HEMATOPOIESIS

Enhanced In Vivo Regenerative Potential of HOXB4-Transduced Hematopoietic Stem Cells With Regulation of Their Pool Size

By Unnur Thorsteinsdottir, Guy Sauvageau, and R. Keith Humphries

After bone marrow transplantation (BMT), there is a rapid regeneration to normal pretransplantation levels in the number of hematopoietic progenitors and mature end cells, whereas hematopoietic stem cell (HSC) numbers recover to only 5% to 10% of normal levels. This suggests that HSC are significantly restricted in their self-renewal behavior and hence in their ability to repopulate the host stem cell compartment. Previously, we have reported that HSC engineered to overexpress the homeobox transcription factor HOXB4 have a large repopulation advantage over untransduced cells as assessed at 4 months in a murine transplantation model (Sauvageau et al, Genes Dev 9:1753, 1995). This phenomenon has now been examined in detail for periods extending to 12 months in cohorts of mice transplanted with various numbers of HOXB4-transduced HSC. In all mice analyzed, HOXB4-transduced HSC were capable of fully reconstituting the HSC compartment, resulting, on average, in some 14-fold greater numbers of HSC than observed when transplanting control, non–HOXB4-transduced bone marrow cells. These data indicate that HOXB4 is a limiting factor in the regeneration of HSC to normal levels after BMT. Furthermore, we show that HOXB4-transduced HSC did not expand above levels normally observed in unmanipulated mice, indicating that its overexpression does not override the regulatory mechanisms that maintain the HSC pool size within normal limits.

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Materials and Methods

Animals. Recipients were 7- to 12-week-old male or female (C57Bl/6J × C3H/HeJ)F1 [(B6C3)F1] mice and donors were (C57Bl/6J × C3H/HeJ)F1 [(B6C3)F1].

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6Ly-Pep3b × C3H/HeJ)F1 [(PepC3)F1] mice. (B6C3)F1 and (PepC3)F1 mice are phenotypically distinguishable by their cell surface expression of different allelic forms of the Ly5 locus; (B6C3)F1 are homozygous for the Ly5.2 allele and (PepC3)F1 are heterozygous for the Ly5.1/Ly5.2 alleles. These mice were bred from parental strain breeders originally obtained from Jackson Laboratories (Bar Harbor, ME), maintained in microisolator cages, and provided with sterilized food and acidified water in the animal facility of the British Columbia Cancer Research Center.

Retroviral generation and infection of primary bone marrow cells. Retroviral vectors carrying the HOXB4 cDNA under the control of the viral long terminal repeat and/or a neomycin gene cassette under the control of an internal PGK promoter were constructed, and high-titer HOXB4 retroviral supernatant from transfected COS cells as prepared in the Terry Fox Laboratory. Loosely adherent and nonadherent bone marrow cells were recovered from the cocultures by repeated washing of dishes and then counted using a hemocytometer. Unless otherwise specified, all media, serum, and growth factors were obtained from StemCell Technologies Inc. (Vancouver, British Columbia, Canada).

Transplantation of retrovirally transduced bone marrow. For bone marrow transplantation procedures, lethally irradiated (950 cGy, 110 cGy/min, 137Cs γ-rays) (B6C3)F1 (Ly5.2) recipients were injected intravenously with 2 × 10^6 bone marrow cells derived from (PepC3)F1 (Ly5.1/Ly5.2) immediately after their cocultivation with HOXB4- or neo-viral producer cells. Donor-derived repopulation in recipients was assessed by flow cytometry to measure the proportion of leukocytes in bone marrow, thymus, spleen, and peripheral blood that expressed the Ly5.1 surface antigen recognized by the fluorescein isothiocyanate (FITC)-conjugated anti-Ly5.1 monoclonal antibody (kindly provided by Dr G. Spangrude, Salt Lake City, UT).

