitors with full lymphohematopoietic potential.

Immunophenotypic and morphologic analysis of hESC-derived and UCB-derived CD34$^+$ cells

CD34$^+$ cells from UCB and hESC showed a number of immunophenotypic differences. UCB contained cells with a relatively homogeneous level of CD34, all of which coexpressed low levels of the pan-leukocyte marker CD45 (**Figure 1c**). In contrast CD34$^+$ cells generated by day 8 from hESC could be divided into CD34^{bright} and CD34^{dim} subsets, neither of which expressed CD45, using a variety of different fluorochrome markers for analysis (**Figure 1d**). Expression of endothelial markers was notably different between the UCB CD34$^+$ cells and the two hESC-CD34$^+$ subsets. The endothelial markers CD31, KDR, and VE-Cadherin were all expressed on hESC-CD34^{bright} but not CD34^{dim} cells (**Figure 1d**). CD34$^+$ cells from UCB coexpressed CD31, but did not express either KDR or VE-Cadherin (**Figure 1c**). In addition, CD143 (BB9) was expressed on both CD34^{bright} and CD34^{dim} cells from hESCs, but only by small fraction of UCB CD34$^+$ cells (**Supplementary Figure S2**). A CD143$^+$CD34-population was also detected in the hEBs but not UCB-derived cells (**Supplementary Figure S2**).

Using imaging flow cytometry, the size and morphology of the two hESC-CD34$^+$ subsets were noted to be significantly different. hESC-CD34^{bright} cells were larger (mean FSC = 250) than both hESC-CD34^{dim} cells (mean FSC = 200) and UCB CD34$^+$ cells (mean FSC = 120) (**Supplementary Figure S3a,b**), and hESC-derived CD34$^+$ cells (particularly CD34^{bright} cells) had a lower nuclear: cytoplasmic ratio than UCB CD34$^+$ cells (**Supplementary Figure S3d**). CD34$^+$ cells derived from hEBs showed higher apoptosis (4.1%) (**Supplementary Figure S3c**), than UCB CD34$^+$ cells [(0.8%), data not shown]. Immunohistochemical analysis of paraffin sections of hEBs revealed that CD34 was expressed on large endothelial cells that line vascular structures within hEBs as well as on smaller single cells with hematopoietic morphology within the lumen of the structures (**Supplementary Figure S4**).

Differential hematopoietic and endothelial potential of hESC-derived CD34$^+$ subsets

Intracellular endothelial nitric oxide synthase was expressed at high levels in most hESC-CD34^{bright} cells, consistent with an endothelial phenotype (**Figure 2a**). Fluorescence-activated cell sorting (FACS) sorted hESC-CD34^{bright} cells were capable of forming tubular structures in endothelial conditions (**Figure 2b**), and CD45$^+$ cells in hematopoietic conditions indicating their bipotential ability (**Figure 2c**). In contrast, hESC-CD34^{dim} cells showed lower endothelial nitric oxide synthase expression and minimal endothelial potential (**Figure 2c**) but did generate CD45$^+$ cells confirming a more hematopoietic-skewed potential.

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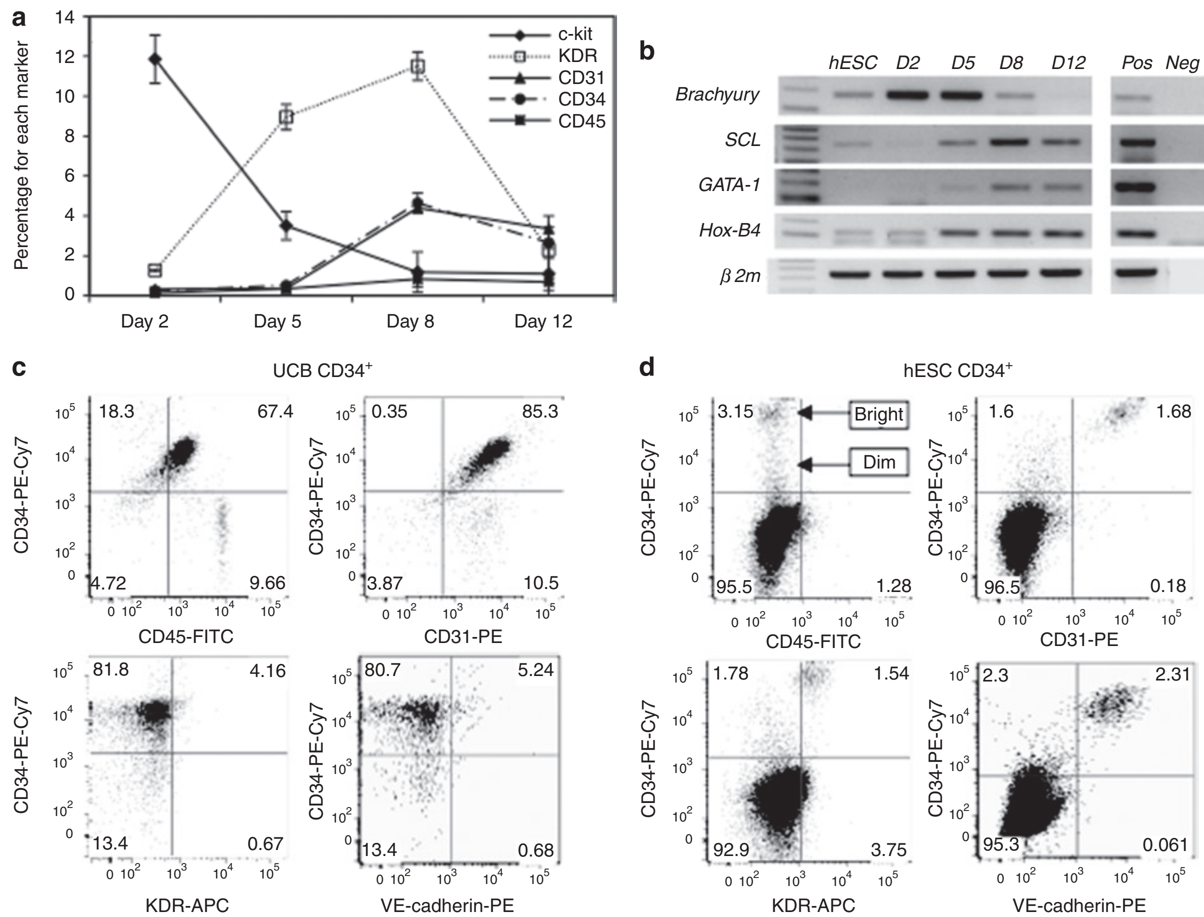

Figure 1 Stimulation with bone morphogenetic protein-4 (BMP-4) and basic fibroblast growth factor (bFGF) in a serum-free embryoid body (EB) system leads to mesoderm specification, hematopoietic differentiation, and generation of CD34$^+$ cells. (**a**) Time line of expression of hematopoietic markers seen during differentiation of human embryonic stem cells (hESCs) cells as EBs, with BMP-4 and bFGF (BF medium). Mean ± SEM (n = 3). Shown on the y-axis are percentages of total hEB-derived cells that express CD34, CD31, or CD45. Since c-kit and KDR were expressed at low levels on most undifferentiated hESCs (data not shown), percentages for these markers reflect only c-kit^{bright} and KDR^{bright} cells. (**b**) Semiquantitative RT-PCR of equal numbers of undifferentiated hESCs and total cells harvested from EBs at days shown of differentiation in BF medium. Pos control (SCL and GATA-1: K562, Hox B4: Mo7e), Neg control (water). A representative gel of three independent experiments is shown (H1: n = 2, H9: n = 1). (**c,d**) CD34$^+$ cells derived from fresh umbilical cord blood (UCB) or day 8 EBs generated in BMP-4 and bFGF were compared by fluorescence-activated cell sorting (FACS) for immunophenotypic differences. (**c**) UCB CD34$^+$ cells are CD45$^+$/CD31$^+$/KDR$^-$/VE-cadherin$^-$ (**d**) hESC-CD34$^+$ cells can be identified as CD34^{bright} cells which are CD45-/CD31$^+$/KDR$^+$/VE-Cadherin$^+$ and CD34^{dim} cells which are CD45$^-$/CD31$^-$/KDR$^-$/VE-Cadherin$^-$. A representative plot (H1 cells) of n = 3 separate experiments is shown (H1: n = 2, H9: n = 1, UCB: n = 3).

Erythromyeloid differentiation from hESC-CD34$^+$ and UCB-CD34$^+$ cells

When replated in myeloerythroid culture conditions, CD34$^+$ cells isolated from both UCB and hESC, generated CD45$^+$ cells that included CD66b$^+$ granulocytes and CD14$^+$/HLA-DR$^+$ monocyte/macrophages, as well as a subset of CD45^{neg} cells that expressed the erythroid marker CD235 (Glycophorin A) (**Figure 3a,b**). Cultures initiated from hESC-CD34$^+$ cells also contained a residual population of CD34$^+$CD45^{neg} cells (**Figure 3b**).

