Deregulated expression of HOXB4 enhances the primitive growth activity of human hematopoietic cells

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Identification of the molecular mechanisms that can promote human hematopoietic stem cell amplification is a major goal in experimental and clinical hematology. Recent data indicate that a variety of regulatory molecules active in early development may also play a role in the maintenance of hematopoietic stem cells with repopulating activity. One important class of early developmental genes determining hematopoietic development are homeobox transcription factors. Here, we report that retrovirally mediated expression of the homeobox gene HOXB4 rapidly triggers an increase in the number of human hematopoietic cord blood cells with stem cell and progenitor cell properties detected both by in vitro and in vivo assays. This growth enhancement extended across primitive myeloid-erythroid and B-lymphoid progenitors but did not lead to alterations in the balance of lymphomyeloid reconstitution in vivo, suggesting that HOXB4 does not affect control of end-cell output. These findings reveal HOXB4 as a novel, positive regulator of the primitive growth activity of human hematopoietic progenitor cells and underline the relevance of early developmental factors for stem cell fate decisions. (Blood. 2002;100:862-868)

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gene transfer was used to force the expression of HOXB4 in primitive human cord blood (CB) cells and their progeny and the biologic effects on subsequent hematopoiesis both in vitro and in vivo were then analyzed. We now show that constitutive expression of HOXB4 rapidly increased the number of cells displaying functional properties of very primitive human hematopoietic cells suggesting a retardation in the exit of cells from this compartment or the reactivation within more differentiated progenitors of a stem cell state.

Materials and methods

Retroviral constructs

A human HOXB4 complementary DNA (cDNA) containing the complete HOXB4 open reading frame was cloned as an EcoRI fragment and inserted upstream of the internal ribosomal entry site (IRES) into a murine stem cell virus (MSCV) 2.1 vector (from R. Hawley, Rockville, MD) containing an IRES-green fluorescent protein (GFP) cassette originally provided by P. Leboulch (Boston, MA; Figure 1A). As a control, the MSCV vector carrying the IRES-GFP cassette alone was used (GFP virus). High-titer, helper-free recombinant retrovirus was generated by transfecting the amphotropic Phoenix packaging cell line and subsequently transducing PG13 packaging cells for pseudotyping with the gibbon ape leukemia virus envelope as previously described.19 The presence of full-length provirus in the PG13 producer cells and transduced primary CB cells was confirmed by Southern blot analysis using standard techniques and a 32P-labeled full-length GFP probe.19 (Figure 1B). HOXB4 transcripts in primary CB cells were demonstrated by reverse transcription–polymerase chain reaction (RT-PCR) and Southern hybridization using a HOXB4-specific probe (data not shown). For proof of protein expression, the HOXB4 cDNA was subcloned in frame 3’ to the FLAG-site of the pSc plasmid (Clontech, Palo Alto, CA). Protein expression of HOXB4 was tested as described previously.23 In brief, protein-lysate of 293 cells was incubated with 0.5 μL of an anti-FLAG M2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and Western blots were developed using horseradish peroxidase-conjugated secondary antibody (Sigma, St Louis, MO) and enhanced chemiluminescence (Amersham, Baie d’Urfe, QC; Figure 1C).

