Overexpression of HOXB4 Enhances the Hematopoietic Potential of Embryonic Stem Cells Differentiated In Vitro

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Little is known about the molecular mechanisms controlling primitive hematopoietic stem cells, especially during embryogenesis. Homeobox genes encode a family of transcription factors that have gained increasing attention as master regulators of developmental processes and recently have been implicated in the differentiation and proliferation of hematopoietic cells. Several Hox homeobox genes are now known to be differentially expressed in various subpopulations of human hematopoietic cells and one such gene, HOXB4, has recently been shown to positively determine the proliferative potential of primitive murine bone marrow cells, including cells with long-term repopulating ability. To determine if this gene might influence hematopoietosis at the earliest stages of development, embryonic stem (ES) cells were genetically modified by retroviral gene transfer to over-express HOXB4 and the effect on their in vitro differentiation was examined. HOXB4 overexpression significantly increased the number of progenitors of mixed erythroid/myeloid colonies and definitive, but not primitive, erythroid colonies derived from embryoid bodies (EBs) at various stages after induction of differentiation. There appeared to be no significant effect on the generation of granulocytic or monocytic progenitors, nor on the efficiency of EB formation or growth rate. Analysis of mRNA from EBs derived from HOXB4 transduced ES cells on different days of primary differentiation showed a significant increase in adult β-globin expression, with no detectable effect on GATA-1 or embryonic globin (βH-1). Thus, HOXB4 enhances the erythropoietic, and possibly more primitive, hematopoietic differentiation potential of ES cells. These results provide new evidence implicating Hox genes in the control of very early stages in the development of the hematopoietic system and highlight the utility of the ES model for gaining insights into the molecular genetic regulation of differentiation and proliferation events.

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exemplified by recent studies on the effect of "knocking out" the vav proto-oncogene. Although the in vitro differentiation of ES cells could be used to identify extrinsic factors that regulate hematopoietic development, more success has been achieved using this system to examine the roles of various transcription factors, including rbtn2, GATA-1, and GATA-2, in hematopoiesis.

To gain further insight into the possible roles of Hox genes in developmental hematopoiesis, we turned to the ES model and examined the effect of engineered overexpression of HOXB4 in ES cells by retroviral transduction. We demonstrate marked enhancement in the generation of definitive erythroid and mixed colony progenitors with no obvious effect on the plating efficiency or growth of the ES-derived embryoid bodies, and thus provide new evidence implicating Hox genes in the control of very early stages of hematopoietic development.

MATERIALS AND METHODS

Cell culture. CCE ES cells (kindly provided by Dr G. Keller, National Jewish Center, Denver, CO) and EFC-1 ES cells (kindly provided by Dr A.G. Smith, Edinburgh Centre for Genomic Research, Edinburgh, UK) were maintained on gelatinized dishes in Dulbecco's modified essential medium (DMEM) supplemented with 10% selected fetal calf serum (FCS), 4mM glutamine, 2% nonessential amino acids, 0.1 mM 2-mercaptoethanol (Sigma Chemical Co, St Louis MO), and leukemia inhibitory factor (LIF) supplied as a supernatant (prepared at the Terry Fox Lab) from Cos cells transfected with an LIF expression vector. Unless otherwise stated, all reagents for cell culture and in vitro differentiation of ES cells were purchased from StemCell Technologies Inc (STI, Vancouver, BC, Canada).

The ecotropic retroviral packaging cell line GPE + 86 was maintained in HXM medium consisting of DMEM supplemented with 10% heat-inactivated (55°C for 30 minutes) newborn calf serum (GIBCO-BRL Canada, Burlington, Ontario), 15 μg/mL hypoxanthine (Sigma), 250 μg/mL xanthine (Sigma), and 25 μg/mL mycophenolic acid (Sigma). Viral-producing cells were cultured in HXM medium containing 1 mg/mL of the neomycin analog G418 (Sigma).

Unless otherwise specified, all cell culture was performed in humidified incubators at 37°C with a mixture of 5% CO2 in air.

