Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells

Kristin Chadwick, Lisheng Wang, Li Li, Pablo Menendez, Barbara Murdoch, Anne Rouleau, and Mickie Bhatia

Human embryonic stem cells (hESCs) randomly differentiate into multiple cell types during embryoid body (EB) development. To date, characterization of specific factors capable of influencing hematopoietic cell fate from hESCs remains elusive. Here, we report that the treatment of hESCs during EB development with a combination of cytokines and bone morphogenetic protein-4 (BMP-4), a ventral mesoderm inducer, strongly promotes hematopoietic differentiation. Hematopoietic progenitors of multiple lineages were generated from EBs and were found to be restricted to the population of progeny expressing cell surface CD45. Addition of BMP-4 had no statistically significant effect on hematopoietic differentiation but enabled significant enhancement in progenitor self-renewal, independent of cytokine treatment. Hematopoietic commitment was characterized as the temporal emergence of single CD45+ cells first detectable after day 10 of culture and was accompanied by expression of hematopoietic transcription factors. Despite the removal of cytokines at day 10, hematopoietic differentiation of hESCs continued, suggesting that cytokines act on hematopoietic precursors as opposed to differentiated hematopoietic cells. Our study establishes the first evidence for the role of cytokines and BMP-4 in promoting hematopoietic differentiation of hESC lines and provides an unprecedented system to study early developmental events that govern the initiation of hematopoiesis in the human.

(Blood. 2003;102:906-915) © 2003 by The American Society of Hematology
of committed cells for therapies involving hematopoietic transplantation.

Materials and methods

hESC cultures

Human embryonic stem cell (ESC) lines H1 and H9 were maintained as undifferentiated cells by passage in feeder-free culture as previously described. We have informed and obtained approval from our local ethics board for the use of these established human ESC lines. hESCs were cultured in 6-well Matrigel (BD Biosciences, Bedford, MA)–coated plates in mouse embryonic fibroblast (MEF)–conditioned medium (MEF-CM) supplemented with 8 ng/mL human recombinant basic fibroblast growth factor (bFGF) (Invitrogen, Burlington, ON, Canada). The MEF-CM was changed daily, and the cells were passaged weekly by dissociation with 200 U/mL collagenase IV (Invitrogen) to maintain undifferentiated growth. The MEF-CM was produced over a 7-day period by daily collection of medium used to feed irradiated (40 Gy) MEF (G1:CF-1RBR; Charles River Canada, St-Constant, Quebec, Canada). Cells were maintained in a humidified atmosphere, differential colony counts were performed based on morphological characteristics. Assessment of colony formation for undifferentiated hESCs was determined up to 40 days following the initiation of methylcellulose culture. For secondary colony-forming unit (CFU) assays, 1 to 5 individual hematopoietic colonies were isolated from the methylcellulose by aspiration at days 10 to 14, placed into suspension, and replated into secondary methylcellulose assay. Colonies were scored after 10 to 14 days, and progenitor self-renewal was calculated as the number of secondary colonies arising from individual primary CFUs.

Formation of EBs

On the day of passage, undifferentiated hESCs at confluence in 6-well plates were treated with collagenase IV and scraped off their Matrigel attachments in strips. They were transferred to 6-well low-attachment plates to allow for EB formation by overnight incubation in differentiation medium consisting of knockout D-MEM supplemented with 20% nonheat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT), 1% nonessential amino acids, 1 mM L-glutamine (all from Invitrogen), 0.1 mM β-mercaptoethanol (Sigma Aldrich, Oakville, ON, Canada), and 4 ng/mL bFGF. After collection, the MEF-CM was filtered through a 0.22-μm sterile membrane (Corning Incorporated Life Sciences, Acton, MA) and stored at −30°C.

SSEA-4 and AP assays

After washing twice with phosphate-buffered saline (PBS), undifferentiated hESCs at 7 days after passage were fixed with 4% paraformaldehyde for 15 minutes at 4°C. The SSEA-4 antigen was detected by immunocytochemistry using a specific SSEA-4 primary monoclonal antibody (MC-813-70; Pharmingen, San Diego, CA) at 10 μg/mL by overnight incubation at 4°C, followed by a biotinylated secondary antibody and an avidin/biotinylated horseradish-peroxidase complex (Vectorstain ABC System; Vector Laboratories, Burlingame, CA). As negative controls, PBS alone or normal mouse IgG (Vector Laboratories) was substituted for the primary SSEA-4 antibody. Alkaline phosphatase (AP) activity was detected histochemically by incubation with “Vector red” substrate (Vector Laboratories) for 30 minutes at room temperature (RT) as described by the manufacturer.

Human ESC and EB dissociation

Single-cell suspensions were made from undifferentiated hESCs or EB-differentiated hESCs, as follows. Adherent hESCs were treated with collagenase IV for 20 minutes, followed by treatment with cell dissociation buffer (Invitrogen) for 20 minutes at a 37°C incubator. Cells were dissociated by gentle pipetting and filtered through a 40-μm cell strainer. EBs were dissociated with 0.4 U/mL collagenase B (Roche Diagnostics, Laval, QC, Canada) for 2 hours in a 37°C incubator, followed by treatment with cell dissociation buffer for 10 minutes in a 37°C water bath, and dissociated by gentle pipetting and passage through a 70-μm cell strainer.

