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Reduced Proliferative Capacity of Hematopoietic Stem Cells Deficient in \textit{Hoxb3} and \textit{Hoxb4}

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Several homeobox transcription factors, such as \textit{HOXB3} and \textit{HOXB4}, have been implicated in regulation of hematopoiesis. In support of this, studies show that overexpression of \textit{HOXB4} strongly enhances hematopoietic stem cell regeneration. Here we find that mice deficient in both \textit{Hoxb3} and \textit{Hoxb4} have defects in endogenous hematopoiesis with reduced cellularity in hematopoietic organs and diminished number of hematopoietic progenitors without perturbing lineage commitment. Analysis of embryonic day 14.5 fetal livers revealed a significant reduction in the hematopoietic stem cell pool, suggesting that the reduction inularity observed postnatally is due to insufficient expansion during fetal development. Primitive Lin-/Sca1+/c-kit+ hematopoietic progenitors lacking \textit{Hoxb3} and \textit{Hoxb4} displayed impaired proliferative capacity in vitro. Similarly, in vivo repopulating studies of \textit{Hoxb3}/\textit{Hoxb4}-deficient hematopoietic cells resulted in lower repopulating capability compared to normal litters. Since no defects in homing were observed, these results suggest a slower regeneration of mutant HSC. Furthermore, treatment with cytostatic drugs demonstrated slower cell cycle kinetics of hematopoietic stem cells deficient in \textit{Hoxb3} and \textit{Hoxb4}, resulting in increased tolerance to antimitotic drugs. Collectively, these data suggest a direct physiological role of \textit{Hoxb4} and \textit{Hoxb3} in regulating stem cell regeneration and that these genes are required for maximal proliferative response.

Class I Homeobox (\textit{Hox}) genes encode a family of 39 transcription factors sharing a highly conserved DNA-binding domain. In mammals they play a major role in specifying position and tissue fate in the embryo, as has been demonstrated by several lack-of-function \textit{Hox} mutant mice that exhibit various developmental abnormalities (see, for example, references 6, 29, 30, 38, 41, 49, and 58). \textit{Hox} genes are also expressed postnatally, and several of them are expressed in primitive hematopoietic cells and committed progenitors but downregulated upon differentiation to mature cells (44).

Murine models have been generated where enforced expression of \textit{Hox} genes is used to determine the effect of overexpression on self-renewal, differentiation, and other cell fate decisions during hematopoiesis (for reviews, see references 10 and 55). Such models include overexpression of \textit{HOXA10}, as well as \textit{HOXA9}, which both affected myelo- and lymphopoiesis and ultimately lead to myeloid leukemia (5, 9, 23, 52, 53). Expression of \textit{HOXB3} and \textit{HOXB4} is found in the primitive CD34+ population that is highly enriched for human hematopoietic stem cells (HSCs) but is rapidly downregulated as the cells differentiate into committed progenitors (44). Despite very similar expression pattern of \textit{HOXB3} and \textit{HOXB4}, suggesting a common role or collaboration between these factors, the consequences from overexpressing these genes are very different. Although enforced expression of \textit{HOXB3} blocks both T- and B-cell development and causes a myeloproliferative disorder (46), overexpression of \textit{HOXB4} greatly enhances the regenerative capacity of HSCs in serial transplantation models and results in selective expansion of \textit{HOXB4}-transduced cells without causing altered lineage decisions or malignant transformations (1, 45, 54). It is noteworthy that this expansion continues until the stem cell pool is normal in size (without overridding it), differing significantly from transplantation of untreated bone marrow (BM) cells, which can only regenerate up to 10% of the number of HSCs found in normal mice. Furthermore, a recent report demonstrates that an ~40-fold net expansion of murine repopulating HSCs can be achieved by enforced expression of \textit{HOXB4} ex vivo for 10 to 14 days (2). This finding is in sharp contrast to ordinary cytokine induced cultures which can support maintenance of HSC numbers or at most expand by a factor of 2 to 4 (4, 12, 28). Less is known about the feasibility of using \textit{HOXB4} for human HSC expansion. Recent findings indicate that \textit{HOXB4} overexpression in human hematopoietic progenitors affects fate decisions in a concentration-dependent manner to determine whether self-renewal, differentiation, or a differentiation block ensues (7a, 8, 46a). These findings emphasize the importance of understanding the physiological effects of \textit{HOXB4} in HSCs in vivo. Interestingly, overexpression studies of \textit{HOXC4} also result in expansion of primitive human hematopoietic progenitors, suggesting a common role for paralog 4 genes on these progenitors (13).