Although qualitatively, myeloerythroid differentiation potential was similar between CD34$^+$ cells from hESCs and UCB, the proliferative potential of CD34$^+$ cells from each source was markedly different. UCB CD34$^+$ cells generated a 138.8 ± 22.8-fold increase in total cell number whereas hESC-CD34$^+$ generated only 4.8 ± 3.8-fold increase in total cells after 2 weeks (n = 3, P < 0.01) (**Figure 3c**). To assess the proliferative defect at a clonal level, limiting dilution analyses were performed in parallel on CD34$^+$ cells from each source. These analyses revealed that the frequency of clonogenic cells within the hESC-CD34$^+$ population was >2 logs lower than in UCB-CD34$^+$ cells, at 1 in 6,315 for hESC-CD34$^+$ cells (95% confidence interval; 1 in 2,968 to 1 in 13,439) versus 1 in 17 for UCB CD34$^+$ cells (95% confidence interval; 1 in 14 to 1 in 20) (**Figure 3d**). Consistent with this data, the clonogenic frequency of hESC-CD34$^+$ cells in methylcellulose was >600-fold lower as compared to UCB CD34$^+$ cells (**Supplementary Figure S5**).

Impaired lymphoid potential from hESC-CD34$^+$ cells

The proliferative potential of hESC-CD34$^+$ cells grown under lymphoid culture conditions, was even more impaired than that seen in myeloerythroid conditions, with cell numbers falling to 10–50% of input number in the first 2 weeks of culture and no

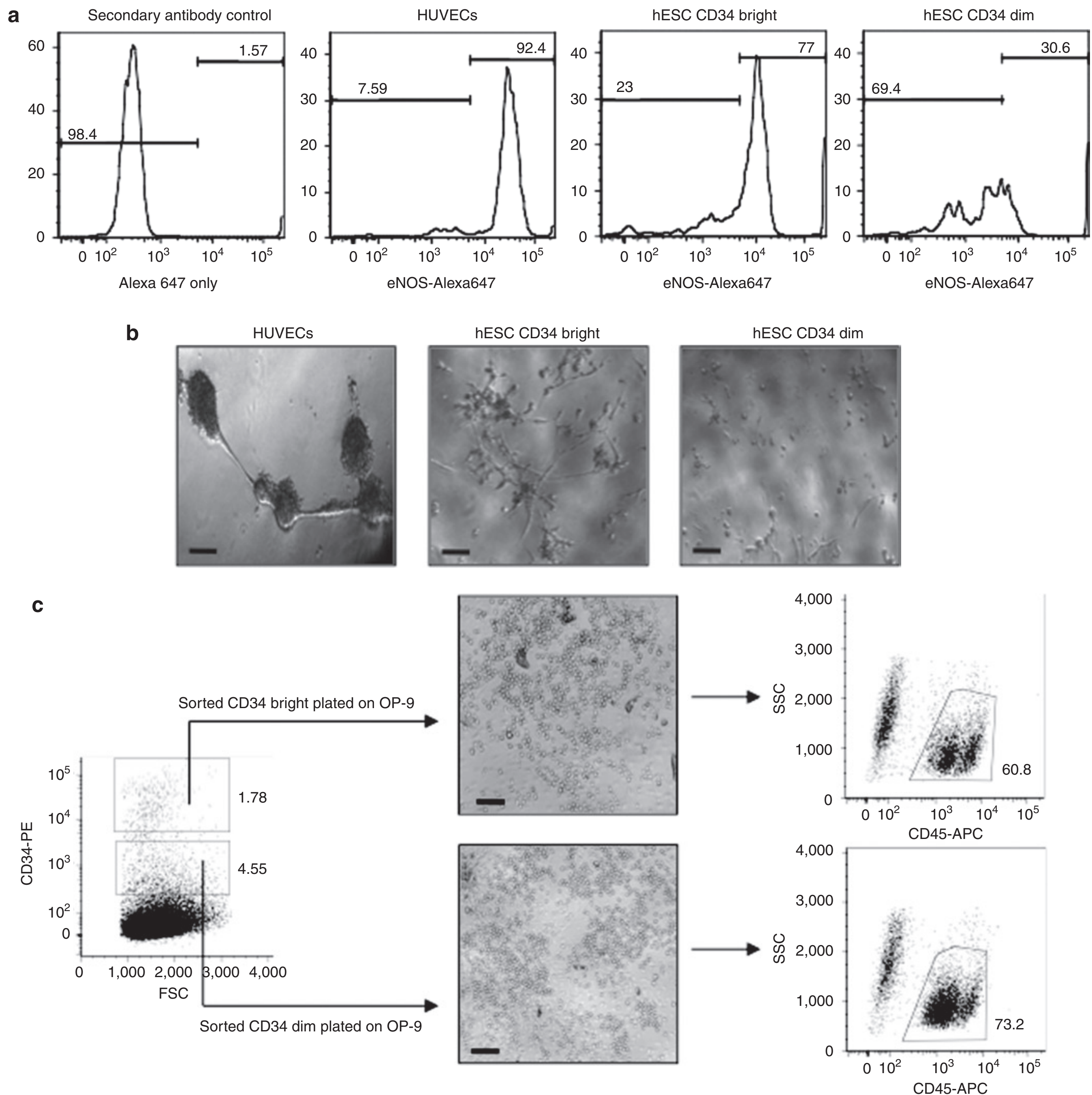

Figure 2 Analysis of hematoendothelial potential in human embryonic stem cells (hESC) CD34+ subsets. (**a**) Intracellular endothelial nitric oxide synthase (eNOS) expression in CD34bright and CD34dim cells sorted from day 8 embryoid body (EBs) (as shown in **c**) and generated in serum-free medium in presence of bone morphogenetic protein-4 (BMP-4), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF) (BFV). Human umbilical vein endothelial (HUVEC) cells were used as a positive control. Specificity was determined by using secondary antibody alone. (**b**) Endothelial tube formation from HUVECs and hESC-CD34bright cells ($n = 2$ H9 cells) in matrigel in complete EGM2 medium (see **Supplementary Materials and Methods**) (original magnification ×40) bar = 100 µm. (**c**) Generation of clusters of hematopoietic cells showing CD45 expression after culture of sorted CD34bright and CD34dim cells on OP-9 medium for 3 weeks in presence of SCF+ FL+TPO + IL-7 and IL-3 (IL-3 added only from day 0–3). Bar = 100 µm. A representative plot (H1 cells) of $n = 3$ separate experiments is shown (H1: $n = 2$, H9: $n = 1$). Images for (**b**) and (**c**) were captured using a Nikon Eclipse Ti-U, inverted microscope (original magnification ×40). eNOS, endothelial nitric oxide synthase.

ability to maintain long-term cultures from these progenitors. In parallel under identical lymphoid conditions, UCB CD34+ cells generated an 87.1 ± 21.1-fold increase of total cells (Figure 4a) ($P < 0.01$) The cloning frequency of hESC-CD34+ cells under lymphoid conditions was less than one hundredth that of UCB CD34+ cells at 1:10,532 (95% confidence interval; 1 in 4,368 to 1 in 25,393) for hESC-CD34+ versus 1 in 68 for UCB CD34+ cells (95% confidence interval; 1 in 51 to 1 in 90) (Figure 4b).

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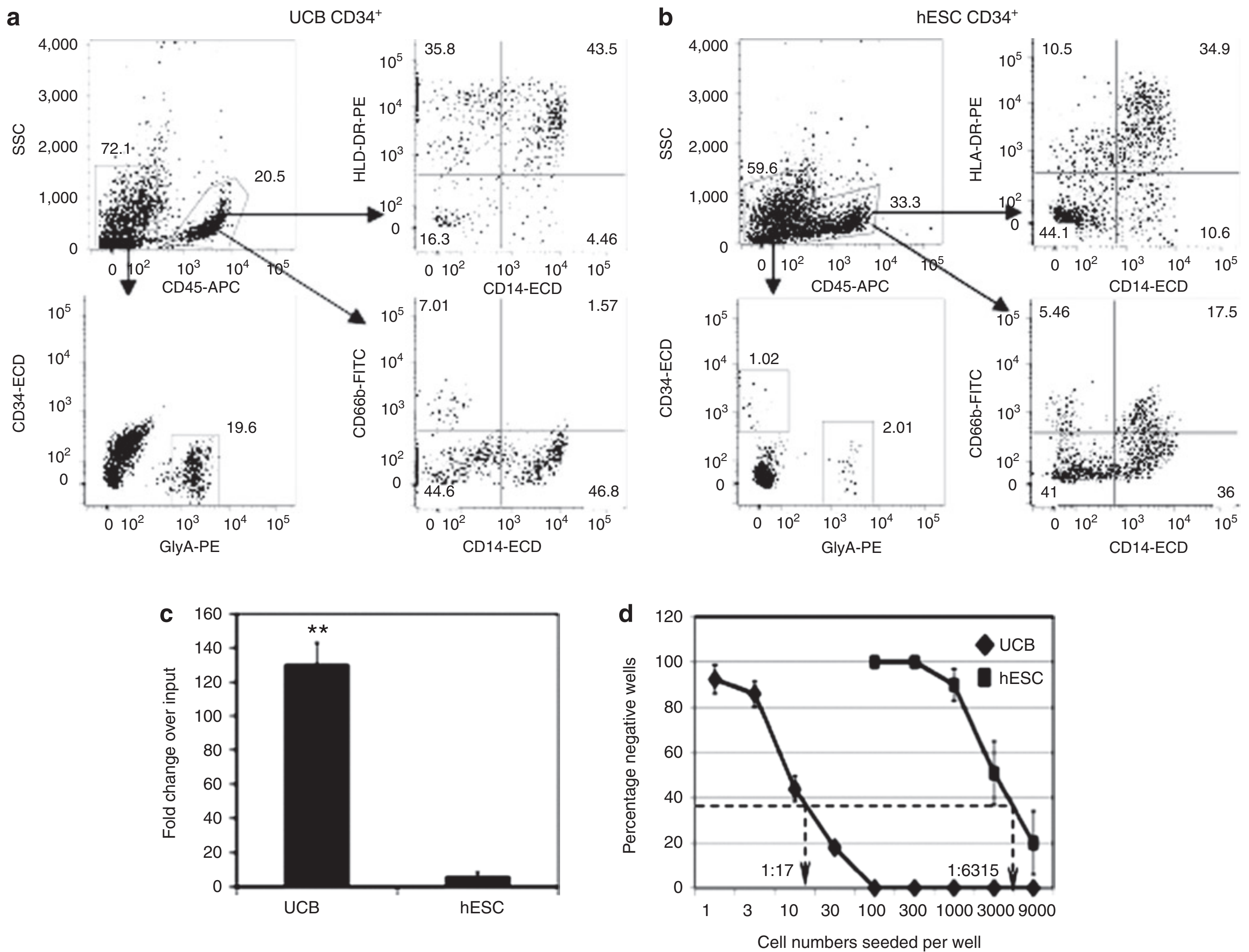