Colony-forming unit assays

Human clonogenic progenitor assays were performed by plating undifferentiated hESCs (1-2 × 10³), differentiated hESCs (1 × 10⁴), or sorted EB-differentiated hESC populations (6 × 10³-3.4 × 10⁴) into methylcellulose H4230 (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with the following recombinant human growth factors: 50 ng/mL stem cell factor (SCF), 3 U/mL erythropoietin (Epo; Amgen), 10 ng/mL granulocyte monocyte colony-stimulating factor (GM-CSF; Novartis, Dorval, QC, Canada), and 10 ng/mL IL-3. After incubation at 37°C and 5% CO₂ for 10 to 14 days in a humidified atmosphere, differential colony counts were performed on primary EBs. Heterogeneity of colonies was determined by picking representative colonies and counting the number of secondary colonies arising from individual primary CFUs. Results were compared to the mean of primary CFUs, and standard deviation (SD) was calculated.

Flow cytometry and FACS

Single cell suspensions of undifferentiated hESC or EB-differentiated hESCs were prepared as detailed in “Formation of EBs” and “Human ESC and EB dissociation” in “Materials and methods.” Colonies from primary and secondary CFU plating assays were isolated from methylcellulose, washed in PBS + 3% FBS (Invitrogen) 3 times, and filtered through 85-μm mesh. For flow cytometry, cells were resuspended at approximately 0.2 to 5.0 × 10⁵ cells/mL with PBS + 3% FBS and stained with fluorochrome-conjugated monoclonal antibodies (mAbs; including CD45 fluorescein isothiocyanate [FITC], CD45 allophycocyanin [APC], CD34 APCs, e-Kit [CD117] phycoerythrin [PE], CD33 FITC, CD13 PE [all from Becton Dickinson Immucyteomtry Systems (BDIS), San Jose, CA], AC133 PE [Miltenyi Biotech, Bergisch Gladbach, Germany], glycophorin A PE [Immunechem, Marseille, France], or their corresponding IgG isotype controls) at a concentration of 5 μg/mL. For detection of SSEA-4, the primary SSEA-4 mAb was preincubated with a secondary FITC-conjugated goat anti–mouse IgG antibody (Immunochem) for 30 minutes before addition to the cell suspensions. Cells were stained for 30 minutes at 4°C or for 15 minutes at RT and washed twice in PBS + 3% FBS. Cells were resuspended in PBS + 3% FBS and were stained with the 7-AAD viability dye (Immunochem) at 15-20 μg/mL for 15 minutes at RT. Live cells identified by 7-aminomucinomycin D (7-AAD) exclusion were analyzed for surface-marker expression using a FACSCalibur (BDIS) and Cell Quest software (BDIS). For isolation of CD45+ and CD45-differentiated hESCs, dissociated EB cells were stained with CD45 APC (BDIS) and 7-AAD and were sorted on a FACSVantage SE (BDIS).

Cytospins and Wright-Giemsa staining

Colonies were isolated from methylcellulose by aspiration, washed in PBS, and spun onto slides using a cytospin apparatus (Cytospin3; Thermo Shandon, Pittsburgh, PA). After fixing in methanol:acetone (80:20 volume), slides were stained with Wright-Giemsa (Sigma Aldrich).

Reverse transcription PCR analysis

For reverse transcription polymerase chain reaction (RT-PCR), mRNA was extracted from cell pellets and reverse transcribed into cDNA using mRNA...
frequencies of c-kit undifferentiated state using feeder-free culture conditions. 20 In semisolid methylcellulose media supplemented with hematopoietic

Established hESC lines H1 and H91 were propagated in an undifferentiated hESCs propagated in feeder-free culture lack hematopoietic cells, we measured hematopoietic entitic progenitor capacity using a multilineage hematopoietic colony-forming assay.26 In this clonogenic assay, cells are seeded into a nitrogen, and stored at ~80°C for CD45, alkaline phosphatase (AP), or CD45/AP double stain. Cryostat sectioning from each specimen yielded 150 serial sections (9 µm). For each staining 3 sections per specimen at a distance of 270 µm (interval of 30 serial sections) were used. Consecutive sections were immunostained with mouse anti-human CD45 monoclonal antibody (1:50; BD PharMingen, San Diego, CA), PBS, or mouse isotype IgG1 mAb (1:50; BD PharMingen) for 2 hours at room temperature, followed by biotin-conjugated horse anti-mouse IgG (1:200; Vector Laboratories). Immunoreactivities were visualized with ABC-AP kit (Vector Laboratories) using “Vector red” substrate (Vector Laboratories). AP activity was detected histochemically by incubation with “Vector blue” substrate (Vector Laboratories). Morphometric analyses of positive CD45 or AP cells were made by computer-assisted image analysis (Image Pro Plus 4.5, Silver Spring, MD). All of the specimens were coded so that the measurements were done blindly for final analysis.