Since \textit{HOXB3} and \textit{HOXB4} are expressed in the stem cell compartment and gain-of-function studies result in enhanced HSC regeneration, we wanted to further analyze the physiological role of these genes in controlling stem cell fate in a lack-of-function mouse model.
have generated important insight into the role of various transcription factors in hematopoiesis. These include targeting of genes such as GATA-2, SCL/tal-1, Rb112l/Mo2, AML1, PU.1/Sp1, Ikaros, Hoxb6, and Hoxa9 (reviewed in references 34 and 51). Hoxa9-deficient mice exhibit prominent defects in erythroid, myeloid, and lymphoid development, including early T-cell development, as well as apparent defects in HSC function, although these have not been fully described (20, 27, 27a). Hoxb6 deficiency mainly affects the erythroid development, increasing the numbers of erythroid progenitors (22). Here we describe a mouse model that is deficient in the contiguous Hoxb3 and Hoxb4 genes. All exons and intermediate sequences of these genes were excised by utilizing the Cre/LoxP technique (18, 43). Homozygous mouse deficient in Hoxb3 and Hoxb4 (Hoxb3/b4−/−) were born at normal Mendelian ratios, showed no major abnormalities in skeletal structure, and remained healthy. However, the hematopoietic organs of Hoxb3/b4−/− mice exhibited a significant reduction in cellularity and reduced numbers of primitive hematopoietic progenitors, in particular of the HSC pool in fetal livers (FLs) from 14.5-day-old embryos. The proliferative capacity of primitive hematopoietic progenitors from mutant mice was diminished in vitro and the regenerative capacity of Hoxb3/b4−/−HSCs was reduced after primary and secondary transplantation. The defects in repopulating abilities were not caused by aberrations in homing but were more likely due to diminished proliferation of HSCs. This is supported by studies after hematopoietic stress, which demonstrated that repopulating Hox3/b4−/−HSCs exhibited slower cell cycle kinetics and a larger proportion of resting stem cells. In summary, these findings show that Hoxb3/b4-deficient HSCs harbor a functional defect, impairing the proliferation capacity when rapid regeneration is required.

**MATERIALS AND METHODS**

**Cloning of Hoxb3 and Hoxb4 and generation of targeting constructs.** A 390-bp (SalI/Fsp1) cDNA fragment from exon 1 of Hoxb4 was used to screen a 129/SvJ mouse genomic BAC (bacteria artificial chromosome) library (Stratagene). A clone containing more than 100 kb of the Hoxb locus was identified and isolated. For generating the Hoxb4 targeting construct, two overlapping subclones were used, a 9.4-kb EcoRI clone (pBS-ERI; Hoxb4 and 3′ sequence) and a 6-kb EagI clone (pBS-EagI; Hoxb4 and 5′ sequence). For generating the Hoxb3 targeting construct, a 7.5-kb NheI fragment (pSL-NheI; Hoxb3 exons III and IV), as well as an overlapping 3′ Apal fragment (pBS-Apal; 3′ sequence) were used. Briefly, for the generation of the Hoxb4 targeting vector, the 6-kb EagI fragment was ligated into a modified pBluescript (pBS-ΔhindIII) and then opened with HindIII and blunt-ended. Into this site the loxP flanked (flxed) neomycin expression cassette, a 1.3-kb XbaI/SalI fragment isolated from pl2neo, was ligated. To the 3′ end of this subclone a 1-kb EagI/HindIII fragment (from pBS-ERI) was ligated, and this construct was then digested with PshAl/CloI, resulting in a 7.2-kb targeting fragment (with a 1.6-kb homologous arm upstream and 4.4-kb arm downstream of the neomycin cassette). The targeting fragment was ligated into a Smal/Clal-cut pBS, resulting in pBS-B4ko. The herpes simplex virus thymidine kinase (tk) gene driven by the PGK promoter was isolated from pPNT by EcoRI and HindIII digestion. The fragment was blunt ligated into the ClaI site in the 3′ polylinker of pBS-BKO, resulting in the final targeting construct, pBS-BKOtk.

**Gene targeting in ES cells.** The targeting constructs were linearized with NotI and purified. A total of 25 to 30 μg of DNA was electroporated (Bio-Rad; 0.2 kV, 250 μF, 1000 V) into ca. 107 RI embryonic stem (ES) cells according to standard procedure (56). The cells were then cultured in selective medium (300 to 500 μg of G418/ml and 4 μM ganciclovir [when appropriate]) for 7 to 9 days. To verify homologous recombination (HR) the surviving colonies were screened with PCR by using the external primers Bsl-est (CCAGATGGGTCACCT CCACAAG) and Bsu-est (GAGAACATCCGCACTCCTGAT) and the internal primers NeomU (TTGGTGGTACACGCGTGTACG) and the B4KO (TC TTGTAGACGTTCCTCAGGGAAT). Positive clones were further analyzed by Southern blot with external probes (data not shown). Southern blotting was also used to verify single integration of the targeting vectors. Genomic DNA was digested with either ApalI or KpnI to screen Hoxb3 and Hoxb4 targeting, respectively, and then probed with a neomycin-specific probe (0.9-kb EcoRI fragment), resulting in a 8.5-kb band from the ApalI digest and a 5.5-kb band from the KpnI digest (Fig. 1). Targeted clones were expanded and ca. 2 × 105 cells were electroporated with 15 μg of the plasmid pC-Cre for excision of the neomycin gene. Resulting neomycin sensitive (Neo+) clones were screened by PCR with the primers P2 (GAAGGTCAATAACTCCCTTCGCT) and P6 (GCTGGCATTCCTCAGTGTCTCCTCA) or the primers P1 (GTTGACATAAC ACTCCCTGCATC) and P5 (TCTCTGGCAGGTTGCTGTTGCTG) to detect the loxP sites at the Hoxb3 and Hoxb4 alleles, respectively (Fig. 1). For analysis of Cre-mediated total deletion of both the Hoxb3 and Hoxb4 genes, primers P1 and P2 were used (Fig. 1). Independently targeted clones of both the Hoxb3/b4-flanked and the Hoxb3/b4-deleted versions were injected into 3.5-day-old C57BL/6 blastocysts and transferred into pseudopregnant (C57BL/6 × CBA)F1 fostermothers by standard techniques (56). Chimeric males were mated to C57BL/6f females, resulting in offspring with a 129Sv/C57BL/6 genetic background. For screening of germ line offspring, DNA was isolated from tail biopsies and analyzed by PCR. Primers P3 (GGAAAGCAAGAAGAGGGAAGAAGAAGGA) and P4 (CATA GTGTTGACAGACGGAGAAG) were used to distinguish between homozygous and heterozygous offspring of Hoxb3/b4-deleted mice. Total RNA was isolated from the peripheral blood of (PB) and BM (LN, thymus, spleen) of Hoxb3/b4 mice and used for reverse transcription-PCR (RT-PCR) analysis to verify the presence or absence of the RNA transcripts.

