Hematopoietic differentiation of human embryonic stem cells progresses through sequential hematoendothelial, primitive, and definitive stages resembling human yolk sac development

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Hematopoietic differentiation of human embryonic stem cells progresses through sequential hematopoietic stages resembling human yolk sac development

Elias T. Zambidis, Bruno Peault, Tea Soon Park, Fred Bunz, and Curt I. Civin

We elucidate the cellular and molecular kinetics of the stepwise differentiation of human embryonic stem cells (hESCs) to primitive and definitive hematopoiesis from human embryonic bodies (hEBs) in serum-free clonogenic assays. Hematopoiesis initiates from CD45 hEB cells with emergence of semiadherent mesodermal-hematopoietic areas in the second to third embryonic weeks with formation of yolk sac foci of nucleated erythroblasts (“megaloblasts”), intimately associated with and surrounded by endothelium. Yolk sac (primitive) blood cells consist of nucleated primitive erythrocytes expressing exclusively embryonic globins (e.g., \(\epsilon_\alpha\) and \(\epsilon_\gamma\) globin chains) and primitive macrophages that arise without detectable monocytic precursors. Following the onset of circulation at about 21 days of development, yolk sac cells are found in embryonic blood. The fetal liver subsequently replaces the yolk sac as the main hematopoietic organ with appearance of definitive enucleate, macrocytic erythrocytes expressing fetal globins (e.g., \(\epsilon_\alpha\) and \(\epsilon_\gamma\) globin chains). Definitive blood cells and hematopoietic stem progenitor cells (HSPCs) can be detected in the fetal liver and embryo beginning at 5 to 6 weeks but have also been assayed as early as 4 to 5 weeks from human yolk sac, suggesting a gradual yolk sac/fetal liver HSPC transition. The adult human long-term repopulating HSPC that ultimately seeds the fetal bone marrow and thymus is the legacy of fetal liver hematopoiesis.

Both yolk sac– and AGM-derived HSPCs are the direct progeny of a bipotential hemangioblast. In contrast to zebrafish and mouse, the early developmental biology of human HSPCs remains difficult to approach due to ethical and technical limitations of studying human embryos. In light of fundamental differences between rodent and human developmental hematopoiesis, including anatomically more

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Introduction

Classic and contemporary anatomic studies of human embryos have revealed that human hematopoiesis begins in the second to third embryonic weeks with formation of mesoderm-derived blood islands in the extraembryonic mesoderm of the developing second to third embryonic weeks. Blood islands develop foci of nucleated erythroblasts (“megaloblasts”), intimately associated with and surrounded by endothelium. Yolk sac (primitive) blood cells consist of nucleated primitive erythrocytes expressing exclusively embryonic globins (e.g., \(\epsilon_\alpha, \epsilon_\gamma\) globin chains) and primitive macrophages that arise without detectable monocytic precursors. Following the onset of circulation at about 21 days of development, yolk sac cells are found in embryonic blood. The fetal liver subsequently replaces the yolk sac as the main hematopoietic organ with appearance of definitive enucleate, macrocytic erythrocytes expressing fetal globins (e.g., \(\epsilon_\alpha, \epsilon_\gamma\) globin chains). Definitive blood cells and hematopoietic stem progenitor cells (HSPCs) can be detected in the fetal liver and embryo beginning at 5 to 6 weeks but have also been assayed as early as 4 to 5 weeks from human yolk sac, suggesting a gradual yolk sac/fetal liver HSPC transition.

The online version of the article contains a data supplement.

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The online version of the article contains a data supplement.
complex primary and secondary yolk sacs, a relatively shortened yolk sac phase,25 and a much earlier onset of medullary hematopoiesis26 in humans, direct extrapolation of murine development to humans may be inappropriate. Furthermore, although studies with scarce human embryo tissues have provided limited insight into the emergence of HSPCs within the 3- to 5-week human embryo,23-24 it remains impossible to study the initial commitment of mesoderm to angiohematopoiesis because human yolk sac blood islands develop as early as 16 to 17 days of development.

