Overexpression of *HOXB4* in hematopoietic cells causes the selective expansion of more primitive populations in vitro and in vivo

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Hox genes were first recognized for their role in embryonic development and may also play important lineage-specific functions in a variety of somatic tissues including the hematopoietic system. We have recently shown that certain members of the Hox A and B clusters, such as *HOXB3* and *HOXB4*, are preferentially expressed in subpopulations of human bone marrow that are highly enriched for the most primitive hematopoietic cell types. To assess the role these genes may play in regulating the proliferation and/or differentiation of such cells, we engineered the overexpression of *HOXB4* in murine bone marrow cells by retroviral gene transfer and analyzed subsequent effects on the behavior of various hematopoietic stem and progenitor cell populations both in vitro and in vivo. Serial transplantation studies revealed a greatly enhanced ability of *HOXB4*-transduced bone marrow cells to regenerate the most primitive hematopoietic stem cell compartment resulting in 50-fold higher numbers of transplantable totipotent hematopoietic stem cells in primary and secondary recipients, compared with serially passaged *neo*-infected control cells. This heightened expansion in vivo of *HOXB4*-transduced hematopoietic stem cells was not accompanied by identifiable anomalies in the peripheral blood of these mice. Enhanced proliferation in vitro of day-12 CFU-S and clonogenic progenitors was also documented. These results indicate *HOXB4* to be an important regulator of very early but not late hematopoietic cell proliferation and suggest a new approach to the controlled amplification of genetically modified hematopoietic stem cell populations.

[*Key Words*: Homeo box genes; bone marrow transplantation; retroviral gene transfer]

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The lifelong maintenance of mature blood cells results from the proliferative activity of a small number of totipotent hematopoietic stem cells (HSCs) that have a high, but perhaps limited, capacity for self-renewal divisions (Harrison 1982; Mauch and Hellman 1989). Recently, much progress has been made in identifying a variety of cytokines that can regulate the cycling status of primitive hematopoietic cells (Ogawa 1993). However, the genetic mechanisms responsible for the intrinsic control of self-renewal and differentiation outcomes of HSC divisions remain largely undefined.

Increasing evidence points to Hox homeo box genes as playing important lineage-specific roles throughout life in a variety of tissues including the hematopoietic system (for review, see Lawrence and Largman 1992). Hox genes encode transcription factors that are structurally related by the presence of a conserved 60-amino-acid sequence that specifies a helix–turn–helix DNA-binding domain (Levine and Hoey 1988). In mammals, 38 Hox genes are found in four clusters (Boncinelli et al. 1989) referred to as A, B, C, and D (Scott 1992) and during embryogenesis exhibit a site and time-specific pattern of expression that correlates with their relative chromosomal position (referred to as spatial and temporal colinearity).

Expression of a large number of *HOXA*, *HOXB*, and *HOXC* (but not *HOXD*) genes in various hematopoietic cell lines of human origin has now been reported by several groups (for review, see Lawrence and Largman 1992). Such observations are consistent with the possibility that Hox gene products participate in the processes of HSC commitment and differentiation. Support for this concept has come from studies indicating a correlation between the expression of specific Hox genes and the phenotype of different human hematopoietic cell lines.

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For example, *HOXB3* has shown specificity of expression in lines showing erythroid features (Magli et al. 1991; Mathews et al. 1991), and *HOXA10* has shown a similar association with cells exhibiting myeloid properties (Lowney et al. 1991). Involvement of Hox genes in the regulation of proliferation of primary hematopoietic cells has also been reported (Perkins and Cory 1993; Carè et al. 1994). Observed differences in the pattern of expression of Hox genes amongst the major subtypes of human leukemia (Celetti et al. 1993; Lawrence et al. 1995) suggest that they may also play a role in leukemogenesis.

We have recently shown that most HoxA and HoxB cluster genes are expressed in the small fraction of normal human bone marrow cells that are $CD34^+$ (<4%; Civin et al. 1984) and contain most if not all hematopoietic progenitors (Sauvageau et al. 1994). Purification of this $CD34^+$ fraction into functionally distinct subpopulations and RT–PCR-based analysis of Hox gene expression in these cells revealed two patterns of expression: one in which the level of expression of a given Hox gene (e.g. *HOXA10, HOXB9, HOXC8*) was essentially invariant in the different subpopulations and the other in which the expression level was much higher (up to 40-fold) in subpopulations containing the most primitive hematopoietic cells (e.g. *HOXB3, HOXB4*). No gene was found to be up-regulated in the more mature $CD34^+$ cell subpopulations. Moreover, comparison of the levels of expression of selected Hox genes in $CD34^+$ and $CD34^-$ cells showed that Hox gene expression was higher in the $CD34^+$ cells and lower or undetectable in the $CD34^-$ cells. Together, these data suggest that Hox genes undergo down-regulation of expression with hematopoietic differentiation and, furthermore, that some, such as *HOXB3* and *HOXB4*, are almost exclusively expressed in the most primitive bone marrow cells. Interestingly, this apparently highly regulated program of Hox gene expression in hematopoiesis has striking parallels with changes in expression associated with embryonic development (3′ to 5′) (Izpisua-Belmonte et al. 1991) On the basis of these results, we hypothesized that the patterns and levels of Hox gene expression play critical roles in determining primitive hematopoietic cell properties.

To test this hypothesis, we engineered the overexpression of *HOXB4* in murine bone marrow cells by retroviral-mediated gene transfer and then analyzed the effect of this manipulation on the subsequent behavior of these cells and their progeny in vitro and in vivo. The results of these experiments show that the enhanced expression of *HOXB4* can profoundly and selectively increase the proliferative potential of primitive hematopoietic cells without detectable effects on the relative or absolute numbers of mature end cells they generate in vivo.

Results

Retroviral-mediated transduction of HOXB4 *to murine bone marrow cells*

In an effort to achieve increased and persistent expression of *HOXB4* in primitive hematopoietic cells, the human *HOXB4* cDNA was introduced into murine bone marrow cells by retroviral-mediated gene transfer. This *HOXB4* cDNA was chosen on the basis of its availability and its derivation from a hematopoietic tissue (Piverali et al. 1990). Of the 361 amino acids found in the HOXB4 protein, only 9 are divergent between human and mouse and none of these occur within the homeo domain. This high degree of similarity (97%) made it very likely that murine and human *HOXB4* would be interchangeable (Bachiller et al. 1994).

The *HOXB4* cDNA (Piverali et al. 1990) was inserted into the murine stem cell virus (MSCV) retroviral vector 5′ to a phosphoglycerate kinase promoter (PGK)-driven *neo* gene such that *HOXB4* expression was driven from the promotor-enhancer sequences contained within the viral long terminal repeat (LTR) (Fig. 1A). The LTR sequences of MSCV were derived from a myeloproliferative sarcoma virus modified to show enhanced activity in embryonic stem cell lines and were therefore likely to have similar activities in primitive hematopoietic cells (Grez et al. 1990; Hawley et al. 1992). High titer polyclonal viral producer cells were generated from the GP+E-86 ecotropic packaging cell line using standard methods. Integrity of the *HOXB4–neo* retrovirus was verified by Northern blot analysis to detect the expected mRNA in viral producer cells (Fig. 1B) and by Western blot analysis to detect HOXB4 protein in transduced murine (FDC-P1) and human (K562) hematopoietic cell lines (Fig. 1C).

The experimental strategy used to study the effects of *HOXB4* overexpression on the behavior of primitive hematopoietic cells and their progeny is depicted schematically in Figure 2.