In vitro clonogenic progenitor assays. In vitro myeloid and pre-B clonogenic progenitor assays were performed as previously reported.9 Competitive repopulation unit (CRU) assay. The CRU assay10 was used to evaluate the regeneration of HSC in neo and HOXB4 mice. Briefly, bone marrow cells from neo or HOXB4 mice that were transplanted earlier with transduced cells derived from (PepC3)F1 (Ly5.1/Ly5.2) mice were injected at different dilutions into lethally irradiated (B6C3)F1 (Ly5.2) mice together with a life-sparing dose of 1 × 10^5 competitor bone marrow cells from (B6C3)F1 (Ly5.2) mice. The level of lymphoid and myeloid repopulation with Ly5.1-neo or Ly5.1-neo-derived cells in these secondary recipients was evaluated more than 13 weeks later by flow cytometry analysis of peripheral blood, as described.11 Recipients with greater than 1% donor-derived peripheral blood lymphoid and myeloid leukocytes as determined by the side scatter distribution of Ly5.1+ cells (ie, lymphoid low side scatter; myeloid high side scatter) were considered to be repopulated by at least 1 lympho-myeloid repopulating (CRU) cell. CRU frequency in the test cell population was then calculated by applying Poisson statistics to the proportion of negative recipients at different dilutions, as described previously.10 Secondary recipients were killed 16 weeks after transplantation.

RESULTS

Retroviral transduction and transplantation of murine bone marrow cells. The MSCV recombinant retroviral vector containing the HOXB4 cDNA (Fig 1A) was used to transfer and overexpress this gene in mouse bone marrow cells. To assess the long-term effects of HOXB4 overexpression on hematopoietic regeneration, lethally irradiated mice were transplanted with HOXB4- or neo-transduced bone marrow cells. Cohorts of mice from 3 independent transplantation experiments (hereafter called HOXB4 and neo mice) were assessed for regeneration of various hematopoietic compartments at different times after transplantation, beginning as soon as 16 weeks and as late as 52 weeks after transplantation (Fig 1B). Each recipient was transplanted with an inoculum of 2 × 10^5 bone marrow cells, as recovered from retroviral infection cultures. This cell dose is estimated to contain approximately 35 HSC, as previously measured for recovered cells under identical infection conditions, using the CRU assay.8 The gene transfer efficiencies to the transplanted bone marrow as assessed by the proportion of G418-resistant clonogenic cells varied between experiments and were 30% to 58% and 70% to 74% for HOXB4- and neo-transduced cells, respectively (Fig 1B). Assuming a retroviral infection efficiency of CRU (HSC) no greater than that of clonogenic progenitor cells, each recipient would have received an estimated maximum of 16 to 24 transduced (neo or HOXB4; see Table 3) CRU, plus an approximately equal number of nontransduced CRU. Findings from the 20-week timepoint in experiment no. 1 have been previously reported9; selected summary data from that timepoint are provided in Figs 2 and 3 to facilitate comparison with the new data from mice of that same transplant cohort now assessed at 52 weeks posttransplantation.

Effect of HOXB4 overexpression on progenitor and mature populations in vivo. Hematopoietic regeneration in both neo and HOXB4 mice, from all 3 transplantation experiments, was essentially completely donor-derived, because greater than 85% of bone marrow, spleen, thymic, and peripheral blood leukocytes were of transplant origin (Ly5.1+) at all time points analyzed. A contribution by transduced cells to this reconstitution was evident by Southern blot analysis that readily detected the neo-or the HOXB4-proviruses in the bone marrow and thymuses of these mice (Fig 1C).

In contrast to differences in HSC levels (see below), the bone marrow, spleen, and thymus nucleated cell counts, as well as the peripheral blood white and red blood cell counts, were similar in HOXB4 and neo mice (Table 1). Furthermore, fluorescence-activated cell sorting (FACS) analysis showed that the absolute release the integrated provirus, or with EcoRI or BamHI, which each cut the provirus once to release DNA fragment(s) specific to the proviral integration site(s). DNA fragments were separated on a 0.9% agarose gel, transferred to nylon membrane (Zeta-Probe; Bio-Rad, Hercules, CA), prehybridized, hybridized, and washed as described.8 Total cellular RNA was isolated using TRIzol (GIBCO BRL), separated on a 1% formaldehyde/agarose gel, transferred to nylon membrane (Zeta-Probe), prehybridized, hybridized, and washed as described.8 Probes used were a Xho I/Sal I fragment of pMC1neo,12 the full-length HOXB4 cDNA, a 2.0-kb Pst I fragment containing the chicken β-actin gene, and the 1.8-kb Kpn I/Hind III genomic fragment of the murine SH2-containing inositol phosphatase (SHIP) gene.13
numbers of bone marrow and splenic myeloid (Mac-1), erythroid (Ter119), B (ie, proB [B220, CD43], immature B [B220, IgM], and mature B [IgM, IgD]), and CD4 and CD8 thymic T cells subpopulations were all also within normal range in HOXB4 mice (Table 2).