**Cell harvest.** Hoxb3/b4 knockout and wild-type littermate mice were sacrificed at ages different (mainly at 10 to 16 weeks), and cells were harvested from the PB, BM, spleen, thymus, and lymph nodes. Various other organs were also removed for pathological analysis. An aliquot of PB was used (Sysmex X1000; TOA Medical Elektronics Co., Ltd.) to measure some of the hematological parameters described in Table 1. Femurs and tibias were crushed in a mortar in the presence of phosphate-buffered saline (Gibco-BRL) containing 2% fetal calf serum (Gibco-BRL) and then filtered through a 70-μm (pore-size) cell strainer. Cells were isolated from the spleen, thymus, and lymph nodes by meshing the organs through a 70-μm (pore-size) cell strainer. FL cells were harvested from embryos at 14.5 days postcoitus for further analysis.

**Cloning assays.** For myeloid clonal progenitor assays, BM cells were cultured in 35-mm petri dishes. For the cell CFU (CFU-C) assay, rich methylcellulose (M3534 containing stem cell factor [SCF]; 50 ng/ml), interleukin-3 (IL-3; 10 ng/ml), and IL-6 (10 ng/ml), Stem Cell Technologies), with the addition of 5 U of human erythropoietin (hEpo; Janssen-Cilag AB) in the culture medium unless indicated otherwise. After washing and resuspending steps, sheep anti-rat immunoglobulin G (Fc)-conjugated immunomagnetic beads (Dynal) were used to deplete lymphoid cells (lin−). For single cell cultures, Lin− c-kit+ Sca− (LSK) cells were used. Briefly, BM cells were treated with ammonium chloride (NH4Cl; Stem Cell Technologies) and then incubated in a lineage antibody cocktail (CD4, CD8, CD5, Gr1, Mac1, B220, and TER119); all antibodies were from BD Pharmingen unless indicated otherwise. After washing and resuspending steps, sheep anti-rat immunoglobulin G (Fc)-conjugated immunomagnetic beads (Dynal) were added, and lineage-positive cells were removed with a magnetic particle concentrator (MPC-6; Dynal). Lin− c-kit+ cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-Sca1 (anti-E13-161.7) and allophycocyanin (APC)-conjugated anti-c-kit (anti-2B8). The cells were washed and stained with Annexin V-FITC and propidium iodide (PI). For single cell analysis, FACSVantage Cell Sorter (Becton Dickinson [BD]) and seeded into Terasaki plates (Nunc) at a concentration of one cell per well in 20 μl of serum free...
FIG. 1. Schematic overview of the Hoxb3/b4 locus and the targeting strategy. The gene targeting strategy successfully removes the Hoxb3 and Hoxb4 genes and generates mice without expression of Hoxb3 and Hoxb4 mRNA. (A) The Hoxb4 (exons shown as black boxes) and Hoxb3 (exons
medium (X-vivo 15; BioWhittaker) supplemented with 1% bovine serum albumin (Stem Cell Technologies), 100 IU of penicillin and 100 μg of streptomycin (Gibco-BRL/ml), 2 mm L-glutamine (Gibco-BRL), and 10^{-4} M 2-mercaptoethanol (Sigma). The following cytokines were used in various combinations (see Results): GM-CSF (Amgen), TPO (Karin), FL-3 ligand (FL: ImmuneX), granulocyte colony stimulating factor (G-CSF; Amgen), and granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-3 (Novartis).

**Cell cycle analysis.** Lin^− cells were isolated as described above. The cells were then stained with c-kit-APC and Scallo-phycocerythrin (PE) and thereafter preserved in 0.4% formaldehyde (LabKemi) and 0.2% Triton-X (Sigma) overnight. The following day, the cells were labeled with Ki67-FITC and 7AAD (Sigma) and analyzed on a FACS Calibur (BD). When cyclophosphamide (Sendoxan; Astra Medica A.G.) treatment was included, the mice were injected intraperitoneally (200 mg/kg) at 96 h prior to BM harvest.

**Transplantation experiments.** For standard competitive transplantation experiments, 2 × 10^7 fresh BM cells from Hoxb3/b4−/− or control littermates (Ly5.2) were mixed together with 8 × 10^7 BM cells from B6.SJL (Ly5.1) cells. The mixture was injected into the tail vein of lethally irradiated B6.SJL recipient mice. To measure reconstitution of Ly5.2-derived cells, PB samples were taken at weeks 6 and 12 and when the mice were sacrificed at weeks 17 to 20. In some cases, 10^7 fresh BM cells from the primary recipients were further transplanted into secondary B6.SJL recipients when 5-fluorouracil (5-FU; Nycomed AB) treatment was included in the transplantation study, Hoxb3/b4−/− and Hoxb3/b4−/+ littermates were injected with 5-FU (150 mg/kg) at days 1 and 5. On day 6, BM cells were harvested, and a 1/10 femur equivalent was mixed together with fresh 2 × 10^7 B6.SJL BM cells. This mixture was then injected intravenously (i.v.) into lethally irradiated B6.SJL recipients. For FL transplantation experiments, 2 × 10^8 Hoxb3−/− or Hoxb3+/− cells (Ly5.2) derived from 14.5-day-old embryos were used in competition with 3 × 10^6 B6.SJL cells (Ly5.1) and transplanted into lethally irradiated (B6.SJL × C57BL/6J)F1 recipients (express both Ly5.1/Ly5.2).