Pluripotent mouse embryonic stem cells (mESCs) have demonstrated capacity to differentiate into hematopoietic progenitors in a manner recapitulating in vivo murine embryonic hematopoiesis,27-31 In vitro differentiation of human embryonic stem cells (hESCs)32,33 similarly provides opportunities to elucidate the origins of human hematopoiesis. Recent studies have demonstrated that hESCs can differentiate to hematopoietic colony-forming cells (CFCs) using either stromal coculture or human embryoid body (hEB)–based systems.34-37 However, whereas primitive and definitive hematopoiesis have been documented in mESC models,27-31 informative hematopoietic colony-forming cells (CFCs) using either stromal coculture or human embryoid body (hEB)–based systems.34-37 However, whereas primitive and definitive hematopoiesis have been documented in mESC models,27-31 sequential and spontaneous waves of primitive and definitive hematopoiesis38-44 have not been delineated from hESCs. This obstacle limits interpretation and usefulness of hESCs as tools for dissecting the earliest cellular/molecular events in human embryonic hematopoiesis, including the search for a clonogenic human hemangioblast. Additionally, unlike mouse ES/EB systems, protocols for efficient commitment to hematopoietic cells thus far appear to require pretreatment of differentiating hEBs with complex cocktails of growth factors that induce, augment, but also likely skew spontaneous hematopoietic commitment of hEB progenitors.34,35

The derivation of genetically modified45,46 hematopoietic progenitors from hESCs may provide a versatile source of both hematopoietic progenitors and endothelial cells for human tissue engineering. More importantly, the basic cellular and genetic mechanisms of human hematopoietic incipience can now be opened to thorough investigation.

Materials and methods

Culture of hESC and hEB differentiation

The hESC line H1 (National Institutes of Health [NIH] code: WA01) was maintained on irradiated primary murine (CF1) primary murine embryonic fibroblasts (PMFs) as previously described32,33 and maintained a continuously pluripotent phenotype (more than 80% to 90% GCTM2/CD9+, SSEA4+, SSEA1+, OCT4+, and alkaline phosphatase positive32,33). Freshly thawed hESCs (fewer than 45 passages) with confirmed normal male karyotype were used for all differentiation experiments. Feeder-free culture on Matrigel prior to hEB formation was avoided.

Our methods for hEB differentiation (Figure S1A, available on the Blood website; see the Supplemental Figures link at the top of the online article) were modified from previous approaches for forming mouse EBs (mEBs) in semisolid medium supplemented with growth factors that induce, augment, but also likely skew spontaneous hematopoietic commitment of hEB progenitors.34,35

Replating assays for hematopoietic colony-forming cell (CFC) or endothelial potential

hEBs were harvested at various time points, washed in PBS, and assayed for clonogenic hematopoietic progenitors. Single-cell suspensions of hEBs were plated in methylcellulose-based medium (mEBs) supplemented with 0.5% EX-CYTE and 5% PFHM-II. Fourteen to 21 days later, colonies were secondarily replated for endothelial differentiation by vigorous washing away of nonadherent cells, trypsinization, and reculture into EGM2 complete endothelial medium (Cambrex Bioscience, Walkersville, MD) on Matrigel-coated plates. Rapidly proliferating cells appeared 4 to 7 days after replating and were analyzed for endothelial function by overnight incubation with 10 μg/mL acetylated DIL-LDL (Molecular Probes, Eugene, OR) or harvested for RNA for RT-PCR analysis.

Flow cytometry analysis of surface markers and hemoglobins and in situ immunofluorescence