Increased proliferative activity in vitro of clonogenic progenitors overexpressing HOXB4

Initial studies examined possible effects of *HOXB4* overexpression in committed clonogenic progenitor cells detected by their ability to give rise to myeloid, erythroid, or myeloid/erythroid colonies in semisolid culture. Bone marrow cells from mice treated 4 days previously with 5-fluorouracil (5-FU) were cocultivated with *HOXB4* or control *neo* viral producer cells and 48 hr postinfection were plated in methylcellulose cultures. In three independent experiments, the gene transfer efficiency to clonogenic progenitors was similar for *HOXB4* and control *neo* viruses, with 58%–70% of colonies showing G418 resistance. *neo*- or *HOXB4*-transduced bone marrow cells did not give rise to colonies in the absence of exogenous growth factors, showing that *HOXB4* overexpression did not render clonogenic cells growth factor independent. Neither were there any differences detected in the proportions of different colony types generated by *HOXB4*- or *neo*-infected cells (assessed by both in situ scoring and by Wright staining of cytospin preparations of individually plucked colonies; data not shown). This suggests that *HOXB4* overexpression also does not alter the ability of committed clonogenic progenitors to complete their differentiation into different types of mature

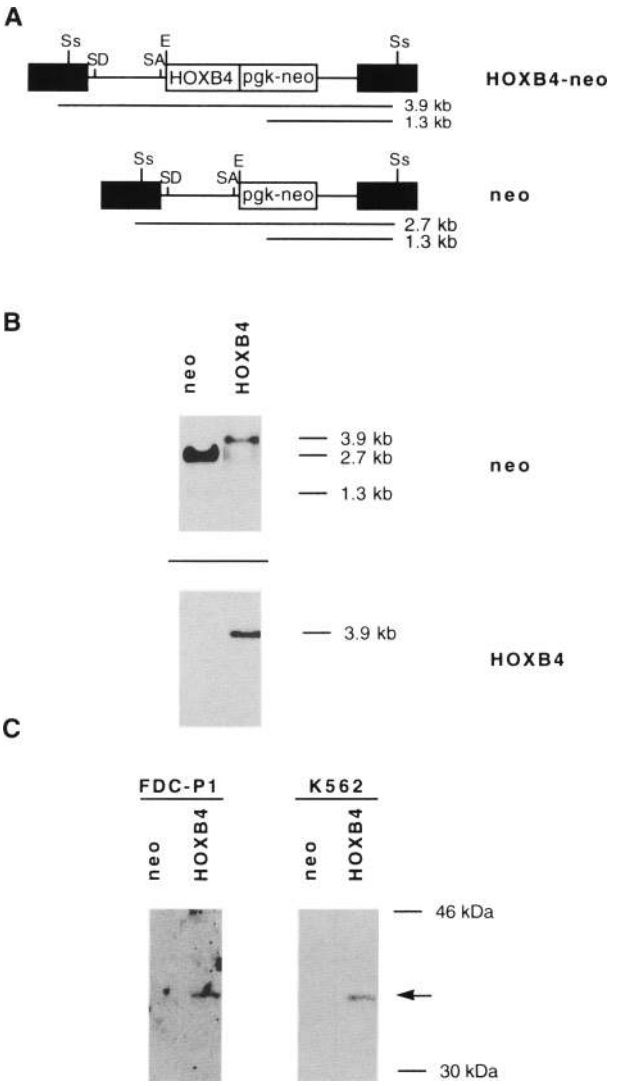

Figure 1. Structure and expression of *HOXB4* and control *neo* retroviruses used in this study. (*A*) Diagrammatic representation of the integrated *HOXB4–neo* and the *neo* proviruses. Expected size of full-length viral transcripts and also those initiated from the PGK promoter are shown. SD and SA denote splice donor and splice acceptor sites. Alternate transcripts derived from these sites are not shown. Restriction sites indicated are *Eco*RI (E) and *Sst*I (Ss). (*B*) Northern blot analysis of the *neo* and *HOXB4–neo* viral producer cell lines. The membrane was sequentially hybridized to a probe specific for *neo* or *HOXB4*. (*C*) Western blot analysis of K562 and FDC-P1 cell lines transduced with the *HOXB4–neo* or the *neo* virus and probed with a polyclonal antibody directed against a *HOXB4* synthetic oligopeptide.

blood cells. However, *HOXB4*-infected cells did give rise to significantly more large (i.e., >1000 cells/colony) granulocyte—macrophage colonies than what was observed in control cultures containing *neo*-transduced cells (41±14% vs. 16±14%, respectively, $n=3$ experiments, $P<0.05$ two-tailed Student *t*-test). The majority of these large colonies had a diffuse morphology. Only rarely could the type of dense colonies with a diffuse halo described by Perkins and Cory (1993), who studied bone marrow cells overexpressing *HoxB8*, be identified in our experiments.

To further characterize the proliferative capacity of the *HOXB4*-infected cells, whole methylcellulose cultures were harvested 7 days after plating and various proportions assayed in secondary methylcellulose cultures. The results obtained from two separate experiments revealed that the cells harvested from the primary assays of *HOXB4*-infected cells were able to generate two- to threefold more secondary colonies than *neo*-transduced control cells obtained from primary assays initiated with the same number of original input cells. Furthermore, one-third (32±8%) of the colonies obtained in the secondary cultures of *HOXB4*-transduced cells were large in size (>1000 cells/colony), whereas the proportion of such colonies in the assays of the replated *neo*-transduced cells was much lower (3.5±0.5%). To assess whether this increase in proliferation reflected a generalized effect of *HOXB4* on the majority of clonogenic progenitors, well-isolated *HOXB4*- and *neo*-transduced G418-resistant colonies were picked at random either 7 (Fig. 3A) or 11 days (Fig. 3B) after initiation of the primary cultures and one-third of each colony was then individually replated into secondary cultures. Under these conditions, <40% of *neo*-transduced colonies replated, yielding a mean of 80 and 180 secondary colonies

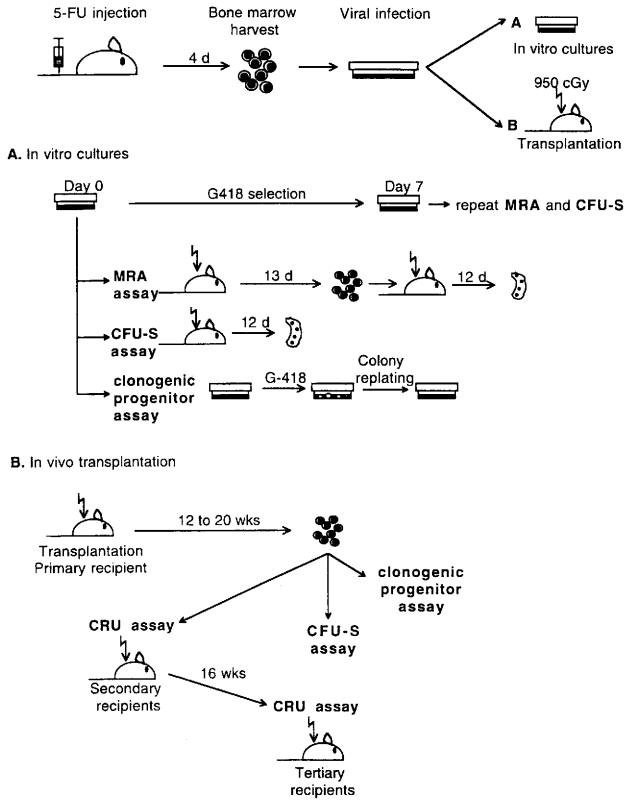

Figure 2. Overview of the experiments and assays used in these studies.

