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Inability of HOXB4 to enhance self-renewal of malignant B cells: Favorable profile for the expansion of autologous hematopoietic stem cells

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Leukemic stem cells share self-renewal properties and slow proliferation with hematopoietic stem cells. Based on expression signatures, it has been suggested that these cells use the same molecular pathways for these processes. However, it is not clear whether leukemic stem cells also respond to factors known to enhance the self-renewal activity of hematopoietic stem cells. The transcription factor homeobox B4 (HOXB4) is known to induce expansion of mouse hematopoietic stem cells. The recombinant TAT-HOXB4 protein also expands human CD34+ cells. In this study we investigated whether overexpression of *HOXB4* could increase leukemic initiating cell numbers, an issue that is crucial to its clinical usage. A transgenic mouse model for E2A-PBX1 induced pre-B acute lymphoblastic leukemia was used in combination with *HOXB4* transgenic mice to test oncogenic interactions between *HOXB4* and *E2A-PBX1*. The frequency of leukemic initiating cells retrovirally overexpressing *HOXB4* was measured by transplantation at limiting dilution and evaluation of leukemia development in recipient mice. Moreover, human B cell lines were evaluated for their colony forming cell potential upon exposure to TAT-HOXB4 protein. Our data with the mouse models show that HOXB4 neither accelerates the generation of *E2A-PBX1* B cell leukemia nor expands the number of leukemia initiating cells. Additionally, the growth or colony forming cell proportions of human B cell lines was not changed by HOXB4, suggesting that human B leukemic initiating cells are not affected by HOXB4.

Bone marrow (BM) transplantation is used to treat leukemia, lymphoma, and other hematologic diseases. The full long-term regeneration of blood components is dependent on the presence of sufficient numbers of hematopoietic stem cells (HSC) in the graft. HSCs are a rare population of cells residing in the BM that can differentiate into specialized blood cells or self-renew to prevent exhaustion. Currently, most transplantations are performed using peripheral blood stem cells either autologous or from allogeneic compatible donors. However, the potential exploitation of mobilized autologous and cord blood stem cells is often limited by the low number of available stem cells to transplant. Therefore, in the last decade, many efforts have been made to expand HSCs ex vivo to produce clinical meaningful doses of HSCs [1]. Initial investigations on panels of cytokines did not translate into improved engraftment in clinical trials. A second wave of studies explored the intrinsic molecular pathways relevant for self-renewal. Indeed, modulation of the Wnt and Notch signalling pathways has shown positive results for ex vivo expansion [2–4]. In addition, overexpression of developmental genes, such as homeobox B4 (*HOXB4*) and, more recently, sal-like protein 4 (*SALL4*), has resulted in substantial expansions of HSCs in vitro [5,6]. The increase in HSCs following activation of Wnt signalling using Wnt3a is associated with elevated levels of *HOXB4* expression [2], suggesting that self-renewal induced by Wnt is mediated by *HOXB4*. Interestingly, a combination of *HOXB4* and Delta-1 ligand generated even higher numbers of repopulating human CD34+ cord blood cells than either of these

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factors alone [7]. So far, *HOXB4* has been widely studied, and it appears to be a critical factor in HSC self-renewal with a high clinical potential either alone or in combination with other factors.

HOXB4 is a member of the class I homeobox (HOX) gene family. These genes are master control regulators of developmental programs including hematopoiesis [8,9]. Multiple *HOX* genes, including *HOXB4*, are highly expressed in the HSC compartment [10–12]. Mice transplanted with HOXB4 transduced HSCs showed a very competitive and robust engraftment without leukemia development, resultant from the expansion of HSCs [13]. Initial reports documented that BM chimeras for *HOXB4* had 50-fold higher numbers of HSCs able to regenerate the hematopoietic compartment after transplantation [14]. *HOXB4* HSCs continue to expand following transfer to secondary and tertiary recipients, resulting in cumulative expansions of 900-fold [1,14,15]. Importantly, HSC numbers did not expand beyond the levels normally found in mice, indicating that *HOXB4* mediated expansion did not affect the function of HSCs to respond to external signals. Of relevance for therapeutic application, mouse HSCs overexpressing *HOXB4* could expand up to 40-fold during a 12-day period of culture with preservation of their regenerative function [5]. In addition, human HSCs also have been shown to expand in vitro with *HOXB4*, although levels of expansion were more modest than those observed in mice [7,16,17]. In vivo studies using a large animal model confirmed that repopulation of *HOXB4* overexpressing cells was superior to competitive control cells, resulting in a faster recovery of all hematopoietic lineages, including platelets and neutrophils, which normally lag behind [17,18]. A shorter delay in establishment of hematopoietic cell numbers to normal levels might dramatically improve the success of BM transplantations because patients remain particularly vulnerable to infections following transplantation until recovery of the immune system. Importantly for its future use in clinical settings, HOXB4 protein has also been shown to enhance mouse and human HSC numbers in vitro [16,19,20]. Our group has shown that recombinant transactivator of transcription (TAT)-HOXB4 protein could expand HSCs. The protein exerted its effect through preservation of symmetrical divisions, while the global number of divisions decreased [19].

Ex vivo expansion of HSCs using recombinant HOXB4 could be used in multiple clinical settings. This is critical, since patients exposed to intensive chemotherapy or radiation therapy cannot mobilize sufficient numbers of HSCs to allow them to undergo autologous transplantation. Stem cell expansion could increase successful transplantation of such patients. However, there is also a risk that the expansion process would increase not only healthy, but also malignant stem cells. Indeed, although radio- and chemotherapy eradicate most leukemic cells in vivo, malignant relapse is the most frequent complication, indicating that leukemia-initiating cells (L-IC), also referred to as leukemic stem cells, can escape these treatments. Both normal and malignant stem cells have the ability to self-renew to prevent their exhaustion. In fact, it has been hypothesized that L-ICs and normal HSCs might use similar molecular pathways [21]. It is thus critical to test whether *HOXB4* could expand malignant cells, in particular L-ICs. We previously demonstrated that *HOXB4* has some activity as collaborator to oncogene *E2A-PBX1* in the induction of T cell leukemia in mice [22]. Pre-B cell leukemia homeobox (PBX) proteins form a complex with HOX proteins and are known to enhance the affinity of HOX proteins for DNA binding [23]. The motif enabling interaction with HOX proteins is retained in the E2A-PBX1 fusion [24] and deregulation of the HOX-PBX pathway is thought to contribute to human *E2A-PBX1* B cell leukemia. Thus it is likely that *E2A-PBX1* positive malignant B cells might be in particular susceptible to HOXB4. Therefore, we evaluated the effect of *HOXB4* overexpression on the L-IC frequency of mouse B cell leukemias induced by E2A-PBX1 [25]. Our results demonstrate that HOXB4 overexpression does not increase the numbers of L-ICs, nor does it accelerate the initiation of E2A-PBX1 B cell leukemia. Additionally, neither *HOXB4* overexpression nor exposure to TAT-HOXB4 protein influenced the proliferation of human leukemic B cell lines independent of their endogenous HOXB4 levels. Thus, the inability of HOXB4 to expand B lineage malignant stem cells corroborates the low incidence of HOXB4 positive human leukemias and is in agreement with earlier observations of HOXB4 as merely weak oncogenic collaborator. These findings identify HOXB4 as a very promising factor for the expansion of human healthy HSCs and are crucial to its clinical usage in the context of B cell malignancies.

Material and methods

Mice

C57Bl/6 inbred wild type mice were purchased from Jackson Laboratories (Bar Harbor, ME). Transgenic mice for the E2A-PBX1 fusion protein and HOXB4 were generated using the pLIT3 vector, containing TCRβ promoter and μ enhancer elements driving the expression of the transgene in lymphoid cells as previously described [22,25]. E2A-PBX1 transgenic mice were kept on a CD3$\varepsilon^{-/-}$ background to prevent development of T cell leukemia [25]. Mice were bred and maintained in a specific pathogen free animal facility of the Maisonneuve-Rosemont Hospital Research Center. All animal protocols were approved by the local Animal Care Committee.

