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

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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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HEMATOPOIESIS IS the process by which mature blood cells are continuously generated throughout adult life from a small number of totipotent hematopoietic stem cells (HSC). The HSCs have the key properties of being able to self-renew and to differentiate into mature cells of both lymphoid and myeloid lineages. Although the genetic mechanisms responsible for the control of self-renewal and differentiation outcomes of HSC divisions remain largely unknown, a number of studies have implicated a variety of transcription factors as key regulatory components of these processes.[1]

Among such factors are the mammalian *Hox* homeobox gene family of transcription factors, consisting of 39 members arranged in 4 clusters (A, B, C, and D), initially described as important regulators of pattern formation in a variety of embryonic tissues.[2] These genes are structurally related by the presence of a 183-bp sequence, the homeobox, that encodes a helix-turn-helix DNA binding motif.[3] Apparent stage- and lineage-specific expression of numerous *HOXA*, *B*, and *C* genes has now been demonstrated for both hematopoietic cell lines[4] and primary hematopoietic cells.[5-7] For example, we have shown that members of the *HOXA* and *HOXB* cluster genes are preferentially expressed in the $CD34^+$ fraction of human bone marrow cells that contains most if not all of the hematopoietic progenitor cells.[7] Further detailed analysis of *Hox* gene expression in functionally distinct subpopulations of $CD34^+$ cells has shown that genes, primarily located at the 3′ end of the clusters (eg, *HOXB3* and *HOXB4*), are preferentially expressed in the subpopulation containing the most primitive hematopoietic cells.[7]

Using a murine bone marrow transplantation (BMT) model, we previously obtained evidence indicating that retroviral overexpression of *HOXB4* in hematopoietic cells can greatly enhance the regeneration of the HSC compartment after BMT,[8] thus implicating *HOXB4* as a regulator of self-renewal divisions of HSC. In these initial studies, the effects of *HOXB4* overexpression were assessed at 20 weeks posttransplantation, at which time the HSC compartment had regenerated to slightly above normal pretransplantation levels or some 47-fold higher than achieved in control transplant recipients. This enhanced regenerative ability was further suggested by a significant expansion of *HOXB4*-transduced HSC after transplantation into secondary recipients. From these limited initial studies it was unclear whether *HOXB4* overexpression in steady-state hematopoiesis would lead to continuing expansion of HSC over time, suggesting that it could override the normal processes that control the population size and self-renewal potential of HSC. Also unanswered was whether *HOXB4* could act on a wide spectrum of transduced HSC or, as opposed, on a limited subset, as would be reflected in polyclonal versus monoclonal or oligoclonal expansion, respectively.

To address these questions, the current studies have examined the size and the degree of polyclonality of the regenerated pool of HSC in mice transplanted with *HOXB4*-transduced bone marrow cells as a function of time for a period extending up to 1 year after transplantation.

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/

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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 allotype and (PepC3)F1 are heterozygous for the Ly5.1/Ly5.2 allotypes. 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 viral producers were generated in the GPE-86 packaging line as previously described.[8] Bone marrow cells were obtained from (PepC3)F1 (Ly5.1) mice who had received 4 days previously an intravenous injection of 150 mg/kg body weight of 5-fluorouracil (5-FU), prestimulated in Dulbecco's modified Eagle medium (DMEM) containing 15% fetal calf serum (FCS), 6 ng/mL of murine interleukin-3 (mIL-3), 100 ng/mL murine Steel factor (mSF), and 10 ng/mL human IL-6 (hIL-6) for 48 hours and then cocultivated on irradiated viral producer cells using identical medium with the addition of 6 µg/mL polybrene for an additional 48 hours. mSF, hIL-6, and mIL-3 were used as diluted 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 2×10^5 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 using 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 assay[10] 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$^+$ donor-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.