In vitro differentiation of ES cells and assay for hematopoietic progenitors in developing embryoid bodies. The methods used for the differentiation of ES cells and the quantitation of progenitors within the EBs were essentially as described by Keller et al. ES cells used in these experiments were maintained in culture for less than 15 passages. Forty-eight hours before differentiation, cells were passaged at low density (5 × 10³ cells per 25-cm² flask) in Iscove's modified essential medium (IMDM) supplemented as described above. Cells were obtained with 0.25% Trypsin-1 mmol/L EDTA (GIBCO-BRL), washed with IMDM containing 5% FCS, and were suspended at 4 to 5 × 10⁶ cells/mL.

Methylcellulose for differentiation consisted of 0.8% α-methylcellulose supplemented with 1% bovine serum albumin (BSA), 15% selected FCS, 2.5 × 10⁻⁴ M 2-mercaptoethanol (Sigma), 160 ng/mL Steel Factor (SF) supplied as a Cos-cell-derived supernatant, and IMDM to volume. In certain experiments (see Fig 3), ES cells were differentiated in 0.9% Iscove's methylcellulose supplemented with 15% selected FCS, 450 μM monothioglycerol (Sigma), 40 ng/mL SF supplied as a Cos-cell-derived supernatant, and IMDM to volume. ES cells were added to the methylcellulose to yield a final density of 300 to 500 cells/mL and 1.0-M aliquots were distributed into 35-mm Petri-style dishes (STI). Cultures were fed at day 8 or 10 of differentiation by layering 0.5 mL of 0.4% methylcellulose containing recombinant human erythropoietin (Epo; 3 U/mL), SF (Cos-supernatant; 160 ng/mL), and either pokeweed mitogen-stimulated murine spleen cell conditioned medium (SCCM; 2%) or 30 ng/mL IL-3 plus 30 ng/mL IL-6 (both supplied as Cos cell supernatants) onto each dish. Primary plating efficiency was determined by calculating the percentage of ES cells which formed EB (ie, number of EB formed divided by number of ES plated multiplied by 100 equals the percent plating efficiency).

At various stages of the primary differentiation EBs were procured for replating in secondary methylcellulose cultures to detect hematopoietic progenitors. Before day 10, EBs were obtained, pelleted, and treated with Trypsin-EDTA for approximately 2 minutes. EBs were disrupted by passage through a 21-gauge needle and the cells were pelleted and counted. After day 10, the isolated EBs were incubated at 37°C for 60 to 90 minutes in a solution of 0.25% collagenase (Sigma) in phosphate-buffered saline (PBS) supplied with 20% FCS. Single-cell suspensions were prepared as described above. The number of cells per culture was determined by dividing the total cell yield by the number of primary differentiation cultures procured.

Cells were plated in either 0.8% α-methylcellulose (STI M3230) or 0.9% Iscove's methylcellulose (STI M4100) supplemented with 3 U/mL Epo, 2% SCCM, 160 ng/mL SF, and IMDM. In some experiments SCCM was replaced by combinations of cytokines supplied as supernatant from transfected Cos cells (Terry Fox Laboratories). Hematopoietic colonies were scored microscopically after 10 to 14 days using standard criteria.21 In some experiments isolated colonies were plucked and centrifuged off slides for cytological analysis.

Retroviral infection of ES cells. Retroviruses were constructed using the MSCV 2.1 retroviral vector22 (kindly provided by Dr R. Hawley, Sunnybrook Research Institute, Toronto, Ontario, Canada). The human HOXB4 cDNA encompassing the entire coding region (kindly provided in a plasmid by E. Boncinelli, Ospedale S. Faffaie, Milan, Italy) was isolated and cloned into the XhoI site directly upstream of the pgk-neo' cassette by blunt-end ligation using standard procedures.23 Stable polyclonal retroviral producer lines were generated by calcium phosphate transfer of DNA (10 μg per con-struct) into GPE + 86 cells followed by selection with 1 mg/mL G418 (GIBCO-BRL). The viral titers of the GPE + 86-pgk-neo' producer cells were determined by transfer of G418 resistance to NIH 3T3 cells23 to be 3 to 5 × 10⁶ colony-forming units (CFU)/mL and 3 to 5 × 10⁵ CFU/mL, respectively.