Figure 3 Proliferation and differentiation of human embryonic stem cells (hESC)-CD34$^+$ and umbilical cord blood (UCB) CD34$^+$ cells in myelo-erythroid conditions. (**a**) UCB CD34$^+$ and (**b**) hESC-derived CD34$^+$ cells, isolated from day 8 hEBs and cultured in myeloid conditions for 4 weeks, generate CD14$^+$/HLA-DR$^+$/CD45$^+$ monocytes, CD66b$^+$ granulocytes, and GlyA$^+$/CD45$^-$ erythroid cells (shown is representative data from n = 4 experiments with H9 cells, and n = 2 experiments with H1 cells). (**c**) Fold change in cell number of UCB and day 8 EB CD34$^+$ cells cultured on OP-9 stroma under myeloid conditions (20%serum + SCF + FL + TPO + IL-3 + EPO). Input cell number for both CD34$^+$ populations was normalized to 1 (n = 3, H9 cells) (mean ± SEM, **P < 0.01 by Student's paired t-test). (**d**) Limiting dilution analysis showing cloning frequency of UCB and hESC-CD34$^+$ cells grown in myeloid conditions (n = 2, H9 cells). EPO, erythropoietin; FL, Flt3 Ligand; SCF, stem cell factor; TPO, thrombopoietin; IL, interleukin.

B lymphoid differentiation from hESC-CD34$^+$ cells was absent. In conditions, which allowed robust differentiation from UCB CD34$^+$ cells into CD19$^+$ B cells and CD56$^+$CD94$^+$ NK cells (**Figure 4c**), hESC-CD34$^+$ cells generated few CD45$^+$ cells and CD19$^+$ B cells were never generated (**Figure 4d**). hESC-derived CD34$^+$ cells were then cultured with higher concentrations of cytokines including c-kit ligand [(stem cell factor (SCF)]; which have been shown to generate lymphoid cells from human aorta-gonad-mesonephros (AGM)-derived cells.[24] Under these conditions, CD56$^+$/CD45$^+$ NK cells could be more readily identified with a subset expressing CD94, (**Figure 4e**) a c-type lectin restricted to the NK lineage.[7] However, again in these conditions, no CD19$^+$ B cells could be generated and the hESC-CD34$^+$ cells primarily differentiated into monocytes (**Figure 4e**). hESC-CD34$^+$ cells were unable to give rise to CD19$^+$ B cells irrespective of the morphogens or serum used during hEB differentiation (**Supplementary Figure S6**). In addition, CD34^{bright} and CD34^{dim} cells separately isolated from hEBs at day 8, and plated on OP-stroma for 4 weeks in lymphoid conditions were also unable to produce B cells (**Supplementary Figure S7**).

B lymphoid potential is defective in hESC-derived CD34$^+$ cells irrespective of culture conditions and time of analysis

We have noted that longer culture of EBs beyond day 8 leads to a rapid loss of CD34$^+$ cells and the generation of more mature monocyte-committed cells (CD14$^+$CD45$^+$) within the EBs (**Supplementary Figure S8**). Thus, in the experiments described to this point, B cell potential of hESC-derived progenitors was assessed using CD34$^+$ cells isolated early (day 8) from EBs, reasoning that this time point would allow analysis of progenitors before commitment and further loss of lineage potential. However, when hematopoietic differentiation from

hESC is induced using stromal coculture in the presence of serum, it is possible to generate CD34$^+$ cells that coexpress CD45 later in culture.[2] Thus to explore further the B cell potential of hESC-derived CD34$^+$ cells which are immunophenotypically more similar to definitive progenitors from UCB, we next tested progenitors derived from hESCs by cocultivation on OP-9 stroma and isolated later in culture after acquisition of CD45. Similar to the previous hEB cultures, two subsets of CD34$^+$ cells were generated from the OP-9 stroma,

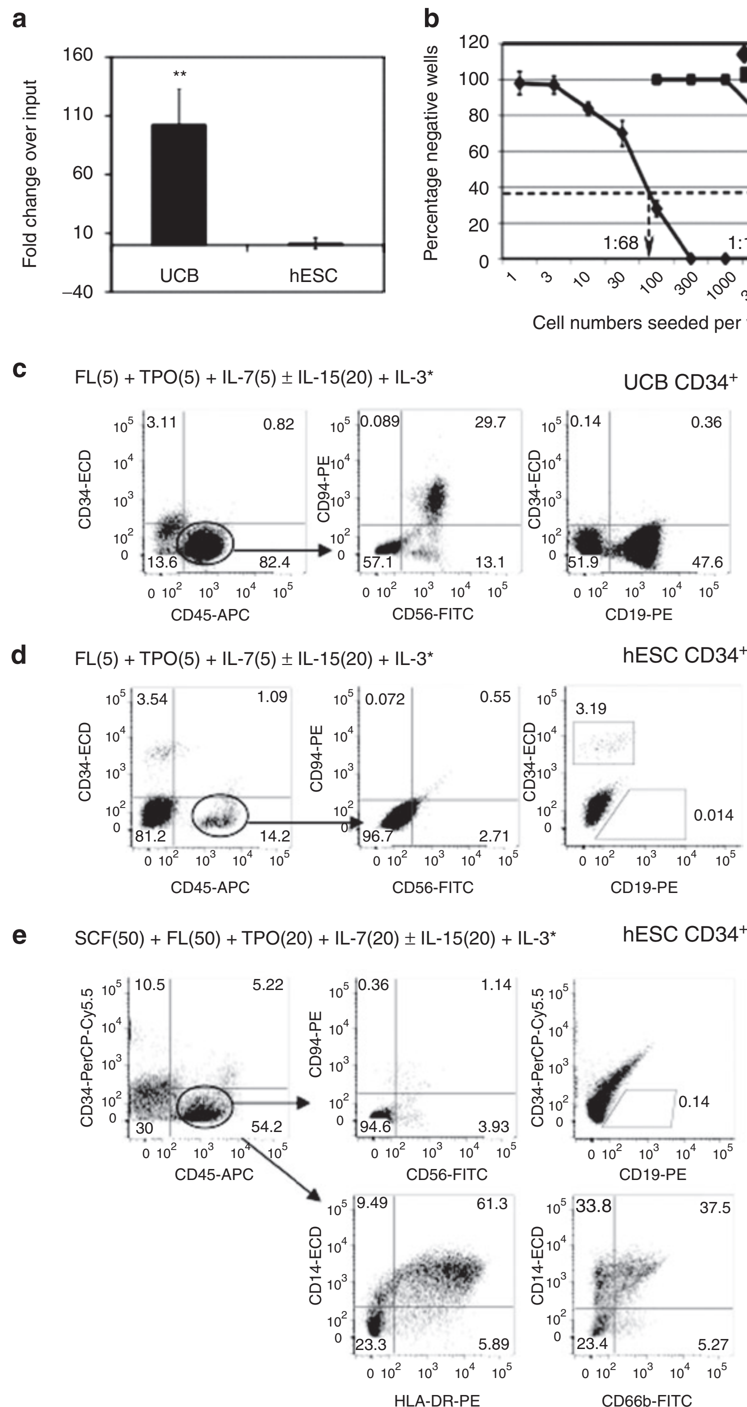

Figure 4 Proliferation and differentiation of human embryonic stem cells (hESC)-CD34$^+$ and umbilical cord blood (UCB) CD34$^+$ cells in lymphoid conditions. (**a**) Fold change in cell number of UCB CD34$^+$ and day 8 EB CD34$^+$ cells cultured on OP-9 stroma under lymphoid conditions as shown in **c,d**. Input cell numbers (9,000/well × 5 wells) normalized to 1 (n = 3, H9 cells) (mean ± SEM, **P < 0.01 by Student's paired t-test). (**b**) Limiting dilution analysis showing cloning frequency of UCB and hESC-CD34$^+$ cells (n = 2, H9 cells). (**c**,**d**,**e**) Fluorescence-activated cell sorting (FACS) analysis of week 4 cultures in lymphoid conditions (5% serum with cytokines shown). *IL-3 (10 ng/ml) was added only for days 0–3. (**c**) Cultures initiated with UCB CD34$^+$ cells, or (**d**,**e**) hESC-CD34$^+$ cells isolated from day 8 embryoid bodies (EBs). Shown is representative data from n = 4 experiments with H9 cells, and n = 2 experiments with H1 cells. EPO, erythropoietin; FL, Flt3 Ligand; SCF, stem cell factor; TPO, thrombopoietin; IL, interleukin.