Hematopoietic progenitors were evaluated from enzymatically dissociated hEB cells or individually picked and pooled hematopoietic colonies at different time points. Fluorochrome-conjugated monoclonal antibodies included CD31-phycocerythrin (PE), CD34-PE, CD45-PE, CD71–phycoerythrin-cyanin 5 (PeCy5), CD13-PE, glycophorin A (CD235A, GlyA)–PE, vascular endothelial (VE)–cadherin (all from Becton Dickinson, San Diego, CA), antivimentin (Lab Vision, Fremont, CA), and Ulex europaeus agglutinin-1–fluorescein isothiocyanate (UEA1–FITC) (Vector Laboratories, Burlingame, CA). Viable cells were gated for analysis and appropriate isotype and secondary antibody controls compared for each sample using a FACSoFlow cytometer and CellQuest software (Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA). Hemoglobin F (HbF), hemoglobin A (HbA), gamma chain, and epsilon chain expression was evaluated by intracellular antigens—fluorescein isothiocyanate (FITC) (Vector Laboratories, Burlingame, CA). Viable cells were gated for analysis and appropriate isotype and secondary antibody controls compared for each sample using a FACSoFlow cytometer and CellQuest software (Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA). Hemoglobin F (HbF), hemoglobin A (HbA), gamma chain, and epsilon chain expression was evaluated by intracellular FACS staining of fixed, permeabilized colony cells (FITC and PERM; Caltag, Burlingame, CA). Viable cells were gated for analysis and appropriate isotype and secondary antibody controls compared for each sample using a FACSoFlow cytometer and CellQuest software (Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA). Hemoglobin F (HbF), hemoglobin A (HbA), gamma chain, and epsilon chain expression was evaluated by intracellular FACS staining of fixed, permeabilized colony cells (FITC and PERM; Caltag, Burlingame, CA). Viable cells were gated for analysis and appropriate isotype and secondary antibody controls compared for each sample using a FACSoFlow cytometer and CellQuest software (Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA). Hemoglobin F (HbF), hemoglobin A (HbA), gamma chain, and epsilon chain expression was evaluated by intracellular FACS staining of fixed, permeabilized colony cells (FITC and PERM; Caltag, Burlingame, CA). Viable cells were gated for analysis and appropriate isotype and secondary antibody controls compared for each sample using a FACSoFlow cytometer and CellQuest software (Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA). Hemoglobin F (HbF), hemoglobin A (HbA), gamma chain, and epsilon chain expression was evaluated by intracellular FACS staining of fixed, permeabilized colony cells (FITC and PERM; Caltag, Burlingame, CA). Viable cells were gated for analysis and appropriate isotype and secondary antibody controls compared for each sample using a FACSoFlow cytometer and CellQuest software (Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA). Hemoglobin F (HbF), hemoglobin A (HbA), gamma chain, and epsilon chain expression was evaluated by intracellular FACS staining of fixed, permeabilized colony cells (FITC and PERM; Caltag, Burlingame, CA). Viable cells were gated for analysis and appropriate isotype and secondary antibody controls compared for each sample using a FACSoFlow cytometer and CellQuest software (Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA). Hemoglobin F (HbF), hemoglobin A (HbA), gamma chain, and epsilon chain expression was evaluated by intracellular FACS staining of fixed, permeabilized colony cells (FITC and PERM; Caltag, Burlingame, CA). Viable cells were gated for analysis and appropriate isotype and secondary antibody controls compared for each sample using a FACSoFlow cytometer and CellQuest software (Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA). Hemoglobin F (HbF), hemoglobin A (HbA), gamma chain, and epsilon chain expression was evaluated by intracellular FACS staining of fixed, permeabilized colony cells (FITC and PERM; Caltag, Burlingame, CA). Viable cells were gated for analysis and appropriate isotype and secondary antibody controls compared for each sample using a FACSoFlow cytometer and CellQuest software (Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA). Hemoglobin F (HbF), hemoglobin A (HbA), gamma chain, and epsilon chain expression was evaluated by intracellular FACS staining of fixed, permeabilized colony cells (FITC and PERM; Caltag, Burlingame, CA). Viable cells were gated for analysis and appropriate isotype and secondary antibody controls compared for each sample using a FACSoFlow cytometer and CellQuest software (Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA). Hemoglobin F (HbF), hemoglobin A (HbA), gamma chain, and epsilon chain expression was evaluated by intracellular FACS staining of fixed, permeabilized colony cells (FITC and PERM; Caltag, Burlingame, CA).
permeabilization (FIX and PERM; Caltag) and immunostaining with FITC- or
stains with erythrosin-B were done on cytospun cells as described.48

NH). Benzidine-hemoglobin and Kleihauer-Betke (K-B) fetal hemoglobin
with Wright-Giemsa reagents (Hema 3 stain; Fisher Scientific, Hampton,
apparatus (Shandon; Theims, Waltham, MA). Cells were fixed and stained
human/mouse cDNA GenBank data using basic local alignment sequence
Supermix reagents and an iCycler thermal cycler and software (Bio-Rad,
RNA samples were treated with RNAse-free DNAse (Qiagen). First-strand
medium was prepared using RNEasy reagents (Qiagen, Valencia, CA).
hESCs, hEB cells, or picked, pooled colonies from methylcellulose CFC
cultures and staining with GCTM249 supernatant (a kind gift from Martin
Pera) plus anti–mouse immunoglobulin M (IgM)–FITC secondary (Becton

Table 1. Sequences of human-specific primers used for qRT-PCR

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*These primers do not distinguish between human and murine HOXB4.
†These primers amplify an amplicon in exon 7A of the AML1 gene that is specific for the a isoform of AML1.50
‡These primers amplify an amplicon in exon 3 of the AML1 gene that is specific for both the a and b isoforms of AML1.50
§These primers amplify an amplicon that spans exon 1 and 2 of the AML1 gene and is specific for the c isoform of AML1.50

Results
hESCs efficiently differentiate into cystic hEBs containing precursors expressing a developmental progression of hematoendothelial surface markers and regulatory genes

Using modified mEB protocols, we differentiated hESCs into hEBs capable of spontaneously generating hematopoietic progenitors without need for recombinant hematopoietic growth factors during hEB differentiation.44,45 About 50% to 60%
of hESC clumps plated became hEBs and acquired a cystic morphology by 8 to 15 days of differentiation (Figure S1B). Differentiating hEB cells rapidly decreased expression of OCT3/4 (POUSF1; Figure S1C) and lost markers characteristic of undifferentiated hESCs22,33,49 (GCTM2, CD9, and TRA-1-60, data not shown) after 3 to 12 days.