Confluent plates of viral producers were incubated for 24 hours in DMEM-10% FCS supplemented as for the ES cells, without LIF. Supernatants were collected, filtered through a 22-μm filter (Millipore, Bedford, MA), and supplemented with LIF and 2 μg/mL polybrene24 before addition to the ES cells.

Freshly passaged ES cells were resuspended at 5 × 10⁴ cells in 5 mL viral supernatant per 25-cm² gelatinized flask and were incubated as usual with 1 or 2 changes of the viral supernatant over 24 hours. Adherent cells were rinsed with PBS and fresh medium was added for a further 24-hour culture period. At 48 hours postinfection ES cells were obtained and transferred to fresh flasks with 0.8 mg/mL G418 (GIBCO-BRL). Polyclonal populations of G418-resistant cells were expanded for further study. In all cases a mock-infected (polybrene only) control was maintained.

To generate sublines from virally infected and parental cell lines, 25 or 50 cells were placed into individual gelatinized wells of a 96-well plate. When colonies of ES cells were visible, cells were obtained and expanded as described above.

RNA extraction, cDNA synthesis, and polymerase chain reaction (PCR) amplification. Single-cell suspensions were derived from parental, neo-transduced, or HOXB4-transduced ES cells at various
stages of the primary differentiation. In addition, pools of erythroid (eight colonies) and GM or GEMM (four colonies) colonies were plated after 8 days in methylcellulose. RNA extraction and cDNA synthesis for each of these sample types were performed as described previously.8

PCR conditions (pH, magnesium, and KCl concentrations, gene 32 protein) were optimized for first-strand cDNA synthesis. This procedure has been shown to quantitatively preserve the differences between frequent and rare transcripts over a wide range for cell numbers ranging from 1,000 to 10,000 cells.9

Southern blot analysis. High-molecular-weight genomic DNA was isolated from undifferentiated ES cells by standard procedures to determine the clonality of retrovirally infected ES cells. The DNA was digested with either Sst I, which cuts once in the viral LTRs to release the proviral genome, or EcoRI, which cuts once in the proviral genome to release fragments unique to the integration site. The digested DNA was separated on a 1% agarose gel and passively transferred onto an ionic nylon membrane (Zeta-probe; Bio-Rad, Mississauga, Ontario, Canada). Similarly, 10-μL aliquots of the PCR-amplified cDNA were electrophoresed and transferred.

Probes were labeled with 32P-dCTP (3,000 Ci/mmol; Amersham, Oakville, Ontario, Canada) by random priming and were purified on Sephadex-G50 columns (Pharmacia Biotech, Uppsala, Sweden) before use. Blots were hybridized for 20 hours at 65°C in 4.4× SSC (SSC: 3 mol/L sodium chloride, 0.3 mol/L tri-sodium citrate), 7.5% formamide, 0.75% sodium dodecyl sulfate (SDS), 1.5 mmol/L EDTA, 0.75% skim milk powder, and 7.5% dextran sulfate. Membranes were washed twice at 65°C for 30 minutes each in 3× SSC, 0.1% SDS, and 1 mg/mL sodium pyrophosphate. To reprobe blots, they were stripped in 0.1% SDS, and 1 mg/mL sodium pyrophosphate. To reprobe blots, they were stripped in

results
Overexpression of HOXB4 enhances the hematopoietic differentiation of CCE ES cells. To determine if Hox genes might be regulators of hematopoiesis during ontogeny, undifferentiated CCE ES cells were transduced with either a HOXB4 or a Neo (control) retrovirus by supernatant infection. Figure 1 demonstrates expression of the HOXB4 message from the retroviral insert in undifferentiated ES cells. Although there did not appear to be endogenous expression of this gene in undifferentiated cells, the more sensitive RT-PCR technique (detailed in Fig 4) suggests that very low levels may be present at day 4 and day 8.

Parental cells and retrovirally transduced cells, selected for G418 resistance, were differentiated in vitro as described. No significant differences in the ability to form EBs in the primary differentiation were detected among the three groups yielding plating efficiencies of 35.0% ± 3.8% for parental, 46.1% ± 15.8% for Neo-transduced, and 32.6% ± 5.0% for HOXB4-transduced cells (mean ± SEM of four determinations). In addition, HOXB4 overexpression did not alter the growth kinetics (number of cells per EB; not shown) or total number of cells generated in these cultures (Fig 2). Thus, HOXB4 altered neither the primary plating efficiency nor the overall yield of cells during the primary differentiation.