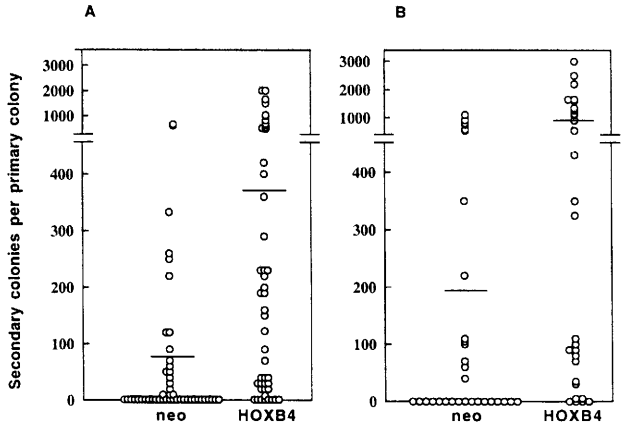

Figure 3. The effect of *HOXB4* overexpression on the ability of individual methylcellulose colonies to form secondary colonies upon replating. In two independent experiments, well-isolated *HOXB4*- or *neo*-transduced colonies from primary cultures were randomly picked after 7 (*A*) or 11 days (*B*) of G418 selection in methylcellulose cultures. Each dot represents the number of secondary colonies generated from each primary colony that was picked and replated in the same culture conditions described above. The calculated mean number of secondary colonies obtained per primary colony is indicated by the broad dash. The difference in means was significant to $P<0.005$ for *A* and *B* (Student *t*-test).

per clone in these two experiments, whereas 80% of the individually analyzed *HOXB4*-transduced colonies replated, generating a mean of 320 and 1000 secondary colonies per clone, respectively, in these two experiments (differences in means significant to $P<0.005$, Student *t*-test).

HOXB4 *effects on the maintenance in vitro of day-12 CFU-S and cells with marrow repopulating ability*

On the basis of the observed increase in the proliferative ability of progenitors with in vitro clonogenic potential following their transduction with *HOXB4*, additional experiments were performed to determine whether similar effects on the behavior of earlier cells detectable as day-12 CFU-S or as cells with marrow repopulating ability (MRA) could be seen. For these studies, infected bone marrow cells were assayed for CFU-S and MRA content immediately after the period of cocultivation with viral producer cells and again after an additional 7 days in liquid culture in the presence of 1.4 mg/ml of G418.

Day-12 CFU-S frequencies of cells harvested immediately after cocultivation with *HOXB4* viral producers were similar to those obtained for the *neo* control (Fig. 4, day 0). However, after maintaining the *HOXB4*- or the *neo*-transduced cells in liquid culture for 1 week, the CFU-S content of the cultures initiated with the *neo*-transduced cells decreased to <1% of input (Fig. 4). In contrast, the day-12 CFU-S content of the cultures initiated with *HOXB4*-transduced cells increased to 200%–500% of input (day 0) values (Fig. 4). As a result, there were ~200-fold more day-12 CFU-S in cultures initiated with the *HOXB4*-transduced bone marrow cells as compared with *neo*-transduced controls at the end of a 7-day period of incubation. Southern blot analysis of DNA extracted from 23 well-isolated spleen colonies produced in recipients of *HOXB4*-transduced cells showed each of the 23 colonies to be uniquely retrovirally marked, indicating that the two- to fivefold net expansion of day-12 CFU-S observed in these cultures was the result of a polyclonal expansion of *HOXB4*-transduced day-12 CFU-S or even an earlier cell type (data not shown). Wright staining of cell preparations obtained from these spleen colonies showed a similar spectrum of late erythroid and myeloid elements compared with *neo* control colonies (data not shown), indicating that *HOXB4* overexpression does not affect the pattern of differentiation that day-12 CFU-S undergo during spleen colony formation in vivo.

The MRA is an assay that measures the ability of a test cell population to regenerate day-12 CFU-S in the bone marrow of a lethally irradiated recipient transplanted 13 days earlier (Fig. 2). The cells thus detected share with HSC a resistance to cycle-active chemotherapeutic drugs (Hodgson and Bradley 1984) and are thought to be precursors of most CFU-S (Mauch and Hellman 1989). MRA measured immediately after infection of bone marrow cells with the *HOXB4* virus (day 0 after cocultivation) was 10-fold higher than that measured for the *neo*-infected cells (Fig. 5). Following 7 days of liquid culture, the MRA content of the *neo*-transduced cells was undetectable (i.e., less than two day-12 CFU-S per femur = background) suggesting that, as for the CFU-S, the maintenance of MRA was compromised under the culture conditions used. In contrast, the MRA of the cells present in the day-7 cultures of *HOXB4*-transduced cells was maintained at readily detectable levels, and although reduced to 25% of input (day 0), this level was

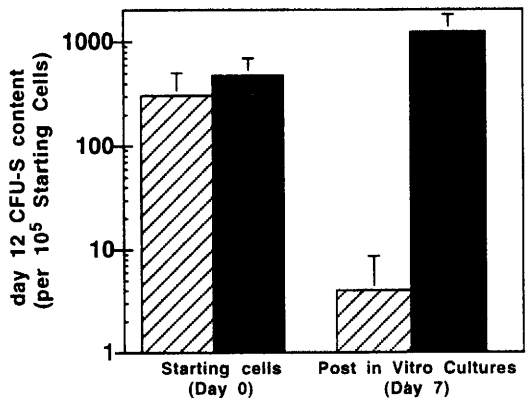

Figure 4. *HOXB4* effects on day-12 CFU-S in vitro. The CFU-S content of recovered cells was assessed immediately after cocultivation (day 0) and also after 7 days in liquid culture and is expressed as day-12 CFU-S colony numbers per 10^5 starting day-0 cells. Results shown represent the mean ±S.D. from four independent experiments.

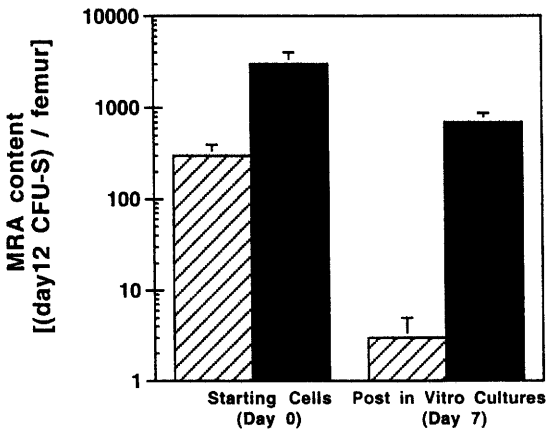

Figure 5. *HOXB4* effects on cells with MRA. MRA was assayed by the content of day-12 CFU-S per femur present in recipients transplanted 13 days previously with *HOXB4*- (solid bar) or *neo*-transduced (hatched bar) bone marrow cells. The MRA of 2×10^5 cells was determined immediately after viral infections (day 0) or after their culture for 7 days. Results shown are the mean ±S.D. of day-12 CFU-S determined in a minimum of 10 recipients.

still >200-fold higher than that present at day-7 in control cultures of *neo*-transduced cells (Fig. 5).

Thus, it appears that the marked (i.e., >2 log) reduction in day-12 CFU-S numbers that occurred when *neo*-transduced cells were maintained in vitro for 7 days was accompanied by a similar reduction in MRA. In contrast, *HOXB4* overexpression reversed this decline leading to a net increase in CFU-S content and a near maintenance of MRA.

HOXB4-*induced expansion in vivo of clonogenic progenitors and day-12 CFU-S*

To assess possible effects of *HOXB4* overexpression on hematopoietic cells maintained for prolonged periods in vivo, *HOXB4*- or *neo*-transduced bone marrow cells were transplanted immediately after infection into lethally irradiated syngeneic recipients and reconstitution of various hematopoietic populations was evaluated (Fig. 2). Each mouse received an innoculum of 2×10^5 marrow cells estimated to contain ~30–40 competitive repopulating units (CRUs), as subsequently determined (see Table 3, below). Gene transfer efficiencies in the transplant marrow as assessed by the proportion of G418-resistant in vitro clonogenic progenitors was 58±8% and 70±7% for *HOXB4*- and *neo*-transduced cells, respectively. This suggests that less than half of the CRUs in the transplant were transduced because frequencies of retroviral infection into these cells are typically lower than into in vitro clonogenic progenitors (Fraser et al. 1993). The extent of donor-derived reconstitution of peripheral blood leukocytes measured 12 or 20 weeks after transplantation was similar (>86%) for animals transplanted with either *HOXB4*- or *neo*-transduced marrow cells. Reconstitution of the hematopoietic system of recipients with both types of transduced cells was confirmed by Southern blot analysis of DNA extracted from bone marrow and spleen cells of mice sacrificed 20 weeks after transplantation (Fig. 6). Northern blot analyses of RNA obtained from these tissues showed high levels of expression of *HOXB4* and *neo* (Fig. 6). Hematopoietic reconstitution by transduced cells was also confirmed by the high frequency of splenic and bone marrow G418-resistant myeloid clonogenic progenitors (75±17% and 42±18%, respectively, for recipients of *HOXB4*- and *neo*-infected cells).

