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ORIGINAL ARTICLE

Evidence for *Hox* and *E2A–PBX1* collaboration in mouse T-cell leukemia

J Bijl[1,2,3], J Krosl[1], C-E Lebert-Ghali[2], J Vacher[4], N Mayotte[1] and G Sauvageau[1,2,3,5]

[1]*Laboratory of Molecular Genetics of Hematopoietic Stem Cells, Institute for Research in Immunology and Cancer (IRIC), Université de Montréal, Montréal, Quebec, Canada;* [2]*Division of Hematology, Research Center of the Maisonneuve-Rosemont Hospital, Montréal, Quebec, Canada;* [3]*Department of Medicine, Université de Montréal, Montréal, Quebec, Canada;* [4]*Section of Cellular Interactions and Development, Clinical Research Institute of Montreal, Montréal, Quebec, Canada and* [5]*Leukemia Cell Bank of Québec, Maisonneuve-Rosemont Hospital, Montréal, Quebec, Canada*

Using murine Moloney leukemia virus (MMLV)-based proviral insertional mutagenesis, we previously showed a preferential targeting of a small region in the *Hoxa* gene locus in *E2A–PBX1*-induced lymphoid leukemia resulting in the overexpression of several *Hoxa* genes including *Hoxa10*, *Hoxa9* and *Hoxa7*. This observation suggested a functional interaction between *Hox* gene overexpression and *E2A–PBX1* in lymphoid tumors. To further explore this possibility, we generated a series of compound *E2A–PBX1* × *Hox* transgenic mice and tested the genetic interaction between these genes in the generation of lymphoid leukemia *in vivo*. Results presented in this report show that the onset of T-cell leukemia is significantly accelerated in *E2A–PBX1* × *Hoxb4* compound transgenic animals when compared with control *E2A–PBX1* or *Hoxb4* littermates. *Hoxa9* appears less potent than *Hoxb4* to accelerate *E2A–PBX1*-induced T-cell leukemia in mice. *E2A–PBX1*-induced T-cell leukemias express much higher levels of *Hoxa* genes than MMLV-induced counterparts, possibly suggesting a contribution of these genes to T-cell transformation by *E2A–PBX1*. Collectively, these data provide the first genetic evidence showing oncogenic collaboration between *E2A–PBX1* and a *Hox* gene in lymphoid malignancies *in vivo* and document the specific deregulation of a subgroup of *Hoxa* genes in these leukemias.
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Introduction

Chromosome translocations and their corresponding gene fusions play a major role in the initiation of malignant transformation. The translocation t(1;19)(q23;p13) encoding the E2A–PBX1 fusion protein is present in about 6% of all B-cell acute lymphocytic leukemias (ALLs), 25% of pediatric pre-B-ALL, and in rare cases of myeloid and T-cell leukemias (Troussard *et al*., 1995; Look, 1997; Armstrong and Look, 2005). The translocation fuses the transactivation domains of the basic helix–loop–helix transcription factor *E2A* with the C-terminal region of the homeobox gene *PBX1* (Kamps *et al*., 1990; Nourse *et al*., 1990). The *E2A* gene products, E12 and E47, are essential for early B- and T-cell development (Bain *et al*., 1994, 1997), and loss of their activity results in the development of T-cell lymphomas (Bain *et al*., 1997). Studies of *Pbx1*$^{-/-}$ mutant mice revealed that *Pbx1* is also required for the generation of common lymphoid precursors of B, NK (natural killer) and T cells (Sanyal *et al*., 2007). Results of *in vitro* studies suggest that fusion with the E2A domain converts the PBX1 homeodomain protein into a constitutive transcriptional transactivator (van Dijk *et al*., 1993; LeBrun and Cleary, 1994; Lu *et al*., 1994), indicating that the transforming potential of the E2A–PBX1 fusion likely results from the combined effects of haploinsufficiency at *E2A* and *PBX1* loci, and the aberrant transcriptional activity of the E2A–PBX1 fusion protein.

PBX1 is a member of the TALE (for three amino-acid loop extension) family of homeodomain proteins, which also includes several MEIS and PREP members. PBX1, but not E2A–PBX1, forms DNA-binding heterodimers with MEIS1/PREP1 (Chang *et al*., 1997). All members of the TALE family, including E2A–PBX1, also interact with HOX proteins to form multiprotein complexes that participate in the regulation of gene expression. The cooperative interaction between PBX1 and HOX proteins was proposed to enhance the DNA-binding affinity and specificity of HOX proteins (Mann, 1995; van Dijk *et al*., 1995), and is required for the execution of some HOX-mediated functions (Azpiazu and Morata, 1998; Kroon *et al*., 1998; Medina-Martinez and Ramirez-Solis, 2003). Moreover, structure/function analyses showed that one of the E2A–PBX1 domains required for cellular transformation is also critical for cooperative DNA binding with HOX proteins and maps to a short region of PBX1 termed *Hox* Cooperativity Motif (HCM; Chang *et al*., 1997), indicating that E2A–PBX1/Hox interactions may play a role in E2A–PBX1-induced leukemias.