Statistical analysis

Results were expressed as mean ± SEM. Statistical significance was determined using an unpaired Student t test. Results were considered significant when P ≤ .05.

Results

Undifferentiated hESCs propagated in feeder-free culture lack phenotypic and functional hematopoietic properties

Established hESC lines H1 and H91 were propagated in an undifferentiated state using feeder-free culture conditions.20 In contrast to the surrounding differentiated stromalike cells, undifferen-

tiated hESCs constituted tightly packed colonies (Figure 1A) that stained positive for AP activity (Figure 1B) and SSEA-4 expression (Figure 1C-D), consistent with previous studies propagating hESC lines on MEF feeder layers12 and in feeder-free culture conditions using MEF-CM.20 To examine whether undifferentiated hESCs cultured under feeder-free conditions possessed any hematopoietic capacity, we characterized hESCs for the expression of the pan-leukocyte marker CD4521,22 and other markers typically associated with primitive human hematopoietic cells, such as CD34,23 c-kit,24 and AC133.25 In 13 independent experiments, undifferen-
tiated hESCs were completely devoid of CD45 expression (Figure 1E) but contained a low frequency of CD34+ cells (Figure 1F) and higher frequencies of c-kit+ and AC133+ cells (Table 1), suggesting that cells of the hematopoietic lineage might be present among undifferen-
tiated hESC populations. To determine whether hESC cultures contained functional hematopoietic cells, we measured hematopoietic progenitor capacity using a multilineage hematopoietic colony-forming assay.26 In this clonogenic assay, cells are seeded into a semisolid methylcellulose media supplemented with hematopoietic growth factors. The localized clonogenic proliferation of a single cell possessing hematopoietic progenitor function will give rise to a hematopoietic colony after 14 days and retrospectively identifies a single hematopoietic progenitor, termed a “colony-forming unit” (CFU). In 16 independent experiments, cultures of 1-2 × 105 undifferentiated hESCs failed to give rise to any hematopoietic colonies in CFU assays for up to 40 days. Based on these results, we conclude that the hESC lines examined do not contain cells committed to the hematopoietic fate.

Cytokines and BMP-4 enhance the derivation of CD45+ hematopoietic cells from hESCs

Since hESCs were devoid of hematopoietic cells, we designed the experimental approach illustrated in Figure 1G to evaluate the hematopoietic potential of hESCs arising through EB differentiation. Spontaneous hematopoietic differentiation within EBs under standard conditions20 (control) was compared to conditions supplemented with a mixture of hematopoietic cytokines (SCF, Flt3L, IL-3, IL-6, and G-CSF); cytokines in combination with the ventral mesoderm inducer BMP-4 (cytokines + BMP-4); or BMP-4 alone without cytokine addition (BMP-4).

Unlike the reduction in SSEA-4 expression that accompanies hESC differentiation (Figure 2A-D), the appearance of CD45+ cells was influenced by cytokine treatment, with or without BMP-4 addition. While a small proportion of spontaneously differentiating
cells expressing CD45 could be identified under control conditions (1.4% ± 0.7%; Figure 2E,I), addition of cytokines increased the frequency of CD45+ cells to 8.0% ± 3.9% of total EB-differentiated hESCs (P ≤ .01 compared to control; Figure 2F,I). Addition of BMP-4 together with cytokines revealed a frequency of 9.3% ± 2.7% of all cells within the EB expressed CD45 (Figure 2G,I), whereas treatment of human EBs with BMP-4 alone only mildly increased the percentage of CD45+ differentiated hESCs to 2.0% ± 0.9% (Figure 2H-I), which was not statistically different from control. The frequency of cells expressing CD34 was similarly modulated to patterns of increased CD45+ cells (Figure 2E-I). Cytokine treatment demonstrated a 5-fold increase in the proportion of CD45+ cells coexpressing CD34, while addition of cytokines + BMP-4 increased the frequency of CD45+CD34+ cells by 6-fold (P ≤ .02; Figure F-G,I). As BMP-4 is known to act as a morphogen, multiple concentrations of BMP-4 (5, 10, 25, and 50 ng/mL) were tested in combination with cytokines and showed no differences in the proportion of CD45+CD34+ hematopoietic cells (data not shown). Since cytokine treatment had no effect on the total cell numbers (Figure 2J) and the cell viability (Figure 2J, inset) between control and treated EBs, the increase in the total yield of CD45+ hematopoietic cells treated with cytokines (Figure 2K) does not likely occur from preferential elimination of most nonhematopoietic cell types within treated EBs. In comparison to the up-regulation of CD45+ and CD45+CD34+ cells derived in response to cytokine and BMP-4 treatment, the frequency of c-kit+ cells was decreased, while AC133 expression did not vary significantly (Table 1). Based on these analyses, cytokine and BMP-4 treatment of human EBs specified the differentiation of cells that possess a CD45+CD34+ phenotype similar to the first definitive hematopoietic cells detected within the wall of the dorsal aorta of human embryos, shown to be highly enriched for hematopoietic progenitors.27-29

### Table 1. Phenotype of undifferentiated hESCs and EB-derived cells differentiated in the presence of extrinsic factors for 15 days