The pre-B and myeloid clonogenic progenitor content of bone marrow 12 weeks after transplantation with *HOXB4*-transduced cells was on average twofold higher than that of recipients of *neo*-infected cells (Table 1). Myeloid clonogenic progenitor numbers in the spleen were also elevated ~10-fold in the one mouse analyzed (Table 1). Twenty weeks after transplantation with *HOXB4*-transduced marrow, an even greater increase in bone marrow and splenic clonogenic progenitor numbers was evident (5- and 32-fold higher, respectively, than in *neo*-transduced marrow recipients). To evaluate whether the increase in clonogenic progenitor cell numbers was accompanied by an expansion of an earlier cell type, day-12 CFU-S frequencies were also assessed in two independent experiments. In each of these, marrow cells from three mice transplanted 16 or 20 weeks earlier with either *HOXB4*- or *neo*-transduced cells were pooled and then assayed. By 16 to 20 weeks, the frequency of CFU-S in the recipients of control cells was back to normal (pretransplantation) levels (i.e., $1.0\pm0.3/10^4$ cells; Chang and Johnson 1991), whereas in mice reconstituted with *HOXB4*-transduced bone marrow cells, the frequency of CFU-S was 5.0- and 7.6-fold higher, respectively, for the two time points.

HOXB4-*induced expansion in vivo of cells with long-term lymphomyeloid repopulating ability*

To determine if *HOXB4* overexpression affects the expansion of the earliest hematopoietic cells, their numbers were quantified by limiting dilution analysis using the CRU assay (Fig. 2) (Szilvassy et al. 1990). For this purpose, various numbers of bone marrow cells pooled from three mice transplanted either with *HOXB4*- or *neo*-transduced marrow cells 12 or 20 weeks earlier were transplanted into lethally irradiated recipients. The presence or absence of lymphomyeloid repopulation (>1%) with donor-derived $Ly5.1^+$ cells in these mice was then evaluated 13 or more weeks later, and CRU frequencies were calculated using Poisson statistics, from the proportion of recipients negative for donor-derived lymphomyeloid repopulation at different cell innocula (Szilvassy et al. 1990).

By 12 weeks post-transplantation, recipients of *neo*-transduced marrow had reconstituted CRU to a frequency of 0.6 in 10^5 bone marrow cells (Table 2) or only 6% that of normal (unmanipulated) mouse marrow (Szilvassy et al. 1990). This low CRU frequency is consistent with the previously well-documented finding that even nonretrovirally infected marrow cells will not

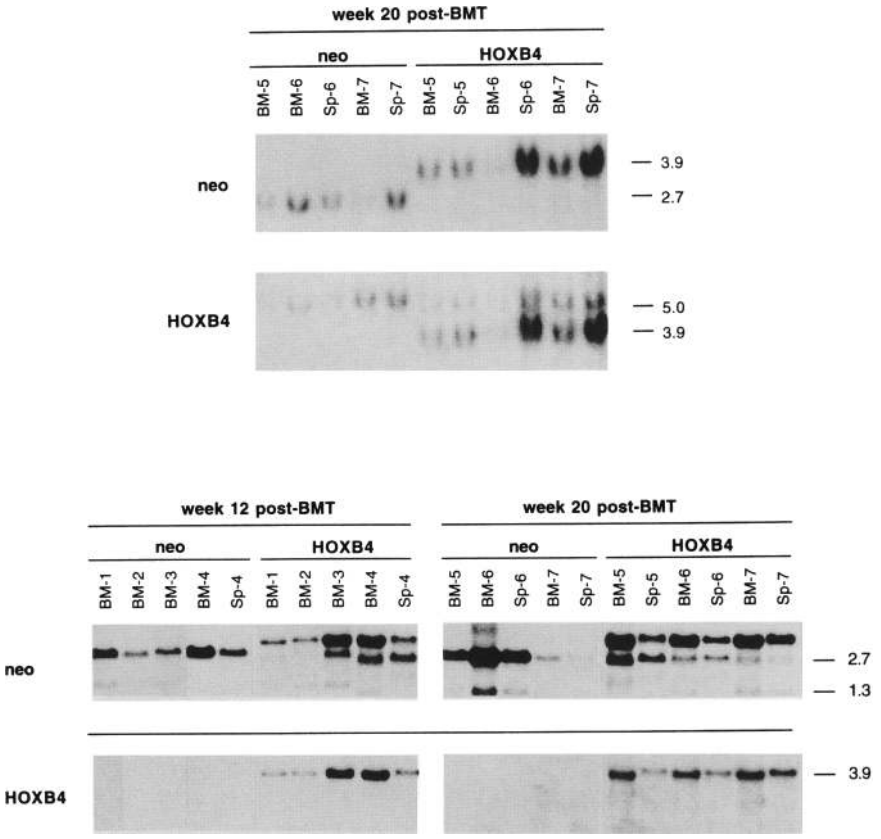

Figure 6. Northern and Southern blot analyses to demonstrate hematopoietic reconstitution by *HOXB4*- or *neo*-transduced bone marrow cells. (*Top*) Southern blot analysis of DNA from bone marrow and spleen cells to demonstrate the presence of integrated virus. DNA was digested with *Sst*I and membranes hybridized to probes specific for *neo* or *HOXB4*. *Sst*I releases the integrated *HOXB4* (3.9 kb) and *neo* (2.7 kb) proviruses. A 5-kb band derived from the endogenous murine *HoxB4* gene cross hybridizing to the human *HOXB4* probe provides a single gene copy control of loading. (*Bottom*) Northern blot analysis to detect expression of transduced *HOXB4* and *neo* genes. Total RNA (5 μg) isolated from bone marrow and spleen cells obtained from mice sacrificed at 12 or 20 weeks post-transplantation was sequentially hybridized to a *neo* and a *HOXB4* probe. In addition to full-length transcripts, an expected 1.3-kb transcript corresponding to the PGK-driven *neo* gene is observed at lower levels in both *neo*- and *HOXB4*-transduced cells. An additional transcript of ~2.7 kb is also observed using a *neo* but not full-length *HOXB4* probe in the cells transduced with the *HOXB4* virus (this transcript is also observed in *HOXB4–neo* viral producer cells; see Fig. 1B); this probably represents a spliced transcipt arising from use of a splice donor site in the MSCV2.1 vector (Hawley et al. 1994). Each number assigned to the various lanes identifies a specific mouse. (BMT) Bone marrow transplantation; (BM) bone marrow; (Sp) spleen.

regenerate CRU numbers to >10% of pretransplant values (Harrison 1982; Harrison et al. 1990; R. Pawliuk and R.K. Humphries, unpubl.). For recipients of *HOXB4*-transduced marrow, a limiting dilution was not reached, suggesting a CRU frequency of >2.9 in 10^5 bone marrow cells, or at least 29% of normal levels, and >4-fold that seen in the recipients of *neo*-infected cells (Table 2).