Southern and Northern blot analyses. DNA was isolated from bone marrow, spleen, and thymus of *neo* and *HOXB4* mice using DNAzol (GIBCO BRL, Burlington, ON, Canada). High molecular weight DNA was digested with *Kpn* I, which cuts in the long terminal repeat region to release the integrated provirus, or with *Eco*RI or *Bam*HI, 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/*Hin*dIII genomic fragment of the murine *SH2-containing inositol phosphatase* (*SHIP*) gene.[13]

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 reported[8]; 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

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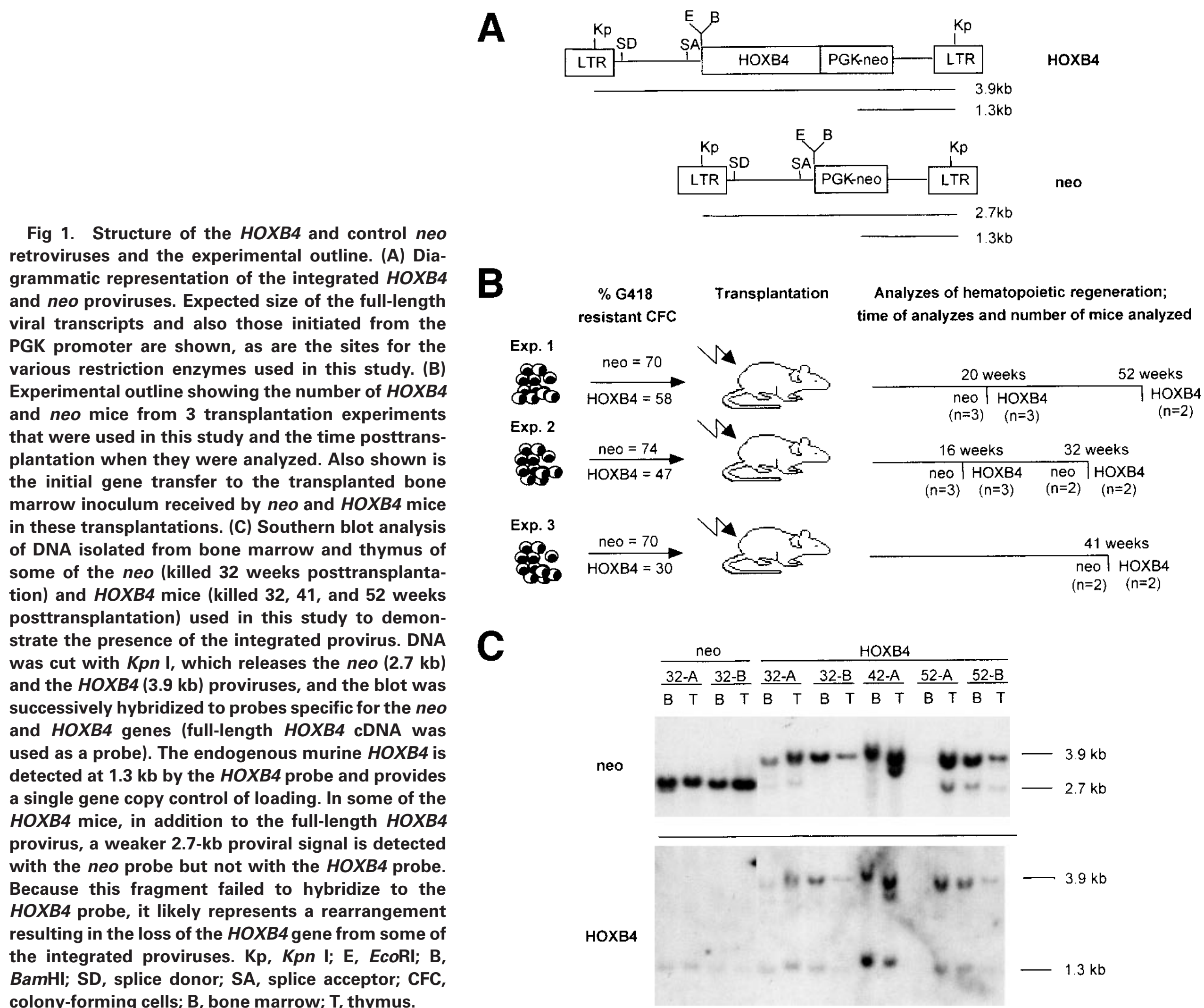