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Undifferentiated hESCs</th>
<th>Control</th>
<th>Cytokines</th>
<th>Cytokines + BMP-4</th>
<th>BMP-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Kit+</td>
<td>32.0 ± 9.2*</td>
<td>7.6 ± 1.5</td>
<td>6.3 ± 2.6</td>
<td>9.1 ± 1.6</td>
<td>8.5 ± 1.5</td>
</tr>
<tr>
<td>c-Kit+ CD34+</td>
<td>1.2 ± 0.4*</td>
<td>3.4 ± 0.9</td>
<td>4.1 ± 2.0</td>
<td>7.3 ± 1.5†</td>
<td>4.2 ± 1.2</td>
</tr>
<tr>
<td>AC133+</td>
<td>25.0 ± 8.2</td>
<td>25.5 ± 3.2</td>
<td>18.9 ± 4.5</td>
<td>19.9 ± 3.0</td>
<td>27.5 ± 5.9</td>
</tr>
<tr>
<td>AC133+ CD34+</td>
<td>24.2 ± 8.1</td>
<td>23.3 ± 2.7</td>
<td>17.0 ± 4.0</td>
<td>16.9 ± 2.3</td>
<td>23.8 ± 4.6</td>
</tr>
</tbody>
</table>

*Significant (P < .01) difference from differentiated hESCs in all treatment based on the cell surface phenotype specified.
†Significant difference (P < .01) from all other treatments of differentiated hESCs.

Values represent the means ± SEM from 4-8 independent experiments. BMP-4 indicates bone morphogenetic protein-4.

Clonogenic hematopoietic progenitors arise from CD45+ cells within cytokine-treated EBs

Based on the expression of CD45 on all known sources of embryonic and adult hematopoietic cells,21,22 we examined whether hematopoietic progenitor function was enriched in CD45+ cells arising from EB-differentiated hESCs. Differentiated hESCs (7-AAD excluding, top left) were divided into 2 populations of...
CD45⁺ (upper gate) and CD45⁻ (lower gate) subsets by fluorescence-activated cell sorting as shown in Figure 3. The gating strategy for subset isolation was based on isotype controls (Figure 3, lower left). In 2 independent experiments using both cytokine and cytokine + BMP-4–treated EBs, only differentiated hESCs within the CD45⁺ subset contained clonogenic hematopoietic progenitors detected by the CFU assay, demonstrating a frequency of progenitor detection of 100% (4 of 4, Table 2). In contrast, when equal or up to 32-fold more of the corresponding CD45⁻ cells were examined, hematopoietic progenitors remained undetectable (0 of 8, Table 2). These results demonstrate that differentiated hESCs with hematopoietic progenitor capacity were restricted to the CD45⁺ population.

Hematopoietic progenitors derived from hESCs are capable of lineage maturation

To determine whether the clonogenic progenitors detected by hematopoietic CFU assays were capable of normal hematopoietic maturation, we examined individual colonies using morphological and phenotypic criteria. Representative colonies arising from differentiated hESCs demonstrated the characteristic morphologies of the various CFU subtypes, including macrophage (Figure 4A), granulocyte (Figure 4B), and erythrocyte (Figure 4C) colonies, as well as multipotent colonies comprised of cells in the granulocytic, erythroid, macrophage, and megakaryocytic lineages (Figure 4D).

Colonies scored by gross morphology to be hematopoietic were examined for commitment and maturation. Individual colonies harvested from the methylcellulose culture by aspiration were examined by Wright-Giemsa staining of cytopsin preparations. In a representative example, colonies identified as granulocytic by light microscopy (Figure 4B) contained granulocytes with segmented nuclei and cytoplasmic granules, including eosinophils and basophils (Figure 4E, inset). Cells comprising these colonies were CD45⁺ and coexpressed CD33 and CD13 markers while lacking glycophorin A expression, indicative of myeloid/granulocytic lineage commitment (Figure 4F-G). Colonies scored as erythroid CFU by their hemoglobinization in culture (Figure 4C) contained cells characteristic of immature erythrocytes with a round cellular morphology, circular dark staining nuclei, basophilic cytoplasm, and a large nucleus-to-cytoplasm ratio (Figure 4H) and also contained mature enucleated erythrocytes (Figure 4H, arrow). Cells within these colonies expressed glycophorin A in the absence of expression of both CD45 and myeloid markers (Figure 4I-J), indicating restriction to the erythroid lineage (Figure 4I). Based on these analyses, our results indicate that the hESCs are capable of giving rise to multiple hematopoietic lineages derived from clonogenic hematopoietic progenitors.

Cytokines and BMP-4 augment the hematopoietic progenitor capacity of hESCs

Figure 4K summarizes the hematopoietic progenitor content of hESCs differentiated under the EB culture conditions indicated. In the absence of cytokines, control cultures were capable of producing an average of 72 CFUs from 1 × 10⁵ differentiated hESCs, whereas supplementing these cultures with cytokines increased the hematopoietic progenitor capacity to an average of 165 colonies (P ≤ .001). The addition of BMP-4 to cytokines further enhanced progenitor capacity and produced an average of 237 colonies (P ≤ .001). Similar to control, EB treatment with BMP-4 alone gave rise to an average of 68 colonies. Regardless of the treatment, all CFU subtypes were observed and were represented in similar proportions (Figure 4L). Our results indicate that cytokine treatment significantly increases the frequency of primitive hematopoietic progenitors in comparison to control and that the generation of hematopoietic progenitors was modestly enhanced by addition of BMP-4 as compared to cytokine treatment alone (P < .05) (Figure 4K).