CRU frequencies in the marrow were also measured in primary recipients sacrificed 20 weeks after transplantation. The CRU frequency of the mice transplanted with

Table 1. *Mice transplanted with* HOXB4*-transduced cells have increased myeloid and pre-B-lymphoid clonogenic progenitors in bone marrow and spleen*

Mouse	Myeloid clonogenic progenitors/femur ($\times 10^3$)	Pre-B clonogenic progenitors/femur	Myeloid clonogenic progenitors/spleen	Pre-B clonogenic progenitors/spleen
		12 weeks post-transplanation		
neo (n = 4)	41 ± 16	2700 ± 1600	595[a]	210[a]
HOXB4 (n = 4)	88 ± 44	5800 ± 800[b]	6650[a]	420[a]
		20 weeks post-transplantation		
neo (n = 3)	41 ± 10		195 ± 47	
HOXB4 (n = 3)	223 ± 30[b]		6018 ± 799[b,c]	

[a]n = 1.
[b]Significantly different from *neo* control ($p < 0.05$).
[c]One mouse was not included in this calculation because it had >100,000 splenic progenitors.

Table 2. *Evaluation by limiting dilution analysis of competitive long-term repopulating cells* (CRU) *in mice transplanted with* HOXB4 *vs.* neo *control-transduced bone marrow cells*

Number of cells injected into secondary recipients	CRU evaluation of primary recipients[a]			
	12 weeks post-transplantation		20 weeks post-transplantation	
	neo	*HOXB4*	*neo*	*HOXB4*
6,700,000	7/7 (30 ± 2)	5/5 (76 ± 21)	N.D.	N.D.
670,000	8/8 (11 ± 8)	5/5 (63 ± 17)	4/4 (20 ± 16)[d]	N.D.
67,000	2/7 (3 ± 0)	3/3 (25 ± 3)	0/6 (<1)	N.D.
33,500	N.D.	N.D.	N.D.	7/7 (37 ± 27)[d]
11,000	N.D.	N.D.	N.D.	5/7 (15 ± 12)
6,600	N.D.	N.D.	N.D.	4/6 (14 ± 20)
CRU frequency per 10^5 cells (range)[b]	0.6 (0.25–1.6)	>2.9	0.3 (0.2–0.5)	14 (10–20)
Relative to normal (%)[c]	6	>29	3	140

Number of cells injected into tertiary recipients	CRU evaluation of secondary recipients[a]	
	16 weeks post-transplantation	
	neo	*HOXB4*
15,000,000	2/3 (3 ± 2)	5/5 (54 ± 5)
1,500,000	3/5 (5 ± 3)	5/5 (31 ± 11)
150,000	1/5 (5 ± 6)	6/6 (8 ± 4)
15,000	N.D.	1/6 (1 ± 1)
1,500	N.D.	0/5 (<1)
CRU frequency per 10^5 cells (range)[b]	0.02 (0.01–0.04)	2.1 (0.8–5.3)
Relative to normal (%)[c]	0.2	21

[a]Results are expressed as number of mice repopulated with donor-derived cells (Ly5.1$^+$) over total. Numbers in parentheses represent the mean % ± S.D. of peripheral blood Ly5.1$^+$ cells found in the transplant recipients.
[b]CRU frequency was calculated using limiting dilution analysis (see Materials and methods)
[c]Compared with control values (Szilvassy et al. 1990).
[d]Mice used for evaluation of CRU amplification in secondary animals were selected from these groups and are identifiable in Fig. 7 as mice number 25.1, 25.2, 25.3, 29.1, 29.2, 29.3, 30.1, and 30.3.

neo-transduced marrow was similar to that obtained at 12 weeks, or ~3% of normal values (Table 2). In contrast, the frequency of CRU in recipients of *HOXB4*-infected marrow was 14 per 10^5 cells. This represents a 1.4-fold increase above normal marrow values and a 47-fold increase above the CRU frequency measured in the marrow of recipients of control cells (Table 2).

Recipient mice used to quantitate CRUs (20 weeks post-transplant; Table 2) were further assessed by Southern blot analysis to identify those repopulated by transduced CRU. Of 14 mice analyzed that received five or fewer CRUs from mice initially transplanted with *HOXB4*-infected marrow, 12 were positive for proviral integration in thymic and/or bone marrow tissue, all of which had been scored previously as positive for donor-derived Ly5.1 lymphomyeloid repopulation in the CRU assay (Fig. 7). Moreover, common proviral integration patterns for thymic and bone marrow tissues were clearly apparent in five of these secondary recipients confirming the lymphomyeloid repopulating potential of the regenerated CRU. The intensities of the proviral integration signals also roughly correlated with the percentages of donor-derived Ly5.1 cells in the peripheral blood [e.g., compare the intense signal for mouse 29.2 (81% Ly5.1$^+$) vs. a much reduced signal for mouse 29.1 (30% Ly-5.1$^+$)], and for two mice (31.3 and 32.1) who were scored as negative for donor-derived lymphomyeloid repopulation, proviral integrants were not detected. This correlation strongly indicates that *HOXB4*-transduced HSCs can terminally differentiate in vivo as shown above for day-12 CFU-S and in vitro clonogenic progenitors. Together these results strongly suggest that the measured CRU expansion in vivo was the result of the selective expansion of *HOXB4*-transduced CRUs. In contrast, four recipients of marrow from primary mice initially transplanted with *neo*-infected bone marrow were all negative for proviral integrants (three of four mice shown; Fig. 7) but positive for donor Ly5.1 cells, indicating that CRU regeneration in the primary mice included nontransduced CRUs.

Self-renewal of *HOXB4*-transduced CRUs was also demonstrated by detection of the same pattern of proviral DNA integration in thymus and bone marrow cells of four different secondary recipients, mice 29.1, 30.3, 33.1,

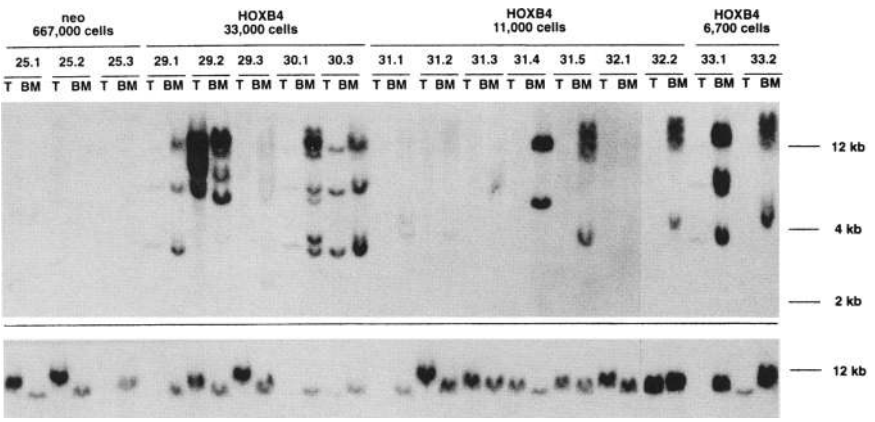

Figure 7. Southern blot analysis of proviral integration patterns in bone marrow and thymic DNA isolated from secondary recipients transplanted with varying numbers of *HOXB4* or *neo*-transduced bone marrow cells. DNA was digested with *Eco*RI that cuts the integrated MSCV provirus once generating DNA fragments specific to each integration site. The membranes were first hybridized to a probe for *neo* (*top*) to identify proviral fragments and subsequently to a probe for the erythropoietin receptor (*bottom*) to provide a control for DNA loading. Exposure times were equivalent for both probes (~1 day). Primary mice used as donors were those sacrificed 20 weeks post-transplantation. The secondary recipients analyzed are as presented in Table 2. The number of bone marrow cells injected into each secondary recipient is indicated at the *top*. Each mouse is identified with a specific identification number. Percentage Ly5.1$^+$ cells in peripheral blood of the mice transplanted with *HOXB4*-transduced marrow are 29.1(30%), 29.2(81%), 29.3(39%), 30.1(62%), 30.3(30%), 31.1(16%), 31.2(3.2%), 31.3(2%), 31.4(29%), 31.5(9%), 32.1(1.1%), 32.2(9%), 33.1(44%), and 33.2(6%). (T) Thymus; (BM) bone marrow.

and 30.1 (Fig. 7). Another uniquely identified totipotent CRU was detected in recipient 29.2. Self-renewal of a CRU with apparent subsequent restriction to the marrow was detected in secondary recipients 31.2, 31.5, 32.2, and 33.2. These latter mice, however, showed lymphomyeloid repopulation by FACS analysis of Ly-5.1-positive peripheral blood leukocytes suggesting that this clone had B-lymphoid and myeloid potential. The degree of self-renewal detected is consistent with the marked expansion of CRUs observed in primary animals (nearly 900-fold, Table 3) and the fact that mice were initially transplanted with small numbers of CRUs (~32, of which at most half would be estimated to have been transduced).