Human B cell lines

Human pre-B cell line 697, naturally harboring the t(1;19) translocation, was given by Dr. Cees Murre (University of California, San Francisco, CA) and cultured in Opti-Mem (Life Technologies, Burlington, ON, Canada) in the presence of 10% Fetal Bovine Serum (FBS) (Hyclone Laboratories, Logan, UT) and 10^{-5} M β-mercaptoethanol (Mallinckrodt Baker Inc., Phillipsburg, NJ). Non-Hodgkin lymphoma (NHL) B cell lines Raji, DHL-16, and

Namalwa were obtained from the ATCC cell bank and were cultured in RPMI-1640 (Wisent, St-Bruno, QC, Canada) medium supplemented with 10% FBS, 2 mmol/L L-Glutamine, 100 U/mL penicillin (Wisent) and 100 μg/mL streptomycin (Wisent). Medium for Raji cells contained additionally: sodium pyruvate 1 mmol/L, HEPES 10 mmol/L, glucose 4.5 g/L, and sodium bicarbonate 1.5 g/L (all from Life Technologies). Cultures with or without 40 nmol/L recombinant TAT-HOXB4 protein (produced as described above [19]) were performed in bags (American Fluoroseal Corporation, Gaithersburg, MD), as adapted from previously described methods [26]. Bags were placed on an orbital shaker in a standard incubator. TAT-HOXB4 or the carrier were kept at 4°C and automatically injected every 4 hours during the entire culturing period [19]. Cultures were initiated at 2 or 4 x 10^5 cells/mL, counted, and restarted at initial concentration every 2 days. For clonogenic progenitor assays, between 150 and 600 cells were plated in complete methylcellulose medium (Methocult, StemCell Technologies, Vancouver, BC, Canada) and evaluated at day 15.

Immunofluorescence

TAT-HOXB4 internalization was measured by immunofluorescence using a monoclonal antibody directed against HOXB4 (Abcam, Toronto, ON, Canada) and revealed using a secondary anti-rabbit AlexaFluor594 coupled antibody and Dapi (Life Technologies) to color nuclei. Fluorescence was detected on a Zeiss FluoView confocal microscope.

Western blot analysis

Cells were lysed in cell lysis buffer (50 mmol/L Tris-HCL pH8.0, 100 mmol/L NaCL, 1% Triton X 100, 5 mmol/L EDTA [Mallinckrodt Baker, Phillipsburg, NJ]) with Protease inhibitor Cocktail (BD Bioscience, Mississauga, ON, Canada). Proteins were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with a monoclonal anti-HOXB4 antibody (1:1000 dilution; Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, Iowa) followed by a secondary polyclonal anti-rat antibody coupled with horseradish peroxydase (Santa Cruz Biotechnologies, Santa Cruz, CA). For Actin detection, membranes were incubated with a monoclonal anti-Actin (1:1000 dilution, Millipore, Temecula, CA) followed by a secondary polyclonal anti-mouse antibody coupled with horseradish peroxydase (Jackson ImmunoResearch Laboratories, Westgrove, PA).

Flow cytometry and cell sorting

Phenotypic characterizations of primary mouse B cell cultures were performed using the following conjugated antibodies: CD45R/B220-APCCy7, CD43-APC, immunoglobulin M (IgM)–biotin, CD11b-PE (BioLegend, San Diego, CA, or BD Pharmingen, Mississauga, ON, Canada). Biotinylated antibodies were detected with PerCP5.5 conjugated streptavidin (BioLegend). Mortality levels were determined using Dapi (Life Technologies). Fluorescence-activated cell sorting (FACS) analyses were performed on a FACS LSRII using FACSDiva software (BD Bioscience) or FACS Calibur (Beckman Coulter, Mississauga, ON, Canada). Data were further analyzed using FlowJo software (Tree Star, Ashland, OR). All cell sorting was performed on a FACS Aria II with FACSDiva software.

B cell cultures and retroviral infections

Nonleukemic and leukemic B cells derived from *E2A-PBX1* transgenic mice were prestimulated overnight, either in the presence of interleukin (IL)-7 (Life Technologies) or a cocktail of IL-7, Flt3 (Orf Genetics, Reykjavik, Iceland) and Steel factor (all at 10 ng/mL), respectively, followed by retroviral gene transfer during 3-day coculture on packaging cell line GP+E-86 [27] engineered to stably express MSCV-*HOXB4*–green fluorescent protein (GFP) or control MSCV-GFP retroviruses. Non-leukemic cells were then cultured in Opti-Mem media supplemented with IL-7 (10 ng/mL), 10% B cell tested FBS (Stem Cell Technologies), 5 x 10^{-5} M β-Mercaptoethanol, 1X Penicillin-Streptomycin and 50 μg/mL Gentamycin. Doubling times were calculated using the Double Time website [28]. Clonogenic progenitor assays were performed as previously described [10,29] by plating 10^3 and 10^4 cells from *HOXB4* or control B cell cultures in Iscove's modified Dulbecco's medium (IMDM; Life Technologies) containing 1% Methocel MC (Sigma-Aldrich, St. Louis, MO) supplemented with 30% FBS selected for B cells, 10^{-4} M β-mercaptoethanol, 2 mmol/L glutamine and 10 ng/mL IL-7.

Limiting dilution assay

Transfected *E2A-PBX1* leukemic B cells were sorted for GFP and transplanted at limiting dilution in sublethally irradiated (600 cGy) C57Bl/6 recipient mice. Mice received one of the following doses: 1000, 500, 100, 50, 20, 10 or 5 cells. For each dose, three or four mice were transplanted. The frequency of L-IC was calculated using the extreme limiting dilution analysis (ELDA) method for stem cells, which is based on Poisson statistics [30,31].

Quantitative RT-PCR

Total RNA was isolated from human B cell lines using Trizol reagent (Life Technologies) and treated with DNase-I (Life Technologies). cDNA was prepared from total RNA using moloney murine leukemia virus (MMLV) reverse transcriptase and random primers (both from Life Technologies) according to the manufacturer's protocols. Quantitative reverse transcriptase (Q-RT)–polymerase chain reaction (PCR) was performed on an ABI 7500 thermal cycler (Applied Biosystems, Streetville, ON, Canada) using SYBR Green (Applied Biosystems). Oligonucleotides for all *HOX* genes were used according to previously validated sequences [32]. Triplicates were accepted in a 0.5 CT range. Copy numbers are calculated according to the following formula $2^{(38-\Delta\Delta CT)}$.

Statistics

Comparisons of survival curves were performed with a logrank test. Difference in L-IC frequencies was determined using a Chi-square test. All other statistical analyses are performed using a Student *t* test with unequal variance (1-tail, unless indicated otherwise).

Results

HOXB4 transduced non-malignant E2A-PBX1 B cell cultures

To fully assess the effect of HOXB4 on the growth of pre-leukemic and leukemic B cells, we used a transgenic mouse model for pre-B cell leukemia induced by the fusion protein E2A-PBX1 in a *CD3ε*$^{-/-}$ background, which has been previously described by us [25]. These E2A-PBX1 transgenic mice produce T cell leukemias in a T cell competent background, and we have demonstrated before that HOXB4 collaborates with E2A-PBX1 in the development of such T cell leukemias

[22]. Thus, we reasoned that, if HOXB4 has any potential to accelerate B cell leukemogenesis, this should be revealed in the context of the oncogene *E2A-PBX1*. To test whether HOXB4 could enhance the growth in response to IL-7 of non-leukemic *E2A-PBX1* B cells in culture, B220+ B cells were sorted from BM of healthy 3-month-old *E2A-PBX1/CD3ε*$^{-/-}$ mice and cocultured on retroviral producers to stably express *HOXB4*-GFP or GFP (Fig. 1A). For both conditions, high transfection efficiencies were obtained (70.35 ± 15.91% for *HOXB4* and 81.30 ± 6.79% for GFP). Cells were seeded at 0.5–1.0 x10^6 cells/mL and grown for 3 weeks in pro-B cell medium. Enforced overexpression of *HOXB4* resulted in an up to 2000-fold net expansion of *E2A-PBX1* pro-B cells in 20 days, while only a maximum of 100-fold expansion over the initial numbers was observed for GFP cultures (Fig. 1B). The higher growth rate of *HOXB4/E2A-PBX1* pro-B cells resulted in a significantly shorter doubling time ($p = 0.023$; Fig. 1C). The enhanced growth of the *HOXB4/E2A-PBX1* pro-B cultures was supported by a higher number of IL-7 responding clonogenic B cell progenitors compared with control cultures. These primitive B cells represent less than 1% of the *HOXB4* or GFP cultures. Even after 3 weeks, the progenitors expanded in the presence of HOXB4, in contrast to a decrease of progenitors in control cultures (Fig. 1D). Transplantation of 1 or 4 x10^6 cells of *HOXB4/E2A-PBX1* or GFP/ *E2A-PBX1* day 16 pro-B cell cultures into sublethally irradiated recipient mice did not result in leukemia development for over 3 months. At 7 weeks, few transplanted GFP+ cells were detected in the periphery (Supplemental Table E1, online only, available at www.exphem.org), and no leukemia was detected during the monitoring period. Thus, *HOXB4* confers an enormous proliferative potential to non-leukemic *E2A-PBX1* B cells in vitro through expansion of clonogenic B cell progenitors but does not transform *E2A-PBX1* transgenic B cells.