**FACS analysis.** Hemopoietic cell suspensions (PB, BM, spleen, thymus, and lymph nodes) were treated with ammonium chloride (Stem Cell Technologies) prior to fluorescence-activated cell sorting (FACS) analysis. For lineage analysis, the cells were stained with FITC-conjugated anti-Mac1, anti-Gr1, anti-B220, and anti-CD4 antibodies, as well as PE-conjugated anti-CD3, anti-CD8, and anti-TER119 antibodies. For a more detailed analysis of B- and T-cell development, APC-conjugated anti-B220, FITC-anti-CD43, PE-(biotin)-anti-IgM, FITC-αβTCR, and PE-βγTCR were also used. For analysis of reconstitution in transplanted mice, PE-conjugated anti-CD45.1 (Ly5.1) and APC-(biotin)-anti-CD45.2 (Ly5.2) antibodies were used. For estimation of LSK CD34lo/−/−/− (LSK CD34) cells, the cells were incubated in the lineage cocktail described above and then labeled with Tri-Color-conjugated goat F(ab')2 anti-rat immunoglobulin G (H + L; Caltag Laboratories) and Sca1-FITC, c-kit-PE, and CD34-(biotin)-APC. For evaluation of LSA (Lin− Sca1+ A44+ ) cells from FL, the antibody against c-kit was replaced by an antibody to A44.1. Analysis was done on FACS Calibur (BD). The results were analyzed with CellQuest software (BD).

**Homing assays.** Fifteen million whole BM cells from four Ly5.2 Hoxb3/b4−/− donors or four littermate control donors were injected into lethally irradiated Ly5.1 recipients. Recipients were sacrificed 24 h later for analysis of competition available (7AAD−) donor-derived cells in the BM and spleen by using a Tri-Color labeled lineage cocktail (CD4, CD8, CD5, Gr1, Mac1, B220, and TER119), PE-conjugated anti-Ly5.1, FITC-conjugated anti-Ly5.2, and APC-conjugated anti-c-kit. For analysis of CFU-S at day 12, 1/10 and 1/20 recipient spleen cells were gated anti-Ly5.1, FITC-conjugated anti-Ly5.2, and APC-conjugated anti-c-kit. For a more detailed analysis of B- and T-cell development, CD45.2 (Ly5.2) antibodies were used. For estimation of LSK CD34lo/−/−/− (LSK CD34) cells, the cells were incubated in the lineage cocktail described above and then labeled with Tri-Color-conjugated goat F(ab')2 anti-rat immunoglobulin G (H + L; Caltag Laboratories) and Sca1-FITC, c-kit-PE, and CD34-(biotin)-APC. For evaluation of LSA (Lin− Sca1+ A44+ ) cells from FL, the antibody against c-kit was replaced by an antibody to A44.1. Analysis was done on FACS Calibur (BD). The results were analyzed with CellQuest software (BD).

**Generation of Hoxb3/b4-deficient mice.** In order to study hematopoiesis in Hoxb3/b4-deficient animals, mice were generated that completely lack expression of Hoxb3 and Hoxb4. The rationale was to generate conditional knockout mice for the contiguous Hoxb3 and Hoxb4 genes to avoid potential developmental abnormalities that could ensue and interfere with examination of postnatal hematopoiesis. LoxP sites were introduced upstream of the Hoxb4 gene and downstream of the Hoxb3 gene by using two different targeting constructs (Fig. 1). After we verified correct targeting in 129Ev ES cells by PCR and Southern blot analysis, the ES cells were used to produce chimeric mice. Mice carrying the floxed (i.e., flanked by loxP sites) Hoxb3/b4 genes were generated in parallel to null mutant mouse lines lacking both genes along with floxing and intermediate regulatory elements (Fig. 1D). Both mouse lines (i.e., the “floxed” and the “deleted”) reproduce normally and are born at normal Mendelian ratios (Table 1). The homozygous Hoxb3/b4−/− mice (Hoxb3/b4x/x) appear completely normal, and the presence of the loxP sites does not disturb the expression of the targeted genes (Fig. 1G). However, no Hoxb3 or Hoxb4 mRNA could be detected in the Hoxb3/b4−/− mice. These mice are slightly smaller (8% lower body weight, P < 0.05) than their healthy littermates at the time of weaning (3 weeks), but at 12 weeks of age this difference is usually negligible.