Fig 1. Structure of the *HOXB4* and control *neo* retroviruses and the experimental outline. (A) Diagrammatic representation of the integrated *HOXB4* and *neo* proviruses. Expected size of the full-length viral transcripts and also those initiated from the PGK promoter are shown, as are the sites for the various restriction enzymes used in this study. (B) Experimental outline showing the number of *HOXB4* and *neo* mice from 3 transplantation experiments that were used in this study and the time posttransplantation when they were analyzed. Also shown is the initial gene transfer to the transplanted bone marrow inoculum received by *neo* and *HOXB4* mice in these transplantations. (C) Southern blot analysis of DNA isolated from bone marrow and thymus of some of the *neo* (killed 32 weeks posttransplantation) and *HOXB4* mice (killed 32, 41, and 52 weeks posttransplantation) used in this study to demonstrate the presence of the integrated provirus. DNA was cut with *Kpn* I, which releases the *neo* (2.7 kb) and the *HOXB4* (3.9 kb) proviruses, and the blot was successively hybridized to probes specific for the *neo* and *HOXB4* genes (full-length *HOXB4* cDNA was used as a probe). The endogenous murine *HOXB4* is detected at 1.3 kb by the *HOXB4* probe and provides a single gene copy control of loading. In some of the *HOXB4* mice, in addition to the full-length *HOXB4* provirus, a weaker 2.7-kb proviral signal is detected with the *neo* probe but not with the *HOXB4* probe. Because this fragment failed to hybridize to the *HOXB4* probe, it likely represents a rearrangement resulting in the loss of the *HOXB4* gene from some of the integrated proviruses. Kp, *Kpn* I; E, *Eco*RI; B, *Bam*HI; SD, splice donor; SA, splice acceptor; CFC, colony-forming cells; B, bone marrow; T, thymus.

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 lympho-myeloid 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

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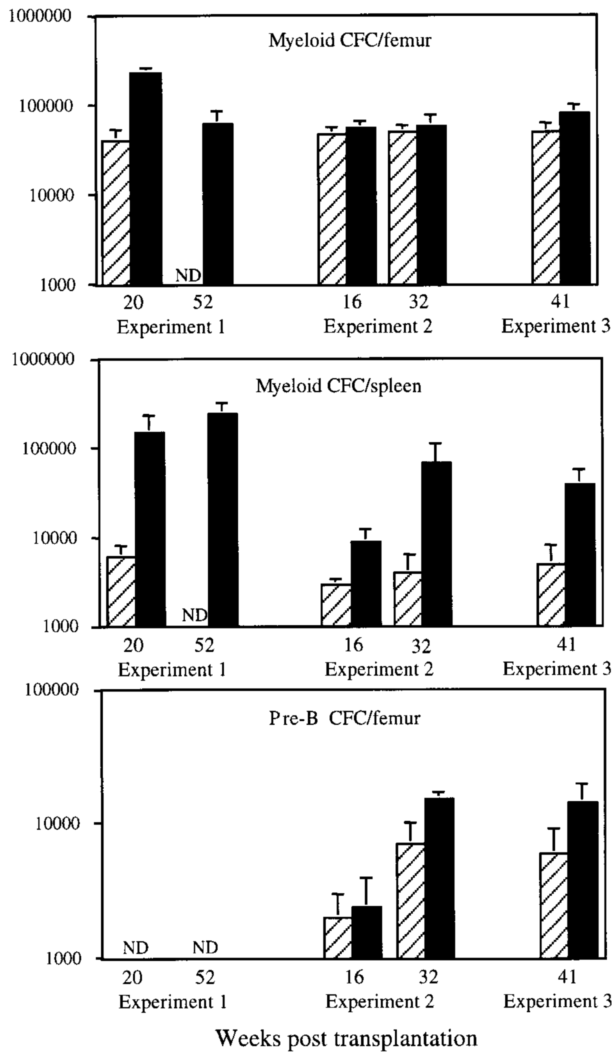