To further assess the regenerative capacity of *HOXB4*-transduced CRUs, their expansion in secondary recipients was also evaluated. Bone marrow cells were harvested from secondary recipients ($n=3$ for *neo* and $n=5$ for *HOXB4*) transplanted 16 weeks earlier with about two to five Ly5.1$^+$ CRUs (Table 2) and CRU frequencies measured by limit dilution analysis in tertiary recipients. CRU frequency in the secondary recipients of *neo*-transduced marrow was 1 in 4.8×10^6 cells (Table 2), or <0.2% of that found in normal unmanipulated marrow, and indicative of a 17-fold expansion over input (summarized in Table 3). In sharp contrast, secondary recipients of *HOXB4*-transduced marrow had a CRU frequency 130 times higher, or ~20% of normal levels, and were indicative of a further 900-fold expansion over input (Table 3).

Despite this dramatic expansion of *HOXB4*-transduced HSCs, it is noteworthy that the relative numbers of the various types of in vitro myeloid clonogenic progenitors (i.e., CFU-GM, BFU-E, and CFU-GEMM) in primary and secondary recipients of *HOXB4*-infected cells were the same as in recipients of control cells. In addition, the total cellularity of the bone marrow was also not different, and the red blood cell, white blood cell, and differential counts were also within the normal range ($n=9$ for each group in the 16- to 34-week observation period; data not shown). Thus, despite a marked and sustained effect of *HOXB4* overexpression on the numbers of myeloid and lymphoid clonogenic progenitors as well as day-12 CFU-S, there was no gross effect on lineage

Table 3. *Expansion of donor-derived CRU in primary and secondary recipients of* HOXB4- *or* neo-*transduced bone marrow cells*

	Donor-derived CRU content per mouse[a]			
	primary recipients (no.)		secondary recipients (no.)	
Virus	CRU transplanted[b]	CRU 20 weeks post-Tx[c]	CRU transplanted	CRU 16 weeks post-Tx[c]
neo	32	600	2	35
HOXB4–neo	32	28,000	5	4700

[a]Results are expressed as number of Ly5.1$^+$ CRU content per mouse considering that one femur represents ~10% of the total marrow of a mouse.
[b]CRU frequency of 5-FU-treated bone marrow cells after cocultivation was measured in a subsequent experiment and was 1 in 6000 cells (95% confidence interval: 1 in 2000 to 1 in 12,000).
[c]Estimated based on data presented in Table 2.

determination nor evidence of a consequent expansion of later cell types. Moreover, despite a significant expansion in the numbers of the most primitive hematopoietic cells, none of the primary recipients ($n=25$) of *HOXB4*-transduced marrow have developed leukemia or any other obvious blood dyscrasia for an observation period now extending to 12 months post-transplantation.

Discussion

Our previous work established that *HOXB4* and several other *HOXA* and *HOXB* genes are preferentially expressed in the most primitive purified subpopulations of $CD34^+$ bone marrow cells. We now demonstrate, using an in vivo murine model, that the engineered overexpression of *HOXB4* can have profound effects on the proliferation of long-term in vivo repopulating HSCs and to a lesser extent on the proliferation of intermediate types of hematopoietic progenitor cells including both lymphoid (pre-B)- and myeloid-restricted populations (Fig. 8). Nevertheless, this deregulation of primitive progenitor cell amplification does not appear to lead to leukemia and is not translated into an altered output of any type of mature blood cell or an altered commitment to any specific blood cell lineage.

Previous studies have shown that even after a single transplantation, the repopulating competence of the regenerated bone marrow cells is reduced ~10-fold (Harrison 1982; Harrison et al. 1990). The reasons for this change are not known, although it has been hypothesized that the sustained proliferative stress imposed on at least some of these cells during the early phase of regeneration of the system may result in a decline in their probability of self-renewal in subsequent divisions. Consistent with these earlier studies, we found that the pool of long-term repopulating stem cells (CRU) regenerated in recipients of *neo*-transduced cells did not recover beyond a level equivalent to 3%–6% of that characteristic of normal mice despite the return to normal levels of bone marrow cellularity and clonogenic progenitors and frequencies.

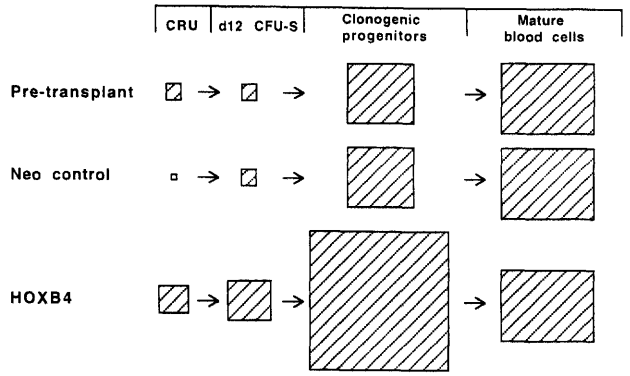

Figure 8. Schematic depiction of the sizes of various hematopoietic populations reconstituted in primary recipients of *HOXB4*- or *neo*-transduced bone marrow cells compared with normal (unmanipulated) mice. Except for the boxes representing the peripheral blood cells that were equivalent in numbers, the surface area of each box is drawn to scale to indicate the relative frequencies of the cell populations.

A different picture emerged in mice transplanted with marrow overexpressing *HOXB4*. Here, CRU numbers recovered to a level that was 1.4-fold higher than the normal value or 47-fold higher than that observed in animals transplanted with *neo*-transduced marrow. The enhanced proliferative capacity of *HOXB4*-transduced CRUs was further demonstrated by serial transplantation studies in which as few as five CRUs transplanted into secondary recipients were shown to be capable of regenerating a significant CRU pool not demonstrably compromised in repopulating ability. In contrast, serial transplantation compromised even further the ability of *neo*-transduced and/or nontransduced CRUs to regenerate CRUs in successive recipients.

Expansion of day 12 CFU-S and in vitro clonogenic progenitors was also documented in the mice transplanted with *HOXB4*-transduced marrow cells. Expansion of these later types of hematopoietic cells might simply be secondary to the expansion of CRUs. However, the fact that clonogenic progenitors transduced with *HOXB4* also replated much better in vitro than those transduced with *neo* strongly suggests that this gene can also directly influence the proliferative capacity of later progenitors. The absence of any perturbation in the proportions of different types of lineage-restricted clonogenic progenitors produced in vivo or the number of mature blood cells present in the circulation of mice repopulated with *HOXB4*-transduced cells strongly suggests that this gene can influence stem cell self-renewal events in the absence of effects on lineage commitment or terminal differentiation. This is consistent with the concept recently proposed by Fairbairn et al. (1993) based on studies with the FDCP-mix cell line that self-renewal and commitment processes may not necessarily be linked at the molecular level. Together with our initial observation showing preferential expression of *HOXB4* in the most primitive bone marrow cell populations (Sauvageau et al. 1994), the present data suggest that the absolute level of *HOXB4* can be a critical determinant of HSC proliferative ability. Interestingly, it was shown recently that inhibition of *HOXB4* expression using antisense oligonucleotides in peripheral blood progenitors compromised the proliferation of these cells (Carè et al. 1994; Giampaolo et al. 1994).