Isolation and transduction of human cells

Low-density cells (< 1.077 g/mL) were isolated by centrifugation of CB obtained with informed consent from mothers undergoing cesarean delivery of healthy, full-term infants and cryopreserved prior to thawing and removal of lineage marker–positive (lin+) cells using a StemSep column (Stem Cell Technologies, Vancouver, BC) as described by the manufacturer. This resulted in a cell suspension containing 59% ± 13% CD34+ cells. These were then transduced as previously described.19,24 Briefly, cells at 2 × 106/mL were prestimulated for 48 hours in Iscoves modified Dulbecco medium (IMDM) containing a serum substitute (BIT; Stem Cell Technologies), 10−4 M mercaptoethanol (Sigma), and 40 μg/mL low-density lipoproteins (Sigma) supplemented with the following recombinant human cytokines: 100 ng/mL Flt-3 ligand (FL, ImmuneX, Seattle, WA), 100 ng/mL Steel factor (SF, prepared and purified in the Terry Fox Laboratory), 20 ng/mL interleukin-3 (IL-3; Novartis, Basel, Switzerland), 20 ng/mL IL-6 (Cangene, Mississauga, ON, Canada), and 20 ng/mL granulocyte-colony stimulating factor (G-CSF; Stem Cell Technologies). After 48 hours, cells were resuspended in filtered virus-containing medium (VCM) supplemented with the same cytokine combination and protamine sulfate (5 μg/mL) on Petri dishes that had been precoated with 5 μg/cm2 fibronectin (Sigma) or on tissue culture dishes, both preloaded twice with VCM, each time for 30 minutes (Corning, Acton, MA). This procedure was repeated on the next 2 consecutive days for a total of 3 infections. For in vivo studies, aliquots of these cells were transferred to fresh-serum-free medium plus the same additives and cytokines and then incubated for an additional 48 hours prior to being stained with Cy5-labeled anti-CD34 antibody (Becton Dickinson, San Jose, CA) and isolation of the GFP+/CD34+ cells on a 3 laser FACStar Plus (Becton Dickinson). For in vitro studies, transduced cells were injected into nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice immediately after transduction (< 24 hours after the last exposure to fresh VCM) without preselection.

In vitro progenitor assays

In vitro colony-forming cell (CFC) activity was assessed by plating aliquots of cells in methylcellulose medium (GF H4434, Stem Cell Technologies) supplemented with 50 μg/mL human SF, 20 ng/mL each of human IL-3, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF; Novartis, Basel, Switzerland), G-CSF, and 3 U/mL erythropoietin (Stem Cell Technologies) and counting erythroid, myeloid, and mixed erythroid-myeloid colonies after 2 weeks of incubation at 37°C. Secondary CFC assays were performed by replating aliquots of cells obtained by harvesting complete 14-day-old primary CFC cultures.19 Cell morphology was assessed on Wright-Giemsa-stained metaphase spreads or individually plucked colonies.

Six-week long-term culture-initiating cell (LTC-IC) assays were carried out using pre-established irradiated murine fibroblasts genetically engineered to produce human IL-3, G-CSF, and SF as feeder layers.25 Both bulk assays and limiting dilution assays, initiated 2 days and 2 to 7 days after termination of transduction, respectively, were performed. In the latter case, cell numbers ranging from 1 to 12 800 GFP+ control or HOXB4-GFP+ transduced cells were sorted into 96-well plates (Nunc, Naperville, IL) and LTC-IC frequencies were calculated using Poisson statistics and the method of maximum likelihood with the assistance of the L-calc software (Stem Cell Technologies). To set up liquid suspension cultures, transduced GFP+ cells were placed in the same cytokine-supplemented serum-free medium described above and then aliquots removed at the times indicated for CFC assays. B-cell progenitor activity was assessed by plating 2 × 104 GFP+CD34+ cells on murine MS-5 cells in RPMI 1640 with 10% fetal calf serum (FCS) and 5% human AB serum with either 50 ng/mL SF, 10 ng/mL IL-2, and 10 ng/mL IL-15 (R & D Systems, Minneapolis, MN) or with these same factors plus 10 ng/mL IL-7 (R & D Systems), 100 ng/mL FL, and 50 ng/mL thrombopoietin (TPO; Genentech, San Francisco, CA), conditions permissive for B-lymphoid development in addition to varying degrees of myeloid cell development. After 3 or 6 weeks, both adherent and
nonadherent cells were collected and analyzed by FACS for the expression of CD34, CD38, myeloid (CD15, CD33), lymphoid (CD3, CD19, CD20), erythroid (glycophorin A [GlyA], CD71), and megakaryocytic (CD41) and natural killer (NK) cell (CD56) markers (see below).