Replating of these cells in conditions to detect hematopoietic progenitors showed that for parental, neo-transduced, and HOXB4-transduced cells the appearance of various hematopoietic progenitors followed a time course similar to
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Fig 2. HOXB4 overexpression does not change the cell yield from primary differentiation cultures. Equivalent numbers (500) of parental, neo-transduced, or HOXB4-transduced CCE ES cells were differentiated in methylcellulose for 8 to 20 days. No significant differences in the number of EBs formed were detected among the three groups. At each time point EBs were obtained and cell yields were determined for parental (- - -), Neo-transduced (---), and HOXB4-transduced (----) cultures. Values are the mean ± SEM of four separate determinations.

that observed by others using the CCE cell line. However, a pronounced influence of HOXB4 overexpression was seen on the numbers of erythroid colony-forming (CFC-E) and mixed-lineage (granulocyte/erythroid/macrophage/megakaryocyte; CFC-GEMM) progenitors detected (Fig 3). As shown in two representative experiments (n = 6), there was no obvious effect on the number of primitive (large, nucleated cells in small colonies) erythroid progenitors detected (day 7 or day 10). However, overexpression of HOXB4 increased the number of definitive erythroid colonies observed at all time points examined between days 12 and 20 of the primary differentiation. There was also a marked increase in the number of CFC-GEMM detected in HOXB4-transduced cultures compared with either Neo-transduced or parental controls. In contrast, overexpression of HOXB4 did not significantly affect the number of CFC-GM observed. Overall, there was a slight increase in the total number of hematopoietic CFC throughout the primary differentiation.

The increase in definitive, but not primitive, erythropoiesis was confirmed by quantitating each type of erythroid colony at day 10 and day 12 when the increase was first observed. On both days there was no significant difference in the num-

Fig 3. HOXB4 overexpression enhances the hematopoietic differentiation of CCE ES cells. Equivalent numbers of parental (- - -), Neo-transduced (---), or HOXB4-transduced (----) CCE ES cells were differentiated in methylcellulose for various periods of time. EBs were obtained and single-cell suspensions were plated in secondary methylcellulose cultures in the presence of 3 U/mL Epo and 160 ng/mL SF supplemented with 30 ng/mL interleukin-3 (IL-3) plus 30 ng/mL IL-6. Results from two representative experiments (total of six) are shown.
Fig 4. Expression of various genes in EBs isolated on different days of primary differentiation. Total RNA was isolated from either 50,000 cells derived from parental (P), neo-transduced (N), or HOXB4-transduced (B4) CCE EBs at various stages of the primary differentiation (A) or from pools of eight erythroid or four GM or GEMM colonies (B). Samples were reverse transcribed and PCR-amplified to generate representative total cDNA populations which were electrophoresed and blotted. The resultant Southern blots were hybridized to probes for HOXB4, Hoxb8, pH-1 globin, adult β-globin, GATA-1, and actin as described.

Table 1 summarizes the results from six experiments comparing the ratios of progenitors detected in HOXB4-transduced cultures to those initiated with parental or Neo-transduced cells in the various experimental conditions after 14 days or 16 days of primary differentiation. Conditions for the detection of hematopoietic progenitors were varied throughout the course of the study to optimize the system and to confirm the specificity of the HOXB4 effect. Regardless of whether a defined cytokine cocktail or SCCM was used for the detection of hematopoietic progenitors similar magnitudes of difference were observed between HOXB4-transduced and control cells. However, greater numbers of progenitors were observed for all groups in the former plating condition, especially in the CFC-E and total CFC compartments. For example, at day 14 the number of erythroid progenitors detected ranged from 316 to 1,840 per culture arising from neo-transduced cells. A similar, though less pronounced, difference was also observed on day 10. The identity of the colonies scored as primitive or definitive was confirmed by plucking and pooling individual colonies to examine globin expression patterns. These experiments confirmed that the primitive erythroid colonies expressed primarily βH-1 globin, whereas colonies scored as definitive erythroid expressed primarily adult β-globin (Fig 4B).