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a CD34^{bright} CD45-subset, which coexpressed the endothelial markers KDR and VE-Cadherin, and a CD34^{dim} subset that expressed the hematopoietic marker CD45 but did not express endothelial markers (**Supplementary Figure S9a**). CD34$^+$CD45$^+$VE-Cadherin-KDR-cells were isolated at day 10, day 16, and day 21 of OP-9 stromal culture and replated on OP-9 stroma with lymphoid cytokines for 4 additional weeks (**Supplementary Figure S9b**). No CD19$^+$CD45$^+$ cells were generated from these CD34$^+$CD45$^+$ cells, and even in these lymphoid conditions most of the CD45$^+$ cells generated expressed the monocytic marker CD14. These experiments indicate that a block in B cell development exists from hESC-derived CD34$^+$ cells irrespective of the induction conditions (EB or stromal coculture) or the timing of their generation in culture.

Gene expression profiles reveal intrinsic differences between hESC-CD34$^+$ and UCB CD34$^+$ cells

To identify the molecular mechanisms underlying the functional defects observed in hESC-CD34$^+$ cells, the expression of genes known to play key roles in definitive hematopoiesis and B lymphoid differentiation was analyzed. We first investigated expression of two transcription factors critical for definitive hematopoiesis, *RUNX1*[25] and *PU.1*.[26] *RUNX1* expression was upregulated in all hESC-CD34$^+$ fractions relative to undifferentiated hESCs (**Figure 5a**). *RUNX1* levels in hESC-CD34$^+$ cells were similar to UCB CD34$^+$CD38$^-$ cells (a population that includes definitive HSCs with full lymphomyeloid potential),[27] and highest levels of *RUNX1* were observed in the presence of vascular endothelial growth factor ("BFV" conditions). *PU.1* expression was similarly increased relative to undifferentiated hESC, reaching levels that were similar to those seen in CD34$^+$CD38$^-$ HSC. *PU.1* expression in UCB subsets was lowest in CD34$^+$CD38$^-$ HSC, threefold higher in CD34$^+$19$^+$ B cell progenitors and 16-fold higher in CD34$^+$CD19$^-$ (which are predominantly myeloid progenitors), consistent with the known *Pu.1* dose-dependant, lineage outcomes with higher levels favoring macrophage development.[28] The high expression of *PU.1* seen in the CD34$^+$CD19$^-$ population, was not seen in any of the hESC-derived CD34$^+$ cells. Thus, expression levels of *RUNX1* and *PU.1* in hESC-CD34$^+$ cells were comparable to CD34$^+$CD38$^-$ definitive HSCs in UCB. The monocyte-skewed differentiation of the hESC-CD34$^+$ subsets shown in functional assays was not associated with high *PU.1* expression.

Positive regulators of B cell development were next analyzed (**Figure 5b–d**). In UCB, none of the genes required for B cell commitment and differentiation viz. *E2A*, *EBF1*, *MB-1*, *IL7Rα*, and *PAX-5*, were expressed in CD34$^+$CD38-HSC, and all were upregulated as expected in B cell committed progenitors (CD34$^+$CD19$^+$ subset) (**Figure 5b–d**). Surprisingly, despite the absence of B cell potential of hESC-CD34$^+$ cells, B lymphoid gene expression was detected, particularly in the CD34^{bright} endothelial subset. *E2A* was expressed at high levels in both hESC-CD34^{bright} and hESC-CD34^{dim} cells generated in all conditions. Expression of *EBF1*, and *IL-7Ra*, both of which act downstream of *E2A* in B cell differentiation, was also detected particularly in the CD34^{bright} progeny of hESC (**Figure 5c**). *MB-1* (CD79a) (B-cell antigen receptor complex-associated protein α-chain precursor), an EBF1 target gene was also expressed particularly in CD34^{bright} cells (**Figure 5d**). However, *PAX-5*, which is absolutely required for

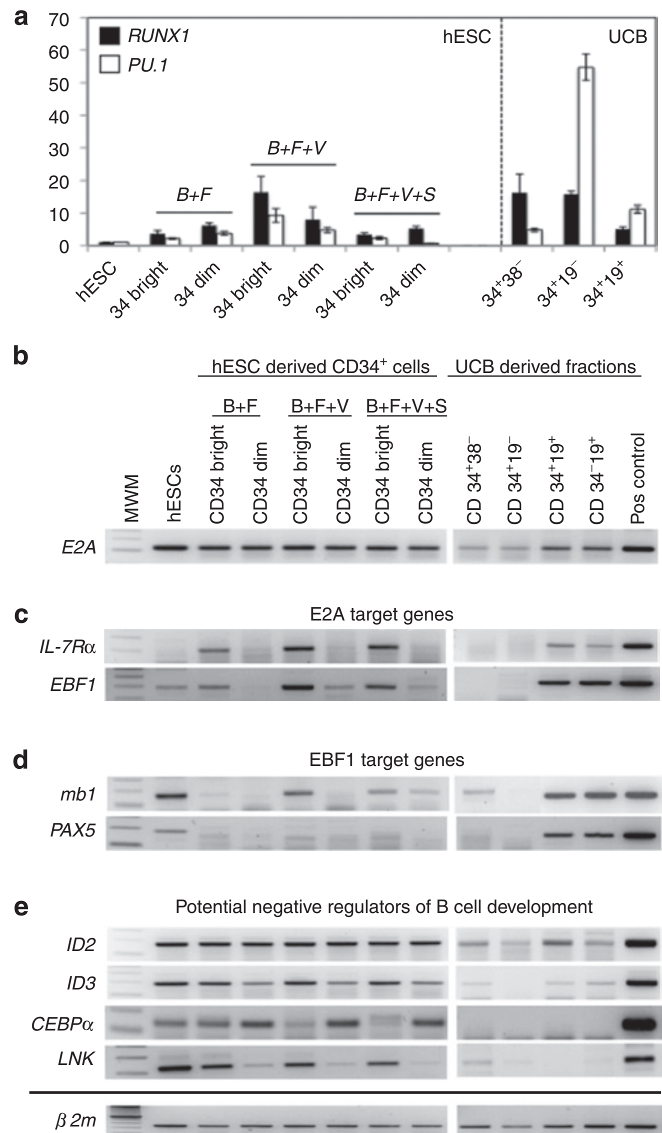

Figure 5 Gene expression profiles of human embryonic stem cells (hESC)-derived and umbilical cord blood (UCB) CD34$^+$ cell subsets. CD34^{bright} and CD34^{dim} cells were isolated by fluorescence-activated cell sorting (FACS) from day 8 embryoid bodies (EBs), after generation in three different conditions: (i) bone morphogenetic protein-4 (BMP-4) and basic fibroblast growth factor (bFGF) (no serum) (B+F), (ii) BMP-4, bFGF, and vascular endothelial growth factor (VEGF) (no serum) (B+F+V), and (iii) BMP-4, bFGF, and VEGF (+10% serum) (B+F+V+S). (**a**) Genes involved in early hematopoietic development (*RUNX1* and *PU.1*) were analyzed by quantitative reverse transcription (qRT)-PCR in undifferentiated hESC, hESC-derived CD34$^+$ subsets, three CD34$^+$ subpopulations of UCB (CD34$^+$CD38$^-$ [containing hematopoietic stem cells (HSCs)], CD34$^+$19$^-$ (which includes myeloid progenitors) and CD34$^+$CD19$^+$ (B cell progenitors), and mature B cells (CD34-CD19$^+$ cells). Data show mean ± SEM for three independent experiments using H9 cells. Y-axis shows fold change in expression is shown after normalization to housekeeping gene RPL-7. (**b–e**) Gene expression analysis by semiquantitative RT-PCR using identical cell numbers from each population shown. (**b**) Expression of *E2A*, a gene involved in early B cell development. (**c**) *E2A* target genes *IL-7Rα* and *EBF1*. (**d**) EBF1 target genes *MB-1* and *PAX-5*. (**e**) Expression of genes that negatively regulate B cell development. β2 microglobulin (β2M) was used as house keeping gene. Results shown are representative of three independent experiments for each gene analyzed (n = 2; H9 cells, n = 1; H1 cells, n = 3 UCB subfractions).