To determine whether cytokines were effective in promoting hematopoietic differentiation in the absence of serum, human EBs were cultured in serum-free conditions for 15 days in the presence of cytokines. Hematopoietic progenitors comprising multiple hematopoietic lineages were detected at an average of 100 CFUs per 10⁵ differentiated hESCs, compared to control conditions containing 20% FCS that gave rise to an average of 72 CFUs (Figure 4K). This observation suggests that hematopoietic differentiation is not dependent on serum and that cytokines are sufficient in replacing a complex mixture of factors present in the FCS used in control conditions. These comparisons further illustrate the role of cytokines in the hematopoietic differentiation of hESCs under defined conditions. Our results indicate that addition of cytokines and BMP-4 to differentiating EBs is capable of strongly promoting hematopoietic differentiation, thereby demonstrating that extrinsic factors are capable of influencing hESC differentiation potential.

BMP-4 promotes self-renewal of hESC-derived hematopoietic progenitors

Self-renewal is functionally defined by the ability of a single hematopoietic progenitor with clonogenic CFU capacity to give rise to daughter cells that retain CFU capacity upon secondary replating. Quantitatively, the absolute number of secondary CFUs generated from a single primary CFU provides a measurement of
the magnitude of self-renewal of individual progenitors. To investigate the self-renewal capacity of hESC-derived hematopoietic progenitors, individual hematopoietic colonies arising from primary CFUs were isolated, dispersed, and cells were replated into secondary progenitor assays as illustrated (Figure 5A). Our results indicate that primary CFUs derived from differentiated hESCs were capable of secondary CFU formation, and therefore possessed properties of self-renewal. Representative examples of secondary macrophage (Figure 5B), granulocyte (Figure 5C), and erythroid (Figure 5D) colonies possessed similar morphologic features as primary CFUs. Multiple secondary colonies representing a variety of colony types were pooled and analyzed by flow cytometry to determine if self-renewing CFUs were maturing into normal hematopoietic lineages. Most cells from secondary CFUs expressed CD45+ (Figure 5E) and were committed to either granulocytic or erythroid maturation, as evidenced by the expression of CD33 and CD13 (Figure 5F-G) or glycophorin A (Figure 5H), respectively. In separate experiments, equal numbers of CFU subtypes were isolated from primary assays to evaluate secondary CFU capacity. Secondary CFU capacity was independent of primary CFU type (data not shown).

The frequency of self-renewal events arising from single primary hematopoietic progenitors by random hESC differentiation (control) was compared to treatments containing cytokines with or without BMP-4 (Table 3). The total number of secondary CFUs generated from individual primary CFUs allowed for a measurement of the self-renewal capacity of primary progenitors (Figure 5I). Self-renewal of hematopoietic progenitors derived from hESCs differentiated under control EB conditions was an infrequent event, occurring from an average of 6.2% of primary CFUs (Table 3). In contrast, treatment of differentiating hESCs with cytokines enhanced the self-renewal capacity to 21.4% of all primary CFUs examined (Table 3). While the frequency of progenitor self-renewal increased with cytokine treatment, the magnitude of self-renewal from both control or cytokine-derived hematopoietic progenitors was minimal, with an average of 0.5 and 0.32

Figure 4. EBs can be induced to form hematopoietic progenitors. Morphology of differentiated hES-derived hematopoietic CFU subtypes detected under all extrinsic treatments, including (A) macrophage, (B) granulocyte, and (C) erythroid colonies; and (D) multipotent colonies containing granulocyte, erythroid, macrophage, and megakaryocyte elements (CFU-GEMM). Scale bars, 100 μm. (E) Wright Giemsa cytospin preparation of cells from a granulocyte colony showing mature neutrophils, basophils (left inset) and eosinophils (right inset). Scale bar, 25 μm. (F-G) Flow cytometry of a representative granulocyte colony, showing staining with CD45 and glycophorin A (F) or CD33 and CD13 (G). (H) Wright Giemsa cytospin preparation of an erythroid colony showing immature erythrocytes and mature enucleated erythrocytes (arrow). Scale bar, 25 μm. (I-J) Flow cytometry of a representative erythroid colony, showing staining with CD45 and glycophorin A (I) or CD33 and CD13 (J); insets in panels F, G, I, and J indicate colonies stained with isotype controls. (K) Hematopoietic progenitor capacity of undifferentiated hESCs and hESCs differentiated as EBs under various treatments for 14 days, detected by placing 1 × 10⁶ cells into CFU assays (n = 4-12, *P < .001 compared to control; error bars indicate SEMs). Wells were examined for colony formation after 10-14 days for EBs. (L) Distribution of colony subtypes derived under each hematopoietic progenitor induction treatment shown in panel K (*P ≤ .001).