In addition to clear effects on earlier hematopoietic cells in vivo, overexpression of *HOXB4* had marked effects on cells cultured in vitro. Of these, the most striking effect was on the enhanced recovery of day-12 CFU-Ss after 7 days of in vitro culture (Fig. 4). It has been reported previously that the CFU-S content of growth factor-stimulated post-5-FU-treated bone marrow cells decreases dramatically with time using similar culture conditions (Bernad et al. 1994). The results obtained here with *neo*-transduced cells are consistent with this report (i.e., there was a decrease of ~2 log in the number of CFU-S at the end of 7 days in culture). However, the CFU-S content of the cultures initiated with *HOXB4*-

transduced cells expanded by two- to fivefold during this same period. Similar differences in the recovery of day 12 CFU-S after in vitro culture were observed in comparisons of non-5-FU-treated bone marrow harvested from mice reconstituted previously with *HOXB4*- versus *neo*-transduced marrows (data not shown). To what extent this difference reflects effects on survival versus self-renewal and/or recruitment from earlier cells remains unresolved at this time. Although the exact mechanisms responsible for this phenomenon are currently unclear, the magnitude of the differences observed suggests that this system could offer a powerful experimental tool for the identification of *HOXB4* target genes.

Expansion of hematopoietic precursors without a concomitant increase in the number of peripheral blood cells has not been observed previously when the effects of overexpression of various hematopoietic growth factors have been studied in a similar model (Johnson et al. 1989; Wong et al. 1989; Chang and Johnson 1991; Tanaka et al. 1991; Hawley et al. 1992; Fraser et al. 1993). In most of these reports, it was found that overexpression of the cytokines studied resulted in an increase in the numbers of peripheral blood cells but that the content of bone marrow clonogenic progenitors was either unchanged or, in some cases, diminished. Interestingly, overexpression of the nonclustered homeo domain-containing gene *TCL-3* (previously called *HOX11*; Hawley et al. 1994) or *HoxB8* (Perkins and Cory 1993) in murine bone marrow cells was also found to have proliferative effects. In both studies, generation of cell lines from transduced bone marrow cells was observed with high frequency in the presence of interleukin-3 (IL-3). In contrast, efforts to generate cell lines in similar conditions (i.e., high IL-3 concentration) were unsuccessful with *HOXB4* (data not shown). Effects on earlier stem cell populations were not directly assessed in these studies. Interestingly and similar to our findings, mice transplanted with *HoxB8*-transduced marrow cells showed increased levels of bone marrow and splenic clonogenic progenitors at 3 months post-transplantation. Because Hox genes found at the 3′ end of the Hox clusters (e.g., *HOXB4*) are known regulators of more 5′ Hox genes (e.g., *HOXB8*, etc.) (Faiella et al. 1994), it is possible that some of the effects described in the present study may also involve the activation of 5′ genes. This possibility is currently under investigation. A significant proportion of mice transplanted with *HoxB8*-transduced cells, however, developed leukemic transformation at ~7 months post-transplantation. In contrast, no leukemic transformation was observed in our mice even after 1 year of observation and despite persistent high levels of expression of *HOXB4*. Thus, although some similarities are observed between these three studies, our results suggest fundamental differences in *HOXB4*-mediated effects.

Taken together, our results suggest that *HOXB4* is a key regulator of proliferation of long-term repopulating HSCs and that overexpression of this gene does not override the regulatory mechanisms involved in lineage determination or in the control of end cell output. These findings demonstrate that it is possible to reverse the decline of HSCs that normally occurs during regeneration of the hematopoietic system after bone marrow transplantation resulting in a dramatic expansion of genetically modified HSCs in vivo. Given the possibility of examining the function of analogous populations of primitive human hematopoietic cells in immunodeficient mice, it will be of interest to analyze such targets using this approach.

Materials and methods

Animals

Mice used as recipients were 7- to 12-week-old (C57Bl/6J×C3H/HeJ) F_1 [(B6C3)F_1], and those used as bone marrow donors (C57Bl/6Ly-Pep3b×C3H/HeJ) F_1 [(PepC3)F_1] male and female mice bred and maintained in the animal facility of British Columbia Cancer Research Center from parental strain breeders originally obtained from The Jackson Laboratories (Bar Harbor, ME). (B6C3)F_1 and (PepC3)F_1 mice are phenotypically distinguishable on the basis of allelic differences at the Ly5 locus: (B6C3)F_1 mice are Ly5.2 homozygotes and (PepC3)F_1 mice are Ly5.1/Ly5.2 heterozygotes. All animals were housed in microisolator cages and provided with sterilized food and acidified water.

Retroviral generation

The MSCV retroviral vector MSCV 2.1 was kindly provided by Dr. R. Hawley (Sunnybrook Research Institute, Toronto, Canada). The *HOXB4* cDNA region encompassing the complete coding sequence was isolated as a *Bam*HI fragment from a plasmid (kindly provided by Dr. E. Boncinelli, Ospedale S. Faffaele, Milan, Italy) and cloned upstream of the PGK–*neo* cassette at the *Xba*I site of MSCV 2.1 by blunt-end ligation using standard procedures (Davis et al. 1994b). Production of high-titer ecotropic helper-free recombinant retroviruses was carried out using standard procedures (Pawliuk et al. 1994) in the ecotropic GP+E-86 (Markowitz et al. 1988b) and amphotropic GP+envAM12 (Markowitz et al. 1988a) packaging cell lines. The viral titers of the GP+E-86-MSCV2.1–PGK–*neo* and GP+E-86-MSCV2.1–*HOXB4*–PGK–*neo* virus (hereafter called *neo* and *HOXB4–neo*) producer cells were 3×10^6 to 5×10^6 CFUs/ml and 3×10^5 to 5×10^5 CFUs/ml, respectively, as assessed by transfer of G418 resistance to NIH-3T3 cells (Cone and Mulligan 1984). Absence of helper virus generation in the *HOXB4–neo* viral producer cells was verified by failure to serially transfer virus conferring G418 resistance to NIH-3T3 cells (Cone and Mulligan 1984). The absence of helper virus in serum of mice transplanted with *HOXB4–neo*-transduced bone marrow was also confirmed using a rescue assay.

Cell lines

The ecotropic packaging cell line GP+E-86 and the amphotropic cell line GP+envAM12 used to generate the recombinant retroviruses were maintained in HXM medium that consists of Dulbecco's modified Eagle medium (DMEM), 10% heat-inactivated (55°C for 30 min) newborn calf serum (NCS) (GIBCO BRL), 15 μg/ml of hypoxanthine (Sigma), 250 μg/ml of xanthine (Sigma), and 25 μg/ml of mycophenolic acid (Sigma). Virus-producing cells were maintained in HXM medium supplemented with 1 mg/ml of neomycin analog G418, and for the amphotropic cell line 200 μg/ml of hygromycin was added (Sigma). Twenty-four hours prior to harvest of viral supernatant or co-

cultivation with bone marrow cells, virus producer cells were cultured in RPMI with 10% fetal calf serum (FCS) or DMEM with 10% NCS, respectively. Unless specified otherwise, all cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. All media, serum, and growth factors unless otherwise specified were obtained from StemCell Technologies Inc. (Vancouver, B.C., Canada).

Retroviral infection of primary bone marrow cells and hematopoietic cell lines

Bone marrow cells were obtained from (PepC3)F1 (Ly5.1/Ly5.2) mice injected intravenously 4 days previously with 150 mg/kg body weight of 5-FU in phosphate-buffered saline, by flushing femurs and tibias with DMEM 2% FCS using a 21-gauge needle. Single-cell suspensions of 1×10^5 to 5×10^5 cells/ml were then cultured on a petri dish for 48 hr in DMEM containing 15% FCS, 10 ng/ml of human IL-6, 6 ng/ml of murine IL-3, and 100 ng/ml of murine Steel factor. All cells were then harvested and plated at 1×10^5 to 2×10^5 cells/ml in the above medium supplemented with 6 μg/ml of polybrene on viral producer cell monolayers irradiated (1500 cGy X ray) the same day at 80%–100% confluence. Cells were cocultured for 48 hr with a medium change after 24 hr. Loosely adherent and nonadherent cells were recovered from the cocultures by agitation and repeated washing of dishes with Hank's balanced salt solution containing 2% FCS. Recovered bone marrow cells were washed once and then counted using a hemocytometer. All growth factors were used as diluted supernatant from transfected COS cells prepared in the Terry Fox Laboratory.