To identify when differentiating hEB cells become competent for generating hematopoietic progenitors, we analyzed hEBs over a 4-week time course for expression of well-characterized hematopoietic progenitor and endothelial markers using FACS and qRT-PCR. Undifferentiated (day 0) hESCs expressed CD117 (FACS data not shown), CD133 (FACS data not shown), and KDR/flk1 (Figure 1A) but had low/undetectable expression of CD34 and CD31 RNA or surface protein (Figure 1A,C). CD34 and CD31 expression both peaked at about 12 to 15 days of hEB development (Figure 1A,C) and were coexpressed on the same hEB progenitors (Figure S2B). CD45 (Figure 1A,C) was expressed on only 1% to 3% of hEB cells and not until about 15 to 30 days (about 1 week after the onset of CD34/CD31 expression).

hEB cells were next evaluated for hematopoietic gene expression by qRT-PCR. A kinetic analysis revealed a progressive expression pattern of genes known (from murine studies) to initiate and regulate hematopoiesis (Figure 1B). Expression of key hematopoietic transcriptional regulators, including SCL/TAL1, CDX4, GATA1, GATA2, EKLF, and PU.1 were all found to increase dramatically after 1 week of hEB differentiation (Figure 1B). Increases in mRNA levels of these transcription factors coincided with similarly increasing expression levels of CD31, CD34, and KDR/flk-1 (Figure 1A). Interestingly, several genes important for regulating HSPC development in MESC and embryos (eg, LMO2, AML1, C-MYB [data not shown], KDR/flk-1 [VEGFR2], and FLT-1 [VEGFR1]) were expressed abundantly at the mRNA level in undifferentiated hESCs as well as in hEBs at all time points (Figure 1A). Although we have not confirmed that mRNA levels correlate directly with protein levels for these genes, these data suggest potentially major differences between mouse and human embryonic regulation of hematopoiesis. Expression of KDR/flk-1 (VEGFR2, a receptor for vascular endothelial growth factor), associated with development of hemangioblasts, was only moderately increased but was relatively high at all time points. This is distinct from mEB differentiation, where a more dramatic expression profile has been described.32,35 Expression levels for these genes peaked at days 6 to 10 of hEB differentiation, suggesting a putative coordinated developmental watershed event in hEB hematopoietic commitment.

Day 7 to 12 hEB cells generate organized, mesodermal-hematopoietelial (MHE) colonies and a robust wave of primitive hematopoiesis correlating with increased expressions of SCL/TAL1, GATA1, GATA2, and CDX4

We reproducibly generated colonies of CFC-mixed, M-CFCs, GM-CFCs, and erythroid colony-forming units (CFU-e’s) (with morphologies similar to definitive colonies produced from cord blood CD34+ cells) from day 14 to 20 hEBs using standard methylcellulose preparations containing FCS, as has already been reported.34,35 hEB single-cell suspensions obtained at earlier time points, however, when peak expression levels for key regulatory genes were observed (days 6 to 12; Figure 1A-B), failed to generate CFCs using standard methylcellulose media. Because many batches of bovine serum have been reported to inhibit the expansion of primitive murine progenitors,27,31,47 we optimized our hEB CFC assays in serum-free semisolid medium. These conditions revealed vigorous primitive hematopoietic CFCs starting at 7 to 9 days of hEB development, beginning with the detection of unique, laterally expanding semiaherent MHE colonies (Figure 2A-B). CFC generation was not detectable from hEB cells differentiated for fewer than 7 days. MHE colonies expanded either as clonalogenic adherent clusters (Figure 2A) or, rarely, from differentiating plastic-adherent secondary hEBs. Early MHE colonies are first detected as adherent clusters of cells with an endothelioid morphology (Figure 2D, left 2 panels) about 1 week after single hEB cell plating, followed by a rapid secondary budding/differentiation of hematopoietic blast colonies several days later (Figures 2A and 3A-B,G). The cellular architecture (Figure 2A) of many MHE cluster colonies differentiated for 2 to 5 weeks was highly reminiscent of human yolk sac blood islands.3