In vivo evaluation of HOXB4 interactions with E2A-PBX1 in B cell leukemia induction

To investigate a potential collaboration between *HOXB4* and *E2A-PBX1* in the development of B cell leukemia, *HOXB4* transgenic mice were crossed with two different lines of *E2A-PBX1/CD3ε*$^{-/-}$ transgenic mice (lines 19 and 23) to obtain compound *HOXB4/E2A-PBX1/CD3ε*$^{-/-}$ mice co-overexpressing *HOXB4* and *E2A-PBX1* in lymphoid cells. Cohorts of single *E2A-PBX1* and compound HOXB4/*E2A-PBX1* transgenic mice were monitored for the development of leukemia. Mice of both *E2A-PBX1*/CD3ε$^{-/-}$ transgenic lines developed B cell leukemia with an average latency time of 293 ± 151 and 410 ± 159 days for lines 19 and 23, respectively (Fig. 2A and 2B). The penetrance of the leukemia in line 19 was complete, although a few mice of line 23 died of non-hematologic causes at an old age, which corresponds to our earlier observations [25] (Fig. 2C). As expected from prior studies [22], single *HOXB4/CD3ε*$^{-/-}$ transgenic mice did not develop leukemia. Interestingly, the age at

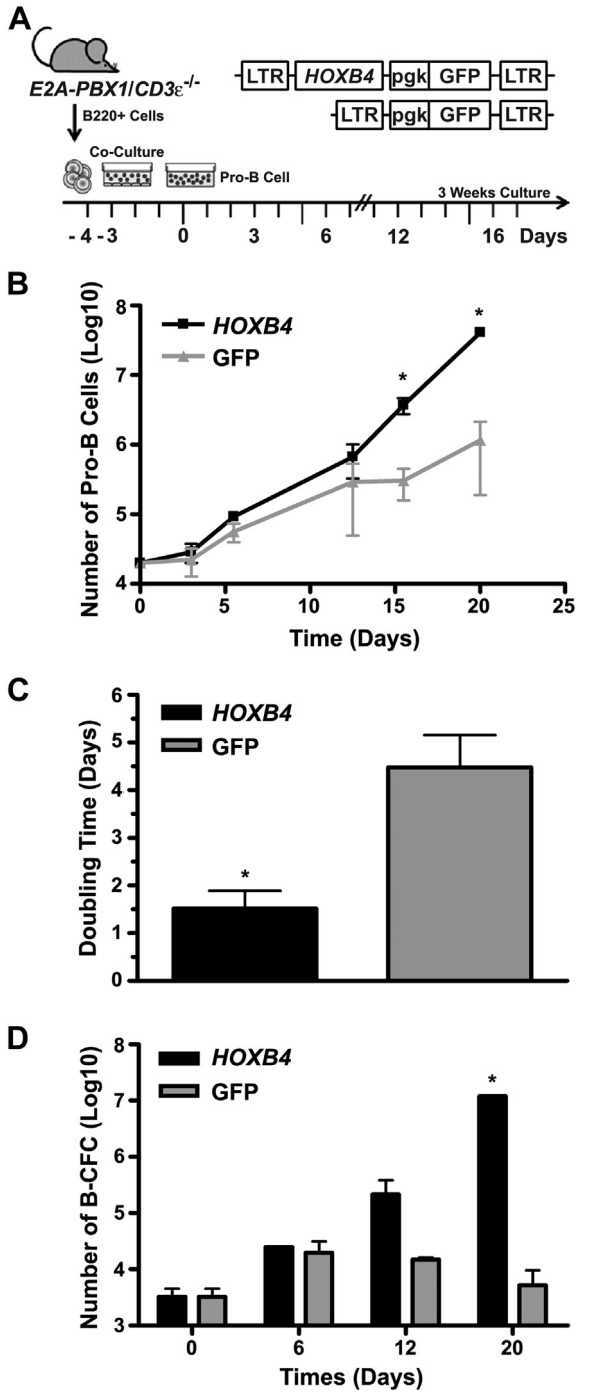
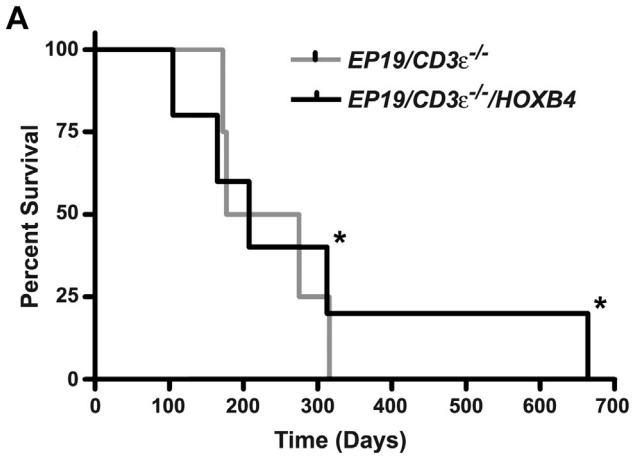
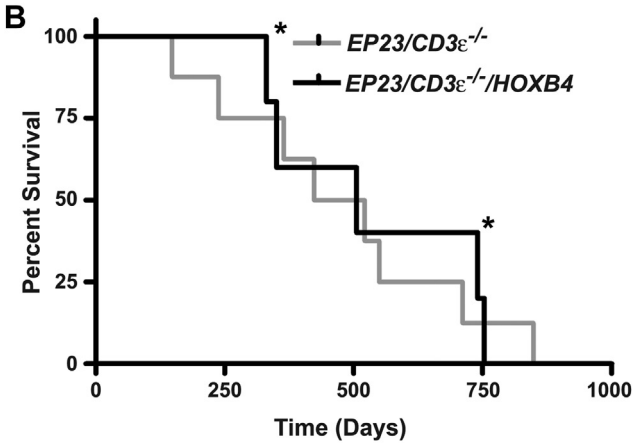


Figure 1. In vitro expansion of *HOXB4* and GFP transduced *E2A-PBX1*/CD3ε$^{-/-}$ B cells. **(A)** Schematic overview of the experimental design and the retroviral vectors. **(B)** Growth curves for *E2A-PBX1* pro-B cell cultures transduced with *HOXB4*-GFP and GFP retroviral vectors (at $t = 15$, $p = 0.04$; $t = 20$, $p = 0.0007$). Numbers were corrected for the fraction of GFP positive cells (>70% for both *HOXB4* and GFP cultures). **(C)** Doubling times for *HOXB4*-GFP and GFP positive *E2A-PBX1*/CD3ε$^{-/-}$ pro-B cell cultures ($p = 0.023$). **(D)** Bar graph depicting the average of B cell progenitors in *HOXB4*-GFP and GFP transduced *E2A-PBX1* pro-B cell cultures (at $t = 20$, $p = 0.0005$). Note that only GFP positive colonies were counted. Data presented in Figure 1 are based on two independent experiments. In total three independent experiments were performed. * $p < 0.05$.