Fig 2. Effects of *HOXB4* overexpression on the number of myeloid and pre-B colony-forming cells after BMT. Results shown are the means ± SD of the numbers of in vitro myeloid colony-forming cells in bone marrow (top) and spleen (middle) and of IL-7–responsive B-lymphoid progenitor cells (pre-B CFC) in the bone marrow (bottom) of individual *neo* (▨) and *HOXB4* (■) mice at various time points after transplantation. The number of *neo* and *HOXB4* mice analyzed at each time point are shown in Fig 1B. Consistent with their preferential derivation from *HOXB4*-transduced cells, a major proportion of myeloid and pre-B lymphoid progenitor cells in *HOXB4* mice were G418-resistant (*HOXB4* mice, 59% ± 9% *v neo* mice, 37% ± 10% for all 3 experiments).

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 pretransplantation 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*

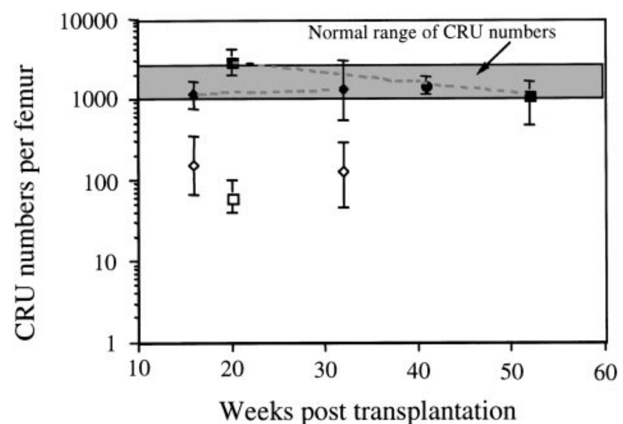

Fig 3. Variation in CRU numbers in recipients of *neo*-and *HOXB4*-transduced bone marrow cells over 1-year period. The number of CRU in femurs of cohorts of *HOXB4* recipients from 3 different experiments ([■] experiment no. 1, [◆] experiment no. 2, and [●] experiment no. 3) and *neo* recipients from 2 different experiments (□ and ◇) were evaluated using the CRU assay. The results shown are expressed as the mean ± 95% confident interval of the CRU numbers in 1 femur of *neo* and *HOXB4* mice at the various time points after transplantation. The number of *neo* and *HOXB4* mice analyzed at each time point are shown in Fig 1B. The shaded area represents the normal number of CRU measured in the femoral cavity of an unmanipulated (C57Bl/6J × C3H/HeJ)F1 mouse.[9]

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

Weeks Post-Tx	Mice	NC/Femur ($\times 10^7$)	NC/Thymus ($\times 10^7$)	NC/Spleen ($\times 10^8$)	Peripheral Blood RBC ($\times 10^9$/mL)	Peripheral Blood WBC ($\times 10^6$/mL)
Experiment no. 1						
20	*neo* (n = 3)	1.9 ± 0.2	ND	3.3 ± 0.5	7.9 ± 0.4	7.8 ± 2.5
	HOXB4 (n = 3)	2.0 ± 0.2	ND	3.6 ± 0.6	7.6 ± 1.0	8.0 ± 2.2
52	*HOXB4* (n = 2)	1.9 ± 0.6	4.5 ± 0.5	7.7 ± 5.0	7.7 ± 1.0	8.2 ± 2.5
Experiment no. 2						
16	*neo* (n = 3)	2.4 ± 0.7	9.1 ± 0.5	3.1 ± 0.5	ND	ND
	HOXB4 (n = 3)	1.8 ± 0.2	8.0 ± 1.5	3.2 ± 0.25	ND	ND
32	*neo* (n = 2)	2.2 ± 0.5	6.0 ± 1.0	2.7 ± 0.2	7.6 ± 0.5	7.5 ± 0.6
	HOXB4 (n = 2)	2.0 ± 0.7	6.4 ± 1.6	2.8 ± 0.1	7.7 ± 0.75	8.0 ± 1.4
Experiment no. 3						
41	*neo* (n = 2)	1.9 ± 0.5	ND	2.2 ± 0.7	ND	ND
	HOXB4 (n = 2)	2.1 ± 0.1	ND	2.4 ± 0.6	ND	ND