Adherent and nonadherent cells of MHE colonies derived from day 7 to 12 hEBs differentiated fully for more than 2 to 4 weeks in methylcellulose with growth factors were picked and analyzed separately by FACS and in situ immunofluorescence. Adherent cells of mature MHE colonies (obtained after vigorous washing of nonadherent cells) took up acetylated Dil-LDL (Figure 3F) and expressed CD31 (more than 50%; Figure 3G) and VE-cadherin (11%; data not shown), but not CD45 (not shown), while budding, loosely associated cells expressed erythromyeloid markers CD71,
immunofluorescent filters.

mesodermal-mesenchymal (nonendothelial) in lineage (D, right 4 panels; magnification 100). MHE clusters can become quite prolific after 3 to 5 weeks (ii; magnification × 200) if refreshed with fresh medium with growth factors every 1 to 2 weeks. Wright stains (iv; magnification × 600, oil) of budding, nonadherent cells from prolific MHE clusters revealed abundant hematopoietic blasts (Bi), primitive nucleated erythrocytes (EryP), foamy primary macrophages (Macro-P), and rare definitive cells including granulocytes and definitive erythroid cells (not shown). (B) Kinetics of MHE colony appearance from differentiating HEBS (mean and standard deviations of 3 independent experiments). Loosely adherent cells from prolific HE clusters were picked and analyzed by FACS (C) and found to express abundant levels of early erythroid (CD71), myeloid (CD13), and panhematopoietic (CD45) markers. Mature (3- to 5-week-old) MHE colonies were extensively washed of budding, nonadherent hematopoietic cells, and remaining plastic-adherent cells were incubated with 10 μg/mL acetylated Dil-LDL overnight and then fixed, permeabilized, and further evaluated for expression of endothelial-specific and mesodermal markers. Approximately 15% to 20% of elongated, plastic-adherent cells that formed the base of MHE colonies were shown by in situ immunofluorescence (D) or FACS analysis (as single cells) (E) to be capable of simultaneously taking up acetylated Dil-LDL and costaining with endothelial-associated markers in lineage (D, right 4 panels; magnification × 100, × 200, respectively). Shown are merged images of phase and red and/or green immunofluorescent filters.

To further characterize these complex MHE colonies, we conducted secondary replating assays of adherent and nonadherent portions of early (about 1 week after replating), developing MHE colonies. Our hypothesis was that the mesodermal progenitor(s), which generates MHE colonies, can secondarily give rise to clonogenic progenitors of either hematopoietic (nonadherent) or endothelial (adherent) lineages. Individual budding hematopoietic blast colonies (Figure 3B) from early MHE colonies (Figure 3A,G) containing few differentiating, hemoglobinizing cells were carefully picked. These colonies were dissociated into single cells and replated for secondary colony analysis (Figure 3D-E). Most (more than 70%) MHE colony-derived hematopoietic blast colonies generated new colonies in methylcellulose with growth factors and readily produced secondary primitive-type mixed erythromyeloid, pure erythroid, or pure macrophage colonies (Figure 3E). This demonstrated that MHE colonies generate multipotent hematopoietic progenitors. In parallel experiments, the adherent cells of individual MHE colonies, which were about 50% to 60% CD31⁺ (Figure 3G) and CD45⁻ (not shown), were enzymatically dissociated into single cells and recultured in EGM2 medium with endothelial growth factors. Adherent cells from 6 of 6 (100%) individual MHE colonies rapidly grew cells of endothelial morphology, had ability to take up acetylated Dil-LDL, and expressed endothelial genes including von Willebrand factor (VWF) and VE-cadherin but not the hematopoietic marker gene CD45 (Figure 3G).

Colonies with a primitive erythroblast morphology expanded in semisolid medium with growth factors from day 7 to 12 hEB cells in the absence of any obvious MHE source. Primitive erythroblast colonies differentiated into either large primitive multiclustered erythroid colonies (Figure 4A, BFU-e-P) or, less commonly, mixed erythromyeloid colonies (Figure 5C; MIXED-P). These erythroblasts produced distinctive brilliant red hemoglobinized, nucleated erythroid colonies (Figure 4A, EryP or BFU-e-P). Macrophage...
colony with a foamy cytoplasmic morphology (Figure 5D, Macrophage-P), and without associated monocytic precursors, were also abundant at this time and were identical on Wright stained smears to macrophages arising from HE clusters (Figure 2A). Primitive erythroblast colonies were picked at early stages (7 to 10 days after hEB replating) for benzidine-hemoglobin staining, which demonstrated the presence of nucleated hemoglobin-containing erythroblasts and erythrocytes intermixed with immature blast cells (Figure 4C). These erythroblasts were determined by K-B stains to express abundant amounts of fetal hemoglobins (Figure 4C).