In vivo assays

The NOD/Scid-scid/scid (NOD/SCID) mice were bred and maintained in the animal facility of the British Columbia Cancer Research Centre (Vancouver, BC, Canada) in microisolator cages containing autoclaved food and water. Test cells were injected intravenously into sublethally irradiated mice (350 cGy from a 137Cs source given at 6-12 weeks of age) and marrow cell aspirates performed 3, 6, and 10 weeks later. Mice were killed 6 to 18 weeks after transplantation, and the cells from both tibiae and femurs of each mouse collected for additional analyses. The absolute number of cells in the marrow of each mouse was calculated assuming that the contents of both femurs and both tibiae represent 25% of the total marrow.

Flow cytometry

Cells were suspended in cold Hanks balanced salt solution, supplemented with 5% pooled normal human serum (HBSS, Stem Cell Technologies) and were then incubated with an antihuman Fc receptor antibody 2.4G2 to block nonspecific antibody binding. The percent positive cells was determined after excluding nonviable (propidium iodide [PI] nonspecific antibody binding. The percent positive cells was determined after excluding nonviable (propidium iodide [PI] nonspecific antibody binding. The percent positive cells was determined after excluding nonviable (propidium iodide [PI] nonspecific antibody binding. After excluding nonviable cells labeled with isotype control antibodies. Separate aliquots of cells were stained for 30 minutes at 4°C with the antihuman CD45-phycoerythrin (PE; Becton Dickinson) and antihuman CD71-PE antibodies (OKT9) to quantitate the total number of human cells present (CD45+/H11001). With antihuman CD34 8G12-Cy5, antihuman CD19-PE, and antihuman CD20-PE (Becton Dickinson) to quantitate the number of human B cells (CD34+/H11002) present, and with antihuman CD15-PE (Becton Dickinson) to quantitate the number of human myeloid cells present. A detection limit of more than 20 CD45+ human cells per 2 × 106 cells analyzed and at least 5 human B cells plus at least 5 human myeloid cells per 2 × 106 cells analyzed was used to identify positively engrafted and lymphomyeloid-engrafted mice, respectively. Additional antibodies used for certain analyses included antihuman GlyA-PE, 10F7 antihuman CD33-PE (Becton Dickinson), antihuman CD41a-PE (Pharmacia Biotech, QC, Canada), and antihuman CD38-PE (Becton Dickinson).

Frequencies of lymphomyeloid stem repopulating (referred to as competitive repopulating units or CRUs)6,7 were calculated from the proportions of mice in a given experiment, or set of identical experiments, that were negative for lymphomyeloid engraftment using Poisson statistics and the method of maximum likelihood with the assistance of the L-calc software (Stem Cell Technologies).

Statistical analysis

Statistical tests were performed using the Student t test (software STATISTICA 5.1, StatSoft, Tulsa, OK).

Results

Retroviral transduction of HOXB4 in human Lin− CB

The Lin− CB cells were transduced with HOXB4 virus-conditioned medium using a 5-day transduction protocol previously optimized for transduction of the control GFP vector used here.24 In the present experiments (n = 14), a mean transduction efficiency of 48% (20%-80%) and 23% (8%-56%) was achieved with the GFP and B4-GFP virus, respectively, with an equivalent frequency of CD34+ cells in the GFP+ fraction in both cases (mean, 12%).

HOXB4 expression increases the production of secondary CFCs both in semisolid and liquid suspension cultures

As an initial test of possible HOXB4 effects on human hematopoietic cell proliferative potential, we examined the ability of transduced (GFP+) CD34+ CB cells to generate colonies of erythroid and myeloid colonies in standard methylcellulose assays. Primary assays did not show any significant differences in either the total number or type of colonies detected after 14 days (48 ± 6 versus 37 ± 6 CFC/200 cells plated from the HOXB4 and control cells, respectively). However, replating of the cells harvested from these primary cultures into secondary CFC assays revealed 5-fold more CFC for HOXB4 cells compared with controls (mean of 4600 ± 1600 secondary CFC/200 initially plated cells versus 983 ± 580 in the controls; n = 6; P = 0.03; Figure 2A). Analysis of the types of cells present in the secondary colonies derived from the HOXB4-transduced cells further showed a selective increase in erythroid colonies (P = 0.03 compared with the control (n = 6; Figure 2B). Notably, in 3 of 6 experiments, there were no detectable secondary erythroid CFC colonies in the control cultures, whereas high numbers were observed in the cultures of HOXB4-transduced cells (> 103) per 200 initially plated cells. Interestingly, both erythroid and myeloid secondary colonies produced showed a normal morphology indistinguishable from those obtained from the cells transduced with the control GFP vector based on microscopic analysis of single plucked Wright-Giemsa–stained colonies and immunophenotyping. Furthermore, enforced HOXB4 expression did not induce formation of secondary blast colonies in any of the experiments in contrast to previous findings with HOXA109 (data not shown). Thus, constitutive expression of HOXB4 significantly increased the proliferative
capacity of transduced human CB cells without apparently blocking terminal differentiation, once it was initiated.