C

Genotype	n	Survival (Days)	Penetrance	Phenotype	Tissue Infiltration
EP23/CD3 ε-/-	24	410 ± 159	88%	B-ALL	yes
EP23/CD3 ε-/-/HOXB4	5	582 ± 221	40%	B-ALL	yes
EP19/CD3 ε-/-	9	293 ± 151	100%	B-ALL	yes
EP19/CD3 ε-/-/HOXB4	5	291 ± 222	60%	B-ALL	yes

Figure 2. Survival curves for compound *E2A-PBX1/HOXB4* transgenic mice. Kaplan-Meyer plots for single *E2A-PBX1* and compound *E2A-PBX1/HOXB4* transgenic mice generated using **(A)** *E2A-PBX1*/CD3ε$^{-/-}$ transgenic line 19 and **(B)** transgenic line 23. Single and compound transgenic mice cohorts included in the survival curves were littermates. *E2A-PBX1* transgenics ($n = 4$; line 19) and ($n = 8$; line 23); *E2A-PBX1/HOXB4* transgenics ($n = 5$; line 19) and ($n = 5$; line 23). **(C)** Details on leukemia for mice cohorts with indicated genotype. Data for the single *E2A-PBX1* transgenic mice from a previously published cohort [25] were additionally incorporated. EP = *E2A-PBX1*. * Mice that were sacrificed for nonhematologic reasons.

which compound *E2A-PBX1/HOXB4/CD3ε*$^{-/-}$ mice succumbed to B cell leukemia was not significantly different from the age single *E2A-PBX1/CD3ε*$^{-/-}$ transgenic mice displayed disease ($p = 0.858$ for line 19 and $p = 0.909$ for line 23). This was not the result of activation of endogenous *HOXB4* expression in *E2A-PBX1* B cell leukemias, which were low to medium (Supplemental Figure E1, online only, available at www.exphem.org). On the contrary, leukemia penetrance tended to be decreased when *HOXB4* was co-overexpressed with *E2A-PBX1* in B cells. These data indicate that *HOXB4* does not collaborate with *E2A-PBX1* in the induction of B cell leukemia.

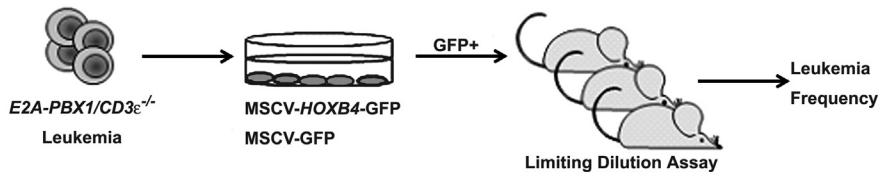
The effect of HOXB4 *on B cell leukemia initiating cells*

Leukemia-initiating cells are thought to cycle slowly and self-renew, as HSCs do. It has been postulated that the same molecular pathways for self-renewal are active in L-ICs as in normal HSCs. Frequently observed relapse of leukemia following current treatments indicates that L-ICs could escape elimination, likely as result of their low proliferation activity. Such leukemia cell persistence and *HOXB4* mediated expansion of residual L-ICs could enhance leukemia relapse. Assessment of changes in the size of the L-IC population induced by *HOXB4* is therefore critical before the use of *HOXB4* in the clinic. To determine whether *HOXB4* could expand L-ICs, the *E2A-PBX1*–induced B-ALL mouse model was further exploited. Leukemic cells derived from BM of a moribund *E2A-PBX1* mouse were transduced with *HOXB4*-GFP or GFP retroviral vectors (Supplemental Figures E2A and E2B, online only, available at www.exphem.org). We sorted GFP+ cells, and cell doses at limiting dilution were transplanted in sublethally irradiated wild type mice to determine the frequency of L-ICs in *HOXB4* and GFP transduced *E2A-PBX1* leukemias (Fig. 3A). Mice that received a graft containing L-ICs became lethally ill between 20 and 60 days. The lowest cell dose that could reinitiate the leukemia in recipient mice and thus contained at least one L-IC was 50 cells for both *HOXB4*-GFP and GFP (Fig. 3B). Two out of seven mice that received 50 GFP-transduced leukemic cells succumbed to leukemia, while only one out of seven mice that received *HOXB4*-GFP leukemic cells became sick. Interestingly, L-IC frequencies in *HOXB4*-GFP leukemic cells were significanlyt lower (1 in 5005) than in GFP transduced leukemic cells (1 in 1111; $p = 0.038$; Chi-square test; Fig. 3C). Through FACS analysis, it was determined that all leukemias expressed the surface markers CD19, B220, and CD43, and were negative for IgM (Fig. 3D). However, retransplantation of *HOXB4*-GFP and GFP *E2A-PBX1* B cell leukemia at limiting dilution showed a higher frequency of L-IC in *HOXB4/E2A-PBX1* leukemia (Supplemental Figure E3, online only, available at www.exphem.org). Thus, although overexpression of *HOXB4* in mouse B cell leukemic cells did not enhance L-IC frequency in vitro, higher numbers of L-ICs were obtained in vivo following leukemia reinitiation.

Evaluation of HOXB4 *overexpression and* HOXB4 *protein exposure on human B cell lines*

The effect of *HOXB4* overexpression was further investigated on human malignant B cell lines to determine whether these correspond to the murine findings. First, the endogenous expression of *HOXB4* mRNA was measured in four human B cell lines derived from pre-B cell leukemia 697,

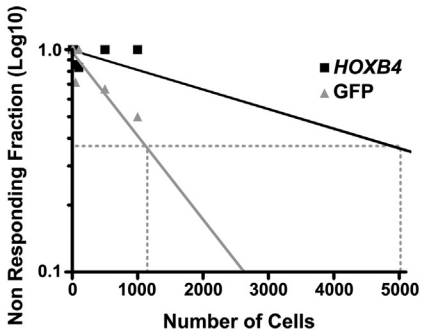
A



B

Vector	Dose	Response	Survival (Days)	Vector	Dose	Response	Survival (Days)
	6 545	1/1	34		20 748	1/1	31
	1 000	0/6	na		1 000	3/6	38.3 ± 1.6
	500	0/6	na		500	2/6	37.5 ± 0.7
HOXB4-GFP	100	1/6	43	GFP	100	0/6	na
	50	1/7	60		50	2/7	41.0 ± 26.9
	20	0/4	na		20	0/4	na
	10	0/4	na		10	0/4	na
	5	0/3	na		5	0/3	na

C



	95 % Confidence		
	Lower	Estimate	Upper
HOXB4-GFP	20 997	5 005	1 193
GFP	2 409	1 111	513

D

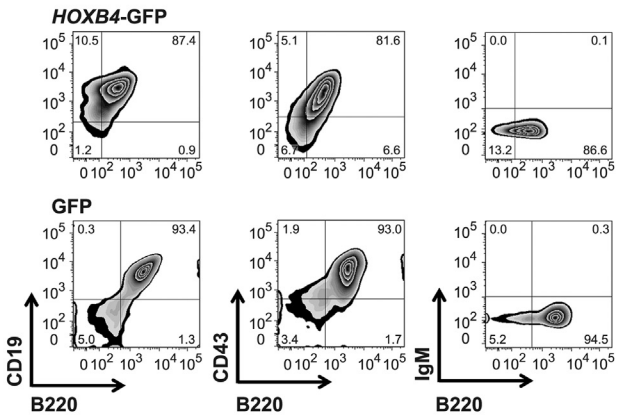


Figure 3. Limiting dilution analyses for *HOXB4*-GFP and GFP *E2A-PBX1*/CD3ε$^{-/-}$ leukemic cells. **(A)** Overview of the experimental strategy used to determine frequency of L-ICs. **(B)** Table showing the fraction of mice that developed leukemia and the average survival time for each dose transplanted with *HOXB4*-GFP (left panel; $n = 21$ total) or GFP (right panel; $n = 21$ total) transduced *E2A-PBX1*/CD3ε$^{-/-}$ leukemic cells. **(C)** Evaluation of L-IC frequency by limiting dilution analysis for *HOXB4*-GFP or GFP *E2A-PBX1*/CD3ε$^{-/-}$ leukemic cells, using Poisson statistics. L-IC is defined as the cell dose that gives 37% of negative mice (where the horizontal dotted line [37%] crosses the curve). Note that the highest dose for each group was used as control for leukemia reinitiation and was excluded for frequency calculations. **(D)** Representative FACS profiles showing the B cell phenotype of *HOXB4* and control leukemia.

which naturally harbours the translocation encoding for *E2A-PBX1*, and NHL (Namalwa, Raji, and DHL-16). Using Q-RT-PCR, it was determined that *HOXB4* was expressed in all four cell lines (Fig. 4A and 4B). Cell lines DHL-16 and 697 expressed low to medium levels of *HOXB4* (CT values 30 and 33, corresponding to 150 and 32 copies per 50 ng total RNA, respectively), while both Raji and Namalwa expressed very high levels of endogenous *HOXB4* transcripts (CT values <25, corresponding to >10000 copies per 50 ng RNA). Protein levels for *HOXB4* correlated with the mRNA expression (Fig. 4B). The low expressing *HOXB4* pre-B cell leukemia line 697 was selected to evaluate whether overexpression of *HOXB4* could enhance its proliferation kinetics. Retrovirally mediated expression of *HOXB4*-GFP in 697 cells resulted in a 200-fold increase in *HOXB4* RNA levels over noninfected cells, resulting in comparable levels of *HOXB4* as measured in Raji cells (Fig. 4A–4C). *HOXB4*-GFP or GFP retroviral transduced 697 cells were seeded in duplicate at a concentration of 10^6 cells/mL and growth was measured for three weeks. In two independent experiments, no differences were observed in the growth rate or doubling times of *HOXB4* and GFP cultures (Fig. 4D and 4E). It is now well recognized that cell lines cultures are not completely uniform, showing some degree of differentiation. Hematopoietic progenitors have the potential to induce colonies, and primary BM cultures are normally supported by the presence of these cells. The ability of cell lines to induce colonies might thus be attributed to more primitive cells on which the maintenance of the cell line depends. Indeed, only 30% of the 697 cells were able