CD19 expression and B cell differentiation,[29] was not expressed in any CD34$^+$ populations derived from hESCs (**Figure 5d**),

Expression of potential negative regulators of definitive hematopoiesis and B cell development was next analyzed (**Figure 5e**). As described previously,[16] *ID2* and *ID3* were expressed in all hESC-CD34$^+$ cells, with higher levels of *ID3* in CD34^{bright} cells than in CD34^{dim} cells (**Figure 5e**). *ID2*, and to a lesser extent *ID3*, were also detected in UCB cells albeit at lower levels than in hESC-CD34$^+$ cells. Two other negative regulators, CCAAT/enhancer-binding protein-α (*CEBPα*) and *LNK* were also expressed at high levels in specific hESC-derived CD34$^+$ subsets. *CEBPα*, which inhibits Pax-5 and CD19 expression and dictates monocytic commitment,[22,30] was expressed in hESC-CD34^{dim} cells, but not detectable in hESC-CD34^{bright} cells or any UCB CD34$^+$ subsets. (**Figure 5e**). *LNK*, a negative regulator of hematopoietic development from the AGM region in mice and of B lymphopoiesis[31,32] was highly expressed in hESC-CD34^{bright} cells, but not hESC-CD34^{dim} or any UCB fractions (**Figure 5e**). Thus, the overall gene expression pattern seen in hESC-CD34$^+$ cells consisted of both positive and negative regulators of B lymphopoiesis and did not correspond to gene expression profiles in either multipotent HSC or more defined B cell progenitors from definitive hematopoiesis.

Aberrant expression of "B lymphoid" and negative regulatory genes precedes the onset of hematopoietic commitment

As several genes detected in hESC-CD34$^+$ subsets were also expressed in undifferentiated hESCs (**Figure 5b–e**), we next explored whether the aberrant molecular signature seen in the hESC-CD34$^+$ cells reflected a transcriptional memory from earlier stages of differentiation not specific to the hematopoietic lineage. We have recently reported that mesoderm commitment from hESC is marked by the downregulation of epithelial cell adhesion molecule (EPCAM aka CD326) and the upregulation of neural cell adhesion molecule (NCAM aka CD56).[23] The CD326-CD56$^+$ human embryonic mesoderm progenitor (hEMP) population appears by day 3.5 in mesoendodermal differentiation conditions, before the upregulation of markers used to define more lineage specific subsets (*e.g.*, CD34, KDR, CD31).[1,8,11] The hEMP population is able to generate hematopoietic, endothelial, mesenchymal, smooth muscle, and cardiac muscle and thus represents an intermediate differentiation stage between hESC and hematoendothelial CD34$^+$ cells.[23] We examined how gene expression levels changed during the generation of CD34$^+$ cells from hESC, by performing quantitative reverse transcription-PCR analysis on isolated populations of undifferentiated hESC, day 3.5 CD326-CD56$^+$ hEMPs, day 8 hESC-CD34^{bright}, and day 8 hESC-CD34^{dim} cells (**Figure 6a**). The BFV differentiation condition (a modification of the original "activin-BVF" conditions,[23] was used for these analyses, as the data in **Figure 5** suggested this combination produced the gene expression pattern most similar to CD34 subsets in UCB.

As expected, following BMP-4 induction the levels of mesoderm-specific genes (*T*, *MIXL1*, and *WNT-3*) (**Figure 6b**), and genes associated with definitive hematopoietic development (*RUNX1* and *PU.1*) (**Figure 6c**) were upregulated at the hEMP stage as compared to undifferentiated hESCs. Surprisingly, *E2A* expression was present and remained unchanged during differentiation from hESC to hEMP and then to CD34$^+$ cells (**Figure 6d**). Paradoxically, genes associated with B cell development which were only detectable at the B cell committed stage (CD34$^+$19$^+$) in the UCB population (*EBF1*, *MB-1*, *IL-7Rα*) (**Figure 5c–d**), were upregulated at the hEMP stage and expression remained high primarily in the CD34^{bright} subset (**Figures 5c–d** and **6d**).

Analysis of negative regulators showed that *ID2* and *ID3* levels were upregulated at the hEMP stage following BMP-4 induction, consistent with the fact that *ID* genes are known targets of BMP-4 stimulation.[33] *ID2* and *ID3* gene expression declined during CD34$^+$ differentiation from hEMP remaining highest in the CD34^{bright} subset (**Figure 6e**). *CEBPα*, which was not detectable in any CD34$^+$ subsets in UCB (**Figure 5e**), was expressed at low levels in hESC, hEMP, and hESC-CD34^{bright} cells, and upregulated in the CD34^{dim} subset (**Figure 6e**). *LNK*, although not expressed in any of the UCB subsets (**Figure 5e**), was persistently expressed at high levels from the undifferentiated hESC stage into the hEMP stage and also in the CD34^{bright} hematoendothelial progenitor stage, becoming finally downregulated in the CD34^{dim} subset.

LNK knockdown increases production of hematopoietic progenitors during hESC differentiation

Since LNK has been shown to be a negative regulator of proliferation as well as hematopoietic development in the mouse AGM,[31] we next analyzed whether knockdown of *LNK* expression could lead to transition from a hematoendothelial to a hematopoietic fate. *LNK* was targeted using a lentiviral-based shRNA strategy. Stable H9 clones expressing sh*LNK*B1 and control shRNA were obtained after selection with puromycin for 3 weeks (**Figure 7a**). RT-PCR analysis demonstrated marked reduction of *LNK* expression in hESC-expressing sh*LNK*B1 as compared to shRNA control vector, demonstrating the specificity of the *LNK* shRNA vector (**Figure 7b**). sh*LNK*B1 and control shRNA-expressing hESC as well as untransduced hESC were cultured on OP-9 stroma in SCF for 15 days, and hematopoietic differentiation analyzed by FACS. *LNK* knockdown resulted in a significant increase in the number of CD34$^+$ cells coexpressing CD45 as compared to untransduced or control shRNA transduced cells (**Figure 7c,d**). The CD34$^+$CD45$^+$ cells did not express VE-Cadherin (**Figure 7c**) consistent with their hematopoietic phenotype. Thus, *LNK* knockdown resulted in a switch from a hematoendothelial (CD34^{bright}/VE-Cadherin$^+$/CD45$^-$) to a hematopoietic phenotype (CD34^{dim}/VE-Cadherin$^-$/CD45$^+$) and a significant increase in the absolute numbers of CD34$^+$/CD45$^+$ cells was seen following *LNK* knockdown (**Figure 7d**). Thus, modulation of negative regulators such as *LNK* may provide a potential approach to enhance hematopoietic commitment during hESC differentiation.

DISCUSSION

CD34 expression is used to identify human definitive hematopoietic stem and progenitor cells that can reconstitute all lymphohematopoietic lineages following transplantation. Our data demonstrate that the CD34$^+$ immunophenotype defines a qualitatively different cell population when applied in the context of hESC-derived hematopoiesis. The major functional differences seen between hESC-CD34$^+$ and UCB-CD34$^+$ cells were in proliferative capacity and B lymphoid potential.

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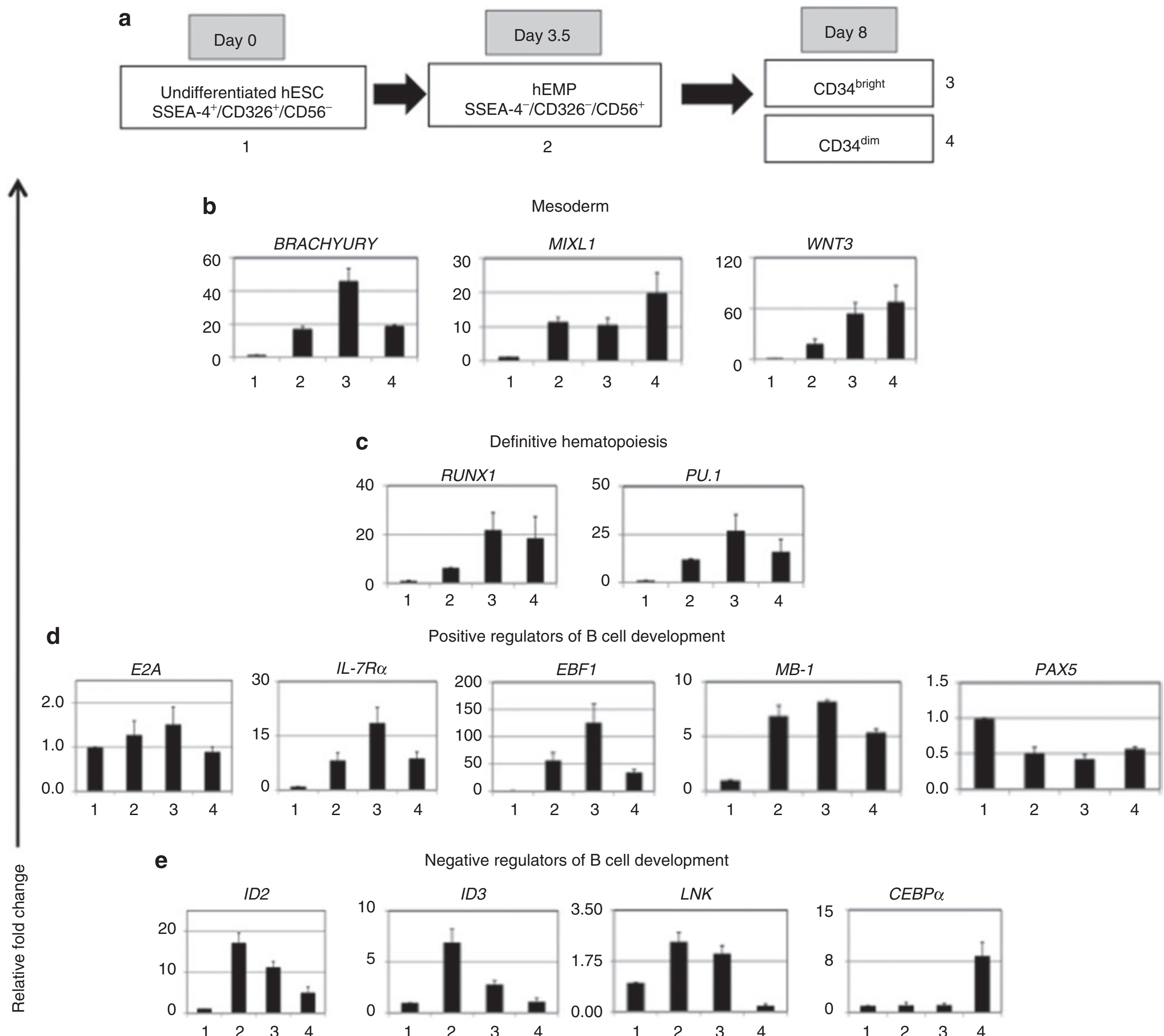