Similar results were obtained when CFC assays were performed on transduced cells maintained in serum-free liquid suspension cultures. These showed no significant difference after 1 week in the number of total nucleated cells present in cultures of \( \text{HOXB4} \) or control \( \text{GFP} \)-transduced cells \( (3.1 \times 10^5 \pm 0.8 \text{ and } 3.4 \times 10^5 \pm 0.3 \text{ at week } 1, \text{ respectively}) \). However, the cultures of \( \text{HOXB4} \)-transduced cells were found to contain significantly greater numbers of CFC (compared with cultures of \( \text{GFP} \) control cells, \( P < .01 \), with a difference ranging from 2-fold after 1 week to 14-fold at 6 weeks Figure 3, \( n = 3 \)). This was associated with a net increase in the number of CFCs present after 6 weeks in the cultures of \( \text{HOXB4} \)-transduced cells, whereas in the \( \text{GFP} \) control arm, the total number of CFCs present declined about 3-fold during the same time period (Figure 3). This increase in CFC numbers compared with the control included myeloid as well as erythroid progenitors throughout the 6-week duration of the experiments (2.2- versus 5.7-fold increases after 1 week, respectively; 14-fold increase in myeloid CFCs and \( 4.2 \times 10^5 \) erythroid CFCs for \( \text{HOXB4} \) versus none for the control after 6 weeks; Figure 3).

**\text{HOXB4} \text{ expression amplifies the number of cells with LTC-IC activity**}

The \( \text{GFP}^+ \) CD34\(^+ \) cells were also assayed for LTC-IC activity to evaluate potential effects of \( \text{HOXB4} \) expression on more primitive hematopoietic cells. Bulk LTC-ICs for the detection of clonogenic progenitor cell output were initiated 2 days after termination of transduction \( (n = 6; \text{ Figure } 4) \). \( \text{HOXB4} \) LTCs contained nearly 10-fold more CFCs after 6 weeks than the control cultures and more than 90% of the CFCs obtained under LTC conditions were granulopoietic in both experimental arms (data not shown). To determine whether the increased CFC output by \( \text{HOXB4} \)-transduced cells was due to the number of cells detectable as LTC-IC or to an enhanced output of CFCs per LTC-IC, LTC-IC frequencies were determined by limiting dilution analysis after 4 to 7 days in vitro culture of CD34\(^+ \)/\( \text{GFP}^+ \) or CD34\(^+ \)/\( \text{HOXB4-GFP}^+ \) cells. As shown in Figure 4, the initial LTC-IC frequency of \( \text{HOXB4} \)-transduced CB cells was significantly increased \( (\sim 5\text{-fold} ; P < .001) \) compared with the control, whereas the progenitor yield per LTC-IC was essentially unchanged. Thus, the major impact of \( \text{HOXB4} \) overexpression was a rapid increase in the number of cells with the primitive functional capacity of LTC-ICs.