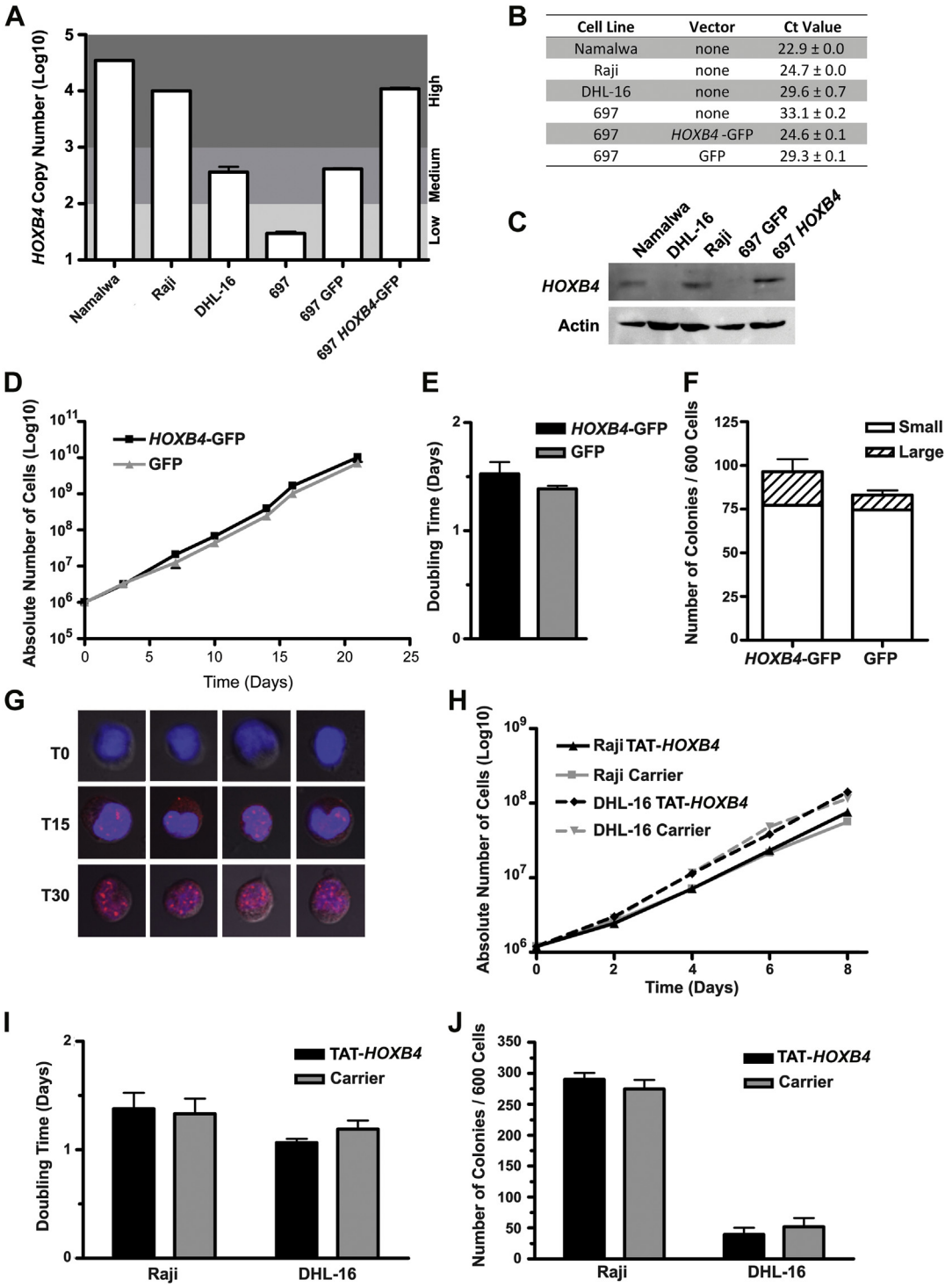


Figure 4. Evaluation of *HOXB4* overexpression or exposure to TAT-HOXB4 on growth of human B cell lines. RNA expression of *HOXB4* in cell lines Namalwa, Raji, DHL16, 697 and 697 transduced with either *HOXB4*-GFP or GFP presented as **(A)** copy numbers or **(B)** Ct values, normalized for GAPDH (Ct = 17). **(C)** Western blotting for *HOXB4*. **(D)** Representative growth curves for *HOXB4*-GFP and GFP transduced B cell line 697. **(E)** Average doubling time of *HOXB4*-GFP and GFP 697 B cells ($n = 3$). **(F)** Average number of CFCs in *HOXB4*-GFP (100 ± 25) and GFP (85 ±17) transduced B cell line 697 per 600 cells ($n = 6$; $p = 0.23$). **(G)** Internalization of TAT-HOXB4 protein by Raji cells at 0 (T0), 15 (T15) and 30 min (T30) following administration. **(H)** Representative growth curves showing absolute number of Raji ($n = 4$) and DHL-16 ($n = 2$) cells treated for 8 days with TAT-HOXB4 protein or carrier as control. **(I)** Average doubling time of Raji ($n = 4$) and DHL-16 ($n = 2$) cells treated with TAT-HOXB4 protein or carrier. **(J)** Number of CFCs per 600 plated Raji ($n = 16$) and DHL16 ($n = 8$) cells treated with TAT-HOXB4 protein or carrier.

to induce colonies in semisolid medium. We took advantage of this property to evaluate the effect of *HOXB4* on the colony forming cell (CFC) potential of 697 cells. Frequencies of CFCs for both GFP and *HOXB4*-GFP–transduced 697 cells were not significantly different (Fig. 4F). Interestingly, a fraction of both GFP and *HOXB4* colonies were clearly larger in size, indicating an increased proliferation potential per cell. The proportion of these large colonies tends to be increased in *HOXB4* cultures (3.9 ± 3.7% for *HOXB4* versus 1.7 ± 1.0% for GFP), but did not reach significance (Fig. 4F, $p = 0.15$). Moreover, the number of cells produced in large colonies of both *HOXB4*-GFP and GFP colonies was equal. Thus, enforced overexpression of *HOXB4* in human pre-B leukemia cell lines did not affect the proliferation rate or the colony forming potential of these cells.

There are still safety issues regarding the use of retroviral vectors in the clinic. Therefore, we have previously generated a recombinant HOXB4 protein to which the TAT sequence of HIV virus was attached, allowing the rapid entry of HOXB4 into the cell. The TAT-HOXB4 recombinant protein conserved the potential to expand mouse [19] as well as human HSCs [26,33]. To test whether HOXB4 expands human malignant B cells, we exposed B cell lines Raji and DHL-16 to TAT-HOXB4 protein, because these lines express different levels of endogenous HOXB4 (Fig. 4A–4C). First, using immunofluorescence, we validated the intracellular localisation of TAT-HOXB4 in Raji cells. Fifteen minutes after the addition of the protein to the medium, TAT-HOXB4 was detected in the cytoplasm and in the nucleus. After 30 min, the localisation of TAT-HOXB4 was predominantly in the nucleus (Fig. 4G). Three independent experiments showed that the growth rate of both B cell lines was not changed in the presence of TAT-HOXB4 during 8 days of culture (the period determined necessary for clinical expansion of HSCs) (Fig. 4H). Doubling times calculated for these cell lines in the presence and absence of HOXB4 were not significantly different (Fig. 4I). Furthermore, the frequency of CFCs was not altered in the presence of TAT-HOXB4 at the end of the culturing period (Fig. 4J). These data show that human malignant B cell lines with different intrinsic levels of HOXB4 expression do not respond to exogenous administered TAT-HOXB4 with increased proliferation or CFC numbers, suggesting that human L-ICs or progenitors from patients with NHL or B cell leukemia will not be enhanced following HSC expansion with HOXB4 protein.