We had previously reported that the bone marrow myeloid progenitor numbers, as assayed in semisolid cultures supplemented with growth factors, were increased by 5-fold in recipients of HOXB4-transduced cells when compared with neo control mice. This increase, initially detected in recipients analyzed at 20 weeks posttransplantation,8 was not observed in HOXB4 mice from transplantation experiments no. 2 or 3 (Fig 2). However, the number of myeloid progenitors in the spleen were always increased in these mice (Fig 2), with an overall increase of total body myeloid progenitor numbers of less than 2-fold in all mice analyzed. Evaluation of bone marrow IL-7-responsive B-lymphoid progenitor cells at 16, 32, and 41 weeks after transplantation showed that their numbers were slightly increased in the HOXB4 mice compared with neo control mice. However, at none of these time points was this increase statistically significant (Fig 2). Together, these data indicate that long-term overexpression of HOXB4 has mild to moderate effects on the number of clonogenic progenitors and essentially no effect on the number of mature end cells in recipient mice.

Effect of HOXB4 overexpression on long-term repopulating cells. To determine the frequency of long-term lymphomyeloid repopulating cells (or HSC) in neo and HOXB4 primary mice, the CRU assay was used,10 which combines principles of limiting dilution together with competitive repopulation. Bone marrow cells were harvested from HOXB4 and neo mice at 5 different time points (Fig 1B), spanning as early as 16 weeks to as late as 52 weeks posttransplant. These cells were then transplanted at several different dilutions into secondary recipients that were themselves analyzed 12 to 15 weeks later for donor-derived (ie, Ly5.1) lympho-myeloid repopulation. At all time points examined and in all experiments, CRU numbers were markedly higher, on average some 14-fold, in HOXB4 mice when compared with neo control mice, whose CRU numbers achieved values that were approximately 10% of normal untransplanted mice (Fig 3). The low recovery of CRU in recipients of neo-transduced bone marrow is consistent with
previous studies using nontransduced unmanipulated adult bone marrow. 14-17 The results with HOXB4-transduced cells thus stand in sharp contrast with recovery to the normal pretreatment level at all times posttransplant analyzed.

Interestingly, once normal levels of CRU were reached in the HOXB4 mice, which is at least as early as 16 weeks, the CRU did not expand further or become exhausted, as indicated by the similar number of CRU cells present at 20 versus 52 weeks posttransplantation in experiment no. 1 (solid box in Fig 3) and at 16 versus 32 weeks in experiment no. 2 (solid diamond in Fig 3). Northern blot analysis of primary recipients (data not shown) and secondary mice used for CRU determination (Fig 4C) confirmed continued expression of the transduced HOXB4 gene. Thus, plateauing of CRU numbers was not associated with extinction of HOXB4 gene expression. Taken together, these results clearly indicate that HOXB4 appears to be a limiting factor in the regeneration of CRU numbers to normal levels after BMT and that its overexpression does not override the regulatory mechanisms that maintain the CRU pool size within normal limits and neither does it lead to rapid exhaustion of CRU.

Polyclonal expansion and hematopoietic regeneration by HOXB4-transduced CRU. To prove that the enhanced regeneration of CRU cells in the HOXB4 mice was indeed caused by preferential expansion of HOXB4-transduced CRU cells and to analyze the degree of polyclonality of the regenerated pool of CRU cells, we performed Southern blot analyses of proviral integration sites in DNA isolated from various hematopoietic tissues of primary and secondary HOXB4 and neo mice. Results from representative primary recipients and their corresponding secondary recipients used for CRU assay are shown in Fig 4.