Day 7 to 12 hEB cells thus contained primitive hematopoietic CFCs, which were observed to arise from hEB-derived endothelial MHE progenitors. These cellular events, indicative of primitive embryonic hematopoiesis, were directly associated with increases of SCL/TAL1, CDX4, GATA1, GATA2, CD31, and CD54 by more than 10- to 10 000-fold in day 7 to 9 cystic hEBs (Figure 1B). Although we have not directly proven it, this critical temporal window may represent a hemangioblastic commitment phase in differentiating hEB progenitors. Importantly, no CD45^+ hEB progenitors were detected by FACS or qRT-PCR (Figure 1A,C) during this time period, although large numbers of CD45^+ cells were clearly produced from day 9 to 12 hEB-derived MHE colonies following reculture of CD45^-hEB cells in semisolid medium with hematopoietic growth factors.

**Definitive hematopoietic cells develop from day 12 to 20 hEBs following primitive erythromyelopoiesis from day 7 to 12 hEBs**

Kinetic analysis of hematopoietic CFCs generated from day 3 to 20 hEB cells in serum-free CFC assay conditions revealed a rich variety of hematopoietic colonies with both primitive and definitive morphologies. Erythroid colonies observed from day 12 to 20 hEBs differed notably in morphology from those observed from day 7 to 12 hEBs. These latter colonies had definitive BFU-e- and CFU-e-derived morphologies, with a salmon red (as opposed to brilliant red) hemoglobinization, similar to erythroid colonies generated from cord blood progenitors.

To further evaluate erythroid colonies scored morphologically as primitive or definitive (Figure 4D), colonies were picked, pooled, and analyzed for hemoglobin expression by qRT-PCR or intracellular FACS analysis. Erythroid colonies from day 7 to 12 hEBs (Figure 4D, BFU-e-P and EryP) expressed embryonic (ε^2^ζ^2^) and fetal (HbF, α^2^γ^2^) but not adult (HbA, α^2^β^2^) hemoglobins (Figures 4F and 6B). The second wave of salmon red-colored erythroid colonies from day 12 to 20 hEBs scored as definitive (Figure 4D, BFU-e-D, CFU-e-D), however, could be shown to express not only embryonic (ε^2^ζ^2^) and fetal (HbF) but also adult (HbA) hemoglobins (Figures 4F and 6B). Thus, day 12 to 20 erythroid colonies are more similar to BFU-e- and CFU-e-derived colonies generated from neonatal CD34^- cord blood cells. Intracytoplasmic staining with an anti–gamma-PE hemoglobin chain antibody gave identical results for all erythroid colonies as staining with the anti-HbF antibody (data not shown). CD71 and GlyA coexpression was found to be comparable between primitive and definitive-type erythroid colonies (Figure 4E).

Two types of mixed-lineage colonies were generated from dissociated hEB cells in serum-free CFC assay conditions. Blast colonies from day 7 to 12 hEBs arising either directly from HE colonies (Figure 4A) or from single hEB cells were observed to...
differentiate into not only BFU-e-P but also into large, mixed primitive colonies (mixed CFC-P) containing nucleated primitive erythroblasts and primitive macrophages (Figure 5A,C). Mixed CFC-P erythromyeloid colonies expressed exclusively embryonic and fetal hemoglobins (Figure 6B) and thus appear to be the progeny of a yolk sac–like hematopoietic progenitor, similar to the blasts budding from MHE colonies. The second type of mixed colony had a more definitive erythromyeloid morphology (Figure 5A,C). Mixed CFC-P erythromyeloid colonies expressed embryonic/fetal and adult (β-globin) hemoglobin chains (Figure 6B). Also generated from day 12 to 20 hEB cultures were definitive-type GM-CFC and M-CFC colonies containing monocytes, macrophages, and rare segmented neutrophils (Figure 5B,F). These definitive myeloid-lineage colonies with abundant monocytic precursors (Figure 5F) contrasted to the primitive macrophage colonies (Figure 5B,D). Macro-P CFC that had a foamy cytoplasm and no apparent monocytic precursors.

Thus, our assay conditions revealed 2 distinct waves of hEB-derived hematopoiesis, with predominance of multipotent primitive hematopoietic CFCs arising from day 7 to 12 hEB cells, followed by a wave of definitive multipotent CFCs arising from day 12 to 20 hEB cells.

**hEB-derived primitive versus definitive colonies display distinct molecular phenotypes**

To further characterize the various clonogenic cell types generated from hEBs, we picked and pooled individual colonies with expression of embryonic/fetal and adult (β-globin) hemoglobin chains (Figure 6B).