Figure 6 Quantitative reverse transcription (RT)-PCR analysis of undifferentiated human embryonic stem cells (hESCs), early mesoderm progenitors (hEMP), and CD34^{bright} and CD34^{dim} cells. (**a**) Schematic model of stages of mesoderm and hematopoietic differentiation from hESCs under mesoderm-specific conditions [(bone morphogenetic protein-4 (BMP-4), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF) (no serum) (B+F+V)], showing immunophenotypic populations isolated for gene expression analysis by qRT-PCR. Day of isolation of each population is shown. Numbers 1–4 next to the boxes correspond to the populations analyzed by qRT-PCR in **b–e**. (**b**) Mesoderm-associated genes. (**c**) Genes associated with definitive hematopoietic development. (**d**) Positive regulators involved in B cell development and maturation. (**e**) Negative regulators that potentially block B cell development. Fold change in expression is shown on y-axis after normalization to housekeeping gene RPL-7. Mean ± SEM for three independent experiments using H9 cells.

To understand the intrinsic differences between hESC-derived hematopoiesis and normal definitive lymphohematopoiesis, we directly analyzed the expression pattern of genes known to be critical for definitive hematopoiesis and B lymphopoiesis in CD34$^+$ cells from each source. Recognizing the functional and immunophenotypic differences between the CD34^{bright} and CD34^{dim} subsets from hESC, it was important to isolate and analyze gene expression in each population separately. The parallel analysis of purified sub-populations from UCB that represent both multipotent HSC (CD34$^+$CD38$^-$ cells) and B lymphoid cells (CD34$^+$CD19$^+$ progenitors and CD34$^-$CD19$^+$ B cells), permitted the interpretation of gene expression patterns in hESC-derived cells in the context of well-defined stages of hematopoietic differentiation. Irrespective of the conditions in which CD34$^+$ cells were generated from hESC, two related themes arose from these studies.

First, promiscuous gene expression by hESC-derived cells significantly complicates attempts to assign hematopoietic lineages using molecular signatures developed from studies of normal adult hematopoietic developmental programs. Functional B lymphoid potential was absent in hESC-derived CD34$^+$ cells, despite the expression of genes in this population that are specific to B lymphoid differentiation during definitive hematopoiesis.

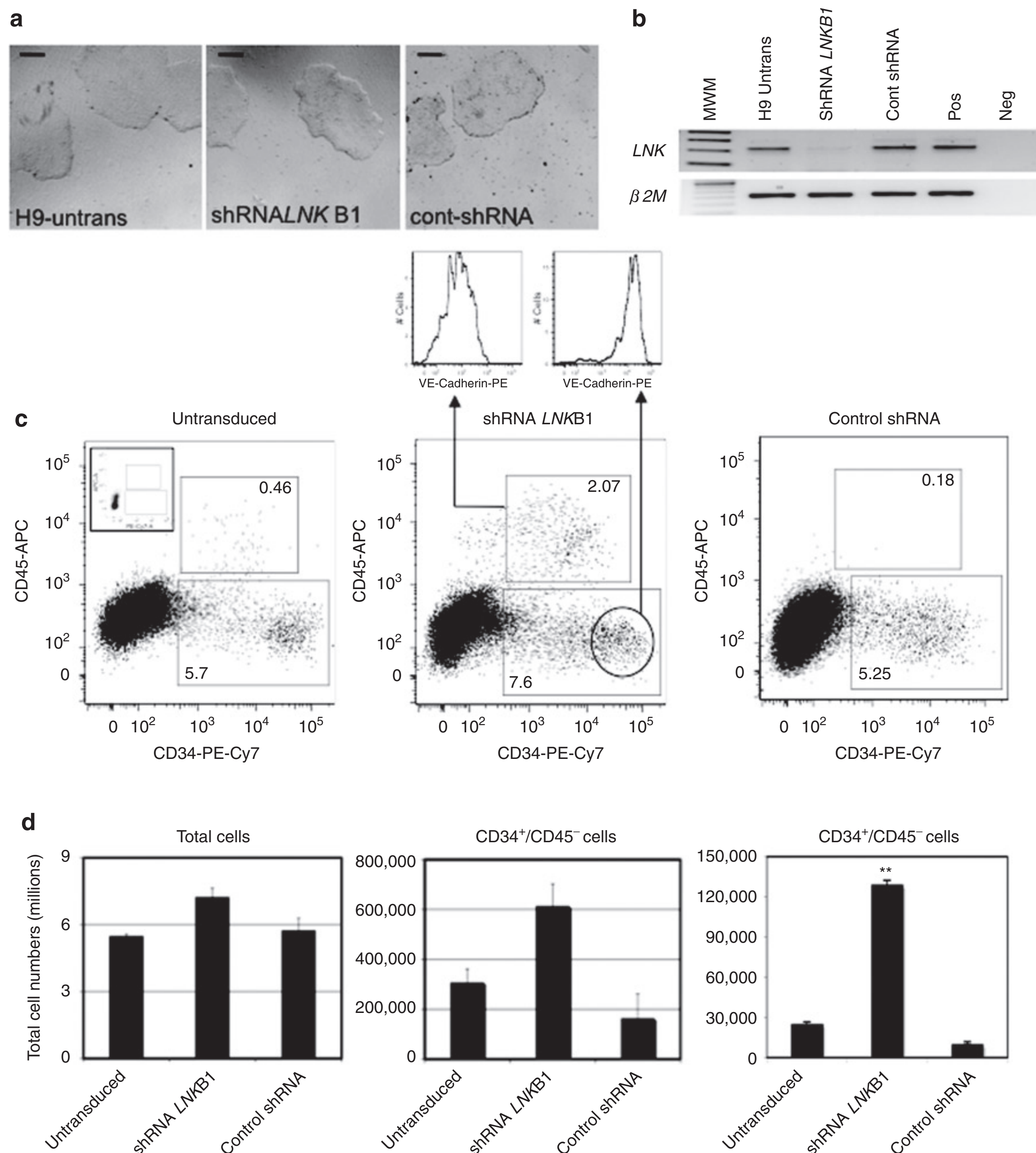

Figure 7 Knockdown of ***LNK*** **leads to an increase in generation hematopoietic progenitor cells.** (**a**) Colonies of untransduced and short hairpin RNA (shRNA) *LNKB1* or control shRNA transduced human embryonic stem cells (hESCs) (H9 cells) on matrigel after selection for 3 weeks in puromycin. Bar = 100 µm. (**b**). Semiquantitative reverse transcription (RT)-PCR showing efficient and specific knockdown of *LNK* in undifferentiated hESC. (Pos control: nalm-6, Neg: water). (**c**) Fluorescence-activated cell sorting (FACS) analysis of hESC-derived cells after culture on OP-9 stroma for 16 days showing expression of hematopoietic and endothelial markers. Insert in untransduced hESC panel on left shows unstained cells. (**d**) Absolute cell numbers of immunophenotypic populations as shown from untransduced, shRNA LNK B1 and control shRNA transduced hESCs. (Mean ± SEM or three independent experiments, H9 cells.) (**$P < 0.01$ by Student's paired *t*-test).

Analysis of the transcription factors *E2A*, *EBF*1, and *MB-1* suggested that unlike the tight restriction of their expression to B lymphopoiesis in UCB, all were expressed in undifferentiated hESC and hESC-CD34$^+$ cells. By extending analysis to the CD326-CD56$^+$ hEMP population recently described as a multipotent mesoderm-specific progenitor that precedes hematopoiesis and CD34 expression,[23] we showed that expression of *E2A* in hESC-CD34$^+$ cells is likely a result of persistence from the undifferentiated stage, and that *EBF1* and *MB-1* are upregulated in hEMP before hematopoietic commitment. It should be noted that both *E2A* and *EBF1* are known to be important in development of nonhematopoietic lineages.[34–37] Thus neither the expression of these transcription factors, nor the expression of *IL-7R*α and *MB-1,* which are downstream targets of E2A and EBF1, respectively, can be reliably used in isolation as proof of B lineage commitment from hESC-derived cells.