Cytospin analysis was performed on colonies derived from day 14 primary differentiation cultures of Neo-transduced and HOXB4-transduced CCE ES cells to confirm colony identification based on in situ scoring. Analysis of 46 randomly plucked
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Table 1. Increased Numbers of CFC-E and CFC-GEMM Are Detected at Days 14 and 16 of Primary Differentiation in Cultures Initiated With HOXB4-Transduced ES Cells

<table>
<thead>
<tr>
<th>Group</th>
<th>Progenitors Relative to Parental ES</th>
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<tr>
<td></td>
<td>CFC-E</td>
</tr>
<tr>
<td>Day 14</td>
<td></td>
</tr>
<tr>
<td>Neo-transduced</td>
<td>0.96 ± 0.11</td>
</tr>
<tr>
<td>HOXB4-transduced</td>
<td>2.19 ± 0.37*</td>
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<tr>
<td>Day 16</td>
<td></td>
</tr>
<tr>
<td>Neo-transduced</td>
<td>0.89 ± 0.13</td>
</tr>
<tr>
<td>HOXB4-transduced</td>
<td>3.48 ± 0.70*</td>
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</tbody>
</table>

Equivalent numbers of parental, Neo-transduced, or HOXB4-transduced CCE ES cells were differentiated in α (n = 4) or Iscove’s (n = 2) methylcellulose for 14 or 16 days. EBs were obtained and single-cell suspensions were plated in secondary methylcellulose cultures in the presence of 3 U/mL Epo and 160 ng/mL SF supplemented with either 2% SCCM (n = 4) or 30 ng/mL IL-3 plus 30 ng/mL IL-6 (n = 4) to detect hematopoietic progenitors. Values presented are the ratio of progenitors, compared with parental, detected in Neo-transduced or HOXB4-transduced cultures. Statistical analysis to compare populations was performed using a Student’s t-test.

* P = .05, † P < .001 compared with Neo-transduced ratios.

colonies derived from HOXB4-transduced cells showed that 27 (59%) were mixed granulocyte/erythroid/macrophage/megakaryocyte (GEMM), 12 (26%) were megakaryocyte-erythroid or erythroid, and 7 (15%) were pure granulocyte/macrophage (GM). In contrast, only 7 of 22 colonies (32%) arising from Neo-transduced cells were multine-lineage (GEMM), while 9% (2 of 22) were erythroid or erythroid-megakaryocyte and the remainder (13 of 22; 59%) were GM. Thus, this examination showed that both cultures contained a variety of hematopoietic cells and confirmed the enhanced number of erythroid and multilineage colonies observed in HOXB4-transduced cultures. No cells with abnormal morphology were detected in the HOXB4 cultures.

Total cellular RNA was isolated at various stages of the primary differentiation from EBs derived from parental, Neo-transduced, and HOXB4-transduced ES cells. After reverse transcription and PCR-amplification, the total cDNA samples were analyzed by Southern blot analysis. Results of a representative hybridization (n = 2) with a variety of probes are shown in Fig 4A.

HOXB4 expression was detectable at very low levels in the EBs at day 4 and day 8 of differentiation. As expected, higher levels of HOXB4 were detected in the HOXB4-transduced population at all times. Interestingly, there was an apparent upregulation of expression at later time points (compare day 8 levels with day 12; Fig 4A), possibly because of increased transcription from the viral LTR in differentiated cells. We examined levels of HOXB4 expression from the viral LTR in various colony types by plucking colonies, isolating RNA, and generating cDNA. As shown in Fig 4B, expression levels were very high in colonies scored as definitive erythroid, GM, and GEMM. Although somewhat lower levels were detectable in primitive erythroid colonies, these levels were still markedly higher than endogenous HOXB4 levels, which were virtually undetectable.