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**Figure 4.** Day 7 to 20 hEB cells contain clonogenic progenitors for both primitive and definitive erythropoiesis. Erythroid colonies with a "brilliant red" hemoglobinization from day 9 hEB-derived BFU-e-P (Ai; magnification × 100) and EryP (ii; magnification × 100) containing nucleated primitive erythrocytes were generated from day 7 to 15 day hEBs. Erythroid colonies containing a brownish, "salmon-red" hemoglobinization were generated from day 12 to 20 day hEBs (Bi BFU-e-D, CFU-e-D). Colonies (3 to 5 pooled) were picked from semisolid medium for staining or FACS analyses. Wright stains from multiclustered BFU-e-P (Ai; magnification × 600) revealed an increased abundance of erythroblasts, while more mature EryP (iv; magnification × 600) contained primarily differentiated nucleated erythrocytes. Primitive erythroblast (Bi) colonies predominate from day 9 to 12 hEBs (Figure 4A, Bi BFU-e-D) and differentiate directly into nucleated erythrocytes positive for hemoglobin by benzidine staining (C, top; magnification × 400). Erythroblast colonies stain brightly positive for fetal hemoglobins by erythrosin-B K-B staining (C, bottom; magnification × 200).

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**Figure 5.** Day 7 to 20 hEB cells contain clonogenic, multilineage erythromyeloid progenitors for both primitive and definitive hematopoiesis. (A) Kinetics of mixed, multipotent erythromyeloid primitive (Mixed CFC-P) or definitive (Mixed CFC-D) colonies. (B) Kinetics of primitive macrophage (Macro-P) and definitive GM-CFC and M-CFC (scored in combination and presented together as "GM-CFC-D"). Shown is a representative experiment performed 3 independent times. Large, loosely packed primitive mixed erythromyeloid colonies (C; MIXED CFC-P, magnification × 100) containing foamy macrophages and primitive erythroblasts (C; Wright stain, MIXED CFC-P, magnification × 600, oil) peak from day 9 to 12 hEBs (along with primitive erythroid CFCs) and differentiate from blast colonies similar to those that arise from MHE clusters. (D) Foamy macrophage colonies with no evidence of monocytic precursors on Wright stains (magnification × 600, oil) also predominated from day 9 to 15 hEBs. (E) Compact colonies containing definitive monocytes/ granulocytes (MIXED CFC-D) as well as GM-CFC-D (F, left and middle; magnification × 1000, oil) and M-CFC-D (F, right; Wright stain, magnification × 600, oil) arose primarily from day 12 to 20 hEBs. (G) Day 9 hEB-derived MIXED CFCs (C) were picked (2 to 3 colonies) and shown by FACS analysis to express abundant amounts of CD71 and CD45 as well as CD34 (as shown). Shown in the bottom row is the FACS profile of day 9 hEB pooled EryP colonies from the same cultures.
identical morphologies for qRT-PCR analysis and compared their gene expression levels to that of pre-CFC assay hEB cells. Colonies characterized and scored as primitive or definitive had different expression profiles of key hematopoietic regulatory genes. Colonies derived from primitive erythroid CFCs (BFU-e-P, EryP) had considerably higher levels of GATA1 and EKLF than did mixed primitive (mixed-P) or mixed definitive (mixed-D) colonies, consistent with the critical roles of these genes in regulating embryonic erythropoiesis (Figure 6A). HOXB4, a homeobox gene associated with self-renewal of HSPCs, was expressed in high amounts preferentially in multilineage mixed colonies (either primitive and definitive). Interestingly, although SCL, LMO2, AML1b, and CDX4 were each expressed in abundant amounts in all types of colonies analyzed, the levels of CDX4 were highest in mixed-D colonies. In addition, C-MYB and AML1c (Figure 6A), known for their role in regulating multilineage definitive hematopoiesis, were expressed in high amounts exclusively in mixed erythromyeloid definitive colonies (which also expressed adult β-globin; Figure 6B).

**Discussion**

ESC differentiation has been accepted as a valid in vitro model for murine yolk sac hematopoiesis. Developing mEBs produce yolk sac–like blood islands coinciding with cyst formation in the EBs. We show herein that a similar developmental process operates in differentiating human EBs, albeit with slower kinetics than for mEBs.