In the bone marrow, spleen, and thymus of primary HOXB4 recipients, which were killed 52 and 32 weeks posttransplantation, multiple proviral integrations could be detected (Fig 4A and B). Variations in the proviral signal intensities detected in most of these tissues are a signature for multiple proviral integrations and thus demonstrate polyclonal regeneration by HOXB4-transduced long-term repopulating cells, rather than multiple proviral integrations into the same cell. In contrast, moderate proviral integration complexity was observed in the primary neo recipients, consistent with oligoclonal repopulation by neo-transduced cells (Fig 4B). This difference between neo and HOXB4 mice cannot be contributed to higher numbers of transduced stem cells being initially transplanted to the HOXB4 mice.
mice, because the neo control and the HOXB4 mice received similar numbers (Table 3), thus underscoring the repopulating advantages of HOXB4-transduced CRU cells over untransduced cells.

Southern blot analysis of proviral integration patterns in bone marrow (myeloid) and thymus (lymphoid) of secondary recipients was used to further analyze the clonality of the regenerated pool of transduced lympho-myeloid repopulating (CRU) cells present in primary HOXB4 and neo mice at the time of their death (Fig 4A and B). In all of the 9 secondary recipients of the primary donor mouse killed at 52 weeks posttransplantation that were scored positive for donor-derived repopulation by FACS (Ly5.1+), HOXB4 proviral integration(s) were detected in their bone marrow and/or thymus (Fig 4A). Furthermore, the intensities of the proviral signals correlated, for most of the mice, with their level of donor derived (Ly5.1+) repopulation (Fig 4A). As can be seen in Fig 4A and summarized in Table 3, a total of at least 15 different HOXB4-transduced clones could be detected in these secondary mice. In addition, at least 4 of these mice had a common proviral integration pattern in their bone marrow and thymus (mice 52-2, 52-4, 52-6, and 52-9 [bone marrow signal very faint due to low amount of DNA]), indicating the lympho-myeloid repopulating potential of the regenerated CRU cells. The lack of detection of common proviral integration patterns both in bone marrow and thymus in the other secondary mice can be explained in some cases by the low amount of DNA analyzed (mouse 52-7 in thymus), the lack of bone marrow sample (mouse 52-3), or very low donor-derived repopulation levels (mouse 52-5, Ly5.1+ PBL only 5%). Thus, even as late as 52 weeks posttransplantation, the hematopoietic regeneration in the primary HOXB4 mice was polyclonal and without apparent dominance of any HOXB4-transduced clone.

Similarly, analysis of the proviral integration sites in the secondary recipients receiving bone marrow cells from 1 of the HOXB4 recipients killed 32 weeks posttransplantation showed a complete concordance between detection of donor-derived hematopoietic regeneration by FACS analysis and contribution to regeneration by HOXB4-transduced cells, again indicating selective or competitive regeneration of the HOXB4-transduced cells over nontransduced cells (Fig 4B). Of the 10 secondary recipients (32-1 through 32-10) that were positive for lympho-myeloid repopulation by FACS, 8 had detectable HOXB4 proviral integration in their bone marrow and thymus (Fig 4B). In the case of the 2 negative mice (32-5 and 32-10), which both had low donor repopulation (Ly5.1+ PBL, 4% and 5%, respectively), the expression of HOXB4 could, however, be detected by Northern blot analysis of their bone marrow (Fig 4C). Several of these secondary recipients, including those that were estimated to receive between 1 and 2 CRU cells (mice 32-6, 32-8, and 32-9), had a common proviral integration in their bone marrow and thymus, thus again confirming the lympho-myeloid repopulating potential of the regenerated HOXB4-transduced CRU cells (Fig 4B). Self-renewal of HOXB4-transduced CRU cells was also demonstrated by detecting a common lympho-myeloid repopulating clone in all of the secondary recipients (32-1 to 32-4) receiving high cell dose (43 CRU/mouse) and in 1 mouse (32-6) receiving fewer CRU cells (2 CRU/mouse). However, interestingly, this clone could not be detected in bone marrow, spleen, or thymus of the primary