Adult β-globin expression was first observed at day 8 of the primary differentiation, with levels increasing greatly between day 8 and day 12. A pronounced enhancement of expression was observed in the HOXB4-transduced cells. More impressively, this difference was still readily apparent at day 18. Thus, there was a correlation between the increased β-globin expression in the primary cultures and the enhancement of erythropoiesis observed after replating of the HOXB4-transduced CCE cell line. In contrast, overexpression of HOXB4 had little or no effect on the levels of embryonic βH1-globin, GATA-1, or Hoxb8, a gene 5′ to Hoxb4, detected in the differentiating EBs.

Hematopoietic progenitors of the EFC-1 ES cell line are increased by overexpression of HOXB4. To rule out that the results obtained with the CCE ES cells were cell-line specific, similar studies were performed with EFC-1 ES cells transduced with either a HOXB4 or a Neo retrovirus. Quantitative examination of the hematopoietic differentiation of this line showed a time course which resembled that of CCE. Similar to the observation with the CCE cell lines, HOXB4 overexpression altered neither the plating efficiency (25.4% ± 2.6%, 16.6% ± 1.5%, and 24.5% ± 3.3% for parental, neo-transduced, and HOXB4-transduced cells, respectively; mean ± SEM for three separate determinations) nor the cell yield (for example, day 14 of primary differentiation cell yields were [×10⁶]: parental, 11.8 ± 1.2; Neo-transduced, 9.6 ± 1.0; and HOXB4-transduced, 11.7 ± 0.3; mean ± SEM for three separate determinations).

EBs derived from the parental and transduced EFC-1 cell lines were disrupted at day 14 of primary differentiation and cells were plated for detection of progenitors. As observed with the CCE ES cells, HOXB4-transduced EFC-1 cells gave rise to an increased number of definitive erythroid CFC and CFC-GEMM leading to an increase in the total number of hematopoietic precursors detected (Fig 5A). No significant differences in the number of CFC-GM were observed among the three lines.

Five sublines were generated from each of the three populations to assess whether the observations might be attributed to clonal variations within the EFC-1 cell line. Selection of unique populations was confirmed by Southern blot analysis to examine retroviral integration sites (Fig 6). RNase protection was used to verify expression of HOXB4 from the viral LTR in each of the sublines (Fig 7).

All sublines were differentiated in a manner similar to the polyclonal populations and the number of hematopoietic CFC present at day 14 was examined by replating in secondary meth-
yel-56-56 cellulose cultures (Fig 5B). As for the CCE cell line, the enhancement of hematopoeisis appeared to be restricted to CFC-E and CFC-GEMM with a fivefold increase in the number of erythroid progenitors (1.296 ± 155 vs 250 ± 31; P = .001; mean ± SEM of five sublines) and a 50-fold increase in the number of CFC-GEMM (161 ± 36 vs 3.1 ± 2.2; P ≤ .05; mean ± SEM of five sublines) detected in the HOXB4-transduced cell lines compared with the neo-transduced cell lines. There was no significant effect on granulocyte-macrophage progenitor (CFC-GM) numbers. Overall there was a marked increase in the total number of hematopoietic CFC detected.

**DISCUSSION**

There is increasing evidence that Hox genes are involved in regulating the maintenance and/or differentiation events within the hematopoietic system. Of particular interest is HOXB4, which is expressed in the mesoderm and fetal liver at stages that correlate with hematopoietic development and differentiation during embryogenesis. In the adult hematopoietic system, HOXB4 is expressed in the more primitive subpopulations of human CD34+ BM cells and its expression is downregulated in more mature subpopulations. Studies with murine BM transduced with a HOXB4 retrovirus suggest that this gene enhances the proliferative potential of hematopoietic cells, including the self-renewal capacity of HSCs, in vivo. Interestingly, there was no apparent effect on differentiative processes and overexpression did not lead to leukemogenesis. In light of these provocative findings with adult BM, we examined the role of HOXB4 on the earliest stages of development and differentiation of the ho-
matopoietic system by inducing overexpression in ES cell lines.