**\text{HOXB4 promotes the development of B cells in vitro**}

To evaluate whether the growth-promoting effects of \( \text{HOXB4} \) might extend to the lymphoid pathway, we examined its effect on the generation of B-lineage cells in vitro. Transduced-positive CD34\(^+ \) CB cells were cultured for 6 weeks on MS-5 fibroblast feeders in media with 2 different growth factor cocktails supportive of B-lymphoid development (SF, IL-2, and IL-15 with or without IL-7, TPO, and FL, respectively) and then assessed by FACS for the number of CD19\(^+ \) cells present in addition to cells of myeloid and NK lineages using a range of markers. Both conditions also supported a significant level of myeloid cell growth and these constituted the dominant cell type in cultures examined at 3 or 6 weeks with no significant difference observed in either total cell number or CD15\(^+ \) myeloid cells or CD56\(^+ \) NK cells. However, as shown in Table 1, under both conditions, the proportion and absolute number of CD19\(^+ \) B cells present was significantly higher in the cultures of \( \text{HOXB4} \)-transduced cells \( (\sim 2\text{- to } 8\text{-fold after 3 weeks and } 3\text{- to } 20\text{-fold after 6 weeks} ; P < .04) \). When we analyzed the effect of constitutive \( \text{HOXB4} \) expression on generation of CD34\(^+ \)/CD19\(^+ \) B-cell precursors, the difference was even more pronounced: at week 3, \( \text{HOXB4} \) induced a 31-fold \( (P < .02) \) and 16-fold \( (P < .0005) \) increase with 3 and 6 cytokines, respectively.

Thus, in culture conditions permissive for B lymphopoiesis, constitutive expression of \( \text{HOXB4} \) promoted production of B cells in sharp distinction to effects observed with \( \text{HOXA10} \).

**\text{HOXB4 expression increases the number of cells with repopulating activity detectable in NOD/SCID mice**}

Because we had seen that constitutive expression of \( \text{HOXB4} \) significantly amplified the number of cells with LTC-IC activity, we assessed whether \( \text{HOXB4} \) would also expand long-term repopulating stem cells using the CRU assay and the xenograft NOD/SCID mouse model. The CRU frequency was determined by limit dilution analysis in 3 cohorts of mice \( (n = 5) \) transplanted with CB infected either with the \( \text{HOXB4} \) or the \( \text{GFP} \) virus. Mice were injected with the CB progeny of an original input of lin\(^- \) cells containing \( 2.5 \times 10^5 \) CD34\(^+ \) cells, one fourth or one eighth of this cell number, respectively, after 24 hours or less in vitro culture after infection. There was no significant difference in the proportion of CD34\(^+ \)/\( \text{GFP}^+ \) cells after transduction between the control and \( \text{B4-GFP} \) transduced CB cells and cells were injected without any
the HOXB4 and the GFP cohort after injection of 2.5 × 10⁷ GFP virus. (A) FACS analysis of a representative lymphomyeloid-engrafted mouse of were injected with different dilutions of CB cells infected either with the HOXB4 or the SCID mice. Analysis of BM aspirates and total BM was performed 3 and 6 weeks after transplantation. The number of human transduced lymphoid (GFP+/CD19+ human B cells) and myeloid (GFP+/CD15+) cells was shown. Human lymphomyeloid engraftment was defined as at least 5 GFP+/CD19+ and GFP+/CD15+ cells/2 × 10⁶ events.† *Proportion of CD19+ B cells expressed as percentage of all cells in the culture. NLymphoid cells were predominantly of myeloid phenotype as assessed by FACS using a range of lineage markers (data not shown).