Discussion

BM transplantation is often the best treatment option for patients with relapsed NHL or other hematopoietic neoplasias. The success of BM transplantation is dependent on the number of HSCs in the graft. In particular, patients with NHL are excellent candidates for transplantation, because they still have healthy HSCs. The major advantage of such autologous BM transplantation is the absence of graft-versus-host disease, a lethal disease in many cases. However, numbers of HSCs obtained from peripheral blood after mobilization are limited and need to be enhanced to provide sufficient numbers to sustain long-term repopulation in adult patients. *HOXB4* is a prominent candidate to clinically expand HSCs, and in this study we evaluated whether the properties of *HOXB4* to augment the HSCs would also apply to L-ICs, thus jeopardizing its use for autologous HSC expansion. Using a transgenic mouse model for *E2A-PBX1*–induced B cell leukemia, we first showed that *HOXB4* does not collaborate with *E2A-PBX1* in the development of B cell leukemia, indicating that activation of the *HOXB4* pathway in B cells does not complement *E2A-PBX1* in the generation of L-ICs. This is quite interesting, as *HOXB4* has shown oncogenic interactions with *E2A-PBX1* in the development of *E2A-PBX1*–induced T cell leukemias [22]. Moreover, we recently demonstrated that another member of the *HOX* gene family, *HOXA9*, does collaborate with *E2A-PBX1* in B cell leukemogenesis [34], but, in contrast to *HOXB4*, this gene was not able to accelerate *E2A-PBX1*–induced T cell leukemias [22]. Also, cooperative oncogenic activity has been demonstrated for *HOXA9* and *E2A-PBX1* in myeloid leukemia [35], but this is not clear for *HOXB4*. The oncogenic activity of HOXB4 is very weak in myeloid leukemia and is only apparent in concert with activation of a limited set of oncogenes, such as Meis1 [36], LMO2 and prdm16 [37]. The fact that occasional myeloid leukemias developed in mice that received BM cells coinfected with *HOXB4* and antisense PBX1 [38] might argue against a cooperative role for *HOXB4* and *E2A-PBX1* in myeloid leukemia. These data indicate that the potential oncogenic activities of *HOXB4* are dependent on the presence of specific oncogenes and in a permissive cellular context.

In addition, our in vitro data with non malignant E2A-PBX1 B cells demonstrated that pro-B cells overexpressing *HOXB4* did not acquire leukemic properties, despite enhanced growth as a result of an increase in IL-7–responding progenitor cells. This is the first time that HOXB4 is shown to have a direct impact on B cell progenitor numbers. Previously, human CD34+ cultures were shown to have increased B cell production in the context of *HOXB4* overexpression as result of the expansion of primitive CD34+ lympho-myelo progenitor cells, while late lymphoid progenitors were unaffected by *HOXB4* [39]. Importantly, retroviral mediated overexpression of HOXB4 in established *E2A-PBX1* B cell leukemias did not increase the frequency of L-IC, nor did *HOXB4* overexpression result in an accelerated re-initiation of the disease. On the contrary, the frequency of L-IC was significantly lower in leukemic cells following transduction with *HOXB4*, compared with those transduced with GFP retroviral vectors. However, a significant increase of *HOXB4* L-ICs was observed following retransplantation of *HOXB4* leukemic cells at limiting dilution (Supplemental Figure E3, online only, available at www.exphem.org), indicating that the microenvironment

might play a critical role in *HOXB4* expansion of L-ICs. Furthermore, exposure or overexpression of HOXB4 in human B cell lines did not affect the overall growth or the capacity of primitive cells to induce colonies in semisolid medium. Thus, although L-ICs are thought to use the same self-renewal pathways as HSCs, our data clearly demonstrate that mouse L-ICs do not respond to HOXB4 with expansion as observed in normal HSCs. Moreover, the inability of HOXB4 to modulate the proportion of the primitive cell fraction in human malignant B cell lines strongly indicates that these results can be extrapolated to human primary B L-ICs as well. The absence of promotion of leukemia also confirms the safety of HSC expansion using HOXB4.

The lack of response to HOXB4 seems to be independent of the activation status of HOXB4, as B cell lines expressing either high or medium/low levels of endogenous *HOXB4* behaved similarly. Of note is that *E2A-PBX1* oncogenic collaborator *HOXA9* was expressed at lower levels than HOXB4 in these tested cell lines (Supplemental Figure E4, online only, available at www.exphem.org). Based on observations from several studies evaluating oncogenic and proliferating properties in different cell types, it now becomes clear that *HOX* gene function requires a permissive cellular milieu. Moreover, the biological function of HOXB4 is not the same in different cell types. For example, the expansion of HSCs by HOXB4 is not simply a result of increased proliferation as seen in fibroblasts, but is more likely the consequence of the promotion of self-renewal divisions, because primitive *HOXB4*+ hematopoietic cells proliferate more slowly than their controls [38]. Support for a difference in the molecular context of L-ICs and HSCs came from a study in which gene expression signatures of HSCs and L-ICs were directly compared [21]. This group reported that numerous genes involved in basic cellular and stem cell functions were aberrantly expressed in L-ICs, which may account for their different response to HOXB4. In addition, it has been recently reported that Notch signalling has a divergent effect on HSCs and L-ICs. Using a retroviral BM transplantation model of Notch-induced T cell leukemia causes an initial expansion of HSC and loss of stem cell quiescence, which was followed by a progressive loss of HSCs. A simultaneous increase in T cell progenitors suggests that high Notch signalling promotes T cell development at the expense of other lineages, as well as stem cell self-renewal. On the other hand, Notch induced the generation and maintenance of L-IC in T cell leukemia, indicating that the requirements for Notch in self-renewal of HSCs and L-ICs are different, which may involve the absence or presence of c-Myc [40].

In conclusion, we showed that L-ICs of B cell origin are not permissive to HOXB4 expansion, which paves the way to clinically expand autologous HSCs from patients suffering B cell malignancies with TAT-HOXB4. However, it still remains to be determined whether leukemic stem cells from other hematopoietic lineages could respond to HOXB4.

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

References

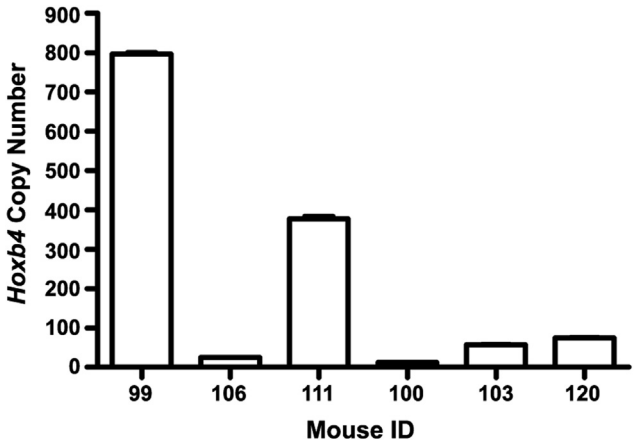
1. Sauvageau G, Iscove NN, Humphries RK. In vitro and in vivo expansion of hematopoietic stem cells. Oncogene. 2004;23:7223–7232.
2. Reya T, Duncan AW, Ailles L, et al. A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature. 2003;423:409–414.
3. Mayani H. Notch signaling: from stem cell expansion to improving cord blood transplantation. Expert Rev Hematol. 2010;3:401–404.
4. Liu J, Sato C, Cerletti M, Wagers A. Notch signaling in the regulation of stem cell self-renewal and differentiation. Curr Top Dev Biol. 2010; 92:367–409.
5. Antonchuk J, Sauvageau G, Humphries RK. HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. Cell. 2002;109:39–45.
6. Aguila JR, Liao W, Yang J, et al. SALL4 is a robust stimulator for the expansion of hematopoietic stem cells. Blood. 2011;118:576–585.
7. Watts KL, Delaney C, Humphries RK, Bernstein ID, Kiem HP. Combination of HOXB4 and Delta-1 ligand improves expansion of cord blood cells. Blood. 2010;116:5859–5866.
8. Argiropoulos B, Humphries RK. Hox genes in hematopoiesis and leukemogenesis. Oncogene. 2007;26:6766–6776.
9. Krumlauf R. Evolution of the vertebrate Hox homeobox genes. Bioessays. 1992;14:245–252.
10. Lebert-Ghali CE, Fournier M, Dickson GJ, Thompson A, Sauvageau G, Bijl JJ. HoxA cluster is haploinsufficient for activity of hematopoietic stem and progenitor cells. Exp Hematol. 2010;38:1074–1086.
11. Bijl J, Thompson A, Ramirez-Solis R, et al. Analysis of HSC activity and compensatory Hox gene expression profile in Hoxb cluster mutant fetal liver cells. Blood. 2006;108:116–122.
12. Sauvageau G, Lansdorp PM, Eaves CJ, et al. Differential expression of homeobox genes in functionally distinct CD34+ subpopulations of human bone marrow cells. Proc Natl Acad Sci USA. 1994;91: 12223–12227.
13. Krosl JK, Faubert A, Sauvageau G. Molecular basis for stem-cell self-renewal. Hematol J. 2004;5(Suppl 3):S118–S121.
14. Sauvageau G, Thorsteinsdottir U, Eaves CJ, et al. Overexpression of HOXB4 in hematopoietic cells causes the selective expansion of more primitive populations in vitro and in vivo. Genes Dev. 1995;9: 1753–1765.
15. Krosl J, Beslu N, Mayotte N, Humphries RK, Sauvageau G. The competitive nature of HOXB4-transduced HSC is limited by PBX1: the generation of ultra-competitive stem cells retaining full differentiation potential. Immunity. 2003;18:561–571.
16. Amsellem S, Pflumio F, Bardinet D, et al. Ex vivo expansion of human hematopoietic stem cells by direct delivery of the HOXB4 homeoprotein. Nat Med. 2003;9:1423–1427.