Under the culture conditions used in these studies, both the CCE and the EFC-1 ES cells differentiated efficiently to give rise to hematopoietic progenitors detectable as early as day 8. In accordance with previous studies, the earliest progenitors detected gave rise to colonies with the morphology typical of the primitive erythroid lineage (small colonies of large, nucleated cells). Throughout the time course of differentiation of both cell lines there was a gradual switch from primitive to definitive erythropoiesis. This switch corresponded with the appearance of myeloid progenitors (CFC-GM and CFC-GEMM). Mast cell progenitors (included within the CFC-GM determinations) were predominant at the latest time points examined (days 18 to 20). The numbers of definitive erythroid and myeloid progenitors detected were similar to those reported by others. We detected fewer primitive erythroid precursors, possibly because of the use of FCS rather than plasma-derived serum in the secondary methylcellulose cultures.

Overexpression of HOXB4 significantly enhanced the number of hematopoietic progenitors detected at various times in the ES differentiation cultures. However, in sharp contrast to its effects in adult BM, HOXB4 expression elicited selective enhancement of specific pools within the ES cell hematopoietic compartment. Although there was no apparent influence on the primitive (yolk-sac like) erythroid progenitors, this lack of effect might be attributed to comparatively low levels of HOXB4 expression from the viral LTR in this cell population. However, because the level of engineered expression was considerably higher than endogenous expression (compare day 8 primitive erythroid of neo-transduced and HOXB4-transduced, Fig 4B) it seems likely that HOXB4 does not influence erythropoiesis at this stage of differentiation. In contrast, the number of definitive erythroid (CFC-E) and myeloid/erythroid (CFC-GEMM) progenitors was significantly increased. The relative lack of effect on CFC-GM progenitors suggests that the most significant consequence of constitutive HOXB4 expression is the enhanced proliferation of a common pluripotent precursor that has differentiated from the pure myeloid pathway toward the erythroid lineage.
Our observation that the erythroid lineage is most significantly enhanced might reflect the relative developmental stages of the populations examined. In contrast to the adult BM-derived populations used previously, it has been suggested that ES-derived hematopoietic progenitors are equivalent in properties to yolk-sac or early fetal liver populations. Because transcription factors are thought to function in a coordinated manner to generate a cascade of events leading to phenotypic changes in the cells of interest, it may only be the ES-derived definitive erythroid and multilineage progenitors that possess the accessory molecules required for a phenotypic response to HOXB4 overexpression. The lack of effect on CFC-GM suggests that these progenitors do not express the necessary cofactors required to induce a proliferative or differentiative response. Alternatively, differences between the in vivo environment and that of isolated stem cells may be reflected by changes in either cell-surface molecules or responsiveness to external stimuli that influence the capacity of a cell for proliferation in a specific lineage. It may be possible to use these differences between the progenitors to elucidate the molecular events involved in the HOXB4 response. However, further clarification of this point awaits the identification and isolation of the hematopoietic stem cell(s) generated in ES differentiation cultures.

Studies of genes expressed in differentiating EBs correlate with the observation of enhanced erythropoiesis in HOXB4-transduced ES cells. Expression of the erythroid-specific gene adult β-globin was dramatically increased in HOXB4-transduced cells, raising the possibility that HOXB4 is involved in its transcription. Although this may be a direct effect, it is also possible that HOXB4 alters the transcription of other factors, including other Hox genes, involved in controlling β-globin expression. Despite the fact that few cellular targets of Hox genes have been identified, there is ample evidence to suggest that cross-regulation among the various proteins may be important for their function. Preliminary results of expression levels in EB suggest that overexpression of HOXB4 does not influence the low level of expression of the more 5′ gene Hoxb8 at any time during the differentiation time course (Fig 4A). Despite this observation, the effect of altered HOXB4 expression on the transcription of other Hox genes in relevant cell populations is certainly one avenue that must be explored when it becomes possible to isolate pure hematopoietic populations from differentiated EB.

We have determined that constitutive HOXB4 expression in ES cells leads to enhanced proliferation of erythroid and mixed erythroid/myeloid progenitors with a reduction in granulocyte/macrophage progenitor yield. These findings suggest a predominantly proliferative effect of HOXB4 rather than one impacting on lineage commitment as observed previously for adult BM. However, differences in both the stage and lineage-specificity of the effects observed in the two systems suggest that the in vitro differentiation of ES cells may provide a system to study the role of Hox proteins in blood development as well as a means for elucidating, at the molecular level, some of the mechanisms by which this family of regulatory proteins influence hematopoiesis.

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