Transplantation of retrovirally transduced bone marrow

Lethally irradiated 7- to 10-week-old (B6C3)F1 (Ly5.2) mice (950 cGy, 110cGy/min, ^{137}Cs gamma rays) were injected intravenously with 2×10^5 bone marrow cells derived from (PepC3)F1 (Ly5.1/Ly5.2) immediately after cocultivation of these cells with *HOXB4–neo* or *neo* viral producer cells. The levels of Ly5.1 donor-derived repopulation in recipients were assessed 12, 20, and 34 weeks post-transplantation by flow cytometric analysis of peripheral blood samples obtained by tail vein puncture (Rebel et al. 1994). In all animals, >86% of the peripheral blood leukocytes were of donor Ly5.1 origin. At these same time points, peripheral blood cell counts and hematocrits were determined for some of these animals.

In vitro clonogenic progenitor assays

For myeloid clonogenic progenitor assays, cells were plated on 35-mm petri dishes (Greiner, Germany) in a 1.1-ml culture mixture containing 0.8% methylcellulose in alpha medium supplemented with 30% FCS, 1% bovine serum albumin (BSA), 10^{-4}M β-mercaptoethanol (β-ME), 3 U/ml of human urinary erythropoietin (Epo), and 2% SCCM in the presence or absence of 1.4 mg/ml of G418. To ensure random colony selection in single colony replating experiments, cultures were also supplemented with 500 ng/ml of murine IL-3, which abrogates the size difference between colonies. Bone marrow cells harvested from the cocultivation with virus producer cells or from reconstituted transplant animals were plated at a concentration of 2×10^3 cells/dish or 2×10^4 to 4×10^4 cells/dish, respectively. Spleen cells from animals transplanted with *HOXB4–neo-* or *neo*-transduced cells were plated 3×10^5 cells/dish, or 3×10^6 cells/dish, respectively. Colonies were scored on day 12 to day 14 of incubation as derived from CFU-GM, BFU-E, or CFU-GEMM according to standard criteria (Humphries et al. 1981). In two experiments, identification of colony types was confirmed by Wright staining of cytospin preparations of colonies. For pre-B clonogenic progenitor assays, cells were plated in 0.8% methylcellulose in alpha medium supplemented with 30% FSC, 10^{-4} M β-ME and 0.2 ng/ml of IL-7. Pre-B colonies were scored on day 7 of incubation.

CFU-S assay

Day-4 5-FU bone marrow cells were harvested after infection by cocultivation with viral producers and injected immediately into lethally irradiated (B6C3)F1 mice or after 1 week culture at an initial density of 1×10^5 to 5×10^5 cells/ml in 30% FCS, 1% BSA, 10^{-4} M β-ME, 3 U/ml of Epo, 2% SCCM with or without 1.4 mg/ml of G418. The number of cells that each mouse received was adjusted to give 10–15 macroscopic spleen colonies (2×10^3 to 4×10^3 bone marrow cells harvested from cocultivation with virus producer cells or a proportion of the 1-week-old liquid cultures, described above, corresponding to 2×10^3 or 1×10^5 *HOXB4-* or *neo*-transduced input cells, respectively). CFU-S content of bone marrow cells obtained from mice transplanted 20 weeks earlier with *neo-* or *HOXB4*-infected cells was also evaluated by intravenous injection of 2×10^5 or 2×10^4 bone marrow cells/mouse, respectively. Untransplanted lethally irradiated mice were tested in each experiment for endogenous CFU-S surviving irradiation and consistently gave no spleen colonies. Twelve days after injection, animals were sacrificed by neck dislocation, and the number of macroscopic colonies on the spleen were evaluated after fixation in Telleyesniczky's solution .

MRA assay

Lethally irradiated (B6C3)F_1 mice were injected intravenously with 2×10^5 day-4 5-FU bone marrow cells directly after they were harvested from the cocultivation with viral producer cells or with a proportion of these cells kept for 7 days in the liquid culture described above, corresponding to 1.5×10^5 *neo-* or *HOXB4*-infected input cells. Thirteen days later, three mice per group were sacrificed and femoral cells were harvested, counted, and pooled. Dilutions corresponding to various proportions of a femur were then injected intravenously into lethally irradiated recipients for macroscopic spleen colony evaluation as described above. As a control to determine endogenous MRA surviving irradiation in primary recipients, half a femur, pooled from two untransplanted lethally irradiated mice, was assayed in three secondary recipients.

CRU assay

Bone marrow cells pooled from three to four mice transplanted 12, 16, or 20 weeks earlier with *neo-* or *HOXB4*-transduced cells derived from (PepC3)F_1 (Ly5.1/Ly5.2) mice were injected at different dilutions into lethally irradiated (B6C3)F_1 (Ly5.2) mice together with a life-sparing dose of 1×10^5 competitor bone marrow cells from (B6C3)F_1 (Ly5.2) mice. The level of lymphomyeloid repopulation with Ly5.1 donor-derived cells in these secondary recipients was evaluated >13 weeks later by flow cytometric analysis of peripheral blood as described (Rebel et al. 1994) in all experiments but for the secondary recipients where it was evaluated at 5 weeks post-reconstitution. Recipients with ≥1% donor (Ly5.1)-derived peripheral blood lymphoid and myeloid leukocytes as determined by the side scatter distribution of Ly5.1$^+$ cells were considered to be repopulated by at least one CRU. CRU frequency in the test cell population was calculated by applying Poisson statistics to the proportion of

negative recipients at different dilutions as described previously (Szilvassy et al. 1990).

DNA and RNA analyses

Southern blot analyses to assess proviral integration were performed as reported previously (Pawliuk et al. 1994) using standard techniques. High-molecular-weight DNA was digested with *Sst*I that cuts in the LTRs to release the proviral genome or with *Eco*RI that cuts the provirus once to release DNA fragments specific to the proviral integration sites. Total cellular RNA was isolated using TRIzol (GIBCO BRL) and separated using formaldehyde/agarose gel electrophoresis. The RNA was transferred to nylon membrane (Zeta-probe; Bio-Rad), prehybridized, hybridized, and washed as described (Davis et al. 1994a). Probes used were a *Xho*I–*Sal*I fragment of pMC1neo (Thomas and Capecchi 1987), *Kpn*I–*Mse*I fragment of pXM(ER)-190 that releases the full-length erythropoietin receptor cDNA (kindly provided by A. D'Andrea, Dana-Farber Cancer Institute, Boston, MA), and full-length *HOXB4* cDNA labeled as described (Sauvageau et al. 1994).

Western analysis

To detect HOXB4 protein, FDC-P1 and K562 transfected cells were harvested and lysed in cracking buffer (1% SDS, 6 M urea, 1% β-ME, 0.01 M sodium phosphate, pH 7.2). Proteins (5 μg) were subjected to SDS-PAGE in a 12.5% gel and transferred to nitrocellulose membrane. Membranes were incubated with a mixture of two polyclonal antisera (1:5000 dilution for each) raised against peptides deduced from the amino-terminal and carboxy-terminal regions flanking the homeo domain of the HOXB4 protein, respectively (BAbCo, Richmond, CA), and incubation with secondary antibody was coupled to alkaline phosphatase. Each peptide antisera was shown previously to specifically detect HOXB4 expressed as a bacterial fusion protein.

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