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.

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

Mice	Bone Marrow B220$^+$ ($\times 10^6$)	B220$^+$/CD43$^+$ ($\times 10^6$)	B220$^+$/IgM$^+$ ($\times 10^6$)	Mac1$^+$ ($\times 10^6$)
neo	7.6 ± 1.5	1.6 ± 0.4	3.2 ± 0.7	5.0 ± 0.5
HOXB4	6.4 ± 0.3	1.5 ± 0.1	2.5 ± 0.4	5.2 ± 1.0
Spleen				
Mice	B220$^+$ ($\times 10^7$)	IgM$^+$/IgD$^+$ ($\times 10^7$)	CD3$^+$ ($\times 10^7$)	Mac1$^+$ ($\times 10^7$)
neo	12.4 ± 2.0	7.5 ± 1.5	3.3 ± 0.4	0.9 ± 0.1
HOXB4	13.2 ± 2.0	8.5 ± 3.0	3.0 ± 0.1	1.1 ± 0.1
Thymus				
Mice	CD4$^-$CD8$^-$ ($\times 10^7$)	CD4$^+$CD8$^+$ ($\times 10^7$)	CD4$^+$CD8$^-$ ($\times 10^7$)	CD4$^-$CD8$^+$ ($\times 10^7$)
neo	0.24 ± 0.11	7.8 ± 0.5	0.83 ± 0.05	0.17 ± 0.08
HOXB4	0.19 ± 0.04	6.9 ± 1.7	0.62 ± 0.14	0.10 ± 0.07

Results shown are the mean ± SD for n = 2 *neo* and n = 2 *HOXB4* mice.

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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.