Figure 5. Self-renewal and expansion of hematopoietic progenitors from differentiated hESCs revealed by secondary colony formation. (A) Colonies derived from primary hematopoietic progenitors (1' CFU) were isolated and placed into secondary colony forming assays (2' CFU) to assess the self-renewal and expansion capacities of hES-derived hematopoietic progenitors. (B-D) Morphologies of typical secondary colonies, including macrophage (B), granulocyte (C), and erythroid (D) colonies; scale bar measures 100 μm. (E-H) Flow cytometry of pooled secondary CFUs, representing all 2' CFU subtypes as indicated by staining with CD45, CD33, CD13, and glycophorin A. Markers for positive cells were determined based on isotype controls (inset). (I) The number of 2' CFUs arising from individual 1' CFU following 2' CFU assay (± SEM), indicating the magnitude of progenitor expansion. The inset shows the 2' CFU formation from primary CB CFU after plating individual or pooled colonies into 2' CFU assays. Progenitor expansion occurs when the 2' CFU output is greater than the 1' CFU input. The magnitude of progenitor expansion was calculated from plating between 1 to 5 individual primary CFUs, corrected to an input of one individual primary CFU.
secondary CFUs detected per primary CFU, respectively (Figure 5I). However, when BMP-4 was used in combination with cytokines, up to 36% of primary CFUs generated secondary CFUs (Table 3). Individual primary CFUs arising from hESCs differentiated in the presence of cytokines + BMP-4 generated an average of 4 secondary CFUs per primary CFU, a magnitude of self-renewal 8-fold higher than control or cytokine treatment alone (Figure 5I).

Although treatment of differentiating hESCs with BMP-4 alone was unable to enhance hematopoietic specification above the control treatment (Figures 2I and 4K), BMP-4 was capable of influencing the self-renewal potential of primary hematopoietic progenitors. More than 50% of primary CFUs generated in the presence of BMP-4 alone were capable of self-renewal (Table 3), with an average capacity to form 10 secondary CFUs per primary CFU (Figure 5I), representing a 20-fold increase in self-renewal capacity over control or cytokine differentiation conditions.

To compare the frequency and magnitude of progenitor self-renewal between hESC-derived hematopoietic progenitors and bona fide sources of committed hematopoietic tissue, primary CFUs arising from human CB samples were assessed for self-renewal capacity under identical conditions (Figure 5I, inset). Only when multiple primary colonies derived from human CB were pooled could secondary progenitors be observed at a low frequency of 0.5 secondary CFU per primary CFU, revealing the rarity of self-renewing progenitors from committed hematopoietic tissue compared to hematopoietic progenitors derived from hESCs.

### Temporal emergence of hematopoietic cell fate in response to cytokines and BMP-4

To better understand the cellular processes by which optimal hematopoietic differentiation was occurring in response to cytokines and BMP-4, we examined the kinetics of hematopoietic cell fate emergence within differentiating EBs. No CD45+ hematopoietic cells were detectable at 3, 7, or 10 days of cytokine + BMP-4 treatment (Figure 6A-B), suggesting that cells remained uncommitted to the hematopoietic cell fate during this time period. However, by day 15, committed hematopoietic cells were observed as defined by a significant increase in the frequency and total yield of CD45+ cells (Figure 6A-B, *P ≤ .01). Continued treatment of differentiating EB cultures to day 22 demonstrated a further increase in the frequency and total yield of CD45+ hematopoietic cells (Figure 6A-B, **P ≤ .01). To examine the primitive and functional status of hematopoietic cells arising from differentiating hESCs, cultures were examined for hematopoietic progenitor capacity at these different time points. A small number of CFUs were detectable at day 10 (Figure 6C) with clonogenic efficiencies of less than 1 in 50,000 cells, whereas by day 15 a large number of CFUs were detected (Figure 6C) with a clonogenic efficiency of 1 in 262. Although there was an increase in the total number of CD45+ cells from day 15 to day 22 (Figure 6B), there was no significant difference in the total number of primitive hematopoietic CFUs beyond day 15 (Figure 6C), suggesting that increases in CD45+ hematopoietic cells between days 15 and 22 occurs concomitantly with differentiation and loss of progenitor function. These results indicate hematopoietic commitment does not occur between day 0 and 10 of EB formation but emerges only after day 10 and is followed by optimal progenitor production between days 15 and 22 of cytokine + BMP-4 treatment.

To further characterize the role of cytokines and BMP-4 in promoting the onset of hematopoietic cell fate commitment, the expression of transcription factors known to play an essential role in adult hematopoiesis (GATA-1, PU.1, and RUNX-1) were compared between control and cytokine + BMP-4-treated EBs at days 3, 10, and 15 of differentiation. Enriched populations of human hematopoietic stem and progenitor cells (CD34+CD38− and CD34+CD38+ CB cells, respectively) expressed all 3 factors (Figure 6D) and were used as positive controls. In contrast, undifferentiated hESCs expressed AML-1/RUNX-1 but were devoid of detectable levels of GATA-1 or PU.1 (Figure 6D). Under control EB conditions, differentiated hESCs did not up-regulate these factors, whereas cytokine treatment with or without BMP-4 induced GATA-1 expression as early as day 3 and PU.1 expression by day 15 (Figure 6D). RUNX-1 expression was unaffected by treatment and served as a positive control for treated cells (Figure 6D).