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Differential *IL-7Rα* expression by CD34^{bright} and CD34^{dim} subsets, could be explained by the endothelial and hematopoietic skewing of these respective populations. *IL-7Rα* was expressed in hESC-CD34^{bright} fractions but not in hESC or CD34^{dim} cells. *IL-7Rα* has been reported to be expressed by human microvascular endothelial cells,[38] thus suggesting this cytokine receptor may be specifically upregulated during hematoendothelial differentiation of hESC cells.

CEBPα is involved in monocytic differentiation of myeloid progenitors[30] and is expressed in CD15$^+$ cells from human bone marrow.[39] When overexpressed, *Cebpα* is sufficient to reprogram murine B cells into monocytes by directly inhibiting *PAX-5*.[22] High *Cebpa* in conjunction with *Pu.1* is able even to reprogram nonhematopoietic fibroblasts to a monocytic fate.[40] *PU.1* expression was not high in the hESC-derived CD34$^+$ cells, but high *CEBPα* expression in hESC-CD34^{dim} cells isolated at such an early stage during hESC differentiation would be likely sufficient to prevent B cell differentiation and drive commitment toward the monocytic lineage in this subset, irrespective of whether the cells were cultured in myeloid or lymphoid conditions.

The second major conclusion from our data is that the persistent expression of transcripts from the hESC stage and/or their upregulation during mesoderm commitment may also contribute to the functional defects of the CD34$^+$ cells generated from these recent precursors. Findings from a previous study suggested that high levels of the ID proteins may block B cell differentiation from hESC.[16] Our data confirms the expression of *ID2* and *ID3* in hESC-CD34$^+$ cells, but also notes that expression begins in undifferentiated hESC with further upregulation during mesoderm commitment. The ID proteins are known to negatively regulate B lymphopoiesis in humans[20] and mice,[21] acting as dominant-negative inhibitors of E2A function by forming dimers with E-proteins which are then unable to bind DNA. Inhibition of E protein function by ID proteins should affect expression of E protein targets. The function of ID proteins is however not restricted to the hematopoietic lineage. ID proteins are known to prevent differentiation during development[41] and *ID* expression has been seen in both epithelial cells and cells undergoing EMT during development,[41] thus explaining their presence in our studies in both undifferentiated hESCs and in the CD326-CD56$^+$ hEMPs. The finding that two downstream targets of E2A, *EBF1* and *IL-7Rα*[42,43] were also expressed in hESC-CD34$^+$ cells, particularly in the hESC-CD34^{bright} subset, argues that high ID expression is not sufficient to explain the block of E2A function in all CD34$^+$ cells derived from hESC. High ID levels are also unlikely to completely account for the proliferative and engraftment defects of hESC-derived CD34$^+$ cells indicating that additional mechanisms are likely responsible for these defects.

The significantly lower proliferation of hESC-CD34$^+$ cells when cultured in the presence of hematopoietic cytokines, suggested a possible role for negative regulators of cytokine signaling. *LNK*, an adapter protein, has been described as a broad inhibitor of signaling pathways in hematopoietic lineages.[44] In our studies, *LNK* was expressed in hESCs, hEMP, and hESC-CD34^{bright} cells, becoming downregulated only in the more mature hematopoietic-restricted hESC-CD34^{dim} cells. *LNK* was not expressed in any of the UCB subsets. *LNK* has been implicated as a negative regulator of hematopoiesis in the AGM region,[31] the site of hematopoiesis in the developing embryo.[24,45] Overexpression of *LNK* in the CD34$^+$CD45$^-$ hematoendothelial progenitors from the murine AGM significantly reduced the generation of CD45$^+$ cells[31] indicating that LNK may block hematoendothelial to hematopoietic transition. In our own studies, knockdown of *LNK* in hESCs using shRNA targeting, lead to an increase in the generation of hematopoietic progenitors (CD34^{dim}/VE-Cadherin$^-$/CD45$^+$) indicating that high *LNK* expression is functionally relevant in the diminished hematopoietic differentiation ability of hESCs. Of note, transient inhibition of *LNK* has been shown to enhance engraftment[46] indicating high *LNK* expression could account for the poor engraftment ability associated with hESC-derived CD34$^+$ cells.[6]

In summary, the finding that transcription factors tightly linked to specific stages of B lymphoid differentiation during normal "adult" hematopoiesis, are also present at the undifferentiated hESC stage and during mesoderm commitment before the onset of hematopoietic differentiation, even less B lymphopoiesis, demonstrates that great caution should be used in assigning lineage potential to hESC-derivatives solely based on gene expression analyses. Furthermore, "transcriptional memory" in the hematopoietic cells derived from the hESC or hEMP stage is likely to contribute to the inability to derive hematopoietic stem cells with normal lymphohematopoietic potential from hESCs. Approaches to improve differentiation methods by altering culture conditions or directly manipulating dysregulated gene expression, particularly of the negative regulators active during mesoderm and hematopoietic differentiation, will be critical in overcoming the functional defects of the hESC-derived cells and to enable the generation of HSCs from hESCs with full lymphohematopoietic reconstitution potential leading to applications in clinical transplantation.

MATERIALS AND METHODS

EB formation and CD34$^+$ cell isolation. To induce EB formation, hESCs were harvested from primary mouse embryonic fibroblasts using collagenase or EZ Passage, pelleted by centrifugation at 1,000 r.p.m. for 5 minutes and resuspended in "serum-free medium" (SFM) comprised of stem line II medium (Sigma, St Louis, MO), CD lipid concentrates (1×), Glutamax (2 mmol/l) insulin transferrin selenium (1×), and penicillin/streptomycin (100 U/100 µg/ml) (all from Invitrogen, Carlsbad, CA), 400 µmol/l monothio glycerol, 50 µg/ml ascorbic acid (both from Sigma). The following morphogens were added to SFM: 10 ng/ml BMP-4 (R&D Systems, Minneapolis, MN) and 5 ng/ml basic fibroblast growth factor (Invitrogen) (BF medium) ± 20 ng/ml vascular endothelial growth factor (R&D Systems) (BFV medium) ± 10% fetal bovine serum (Thermo Scientific Hyclone, Logan, UT) (BFVS medium) were added to SFM on day 0. EB formation was induced in ultra-low attachment 60- or 100-mm dishes (Corning Life Sciences, Lowell, MA) with medium changes every 3 days. For FACS analysis, single-cell suspensions of hEBs were produced by trypsinization (0.05%) for 5 minutes. Cells were stained with CD34-PE or CD34-PE-Cy7 (Becton Dickinson, San Jose, CA). Two fractions, CD34^{bright} and CD34^{dim} could be best identified when a bright fluorochrome-like PE-Cy-7 or PE was used. Cells were sorted on a FACSAria (Becton Dickinson).

Generation and isolation of CD326-CD56$^+$ hEMPs. To induce mesoderm differentiation, colonies of H9, cells were cut into uniform-sized pieces using the StemPro EZ Passage tool (Invitrogen), transferred into 6-well plates precoated with Matrigel (1:20 dilution, growth factor reduced, no phenol red; BD Biosciences), and cultured initially in mTESR medium (Stem Cell Technologies, Vancouver, British Columbia, Canada) until

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50–60% confluent (typically 2 days). To induce differentiation, mTESR medium was replaced with SFM used for EB formation supplemented with BMP-4, basic fibroblast growth factor, and vascular endothelial growth factor. These conditions were a modification of the activin-BVF/matrigel conditions previously described.[24] After mesoderm induction, CD326–CD56$^+$ (hEMP) cells were isolated by FACS at day 3.5 for further gene expression analysis by quantitative PCR.

Myeloid cell cultures. hESC-derived CD34$^+$ and UCB-CD34$^+$ cells were cocultured on OP-9 stroma in 48 well plates (Corning Life Sciences) in myeloid differentiation conditions *i.e.*, IMDM (Invitrogen) with 20% fetal bovine serum and SCF (50 ng/ml), Flt3 Ligand (10 ng/ml), thrombopoietin (5 ng/ml), interleukin-3 (IL-3) (10 ng/ml), and erythropoietin (2 U/ml) (all from R&D Systems). For short-term proliferation assays, equal numbers (9,000 cells/well × 5) of hESC and UCB-CD34$^+$ cells were plated on OP-9 stroma with half media change every 4 days. On day 14, cells were labeled with CD45-PE and CD34-PE-Cy7, analyzed using BD LSRII, and absolute numbers of CD45 and CD34$^+$ cells calculated. As UCB CD34$^+$ cells proliferated rapidly, for differentiation assays hESC-derived CD34$^+$ cells were plated at 5,000–10,000 cells/well and UCB, CD34$^+$ cells at 500 cells/well. Expression of erythroid and myeloid markers was analyzed after 4 weeks.