### Table 1. Hematological Parameters in HOXB4 and neo Mice 16 to 52 Weeks Posttransplantation

<table>
<thead>
<tr>
<th>Weeks Post-Tx</th>
<th>Mice</th>
<th>NC/Femur (x 10^7)</th>
<th>NC/Thymus (x 10^7)</th>
<th>NC/Spleen (x 10^7)</th>
<th>RBC (x 10^10/mL)</th>
<th>WBC (x 10^10/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>neo (n = 3)</td>
<td>1.9 ± 0.2</td>
<td>ND</td>
<td>3.3 ± 0.5</td>
<td>7.9 ± 0.4</td>
<td>7.8 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>HOXB4 (n = 3)</td>
<td>2.0 ± 0.2</td>
<td>ND</td>
<td>3.6 ± 0.6</td>
<td>7.6 ± 1.0</td>
<td>8.0 ± 2.2</td>
</tr>
<tr>
<td>52</td>
<td>HOXB4 (n = 2)</td>
<td>1.9 ± 0.6</td>
<td>4.5 ± 0.5</td>
<td>7.7 ± 5.0</td>
<td>7.7 ± 1.0</td>
<td>8.2 ± 2.5</td>
</tr>
<tr>
<td>16</td>
<td>neo (n = 3)</td>
<td>2.4 ± 0.7</td>
<td>9.1 ± 0.5</td>
<td>3.1 ± 0.5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>HOXB4 (n = 3)</td>
<td>1.8 ± 0.2</td>
<td>8.0 ± 1.5</td>
<td>3.2 ± 0.25</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>32</td>
<td>neo (n = 2)</td>
<td>2.2 ± 0.5</td>
<td>6.0 ± 1.0</td>
<td>2.7 ± 0.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>HOXB4 (n = 2)</td>
<td>2.0 ± 0.7</td>
<td>6.4 ± 1.6</td>
<td>2.8 ± 0.1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>41</td>
<td>neo (n = 2)</td>
<td>1.9 ± 0.5</td>
<td>ND</td>
<td>2.2 ± 0.7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>HOXB4 (n = 2)</td>
<td>2.1 ± 0.1</td>
<td>ND</td>
<td>2.4 ± 0.6</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Results shown are the mean ± SD for the indicated number of neo and HOXB4 mice.

Abbreviations: Tx, transplantation; NC, nucleated cell counts; RBC, red blood cells; WBC, white blood cells.

### Table 2. Absolute Numbers of Various Phenotypically Defined Hematopoietic Populations in neo and HOXB4 Mice 32 Weeks After Transplantation

<table>
<thead>
<tr>
<th>Mice</th>
<th>Bone Marrow</th>
<th>Peripheral Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B220+</td>
<td>B220+/CD43+</td>
</tr>
<tr>
<td>neo</td>
<td>7.6 ± 1.5</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>HOXB4</td>
<td>6.4 ± 0.3</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>B220+</td>
<td>IgM+/IgD+</td>
</tr>
<tr>
<td>neo</td>
<td>12.4 ± 2.0</td>
<td>7.5 ± 1.5</td>
</tr>
<tr>
<td>HOXB4</td>
<td>13.2 ± 2.0</td>
<td>8.5 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>CD4+</td>
<td>CD8+</td>
</tr>
<tr>
<td>neo</td>
<td>0.24 ± 0.11</td>
<td>7.8 ± 0.5</td>
</tr>
<tr>
<td>HOXB4</td>
<td>0.19 ± 0.04</td>
<td>6.9 ± 1.7</td>
</tr>
</tbody>
</table>

Results shown are the mean ± SD for n = 2 neo and n = 2 HOXB4 mice.
HOXB4 mouse (Fig 4B), indicating that this cell, despite extensive self-renewal division, did not contribute significantly to bone marrow, spleen, or thymic repopulation in this primary HOXB4 recipient at the time of death. The detection of 4 different HOXB4-transduced lympho-myeloid repopulating clones and at least 9 others with either lymphoid or myeloid potential in these secondary HOXB4 mice (Fig 4B and Table 3) strongly suggest that the enhanced CRU regeneration in the primary HOXB4 mouse killed 32 weeks posttransplantation was also a polyclonal event.
HOXB4 EFFECTS ON STEM CELL REGENERATION