17. Zhang XB, Schwartz JL, Humphries RK, Kiem HP. Effects of HOXB4 overexpression on ex vivo expansion and immortalization of hematopoietic cells from different species. Stem Cells. 2007; 25:2074–2081.
18. Watts KL, Nelson V, Wood BL, et al. Hematopoietic stem cell expansion facilitates multilineage engraftment in a nonhuman primate cord blood transplantation model. Exp Hematol. 2012;40:187–196.
19. Krosl J, Austin P, Beslu N, Kroon E, Humphries RK, Sauvageau G. In vitro expansion of hematopoietic stem cells by recombinant TAT-HOXB4 protein. Nat Med. 2003;9:1428–1432.
20. Tang Y, Chen J, Young NS. Expansion of haematopoietic stem cells from normal donors and bone marrow failure patients by recombinant hoxb4. Br J Haematol. 2009;144:603–612.
21. Majeti R, Becker MW, Tian Q, et al. Dysregulated gene expression networks in human acute myelogenous leukemia stem cells. Proc Natl Acad Sci USA. 2009;106:3396–3401.
22. Bijl J, Krosl J, Lebert-Ghali CE, Vacher J, Mayotte N, Sauvageau G. Evidence for Hox and E2A–PBX1 collaboration in mouse T-cell leukemia. Oncogene. 2008;27:6356–6364.
23. Chang CP, Brocchieri L, Shen WF, Largman C, Cleary ML. Pbx modulation of Hox homeodomain amino-terminal arms establishes different DNA-binding specificities across the Hox locus. Mol Cell Biol. 1996;16:1734–1745.
24. Kamps MP. E2A-Pbx1 induces growth, blocks differentiation, and interacts with other homeodomain proteins regulating normal differentiation. Curr Top Microbiol Immunol. 1997;220:25–43.
25. Bijl J, Sauvageau M, Thompson A, Sauvageau G. High incidence of proviral integrations in the Hoxa locus in a new model of E2a-PBX1-induced B-cell leukemia. Genes Dev. 2005;19:224–233.
26. Csaszar E, Gavigan G, Ungrin M, et al. An automated system for delivery of an unstable transcription factor to hematopoietic stem cell cultures. Biotechnol Bioeng. 2009;103:402–412.
27. Markowitz D, Hesdorffer C, Ward M, Goff S, Bank A. Retroviral gene transfer using safe and efficient packaging cell lines. Ann NY Acad Sci. 1990;612:407–414.
28. Roth V. Doubling Time. 2006. Available at: http://www.doubling-time.com/compute.php.
29. Fournier M, Lebert-Ghali CE, Krosl G, Bijl JJ. HOXA4 induces expansion of hematopoietic stem cells in vitro and confers enhancement of pro-B-cells in vivo. Stem Cells Dev. 2012;21:133–142.
30. Szilvassy SJ, Humphries RK, Lansdorp PM, Eaves AC, Eaves CJ. Quantitative assay for totipotent reconstituting hematopoietic stem cells by a competitive repopulation strategy. Proc Natl Acad Sci USA. 1990;87:8736–8740.
31. Hu Y, Smyth GK. ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. J Immunol Methods. 2009;347:70–78.
32. Thompson A, Quinn MF, Grimwade D, et al. Global down-regulation of HOX gene expression in PML-RARalpha + acute promyelocytic leukemia identified by small-array real-time PCR. Blood. 2003;101: 1558–1565.
33. Huang CH, Chen PM, Lu TC, et al. Purified recombinant TAT-homeobox B4 expands CD34(+) umbilical cord blood and peripheral blood progenitor cells ex vivo. Tissue Eng Part C Methods. 2010;16: 487–496.
34. Hassawi M, Shestakova EA, Fournier M, et al. Hoxa9 collaborates with E2A-PBX1 in mouse B cell leukemia in association with Flt3 activation and decrease of B cell gene expression. Dev Dyn. 2014; 243:145–158.
35. Thorsteinsdottir U, Krosl J, Kroon E, Haman A, Hoang T, Sauvageau G. The oncoprotein E2A-Pbx1a collaborates with Hoxa9 to acutely transform primary bone marrow cells. Mol Cell Biol. 1999;19:6355–6366.
36. Pineault N, Abramovich C, Ohta H, Humphries RK. Differential and common leukemogenic potentials of multiple NUP98-Hox fusion proteins alone or with Meis1. Mol Cell Biol. 2004;24: 1907–1917.
37. Zhang XB, Beard BC, Trobridge GD, et al. High incidence of leukemia in large animals after stem cell gene therapy with a HOXB4-expressing retroviral vector. J Clin Invest. 2008;118:1502–1510.
38. Cellot S, Krosl J, Chagraoui J, Meloche S, Humphries RK, Sauvageau G. Sustained in vitro trigger of self-renewal divisions in Hoxb4hiPbx1(10) hematopoietic stem cells. Exp Hematol. 2007; 35:802–816.
39. Haddad R, Pflumio F, Vigon I, et al. The HOXB4 homeoprotein differentially promotes ex vivo expansion of early human lymphoid progenitors. Stem Cells. 2008;26:312–322.
40. Chiang MY, Shestova O, Xu L, Aster JC, Pear WS. Divergent effects of supraphysiological Notch signals on leukemia stem cells and hematopoietic stem cells. Blood. 2013;121:905–917.