To assess whether cytokines and BMP-4 are enhancing hematopoietic differentiation or by contrast are promoting survival, we analyzed the spatial emergence of hematopoietic cells within treated EBs. Similar approaches have been used to examine

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**Table 3. Frequency of 2′ CFU formation following replating of 1′ CFU from various treatments**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Frequency of 2′ CFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.2%</td>
</tr>
<tr>
<td>Cytokines</td>
<td>21.4%</td>
</tr>
<tr>
<td>Cytokines + BMP-4</td>
<td>36.4%</td>
</tr>
<tr>
<td>BMP-4</td>
<td>57.1%</td>
</tr>
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n = 7-16 for each treatment.
patterns of neural cell fate emergence within EBs in the murine system. Here, using EBs generated from hESCs, individual EBs treated with cytokines + BMP-4 were harvested, sectioned, and stained for CD45 expression and AP activity at days 3, 7, 10, and 15. CD45+ cells were undetectable from days 3 to 10, whereas EBs contained a large number of undifferentiated AP+ cells at these time points. By day 15, CD45+ cells were detected within EBs and occurred concomitantly with the loss of AP+ cells, suggestive of increased differentiation (Figure 7A). Detailed spatial analysis of CD45+ cells within sections of treated EBs indicated that CD45+ cells appeared as single clonal events (Figure 7B) surrounded by differentiated, nonhematopoietic cells that are devoid of AP expression (middle panel, Figure 7B). Differentiated CD45+ cells do not express AP (right panel, Figure 7B). The observation of multiple clonal CD45+ events arising within cytokine- and BMP-4-treated EBs could be due to (1) multiple independent points of hematopoietic emergence indicative of the directed differentiation of uncommitted precursors toward the hematopoietic lineage or,

alternatively, (2) a single point of origin for hematopoietic commitment followed by the rapid proliferation and migration of committed CD45+ cells throughout the EB.

Cytokine treatment prior to emergence of hematopoietic cell fate within human EBs is sufficient and necessary to promote enhanced hematopoietic differentiation

Since CD45+ cells and functional hematopoietic progenitors (CFUs) arise only after 10 days of EB development (Figure 6), 2 stages of human EB differentiation can be operationally defined in the context of hematopoietic potential (Figure 8A). Stage I (day 0 to day 10) is characterized by the loss of undifferentiated AP+ and SSEA-4+ hESCs (Figure 2A-D and Figure 7A) and the presence of differentiated cells devoid of either hematopoietic marker expression (CD45) or hematopoietic progenitor ability (Figure 6). Stage II (day 10 to day 15) is defined as the period of hematopoietic emergence, as evidenced by the appearance of CD45+ cells and hematopoietic progenitors of multiple lineages (Figures 6-7). Based on these defined stages, we further characterized the actions of cytokines by restricting the period of cytokine exposure during EB differentiation to stage I alone (Figure 8A, +/−) compared to control differentiation during both stages I and II (Figure 8A, −/−).

The presence of hematopoietic cells was evaluated 2.5 (day 12.5) and 5 days (day 15) after removal of cytokines from differentiating EBs and compared to control differentiation conditions. By day 12.5, EBs treated with cytokines during stage I alone (+/−) contained greater numbers of CD45+ and primitive CD45+CD34+ cells than EBs cultured in the absence of cytokines (−/−) (Figure 8B-C). By day 15 EBs treated with cytokines during stage I alone (+/−) gave rise to a significantly higher frequency of both CD45+ and primitive hematopoietic CD45+CD34+ cells compared to control (Figure 8D-E, *P ≤ .01). A representative example of day-15 analysis is shown in Figure 8F-G. These results provide evidence to suggest that prior to the emergence of committed CD45+ hematopoietic cells, cytokine treatment of hESCs is sufficient and necessary to promote enhanced hematopoietic differentiation. Since cytokines are capable of influencing hematopoietic potential during stage I of hESC differentiation, we suggest that cytokines are able to either (1) direct the differentiation of uncommitted hESCs, and/or (2) induce hematopoietic cell fate commitment from currently uncharacterized hematopoietic precursors present during stage I of EB development.
Discussion

Our study represents the first report to characterize the factors required for hematopoietic differentiation from hESCs. We demonstrate that hematopoietic cytokines, together with BMP-4, strongly promote hematopoietic commitment from EB-differentiated hESCs in the form of progenitors capable of multilineage hematopoietic maturation. These progenitors were restricted to the subpopulation of differentiated hESCs expressing the pan-leukocyte marker CD45, in contrast to a previous report demonstrating that hESCs differentiated into cells with hematopoietic properties by stromal coculture do not express CD45.19 CD45+ hematopoietic progenitors differentiated in our system are phenotypically similar to both committed adult hematopoietic tissue21,22 and to the first definitive hematopoietic cells detected within the aorta-gonad-mesonephros (AGM) region from 3- to 5-week-old human embryos coexpressing both CD45 and CD34.27,29