Although blood elements have recently been reported from differentiated hESCs, neither the delineated phases of primitive and definitive hematopoiesis nor a clonogenic human hemangioblast (which directly precedes primitive erythropoiesis in mEB systems) have yet been described. Evidence for a clonogenic mouse hemangioblast has been provided with characterization of a bipotential yolk sac–like progenitor termed the “blast colony-forming cell” (BL-CFC). This transient, VEGF-responsive, mEB-derived progenitor is believed to arise physiologically from mesoderm in vertebrate yolk sac and has been shown to be a common progenitor for primitive erythroblasts as well as definitive erythroyeloid blood cells. In mice, hemangioblasts are likely contained within a subset of KDR/flk-1+ mesoderm cells, and in developing mEBs they can be enriched with this marker.

Our studies have not yet demonstrated a clonogenic bipotential human hemangioblast. As a first step toward this goal, however, we have herein described an hEB-based system where both primitive and definitive human hematopoiesis are generated robustly without requirement for supplemental growth factors or xenogeneic stromal cocultures. Using serum-free CFC assay conditions, we have delineated 2 discrete, sequential waves of hematopoiesis resembling the primitive and definitive hematopoietic patterns occurring during human yolk sac development. This process initiates with primitive erythropoiesis and can be seen to arise secondarily from adherent HME progenitors. A wave of primitive hematopoiesis arose from day 7 to 12 hEBs, and a distinct second wave of definitive hematopoiesis subsequently arose from day 12 to 20 hEBs. These observations indirectly suggest the development of a hematopoietic stem cell at days 6 to 9 of hEB differentiation that either precedes or is concomitant with the burst of primitive erythropoiesis. The direct visualization of MHE colony-derived primitive hematopoietic blasts arising in intimate association with adherent CD31+ VE-cadherin+ endothelial cells during this time is consistent with this hypothesis. Also in support of this hypothesis, the emergence of MHE colonies and primitive erythroid colonies coincides with a dramatic increase in expression of SCL/MEIS2, GATA1, GATA2, EKLF, PU.1, C-MYB, and CDX4 in day 6 to 9 hEB cells (consistent with the known importance of these genes in the commitment of mesoderm to angiohematopoiesis). Furthermore, expression of early hematopoietic surface markers CD31, CD34, and KDR, which all increased prior to onset of CD45 expression during day 6 to 12 hEB development, also accompanies (or probably closely follows) hematopoietic transcription factor expression. These events are summarized schematically in Figure 7.

The ontogeny of our novel MHE colonies and their relationship to clonal bipotential hemangioblasts or more upstream mesodermal progenitors currently remains unclear. Because we have observed...
been noted in the mouse precirculatory yolk sac. Interestingly, these same “hematocytoblasts” gave rise to more definitive erythroid cells in all vertebrate species studied, including humans, thus suggesting a common hemangioblast for both locations. A recent study has provided evidence that hematopoiesis from hESCs may similarly arise from hESC-derived endothelial progenitors with a CD31+KDR+VE-cadherin+ phenotype. Our preliminary data (Figure S2) similarly reveal that day 9 to 10 CD45−CD34−CD31+hESC cells give rise to (at least) definitive lymphohematopoietic cells.

Our results also provide insight into the role key hematopoietic genes may play in human embryonic development and introduce experimental approaches for studying mechanisms involved in normal and genetically modified hESCs. The highly conserved basic helix-loop-helix (bHLH) transcription factor SCL/TAL1 (stem cell leukemia protein), for example, has been shown to play a crucial role in patterning of mesoderm into blood and endothelial lineages by regulating the development of the hemangioblast. Although its importance in T-cell leukemia is established, the role SCL/TAL1 plays in normal human developmental hematopoiesis remains obscure. Our data revealed that SCL/TAL1 was the first and most dramatically up-regulated gene coinciding with emergence of primitive hematopoiesis and was expressed abundantly in all hematopoietic colonies. In contrast, other key regulators of mouse hematopoiesis such as LMO2 (LIM domain only protein 2), a LIM-domain binding protein that physically forms a heterodimer with SCL/TAL1 and acute myeloid leukemia-1 (AML1), were expressed in large amounts in undifferentiated hESCs, and throughout hESC differentiation, without correlation to CFC emergence. Expression analysis of the 3 isoforms of AML1 further revealed that although AML1b is widely expressed in all CFCs as well as undifferentiated hESC cells, only the AML1c isoform was expressed in mixed-definitive colonies. This result is consistent with the hypothesized role of AML1c in mouse definitive hematopoiesis.

In summary, we have described an experimental system for the direct analysis of human embryonic hematopoietic development that proceeds in a manner appearing to model human yolk sac development. The key molecular and cellular events of hematopoietic genesis can be delineated in a manner that was previously impossible due to inaccessibility of human fetal tissue. Furthermore, the potential expansion and transplantation of hematendothelial stem cells may provide unprecedented translational opportunities for human tissue engineering.

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