**RESULTS**

<table>
<thead>
<tr>
<th>Mouse genotype</th>
<th>No. of pups (% frequency)</th>
<th>Avg wt (g) ± SD b:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoxb3/b4−/−</td>
<td>45 (23.3)</td>
<td>16.5 ± 0.5 *</td>
</tr>
<tr>
<td>Hoxb3/b4+/−</td>
<td>99 (51.3)</td>
<td>16.3 ± 0.5</td>
</tr>
<tr>
<td>Hoxb3/b4−/−</td>
<td>49 (25.4)</td>
<td>15.2 ± 0.5 *</td>
</tr>
</tbody>
</table>

| b: Ten pups per genotype, P < 0.05; †: P > 0.2. |

**TABLE 1. Hoxb3/b4-deficient mice are born in normal Mendelian ratios**

![Image of a table showing the results of the study](image-url)
Biochemical analysis of the thorax structure of HOXB4-deficient mice (see Discussion). HOXB3/b4−/− mice exhibit reduced cellularity in hematopoietic organs. Pathological examination of hematopoietic tissues of the HOXB3/b4−/− mice (BM, spleen, thymus, and lymph nodes) did not reveal any abnormalities. However, the mice displayed significantly reduced cellularity in hematopoietic organs (Table 2). The spleen weight and cellularity was reduced by 30% (P < 0.001) compared to normal littermates, and BM cellularity was reduced by ca. 25% (P < 0.003). A reduction in the red blood cell count and hemoglobin values was also observed, but the white blood cell count was normal. For comparative studies involving the knockout mice, littermate controls were used in all of our experiments. The observed reduction in cellularity led us to ask whether the reduction was restricted to a specific lineage or compartment within the hematopoietic hierarchy. FACS was performed on cells derived from PB, BM, spleen, and thymus with antibodies to Gr1, Mac1, CD4, CD8, B220, CD3, and TER119. The results did not show any significant difference in the lineage distribution of the hematopoietic cells derived from HOXB3/b4−/− mice compared to HOXB3/b4+/+ littermates (Fig. 2). In light of previously published data on the blocking effect of overexpression of HOXB3 on B- and T-cell maturation (46), these populations were analyzed in more detail by FACS analysis. Antibodies to B220 were used in combination with CD43 and IgM for B-cell development, and CD4/CD8 were used in combination with αβTCR or γδTCR for T-cell analysis; however, no difference was observed between HOXB3/b4−/− cells and control cells (data not shown). Thus, the reduction observed in hematopoietic cell numbers suggested that the phenotype might arise from the primitive progenitor and stem cell compartment. The hematopoietic progenitor cell pool is reduced in HOXB3/b4−/− deficient mice. In order to analyze colony-forming abilities of HOXB3/b4−/−-derived progenitor cells, fresh BM cells were plated out in methylcellulose optimized for either CFU-C or BFU-E assay. BFU-Es and CFU-Cs were enumerated on day 8 and on days 10 and 11, respectively. HOXB3/b4−/− BM cells showed a nonsignificant reduction in the frequency of hematopoietic progenitor colonies compared to HOXB3/b4+/+ littermates, both for CFU-C (colonies/10,000 BM cells = 18 ± 4 versus 21 ± 3 [P = 0.2]) and for BFU-E (colonies/100,000 BM cells = 11 ± 3 versus 13 ± 3 [P = 0.3]). However, due to reduced BM cellularity of the HOXB3/b4−/−, the absolute numbers of CFU-C were significantly reduced (Fig. 3A, P < 0.01) and the numbers of BFU-E were also reduced, although not significantly (Fig. 3A, P < 0.07). The frequency of day 12 CFU-S colonies in HOXB3/b4−/− mice was also similar to that for control littermates (colonies/50,000 BM cells; 5.2 ± 0.9 for knockout mice versus 5.5 ± 1.4 for wild-type mice [P = 0.8; n = seven mice/group]), whereas the absolute number of CFU-S at day 12 was slightly reduced (Fig. 3A, P = 0.11).

Normal cell cycle distribution but reduced absolute numbers of primitive hematopoietic progenitors in HOXB3/b4−/− deficient mice. Because enforced expression of HOXB4 has been shown to increase expansion and regeneration of HSCs, we analyzed whether there was a reduction in the proportion of cycling HSCs in the HOXB3/b4−/− mice. The proportion of LSK

### Table 2. Cellularity in hematopoietic organs of HOXB3/b4-deficient mice

<table>
<thead>
<tr>
<th>Mouse genotype</th>
<th>Spleen wt (mg)</th>
<th>Spleen cellularity (10⁶)</th>
<th>RBC count (10¹²/liter)</th>
<th>Hemoglobin level (g/liter)</th>
<th>WBC count (10⁹/liter)</th>
<th>BM cellularity (two femurs × 10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOXB3/b4+/+</td>
<td>103 ± 14</td>
<td>187 ± 30</td>
<td>8.1 ± 0.8</td>
<td>151 ± 10</td>
<td>4.1 ± 1</td>
<td>45 ± 6</td>
</tr>
<tr>
<td>HOXB3/b4−/−</td>
<td>70 ± 7</td>
<td>127 ± 24</td>
<td>7.4 ± 0.7</td>
<td>139 ± 10</td>
<td>3.8 ± 1</td>
<td>34 ± 5</td>
</tr>
</tbody>
</table>

*a* Ten mice per genotype, each at 12 to 16 weeks of age.

*b* The P values for the hematopoietic parameters were as follows: spleen weight, <0.001; spleen cellularity, 0.001; red blood cell (RBC) count, 0.05; hemoglobin level, 0.03; white blood cell (WBC) count, 0.5; and BM cellularity, 0.003.