Our results indicate that committed hematopoietic cells emerge at a defined stage of human EB differentiation, suggesting that a temporally regulated process governs the commitment of cells toward hematopoiesis in the presence of cytokines and BMP-4. This defined differentiation period is reminiscent of the work of Keller’s group, which characterized a specific period in which cells with hematopoietic potential arose during mouse EB development.33 Although the specific mode of action through which cytokines and BMP-4 promote hematopoietic emergence within mouse and human EBs remains to be fully elucidated, our data demonstrate that restricted hematopoietic cytokine treatment prior to the onset of hematopoietic emergence was sufficient to promote hematopoietic differentiation. Detection of hematopoietic cells was accompanied by up-regulation of hematopoietic transcription factors and the emergence of single CD45+ hematopoietic cells within cytokine- and BMP-4–treated EBs. Taken together, our data suggest that cytokines are able to either (1) direct the differentiation of uncommitted hESCs, and/or (2) induce hematopoietic cell fate from currently uncharacterized hematopoietic precursors arising during stage I of EB development. Given the identification of factors required for hematopoietic differentiation combined with the characterization of the kinetics of hematopoietic cell emergence provided by our current study, single cell tracking experiments now can be explored to define whether cytokines are capable of directing hematopoietic cell fate commitment from responsive hESCs, together with studies to identify the potential presence of hematopoietic precursors within differentiating EBs. Common precursors possessing both hematopoietic and endothelial cell fate potential, arising during a precise window of cellular development, already have been identified using differentiated murine embryonic stem cells (mESCs) cells in vitro.34 In addition, hematopoietic precursors have been found to arise from cells expressing endothelial markers within the AGM region of the developing human embryo.28,35

Surprisingly, despite an established inductive role for BMP-4 in hematopoietic development during embryogenesis36 and the generation of hematopoietic cells from primate and murine ESC lines,36,37 BMP-4 alone or in combination with cytokines had only a modest effect on the frequency or total number of hematopoietic progenitors derived from treated human EBs under our conditions. However, BMP-4 treatment allowed for the generation of unique hematopoietic progenitors that possessed greater self-renewal capacity than progenitors derived from control or cytokine-containing cultures. Since hematopoietic progenitors arising from BMP-4 treatment do not continue to receive BMP-4 subsequent to EB dissociation yet demonstrate self-renewal properties in secondary progenitor assays weeks after BMP-4 exposure, we suggest that BMP-4 causes the activation of developmental programs responsible for progenitor self-renewal. Our findings provide the foundation to further characterize the role of BMP-4 during human EB differentiation, similar to studies using mouse embryonic stem (ES) cell systems.37

A correlation between the quantity of hematopoietic progenitors transplanted and success of hematologic recovery of patients to combat both neutropenia and megakaryocytic anemia is widely accepted.11,38 Generation of adequate numbers of hematopoietic progenitors from committed sources of hematopoietic tissue such as human CB or M-PB sources is a continuing concern for hematopoietic cell transplantation. Human39 ESCs have been suggested to supply an alternative source of hematopoietic progenitors for transplantation.40 Accordingly, we compared the absolute numbers of progenitors that can be produced from hESCs to human CB and M-PB sources. Using 1 × 10^7 differentiated hESCs and identical numbers of CB or M-PB mononuclear cells, our results indicate that a total of 237 hematopoietic progenitors could be derived from hESCs treated with cytokines and BMP-4, as compared to 182 CB and 249 M-PB–derived progenitors. Given that the total number of cells procurable from a single CB or M-PB sample is finite, in contrast to the immense expansion capacity of hESCs for hematopoietic progenitor production, our present findings suggest that differentiation of hESCs could provide an alternative supply of hematopoietic progenitors to those commonly used in the clinic, thereby emphasizing the importance of studying human hematopoietic cells capable of in vivo reconstitution39 that are derived from hESC lines.

Given the considerable difficulties encountered in detecting analogous cell types from mESCs, the identification of human hematopoietic repopulating cells differentiated from hESCs is anticipated to be arduous. Initial studies detecting murine hematopoietic progenitors in vitro from differentiated mESCs33,41 were followed by nearly a decade of attempts to detect mESC-derived cells with hematopoietic lympho- myeloid repopulating function.42,43 Recently, hematopoietic cells capable of repopulation have been derived from mESCs. These repopulating stem cells arise from a precise window during mESC differentiation44 and require genetic manipulation to overexpress genes previously implicated in leukemic growth44,45 in order to reproducibly detect repopulating function. Based on the identification of the period of cytokine action and the time point of hematopoietic emergence defined in our current study, preliminary experiments are under way in our laboratory to derive human in vivo hematopoietic repopulating cells from hESCs that are distinct from the hematopoietic progenitors detected by in vitro CFU assays.46

Acknowledgments

We would like to thank Claire Heslop for performing the RT-PCR reactions described in this study, and Melissa Carpenter and Jane Lebowski of Geron Corporation for their scientific insights and assistance in the maintenance of human ESC lines.
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