**Table 1. HOXB4 enhances production of B cells in vitro**

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<th>Total CD19+ B cells GFP (× 10⁶)</th>
<th>Total CD19+ B cells B4 (× 10⁶)</th>
<th>Total CD34+/CD19+ B-cell precursors GFP (× 10⁶)</th>
<th>Total CD34+/CD19+ B-cell precursors B4 (× 10⁶)</th>
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<tr>
<td>3 GFs</td>
<td>3 wk 55.5 ± 39 (9.5% ± 6.4%)*</td>
<td>94 ± 45</td>
<td>0.09 ± 0.09</td>
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<td>6 wk 1.6 ± 0.2 (3.7% ± 0.4%)*</td>
<td>31.2 ± 3.5†</td>
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<td>0.5 ± 0.5†</td>
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<tr>
<td>6 GFs</td>
<td>3 wk 8.6 ± 7.2 (0.2% ± 0.1%)*</td>
<td>11.3 ± 2.2%*</td>
<td>0.2 ± 0.06</td>
<td>4 ± 0.09†</td>
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<tr>
<td></td>
<td>6 wk 1.6 ± 0.7 (0.1% ± 0.03%)*</td>
<td>4.3 ± 0.1†</td>
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HOXB4-GFP and GFP-transduced CB cells were seeded on MS-5 murine fibroblasts and cultured with 3 (SCF, IL-2, and IL-15) or 6 (SCF, IL-2, IL-15, IL-7, FL, and TPO) growth factors permissive for B lymphoid in addition to variable degrees of myeloid cell growth (6 GFs > 3 GFs for myeloid growth). Hematopoietic differentiation into the lymphoid lineage was quantified by CD19 expression or CD34/CD19 coexpression (n = 2).

GF indicates growth factor.

*Proportion of CD19+ B cells expressed as percentage of all cells in the culture. Nonlymphoid cells were predominantly of myeloid phenotype as assessed by FACS using a range of lineage markers (data not shown).

†Statistically significant P < .02.

In contrast to its effect on the number of primitive repopulating hematopoietic cells, constitutive expression of HOXB4 (as documented by the continued expression of the linked GFP gene at readily detectable levels through the observation period [Figure 5A]) did not alter the normal differentiation program of human progenitor cells in vivo; proportions of engrafted lymphoid (CD19+/CD45+), myeloid (CD15+/CD45+), megakaryocytic (CD41+/CD45+), and erythroid (glycophorin+/CD45+) human cells were determined at weeks 3, 6, and 10 after transplantation by femoral bone marrow aspiration and again at 18 weeks when the mice were killed (n = 3). Constitutive expression of HOXB4 did not alter the proportions of the different lineages at any time point compared to the nontransduced compartment of the HOXB4 mice and the transduced and nontransduced compartment of the control mice. This was confirmed when absolute cell numbers were calculated after sacrificing the animals (data not shown). Thus, enhanced and extended expression of HOXB4 significantly augmented the number of primitive cells with repopulating activity, but did not alter differentiation in vivo.

**Discussion**

The characterization of molecular mechanisms to expand human stem cells has major medical implications with respect to therapeutic strategies based on stem cell transplantation. Earlier studies have shown that cytokine-induced proliferation of progenitor-enriched populations is characterized by induction of lineage commitment and terminal differentiation accompanied by a rapid loss of stem cell activity. An intriguing alternative to use of extrinsic growth factors is to harness the function of intrinsic regulators that may be upstream of cytokine receptor–mediated signal transduction pathways. We now show that constitutive expression of the clustered homeobox gene HOXB4 can rapidly

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**Figure 5. Lymphomyeloid repopulation in NOD-SCID mice transplanted with HOXB4-GFP– and GFP-transduced CB cells.** Three cohorts of NOD/SCID mice were injected with different dilutions of CB cells infected either with the HOXB4 or the GFP virus. (A) FACS analysis of a representative lymphomyeloid-engrafted mouse of the HOXB4 and the GFP cohort after injection of 2.5 × 10⁷ CD34+ cells 6 weeks after transplantation. The number of human transduced lymphoid (GFP+/CD19+) and myeloid (GFP+/CD15+) cells is shown. Human lymphomyeloid engraftment was defined as at least 5 GFP+/CD19+ and GFP+/CD15+ cells/2 × 10⁶ events.† (B) CRU frequency among HOXB4-GFP– and GFP-transduced CB cells in NOD/SCID mice. Analysis of BM aspirates and total BM was performed 3 and 6 weeks after transplantation and analyzed with Poisson statistics. *P = .02.
lead to increased numbers in vitro of cells detectable at the level of NOD/SCID repopulating cells, LTC-ICs, and committed clonogenic progenitors. Furthermore, enforced expression did not block terminal differentiation or changed lineage distribution in vivo. These data thus reveal HOXB4 as a novel growth stimulatory regulator of primitive human hematopoietic cells.