![Fig. 2. HOXB3/b4-deficient mice exhibit normal distribution of hematopoietic lineages in all hematopoietic organs. Representative data from FACS analysis of hematopoietic cells within BM, PB, spleen, and thymus with the specific antibodies stated in the figure. The frequency (%) of each population is given outside the respective quadrant.](image-url)
Reduced proliferative capacity of primitive hematopoietic progenitors in vitro. Due to the reduction in hematopoietic cellularity and a mild reduction in the hematopoietic progenitor cell pool caused by the loss of Hoxb3/b4, we sought to determine whether proliferation recruitment of primitive hematopoietic progenitors was affected in Hoxb3/b4−/− mice. LSK cells were sorted out from the BM and plated in serum-free medium in a single cell assay to evaluate survival and proliferation. The cells were plated out in medium containing either TPO or SCF alone and, on day 6, a multicytokine mixture (SCF, FL, TPO, G-CSF, IL-3, and GM-CSF) was added to test the viability, which was found to be normal for the mutant cells. Recruitment into proliferation of single LSK cells was tested by supplementing the medium at day 1 with SCF alone, SCF-TPO, SCF-TPO-FL, or the multicytokine mixture described above. Low stimulatory growth conditions (few cytokines) did not give the mutant cells a proliferative disadvantage compared to control cells, but a significant difference in proliferation potential was detected between the Hoxb3/b4−/− and Hoxb3/b4+/− littersmates, when the multicytokine combination was used (Fig. 4 and data not shown). A significantly reduced number of LSK Hoxb3/b4−/− cell clones with high proliferative

capacity was evaluated (Fig. 4 and data not shown). A significantly reduced number of LSK Hoxb3/b4−/− cell clones with high proliferative
capacity (progeny cells cover ≥50% of the well) was observed compared to Hoxb3/b4+/+ littermates (P < 0.001). Similarly, there was a reduction in the total number of responding clones from the mutant cells, although this was not statistically significant (Fig. 4, P < 0.07). These results indicate that primitive LSK Hoxb3/b4−/− derived cells proliferate less effectively than their normal counterparts in vitro and that the effects due to lack of Hoxb3 and Hoxb4 are most prominent in settings in which there is extensive pressure for proliferation on this primitive cell population.

**Normal homing of Hoxb3/b4-deficient BM cells.** Before evaluating the regeneration capacity of Hoxb3/b4-deficient repopulating HSCs, we sought to determine whether Hoxb3/b4−/− BM cells were defective in homing to hematopoietic sites. Fifteen million Hoxb3/b4−/− and control BM cells were transplanted into irradiated recipients, and homing into BM and spleen was evaluated by FACS. The homing capacity of total, Lin−, and Lin− c-kit+ Hoxb3/b4-deficient donor cells was not affected, and this included primitive, multipotent myeloid (CFU-S at day 12) progenitors (Fig. 5A). There was also no difference in the ability of normal donor cells to home into the BM stroma and spleens of Hoxb3/b4−/− mice compared to control mice, suggesting that the reduced cellularity of hematopoietic organs in Hoxb3/b4−/− mice was not simply due to an impaired ability to retain hematopoietic cells (Fig. 5B).

**Repopulating HSCs from Hoxb3/b4-deficient mice have reduced regenerative capacity.** In order to determine whether the lack of Hoxb3/b4 affected the function of repopulating HSCs after transplantation to lethally irradiated mice, a competitive transplantation experiment was performed. Fresh FL cells from Hoxb3/b4−/− mice or normal littermates (expressing the Ly5.2 marker) were transplanted together with B6.SJL competitor cells (expressing Ly5.1) into lethally irradiated B6.SJL recipient mice. PB samples were taken 3, 6, and 12 weeks posttransplant, and the level of reconstitution by the Ly5.2 cells was analyzed. Repopulation by Hoxb3/b4−/− cells lagged behind that of controls at 3 and 6 weeks (Fig. 6A), although by 12 weeks equivalent levels were reached. This finding prompted us to test the regenerative capacity of adult BM HSCs by the same approach. The Hoxb3/b4−/− derived BM cells showed significantly lower regenerative capacity than their Hoxb3/b4+/− counterparts 6 weeks posttransplantation (P = 0.01), but in long-term reconstituted mice at 17 weeks this difference was less prominent and not significant (P = 0.12; Fig. 6B). The animals were sacrificed at 17 weeks posttransplantation, and the lineage distribution of Ly5.2 cells in BM and PB was analyzed. All transplanted mice showed reconstitution of all hematopoietic lineages, and the lineage distribution was normal in mice transplanted with Hoxb3/b4−/− BM cells (data not shown). Since the Hoxb3/b4−/− cells exhibited...
lower reconstitution ability in the primary recipients, we sought to determine whether these findings would be more prominent upon further proliferative stress after transplantation to secondary recipients. Reconstitution of Hoxb3/b4-/- cells in the secondary recipients was significantly lower compared to Hoxb3/b4+/- cells, at 6 and 17 weeks posttransplant (Fig. 6C). These observations strongly suggest that deficiency of Hoxb3/b4 negatively affects the regenerative capacity of adult BM repopulating HSCs.