Table 3. Summary of the Clonal Analyses of Primary and Secondary Recipients of neo- or HOXB4-Transduced Cells Presented in Fig 4, Demonstrating Enhanced Polyclonal Long-Term Repopulation by HOXB4-Transduced Stem Cells

<table>
<thead>
<tr>
<th>Mice</th>
<th>Estimated Numbers of Transduced CRU Injected/1° Mouse*</th>
<th>No. of Transduced Clones Detected in 1° Mice</th>
<th>Minimal Life-Span of Each Transduced Clone (wks)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>neo-32</td>
<td>26</td>
<td>4</td>
<td>48</td>
</tr>
<tr>
<td>HOXB4-32</td>
<td>16</td>
<td>13</td>
<td>48</td>
</tr>
<tr>
<td>HOXB4-52</td>
<td>20</td>
<td>15</td>
<td>68</td>
</tr>
</tbody>
</table>

*The number of transduced CRU injected per mouse was estimated based on our previous results that determined the frequency of CRU at 1 in 6,000 bone marrow cells as harvested from identical retroviral infection cultures as used in this study and the estimation of gene transfer to CRU equal to that of CFC presented in Fig 1B.
†The minimal life-span of each clone detected is the sum of the life-span, after transplantation, of primary (32 weeks for the neo mouse and 32 and 52 weeks for the 2 HOXB4 mice) and secondary recipients (16 weeks for all mice).

In contrast to the HOXB4 mice, those secondary neo recipients that were scored positive for donor-derived lymphomyeloid repopulation (estimated to receive ~5 CRU cells) were only positive for proviral integrations in their bone marrow (Fig 4B). These data for the neo mice thus stand in sharp contrast to that of the HOXB4 mice, in which transduced cells with lymphoid-myeloid repopulating potential can be detected in secondary mice that received approximately 70 times lower number of bone marrow cells than these secondary neo mice (1.4 × 10⁶ cells for the neo; Fig 4A and B).

DISCUSSION

Using retroviral gene transfer and the murine BMT model, we have previously obtained evidence that HOXB4 overexpression can markedly enhance CRU regeneration as assessed 5 months posttransplantation of transduced bone marrow cells. In this current study, we extend those initial observations to provide significant new insights into the kinetics, control, and magnitude of this phenomenon. Our current findings show that the enhanced regeneration of the CRU compartment by the HOXB4-transduced CRU is evident as early as 16 weeks posttransplantation and persists for at least 1 year after transplantation. This increase in regenerative behavior is such that normal, pretransplantation levels of CRU are achieved and maintained—levels some 14-fold higher than observed with non-HOXB4-transduced stem cells. Moreover, the apparent stabilization in the CRU pool at normal pretransplantation levels suggests that the expansion of HOXB4-transduced CRU in these mice is ultimately subjected to existing in vivo control mechanisms. HOXB4 was also demonstrated to act on multiple CRUs, because the regenerated pool of HOXB4-transduced CRUs in the transplanted mice was highly polyclonal even as late as 1 year after transplantation. Furthermore, detailed analysis of various mature cell populations in these mice strongly suggests that overexpression of HOXB4 neither alters myeloid or lymphoid differentiation nor leads to dominant outgrowth of any type of hematopoietic cells.

Serial transplantation studies have suggested that the transplantable HSCs may fail to fully regenerate the HSC compartment, because the self-renewal capacity of HSCs may be intrinsically limited or at least subjected to exhaustion. In contrast to depletion of the HSC pool, recent studies have suggested that the failure to fully regenerate the HSC compartment after transplantation could be the result of negative feedback mechanisms activated when progenitors and mature cells have been regenerated to their normal levels, which prematurely inhibit further HSC expansion. HOXB4 overexpression might thus render HSC less sensitive to this negative feedback mechanism.

Using fibroblasts engineered to overexpress HOXB4, it was recently shown that these cells acquire the capacity to grow in low concentrations of serum. An alternative explanation for the HOXB4 effect described in the present studies is that HOXB4 might alter the sensitivity of HSC to extrinsic factors acting during hematopoietic regeneration, thus allowing for a greater expansion of the HOXB4-transduced HSC compartment.