Intriguingly, constitutive expression of HOXB4 induced a rapid and significant increase in the number of cells with LTC-IC activity because cells were plated into the LTC-IC detection assay as soon as 48 hours as well as 7 days after transduction. This rapid onset of a HOXB4 effect on the number of human primitive hematopoietic cells was confirmed in the CRU assay in which HOXB4-expressing cells were injected into NOD/SCID mice within 24 hours after transduction and by this time CRU numbers were increased by some 4-fold over control transduced cells, as determined by limit dilution assay. Constitutive expression of HOXB4 might influence the growth kinetics of primitive progenitor cells, for example, by shortening the doubling time, or enhance self-renewal of HSCs. However, these data also suggest the interesting possibility of recruitment of cells, which have already left the primitive progenitor compartment, thus enhancing the number of cells detectable as LTC-ICs or CRUs. Both explanations are consistent with previous findings in the murine system, in which constitutive expression of HOXB4 shortened the doubling time of hematopoietic progenitor cells and induced an accelerated regeneration of CRUs in lethally irradiated recipient mice (eg, 25% of normal CRU level versus 0.2% in the control 2 weeks after transplantation). The striking effect on CRU numbers observed after only 24 hours after infection culture will also make it of interest to further assess in future experiments, the potential to achieve significant in vitro expansion of human CRUs with more extended culture periods as has recently been demonstrated in the murine model.

The impact of the 5′-located Antennapedia-like HOXB4 on early human hematopoietic development contrasts with effects so far described for other HOX genes on human hematopoietic progenitor cells. Thus, constitutive expression of the 5′-located Abdominal-B-like Hox gene HOXA10 resulted in competitive growth advantage of myeloid cells and blockage of differentiation with formation of blast colonies in vitro and ex vivo. The differential impact of HOXB4 and HOXA10 is furthermore highlighted in their opposite effects on B-cell differentiation and erythropoiesis with HOXA10 overexpression impairing B-cell development, whereas HOXB4 induced a marked enhancement of B lymphopoiesis. The mechanisms that lead to the differential gene effect are not known, but data point to a pivotal role of the TALE homeobox genes and Hox cofactors Meis1 and Pbx1 for the specification of Hox gene effects in the hematopoietic system. Importantly, HOXB4 cannot interact directly with Meis1 but only with Pbx1 in contrast to HOXA10, which can interact directly with both proteins. Furthermore, HOX5 overexpression, also in contrast to HOXB4, has been shown to impair erythroid differentiation and enhance formation of colonies with undifferentiated blasts. Furthermore, HOXB7 overexpression has been associated with the induction of persistent proliferation of a blast population in vitro, but without modifying the total number of hematopoietic progenitor cells.

The effect of HOXB4 is somewhat reminiscent of effects recently reported for BMP-4 or SHH, which were originally described for their essential role in early developmental cell fate decisions; both factors are able to maintain or increase human stem cells with repopulating capacity in vitro without altering lineage differentiation. Interestingly, both genes are linked to homeobox genes: SHH induces expression of Bmp-4 and of the 5′-located Hox genes Hoxd-11 as well as Hoxd-13, while Hoxd-12 regulates SHH expression in a positive feedback loop. BMP-4 regulates Hoxc-8 expression and acts together with the homeobox gene Mix.1 in inducing embryonic hematopoiesis.

Our data characterize HOXB4 as a potentially powerful positive mediator of the maintenance and expansion of human stem cells and provide a new avenue to manipulate and further elucidate the basis for human hematopoietic stem cell fate decisions. These in vitro and in vivo models will facilitate the dissection of the molecular mechanisms underlying the HOXB4-induced stem cell proliferation in the human cellular milieu. Furthermore, they will allow tests of whether stem cell amplification by HOXB4 can be further augmented by mutating distinct motifs of the gene such as the PBX YPWM interacting motif as reported previously in the murine system.

Acknowledgments

The expert assistance of Ms Patty Rosten for technical support and Ms Colleen MacKinnon in the preparation of the manuscript is gratefully acknowledged.

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