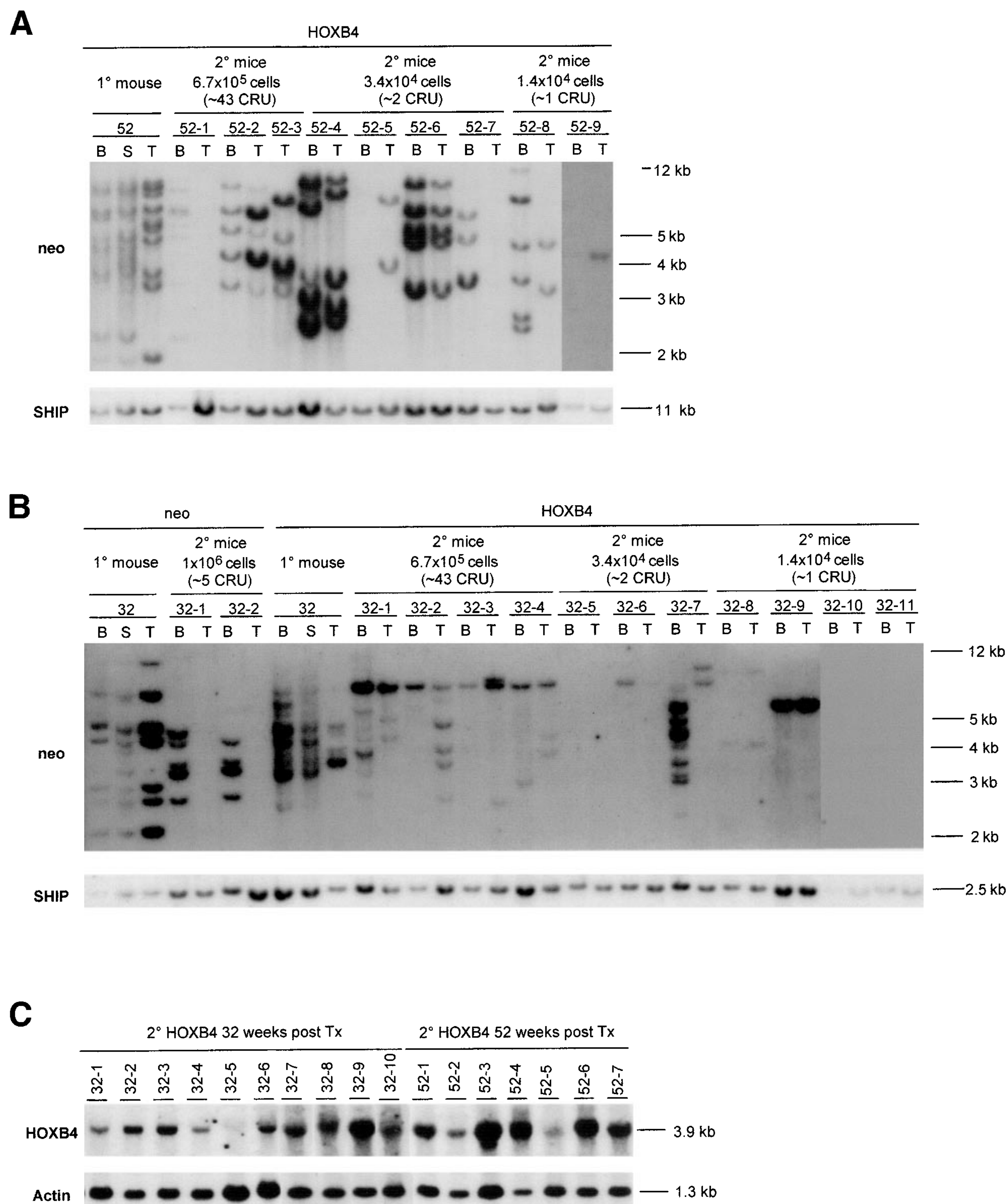

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Table 3. Summary of the Clonal Analyses of Primary and Secondary Recipients of *neo*- or *HOXB4*-Transduced Cells Presented in Fig 4, Demonstrating Enhanced Polyclonal Long-Term Repopulation by *HOXB4*-Transduced Stem Cells

Mice	Estimated Numbers of Transduced CRU Injected/ 1° Mouse*	No. of Transduced Clones in 1° Mice, Detected in 2° Mice	Minimal Life-Span of Each Transduced Clone (wks)†
neo-32	26	4	48
HOXB4-32	16	13	48
HOXB4-52	20	15	68

*The number of transduced CRU injected per mouse were 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[8] 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 (1.4×10^4 cells for the *HOXB4 v* 1×10^6 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.[8] 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.[14,15] 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.[16,17] *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.[18] 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 appears to increase, possibly due to accumulation of genetic lesions.[19] 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.[20] 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 *Eco*RI and then *Bam*HI, 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 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 (30%), and 52-9 (20%). B, bone marrow; S, spleen; T, thymus.

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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 *HOXA*10 in a similar transplantation model.[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 *HOXA*10, 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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REFERENCES

1. Shivdasani RA, Orkin SH: The transcriptional control of hematopoiesis. Blood 87:4025, 1996

2. Krumlauf R: Hox genes in vertebrate development (review). Cell 78:191, 1994

3. Levine M, Hoey T: Homeobox proteins as sequence-specific transcription factors. Cell 55:537, 1998

4. Lawrence HJ, Sauvageau G, Humphries RK, Largman C: The role of Hox Homeobox genes in normal and leukemic hematopoiesis. Stem Cells 14:281, 1996