Hoxb3/b4-/- HSCs exhibit increased tolerance to 5-FU. To further investigate the effects of Hoxb3/b4 deficiency on the repopulating HSC compartment in adult mice, we sought to determine whether mutant HSCs exhibited abnormal proliferation kinetics. Therefore, the mice were treated with 5-FU twice (on day 1 and on day 5) to test the hypothesis that slower proliferation kinetics result from Hoxb3/b4 deficiency. The first treatment of 5-FU eliminated actively cycling cells, thereby forcing the primitive resting population into cycle. A second treatment of 5-FU then killed the primitive cells that had been recruited into proliferation as a response to the previous hit. Hoxb3/b4-/- and Hoxb3/b4+/- littermates were injected i.v. with 5-FU (150 mg/kg) on day 1 and on day 5 (96 h apart), BM was harvested the following day and transplanted together with fresh B6.SJL cells into lethally irradiated B6.SJL recipients. FACS analysis showed no difference in lineage distribution between the knockout donor cells and their normal counterparts (data not shown). However, there was a clear and significant difference in overall reconstitution of the recipient mice when the Hoxb3/b4-/- cells were compared to the Hoxb3/b4+/- cells. After two hits with 5-FU, the Hoxb3/b4-/- cells now had a clear advantage over the Hoxb3/b4+/- derived cells, showing two- to threefold-higher levels of reconstitution at 6 weeks posttransplantation ($P < 0.001$) and, on average, 40% higher reconstitution after 20 weeks ($P = 0.001$; Fig. 7A). These data indicate that the primitive compartment is less activated after the first 5-FU treatment in Hoxb3/b4-/- mice than in the normal littermates, resulting in a higher proportion of noncycling, protected stem and progenitor cells that are not affected by the second 5-FU hit.

Activation of Hoxb3/b4-deficient HSCs from $G_0$ into cell division is delayed after hematopoietic stress. In order to support the hypothesis that primitive Hoxb3/b4+/- cells are less activated than control cells after treatment with cytotoxic drugs such as 5-FU, we performed cell cycle analysis after cyclophosphamide treatment. Cyclophosphamide acts similarly to 5-FU; however, it does not alter the expression of cellular markers such as c-kit and Mac1 posttreatment, as has been observed after 5-FU treatment (39, 50). Therefore, the primitive hematopoietic LSK cells have an unaltered immunophenotype after cyclophosphamide treatment. Hoxb3/b4-deficient mice and normal littermates were treated with cyclophosphamide (200 mg/kg, given intraperitoneally); 96 h later, the BM cells were harvested and the LSK population was stained with K167 and 7AAD for an analysis of the cell cycle distribution. A significantly higher proportion of Hoxb3/b4+/- LSK cells were found in the noncycling $G_0$ phase ($P = 0.04$), and a significantly lower proportion was in active (S/G2M) cycle compared to control cells ($P = 0.04$; Fig. 7B). The frequencies of cells in $G_1$ were the same in both groups. Collectively, these findings demonstrate that there is delayed activation and recruitment into proliferation of Hoxb3/b4-deficient HSCs after hematopoietic stress that is induced by cytotoxic drugs.
FIG. 7. Hoxb3/Hoxb4-deficient HSCs exhibit slower proliferation kinetics and delayed activation into active cell cycle after hematopoietic stress induced by cytotoxic agents. (A) Effects of two hits of 5-FU (150 mg/kg, given i.v.) on the reconstitution of Hoxb3/b4−/− (shaded bars) and Hoxb3/b4+/− (littermates, solid bars) derived cells. Donor mice (Ly5.2) were given 5-FU on day 1 and on day 5. On day 6, the BM was harvested and 1/10 femur equivalent was transplanted, along with fresh competitor B6.SJL cells (Ly5.1), into lethally irradiated recipients. The level of reconstitution was measured in PB cells at weeks 6 and 20 posttransplantation. Data from two independent experiments were pooled. Each bar represents 20 recipient mice, originating from and 20 posttransplantation. Data from two independent experiments.

DISCUSSION

Accumulating data show that Hox transcription factors play an important role in hematopoiesis. Here we report that deficiency of Hoxb4 and Hoxb3 decreases proliferation capacity of hematopoietic cells with repopulating ability and causes significantly reduced cellularity in hematopoietic organs. The reduction in cellularity is most pronounced in the spleen (30%) and in the BM (25%), with a more marginal reduction observed in the PB. The reduction is not caused by alterations in lineage commitment of hematopoietic cells, but rather the defect seems to be at a more primitive level, within the multipotent progenitor and stem cell compartment. The colony-forming ability of clonogenic progenitors seems not to be significantly affected by the deficiency of Hoxb3 and Hoxb4; however, their absolute numbers were reduced. Interestingly, the cell cycle distribution of endogenous LSK CD34 Hoxb3/b4−/− cells was normal; however, absolute numbers of this primitive population were mildly reduced. These findings suggest that the numbers of primitive hematopoietic progenitors may be reduced during embryogenesis. During fetal hematopoiesis a robust expansion of stem cells and progenitors occurs in the FL at days 11 to 15 (32). We therefore analyzed the FL of 14.5-day-old embryos and observed a significant reduction in the absolute number of the repopulating Lin− Sca1+ AA4.1+ cells (21) in Hoxb3/b4−/− embryos compared to controls and, moreover, a competitive transplantation assay demonstrated a slower regeneration capacity of FL HSCs. Furthermore, competitive repopulation assays with BM-derived cells demonstrated an impaired regeneration rate of Hoxb3/4−/− HSCs in primary recipients, and this effect is amplified somewhat in secondary recipients, possibly in part due to a mild reduction of HSCs in the primary recipient. Collectively, these findings support the notion that deficiency of Hoxb3 and Hoxb4 negatively affects cycling of multipotent progenitors and stem cells undergoing rapid proliferation. However, other important parameters such as survival, differentiation, and homing of these primitive cells seem not to be affected. We have also generated mice with a deficiency in Hoxb4 alone (complete deletion), where the impact of the deficiency is qualitatively similar but is less pronounced quantitatively (5a). Therefore, the additional deletion of Hoxb3 seems only to enhance the effects seen without causing a new phenotype, a dose-dependent phenomenon that has been observed in other compound Hox knockout models (see discussion below).