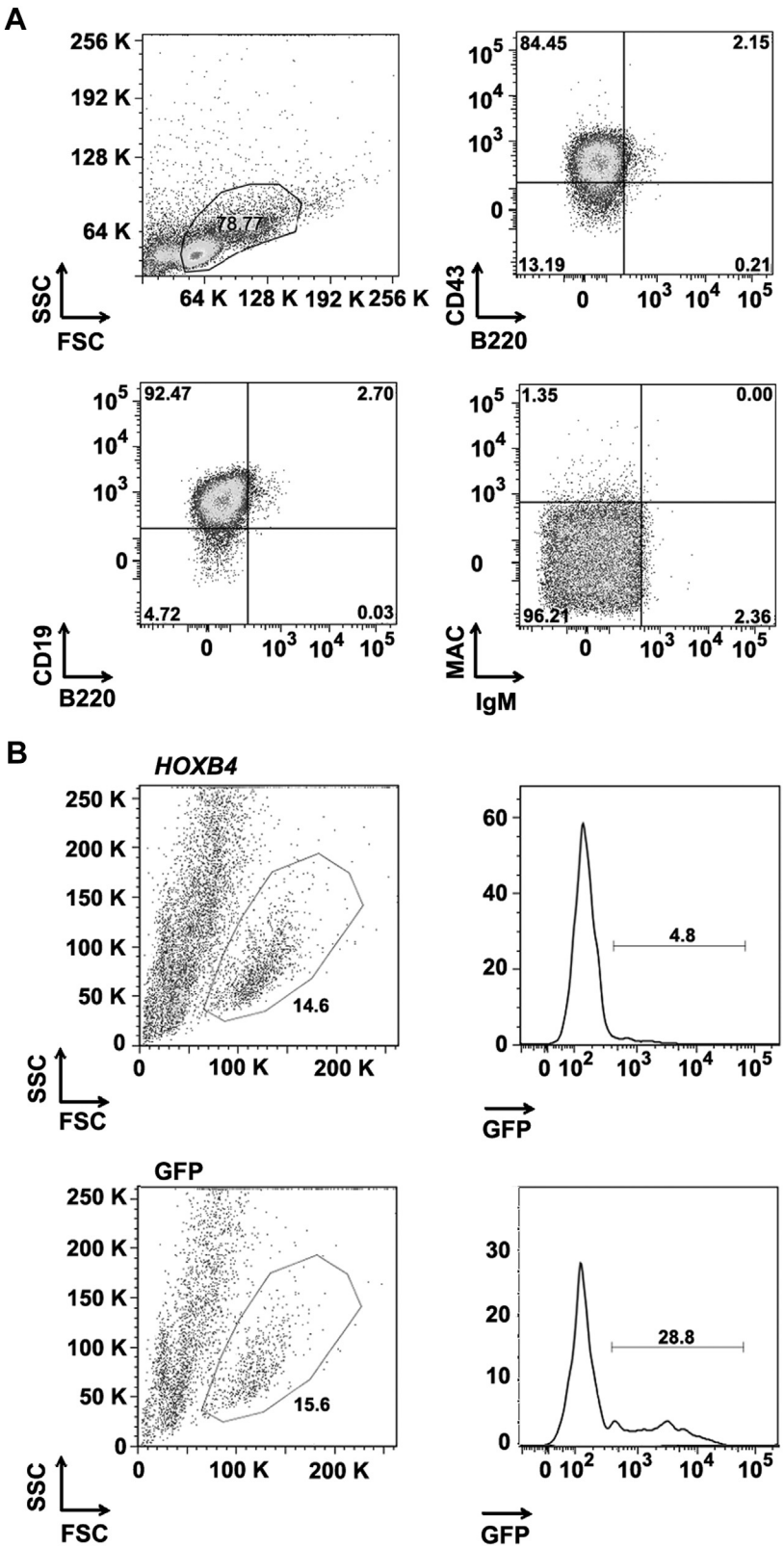
Supplemental Table E1. Monitoring of transplanted *HOXB4* and GFP *E2A-PBX1* pro-B cells in peripheral blood

Cell type	Vector	Dose	n	Survival	GFP percentage	
					Week 3	Week 7
Day 16	MSCV-*HOXB4*-GFP	1.0 x 10^6	3	100%	0.29 ± 0.27	0.10 ± 0.04
Pro-B Cells	MSCV-*HOXB4*-GFP	4.0 x 10^6	2	50%[a]	0.18 ± 0.26	0.16
	MSCV-GFP	1.0 x 10^6	2	100%	0.81 ± 0.86	0.38 ± 0.25
	MSCV-GFP	0.8 x 10^6	1	100%	0.46	0.13

[a]No GFP+ cells detected in hematopoietic organs.



Supplemental Figure E1. Endogenous levels of *HOXB4* expression in *E2A-PBX1* B cell leukemia. mRNA expression levels of endogenous *HOXB4* given in copy numbers for 6 individual *E2A-PBX1* B cell leukemia, normalized for *Gapdh* (Ct = 19). ID = Identity.

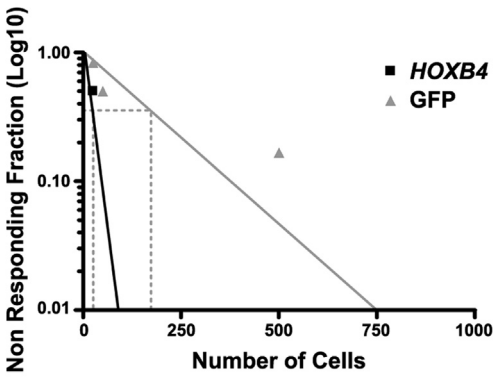


Supplemental Figure E2. Phenotype and transduction efficiency of primary *E2A-PBX1* B cell leukemia. **(A)** FACS profiles showing the B cell phenotype of the primary *E2A-PBX1* leukemia used in *HOXB4*-GFP and GFP limiting dilution assays. **(B)** Sorting plots showing the *HOXB4*-GFP (upper panel) and GFP (lower panel) transduction efficiency of *E2A-PBX1* leukemia.

A

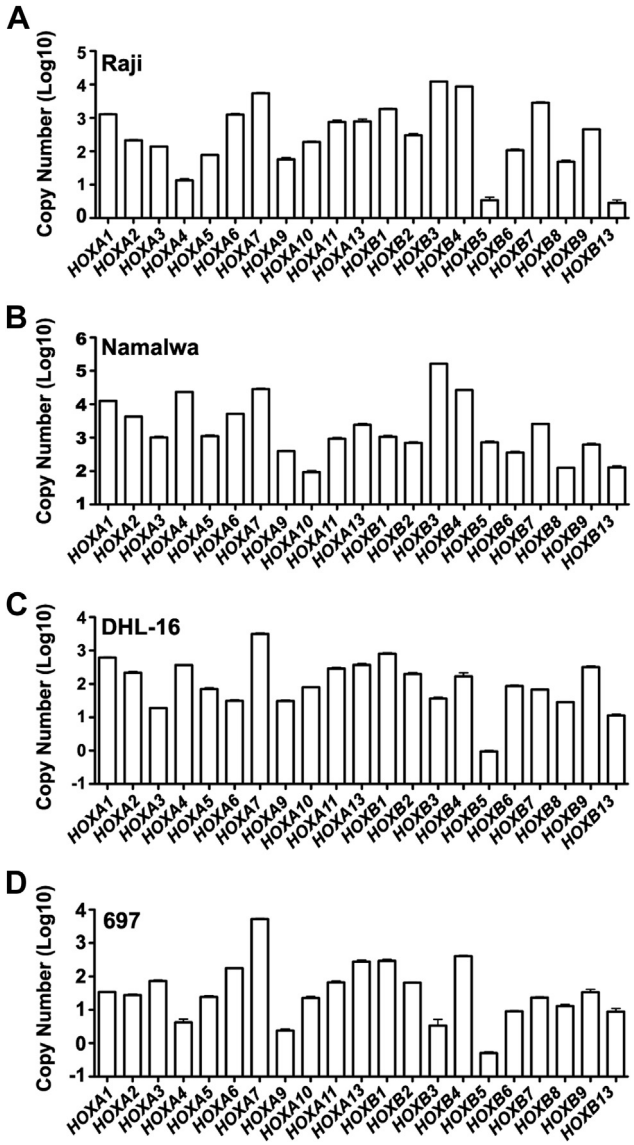
Vector	Dose	Response	Survival (Days)	Vector	Dose	Response	Survival (Days)
HOXB4	1 000	6/6	23.7 ± 1.8	GFP	1 000	6/6	19.0 ± 0.0
	500	6/6	25.2 ± 3.8		500	5/6	18.8 ± 0.4
	50	6/6	32.7 ± 7.5		50	3/6	20.7 ± 2.9
	25	3/6	41.0 ± 5.3		25	1/6	35.0

B



	95 % Confidence		
	Lower	Estimate	Upper
HOXB4-GFP	47.6	23.3	77.6
GFP	389.2	173.6	77.6

Supplemental Figure E3. Limiting dilution analysis for *HOXB4*-GFP and GFP *E2A-PBX1*/CD3ε$^{-/-}$ secondary leukemia. **(A)** Table showing the fraction of mice that developed leukemia and the average survival time for each dose of *HOXB4*-GFP (left panel; $n = 24$) and GFP (right panel; $n = 24$) secondary leukemia. **(B)** Evaluation of L-IC frequency by limiting dilution analysis for *HOXB4*-GFP and GFP secondary *E2A-PBX1*/CD3ε$^{-/-}$ leukemic cells, using Poisson statistics. L-IC is defined as the cell dose that gives 37% of negative mice (where the horizontal dotted line [37%] crosses the curve) ($p = 0.00027$).



Supplemental Figure E4. Endogenous levels of *HOXA* and *HOXB* genes expression in human B cell lines. *HOXA* and *HOXB* mRNA expression in copy number in cell lines **(A)** Raji, **(B)** Namalwa, **(C)** DHL-16, and **(D)** 697, normalized for *Gapdh* (Ct = 17).