Lymphoid cultures. hESC-derived CD34$^+$ cells were plated at 5,000–10,000 cells/well and UCB CD34$^+$ cells at 500/well in RPMI with 5% fetal bovine serum under two different cytokine conditions (i) Flt3 Ligand (5 ng/ml), thrombopoietin (5 ng/ml), and IL-7 (5 ng/ml) ± IL-15 with IL-3* or (ii) SCF (50 ng/ml), Flt3 Ligand (50 ng/ml), thrombopoietin (25 ng/ml), IL-7 (20 ng/ml) ± IL-15 (for NK) with IL-3* (*10 ng/ml for only the first 3 days) (all from R&D Systems). Proliferation assays as described above were also performed under lymphoid conditions. Expression of lymphoid markers was analyzed after 4 weeks.

Hematoendothelial differentiation assays. 30,000 CD34^{bright} and CD34^{dim} or human umbilical vein endothelial cells were plated on Matrigel in 96-well plates, in 100-μl complete EGM2 medium (Lonza, Allendale, NJ). After 72 hours, tube formation was assessed on a Nikon Eclipse Ti-U, inverted microscope (Nikon Instruments, Melville, NY). Images were captured on a Nikon digital sight camera, using the NIS-Elements BR 3.00 imaging software. Hematopoietic ability of CD34^{bright} and CD34^{dim} was also analyzed (see **Supplementary Materials and Methods**).

Cloning frequency in limiting dilution assay. For limiting dilution assay 100, 300, 1,000, 3,000, and 9,000 FACS-sorted hESC-CD34$^+$ cells, were plated on OP-9 stroma seeded 96-well plates using the automated cell deposition unit. For UCB, in addition to the above cell numbers, 1, 3, 10, 30, and 100 cells/well were plated. For higher cell numbers (1,000, 3,000, and 9,000) 10 wells were plated per each cell number. For cell numbers 1–300, a total of 96 wells were plated per each cell number under both myeloid and lymphoid conditions. Wells showing growth after 14 days were scored as positive. Clonogenic frequency with 95% confidence interval was determined using the L-Calc Software (Stem Cell Technologies, Vancouver, British Columbia, Canada).

FACS analysis and cell sorting. FACS analysis was performed on FACSCalibur and BD and cell sorting on a FACSVantage or FACSAria (Becton Dickinson) by direct immunofluorescence staining with human-specific monoclonal antibodies. Nonspecific binding was blocked with intravenous immunoglobulin (0.1%) (IVIG; Cutter, Berkley, CA) before staining with fluorochrome-conjugated antibodies. Cell acquisition used FACSDiva (Becton Dickinson) and analysis was performed using Cell Quest Pro (Becton Dickinson) or FlowJo (Tree Star, Ashland, OR). The following antibodies; (conjugated to APC): KDR, CD45; (conjugated to PE): c-kit, KDR, CD31, CD34, HLA-DR, CD94, CD19, CD235, CD143, VE-Cadherin; (conjugated to FITC): CD31, CD34, CD45, CD56, CD66b; (conjugated to ECD): CD14, CD34; (conjugated to PE-Cy-7): CD34, CD45; (conjugated to PE-Cy5.5): CD34; (conjugated to PerCP-Cy5.5): CD326 (all from Becton Dickinson) were used. Unstained cells were used as negative control. For intracellular staining sorted CD34^{bright} and CD34^{dim} cells were treated with fixation/permeabilization and permeabilization buffers (eBiosciences, San Diego, CA) as per manufacturer's instructions, stained with primary rabbit antihuman antiendothelial nitric oxide synthase antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by goat anti-rabbit secondary antibody (conjugated to Alexa 647) (Invitrogen). To correlate morphology and immunophenotype, differences in hESC and UCB-CD34$^+$ cells were also analyzed using imaging flow cytometry (see **Supplementary Materials and Methods** for details).

RT-PCR analysis. 40,000 cells were sorted from each of the following populations: (i) CD34^{bright} and CD34^{dim} isolated from day 8 EBs generated in SFM-BF, SFM-BFV, or 10%Serum+BFV, (ii) SSEA-4$^+$ sorted undifferentiated hESCs, and (iii) UCB-derived CD34$^+$CD38$^-$, CD34$^+$CD19$^-$, CD34$^+$CD19$^+$, and CD34$^-$CD19$^+$ cells. RNA was extracted using the RNeasy microkit (Qiagen, Valencia CA) and converted to complementary DNA using oligo dT primers. 2 μl of complementary DNA (corresponding to 2,000 cell equivalent per PCR) was used for each reaction. RT-PCR was performed for the genes indicated (**Supplementary Figure S10**). For *CEBPα*, an intronless gene, RNA samples were treated with RNAse free DNAse 1 (Qiagen) to confirm that no product was obtained in the absence of RT enzyme. The PCR products were analyzed on a 2% agarose gel and stained with SyBr safe (Invitrogen). All semiquantitative RT-PCR data was generated from three independent experiments. For quantitative RT-PCR using SyBrGreen, samples were run in 96-well plates, in triplicates and subjected to PCR in a 7,900 or 7,500 real-time PCR systems (Life Technologies, Carlsbad, CA). The comparative threshold cycle (C_t) method was used to analyze data with gene expression levels normalized, to the housekeeping gene *RPL-7*[23] that has been shown to be highly reliable in gene expression analysis of undifferentiated and differentiated progeny of hESCs.[47] All quantitative RT-PCR data was derived from cells isolated from at least three independent experiments.

Targeting LNK expression in hESCs using lentiviral-based shRNAs. A lentiviral vector pLKO.1-U6-LnkshRNA-PGK-Puro-expressing *LNK* shRNA from the U6 promoter was obtained from Dr Philip Koeffler (Cedars Sinai Medical Center, Los Angeles, CA). hESCs (H9 cell line) were transduced on matrigel in mTESR medium for 8 hours with lentiviral vectors expressing the shRNAs designated as sh*LNK*B1. A lentiviral vector expressing control (nonspecific) shRNA was used in parallel to determine the specificity of *LNK* knockdown. Following transduction, fresh mTESR medium was added to the wells. After 48 hours, hESCs were split onto matrigel again to prevent the cells from becoming confluent. Puromycin selection (10 μg/ml) (Invitrogen) was started 24 hours after replating hESCs. After 4 weeks of passaging on matrigel in mTESR medium with constant selection with puromycin (10 μg/ml), resistant hESC clones designated H9-LnkB1 were obtained. Knockdown of *LNK* expression was determined by semiquantitative RT-PCR analysis of equal numbers (10,000) of untransduced H9 cells and H9 LnkB1 as well as H9-control shRNA. Near complete knockdown of *LNK* expression was seen in the shRNA vector as compared to the untransduced or control shRNA transduced hESCs (**Figure 7**). The *LNK* knockdown clones were subsequently transferred on primary mouse embryonic fibroblasts and maintained as described for hESCs. To analyze the effects of LNK knockdown on hematopoietic differentiation, hESC clones were transferred to OP-9 stroma in αMEM + 10% fetal bovine serum (Hyclone)[2] + 100 ng/ml SCF and analyzed by cell counting and FACS (for CD34, CD45, KDR, VE-Cadherin expression after 15 days.

Stromal-based coculture for hematopoietic differentiation. hESCs (H9) cells were plated on confluent layers of OP-9 stroma as described[2] with minor modifications. SCF (50 ng/ml) was added to the medium and half medium was changed every 3 days. For time course experiments cocultures were harvested as described[2] using sequential collagenase IV (Stem

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Cell Technologies) and Trypsin (Invitrogen) digestion on day 10, day 16, and day 21. Cells were stained with antibodies to CD34, CD45, KDR, VE-Cadherin as described above in "FACS analysis and sorting" section. $CD34^+$ $CD45^+$ cells FACS isolated at different time points were replated on OP-9 for 4 weeks with lymphoid cytokines as described for "lymphoid cultures." After 4 weeks, cells were harvested and stained for the expression of CD19 (B cell marker), CD14 (monocytic marker), and CD45 (pan-leukocyte marker). Hamster anti-mouse CD29 (anti-β1 integrin antibody tagged to Alexa 488) (Serotec, Raleigh, NC) was included to distinguish human cells from mouse OP-9 stroma.

SUPPLEMENTARY MATERIAL

Figure S1. Undifferentiated hESCs express pluripotency markers and have a normal karyotype.
Figure S2. Phenotypic analysis of UCB- and hESC-derived $CD34^+$ cells.
Figure S3. Imaging flow cytometric analysis for cellular morphology, apoptotic cells, and N/C ratio of hESC-derived $CD34^+$ cells and UCB-derived $CD34^+$ cells.
Figure S4. Spatial distribution of $CD34^+$ cells within EBs.
Figure S5. hESC-$CD34^+$ cells are less clonogenic than UCB $CD34^+$ cells.
Figure S6. hESC-derived $CD34^+$ cells are impaired in B cell differentiation irrespective of morphogens used during EB formation.
Figure S7. Lack of B cell development from hematoendothelial and hematopoietic cells derived from hESCs.
Figure S8. Spontaneous differentiation leading to generation of mature monocytic cells within the EBs over time.
Figure S9. B lymphoid potential is defective in hESC-derived $CD34^+CD45^+$VE-cadherin-cells generated in OP-9 stromal coculture.
Figure S10. Forward and reverse primers used for RT-PCR and qRT-PCR analysis.
Materials and Methods.

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