It is of interest to compare the findings presented here with those of previous reports that have used gain-of-function models to define the role of Hoxb4 and Hoxb3 in hematopoiesis. The loss of Hoxb3 expression did not affect B- or T-cell maturation and had no aberrant effects on the myeloid lineages, as reported by Sauvageau et al. in a retrovirally engineered overexpression study (46). This would indicate that Hoxb3 expression is redundant but that downregulation of this gene is important for normal differentiation. The loss of Hoxb3 and Hoxb4 does not seem to affect the hematopoietic lineage commitment pathways but rather reduces the proliferation capacity of stem cells, although the effects of deficiency are clearly not as dramatic as enforced HOXB4 expression resulting in 50-fold expansion of long-term repopulating HSCs (1, 45, 54). In a recent, elegant overexpression study, a new additional function of HOXB4 is suggested in promoting primitive HSCs, derived from yolk sac as well as from ES cells, to become definitive (26, 40). However, this promotion cannot entirely depend on Hoxb4 since the lack of this gene does not block the onset of definitive hematopoiesis. These effects could also be dose dependent, where exceeding a certain threshold-level changes the functional effect of the transcription factor, as has been seen for GATA-1, where the lineage outcome is correlated with the level of GATA-1 expression (25).

Given the functional complexity of the Hox gene clusters with regard to redundancy, as well as shared internal regulation, the deletion of a single gene might not give an accurate
picture of its role since a neighboring or a paralog gene(s) might rescue the phenotype. Indeed, compound knockouts of paralog genes can display dose-dependent degrees of synergism in the homeotic transformations observed, based on the mutant combinations analyzed (19, 59). Surprisingly, in our double-knockout model, homozygous Hoxb3/b4−/− mice were born at a normal Mendelian ratios without signs of any life-threatening phenotype as reported for Hoxb4 mutants, which died at or around the time of birth due to a split sternum (38).

The penetrance of this lethal phenotype was reported to be stronger (100%) in a pure 129SvEv genetic background but is still quite significant (50%) in the mixed C57BL/6J × 129SvEv background, which is the one that we have mainly used. The absence of this phenotype in our model is intriguing. A possible explanation could lie within the very different targeting strategies used since in the study by Ramirez-Solis et al., Hoxb4 mutants were generated either by disrupting the first exon by insertion of a double selection cassette and stop codons or by inserting a stop codon in the second exon. The split sternum phenotype was only observed in the first model (38). This might indicate that aberrant splice variants or the presence of a selection cassette driven by strong promoters within the complex-regulated Hox cluster could affect the observed phenotype (i.e., see reference 6). In the present study, all exons and introns of Hoxb4 and Hoxb3 are completely removed, along with intermediate and flanking sequences containing specific and shared regulatory elements (i.e., see references 15, 16, 17, 31, and 47). This leaves no possibility for expression of abnormal splice variants or truncated proteins with aberrant function. However, the deletion brings Hoxb2 and Hoxb5 together, thereby possibly altering the sequential expression pattern of the Hox cluster during embryogenesis (as well as in hematopoiesis).

The mechanism by which Hox genes affect transcription remain largely unknown, as well as their target genes. In addition to classical transcriptional activities, including DNA binding or direct involvement in transcription complexes, recent studies suggest that these genes might also be involved in chromatin modulation by affecting the acetylation of histones, thereby either functioning as repressors or activators (14a, 42, 48). With regard to Hoxb4, cellular proliferation induced by Hoxb4 has been reported to cause increased activity of the AP-1 complex and higher levels of cyclin D1, both directly involved in cell cycle regulation (24). Hoxb4 has also been reported to bind to and participate in downregulation of c-myc, resulting in differentiation of a promyelocytic leukemia cell line (35, 36).

Regulating maintenance and expansion of the stem cell pool involves a very complex mixture of internal and external signals. In addition to Hoxb4, a number of other molecules have also been reported to have an important role in this scheme. Among these is the cyclin-dependent kinase inhibitor, p21Cip1/waf1, which is necessary for maintaining stem cells in a quiescent state, and deficiency of this molecule leads to stem cell exhaustion (11). Interestingly, p21Cip1/waf1 has been suggested to be a transcriptional target of another Hox transcription factor, namely, HOXA10 (7). Other factors important for maintenance of hematopoietic activity include molecules such as Pbx1 (14) and Rae28 (33), both with strong connections to Hox genes. A few candidate genes with the ability to expand stem cells have been reported. An example of this is HOXA9 (52); however, long-term overexpression of this gene leads to AML (23). Other examples include Hedgehog and Sonic hedgehog, which enhance proliferation of primitive human hematopoietic cells via BMP regulation (3), and Notch1 (57) and the homeobox gene Lhx2 (37), which have been successfully used to generate immortalized HSCs.

In summary, the data presented here suggest that proliferation, but not commitment of true stem cells, is negatively affected by Hoxb3/b4 deficiency. Furthermore, it appears that Hoxb3 and Hoxb4 are mainly important under conditions that call for a rapid proliferation response and are dispensable for normal, steady-state hematopoiesis. These findings are important for understanding the regulatory mechanisms that control fate, particularly self-renewal, of HSCs. Further studies are required to elucidate fully the mechanism of Hoxb4 action in HSCs in order to determine whether enforced expression of Hoxb4 can be used safely to generate or expand stem cells ex vivo for cell or gene therapy.

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