5. Giampaolo A, Sterpetti P, Bulocrini D, Samoggia P, Pelosi P, Valtieri F, Peschle C: Key functional role and lineage-specific expression of *HOXB* cluster genes in purified hematopoietic progenitor differentiation. Blood 84:3637, 1994

6. Moretti P, Simmons P, Thomas P, Haylock D, Rathjen P, Vadas M, D'Andrea R: Identification of homeobox genes expressed in human haemopoietic progenitor cells. Gene 144:213, 1994

7. Sauvageau G, Lansdorp PM, Eaves CJ, Hogge DE, Dragowska WH, Reid DS, Largman C, Lawrence HJ, Humphries RK: Differential expression of homeobox genes in functionally distinct CD34+ subpopulations of human bone marrow cells. Proc Natl Acad Sci USA 91:12223, 1994

8. Sauvageau G, Thorsteinsdottir U, Eaves CJ, Lawrence HJ, Largman C, Lansdorp PM, Humphries RK: Overexpression of *HOXB4* in hematopoietic cells causes the selective expansion of more primitive populations in vitro and *in vivo*. Genes Dev 9:1753, 1995

9. Thorsteinsdottir U, Sauvageau G, Hough MR, Lawrence HJ, Largman C, Humphries RK: Overexpression of *HOXA*10 in murine hematopoietic cells perturbs both myeloid and lymphoid differentiation and leads to acute myeloid leukemia. Mol Cell Biol 17:495, 1997

10. Szilvassy SJ, Humphries RK, Lansdorp PM, Eaves AC, Eaves CJ: Quantitative assay for totipotent reconstituting hematopoietic stem cells by a competitive repopulations strategy. Proc Natl Acad Sci USA 87:8736, 1990

11. Rebel VI, Miller CL, Thornbury GR, Dragowska WH, Eaves CJ, Lansdorp PM: A comparison of long-term repopulating hematopoietic stem cells in fetal liver and adult bone marrow from the mouse. Exp Hematol 24:638, 1996

12. Thomas KR, Capecchi MR: Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell 51:503, 1987

13. Damen JE, Liu L, Rosten PM, Humphries RK, Jefferson AB, Majerus PW, Krystal G: The 145-kDa protein induced to associate with Shc by multiple cytokines is an inositol tetraphosphate and phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase. Proc Natl Acad Sci USA 93:1689, 1996

14. Harrison DE: Loss of stem cell repopulating ability upon transplantation. Effects of donor age, cell number and transplantation procedure. J Exp Med 156:1767, 1982

15. Harrison DE, Stone M, Astle CM: Effects of transplantation on the primitive immunohematopoietic stem cell. J Exp Med 3:119, 1990

16. Pawliuk R, Eaves C, Humphries RK: Evidence of both ontogeny and transplant dose-regulated expansion of hematopoietic stem cells *in vivo*. Blood 88:2852, 1996

17. Iscove NN, Nawa K: Hematopoietic stem cells expand during serial transplantation *in vivo* without apparent exhaustion. Curr Biol 7:805, 1997

18. Krosl J, Baban S, Krosl G, Rozenfeld S, Largman C, Sauvageau G: Proliferation and transformation by *HOXB4* and *HOXB3* genes involves cooperation with PBX. Oncogene 16:3403, 1998

19. Morrison SJ, Wandycz AM, Akashi K, Globerson A, Weissman IL: The aging of hematopoietic stem cells. Nat Med 2:1011, 1996

20. Harrison DE, Lerner CP: Most primitive hematopoietic stem cells are stimulated to cycle rapidly following treatment with 5-fluorouracil. Blood 78:1237, 1991

21. Sauvageau G, Thorsteinsdottir U, Hough MR, Hugo P, Lawrence HJ, Largman C, Humphries RK: Over-expression of *HOXB3* in hematopoietic cells causes defective lymphoid development and progressive myeloproliferation. Immunity 6:13, 1997

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