Several studies have indicated that, in steady-state hematopoiesis, the proliferation of HSC is tightly controlled. In mice, HSC numbers remain relatively constant throughout most of their adult life, although in very old mice (>2 years) their numbers appear to increase, possibly due to accumulation of genetic lesions. The HSC population in mice has also been demonstrated to be quiescent (or slowly cycling), because the vast majority of these cells are resistant to cytotoxic agents such as 5-FU or hydroxyurea. The stabilization of the CRU pool in HOXB4 mice at normal levels suggests that, although CRU cells overexpressing HOXB4 have enhanced regenerative potential, their ability to respond to this regulatory mechanism is not altered. However, because the cycling status of CRU cells in

Fig 4. Southern blot analysis of proviral integration patterns in primary and secondary recipients of neo- and HOXB4-transduced bone marrow cells. DNA samples isolated from various hematopoietic organs of primary recipients killed 52 (A) or 32 (B) weeks posttransplantation and their secondary recipients (killed 16 weeks posttransplantation) were first digested with EcoRI and then BamHI, both of which cut the integrated provirus once, generating a DNA fragment specific for each proviral integration site. The number of transduced clones detected with either enzyme were the same; thus, the results for only 1 of the enzymes is shown. The membranes were first hybridized to a neo-specific probe for detection of proviral fragments and subsequently with a probe specific for the SHIP gene to provide a single copy control of loading. Exposure times were 48 hours for the neo and SHIP probes. To demonstrate that the proviral fragments contained the HOXB4 cDNA, the blots were also hybridized with full-length HOXB4 cDNA probe, which generated the same proviral banding pattern as the neo probe (data not shown). Each mouse is identified with a specific number derived from the time that the primary recipient was killed, and indicated above that number are the estimated number of bone marrow cells received by each secondary recipient as well as the estimated number of CRU cells that they received. Expression of the 3.9-kb HOXB4-containing message in the bone marrow of secondary recipients is shown in (C). The percentages of the donor-derived repopulation (ie, Ly5.1⁺) in the secondary neo mice were as follows: 32-1 (44%) and 32-2 (18%). In secondary HOXB4 mice, the percentages were as follows: 32-1 (60%), 32-2 (76%), 32-3 (83%), 32-4 (52%), 32-5 (4%), 32-6 (23%), 32-7 (18%), 32-8 (8%), 32-9 (51%), 32-10 (5%), 32-11 (0%), 52-1 (20%), 52-2 (69%), 52-3 (73%), 52-4 (47%), 52-5 (5%), 52-6 (36%), 52-7 (47%), 52-8 (39%), and 52-9 (20%). B, bone marrow; S, spleen; T, thymus.
HOXB4 recipients is currently unknown, other regulatory mechanisms acting to maintain stable levels of HOXB4-transduced CRU cells cannot be ruled out. Despite a profound and consistent effect on the expansion of primitive hematopoietic cells, overexpression of HOXB4 did not promote preferential expansion along any hematopoietic lineage or lead to leukemia, despite evidence of persistent HOXB4 expression for at least 52 weeks. These results stand in sharp contrast to our published data for the retroviral overexpression of either HOXB3 or HOXA10 in a similar transplantation model.\textsuperscript{9,21} The different outcomes on hemopoiesis generated by overexpression of these 3 different HOX genes strongly indicate that these proteins activate and/or repress different sets of target genes in hematopoietic cells. The effects of HOXB4 overexpression during hematopoietic regeneration can thus be viewed as more restricted than those generated by overexpression of either HOXB3 or HOXA10, suggesting that targets open to HOXB4 are restricted to primitive hematopoietic cells.

Together, the results presented in this report document that as few as 10 to 20 HOXB4-transduced HSC are sufficient to regenerate the HSC pool size to pretransplantation levels and, more importantly, once that level is reached, the number of HOXB4-transduced HSC is controlled over time. These findings now suggest that it is possible to engineer HSC that possess repopulating potential 10- to 20-fold higher than that of unmanipulated HSC. In addition, these findings provide important insights into the control and molecular mechanism of stem cell self-renewal and point to potential application in stem cell-mediated therapies such as those where HSC numbers may be limiting.

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