Correspondence: Dr J Bijl, Centre de Recherche Hôpital Maisonneuve-Rosemont, 5415 Boul. De l'Assomption, Montréal, Quebec, Canada H1T 2M4.
E-mail: jbijl.hmr@ssss.gouv.qc.ca
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In a mouse bone marrow transplantation model, overexpression of *E2A–PBX1* resulted in the development of myeloid leukemias with long latency (Kamps and Baltimore, 1993). Supporting the role for *E2A–PBX1*/*Hox* interactions in leukemogenesis, our studies have shown that co-overexpression of *E2A–PBX1* and *Hoxa9* or *Hoxb3* significantly accelerates the onset of the disease (Thorsteinsdottir *et al*., 1999). Lymphoid-restricted overexpression of *E2A–PBX1* induces T-cell lymphomas after approximately 5 months, clearly indicating a requirement for additional genetic events (Dedera *et al*., 1993; Bijl *et al*., 2005). Using murine Moloney leukemia virus (MMLV) insertional mutagenesis in a transgenic mouse model of *E2A–PBX1*-induced pre-B-cell leukemia, we recently reported that all leukemias analysed harbored proviral integrations in a small region of the *Hoxa* locus between *Hoxa7* and *Hoxa9* (Bijl *et al*., 2005). These leukemias expressed high levels of several *Hoxa* genes, suggesting that *Hox* gene products potentially collaborate with *E2A–PBX1* in this disease.

In this study, we directly address whether *E2A–PBX1* genetically interacts with *Hox* genes when co-overexpressed in lymphoid cells.

Results

Description of transgenic mice used in these studies

Genetic interactions between *E2A–PBX1* and *Hoxa9* or *Hoxb4* were studied using transgenic mice that express these transgenes in lymphoid cells. *Hoxa9* and *Hoxb4* were chosen as members of the *Hox* gene family showing potent and weak oncogenic potential, respectively (Kroon *et al*., 1998, 2001; Sauvageau *et al*., 2001; Thorsteinsdottir *et al*., 2002; Pineault *et al*., 2004). A brief summary of the different transgenics lines used in this study is provided in Table 1. *Hoxa9* and *E2A–PBX1* (isoform 1a) transgenic mice were previously described (Thorsteinsdottir *et al*., 2002; Bijl *et al*., 2005). *Hoxb4* transgenic mice were never reported before and they are further described below. All mice used in these experiments were backcrossed over 10 generations and, except for *E2A–PBX1* transgenics, they do not develop spontaneous tumors unless exposed to mutagens such as irradiation (data not shown) or MMLV (see Table 1 and below).

Hoxb4 transgenic mice were generated using the same regulatory elements as described previously for the *Hoxa9* and *E2A–PBX1* lines (see also Figure 1a). Western blot analyses revealed high levels of HOXB4 in the thymus, moderate levels in the spleen, but no detectable expression in the bone marrow (Figure 1b). This expression pattern is reminiscent to that observed with HOXA9 in the lines previously generated in our laboratory (Thorsteinsdottir *et al*., 2002). The impact of *Hoxb4* overexpression on B- and T-cell development was first analysed in adult (>3-month-old) transgenic mice. Fluorescence-activated cell sorting (FACS) analyses revealed that the size of the lymphoid populations were comparable between *Hoxb4* transgenic mice and littermate controls (Figure 1c and data not shown). In addition, no differences were observed in the proportions and absolute numbers of pre-B ($B220^+CD43^+$) and maturing B-cell populations ($B220^+IgM^+$), or in subpopulations of thymocytes ($CD8^-CD4^-$, $CD8^+CD4^+$, $CD8^+CD4^-$ and $CD8^-CD4^+$). The double-negative (DN) populations ($CD8^-CD4^-Lin^-$) were slightly more abundant in the *Hoxb4* transgenic thymus, but the proportions in DN1–4 (from more primitive_1 to mature_4) subpopulations were comparable between *Hoxb4* and control littermates (Supplementary Table 1). In accordance with these observations, similar frequencies of bone marrow cells capable of initiating long-term B-cell cultures (WW-IC) and long-term myeloid cultures (LTC-IC), as well as myeloid clonogenic progenitors were determined for *Hoxb4* and control mice (Figure 1d and Supplementary Table 2). Despite normal mature B-cell populations, a tendency toward reduced B-cell progenitor numbers was observed in *Hoxb4* transgenic mice ($P=0.064$, *t*-test, Figure 1d). Together and within the power of this study, these results suggest that lymphoid and myeloid cell development appears rather normal in our *Hoxb4* transgenic mice.

Proviral insertional mutagenesis in Hoxb4 *and* Hoxa9 *transgenic mice*

Using proviral insertional mutagenesis, we previously reported a high frequency of MMLV integrations in the *Hoxa* locus of lymphoid tumors that developed in our

Table 1 Description of transgenic lines used in this study

Transgenic lines	*No. of lines*	*Previously described*	*Spontaneous leukemia*	*Mean time to leukemia (days)*	*Acceleration of leukemia onset*[a]	
					Irradiation	*MMLV injection*
E2A-PBX1	2	Yes[b]	Yes	188[c]	Yes	Yes
HOXA9	1	Yes[d]	No	>500	Yes	Yes
HOXB4	1	No	No	>500	No	Yes
E2A-PBX1.23/HOXA9	1	No	Yes	149	ND	ND
E2A-PBX1.19/HOXB4	1	No	Yes	103	ND	ND
E2A-PBX1.23/HOXB4	1	No	Yes	90	ND	ND

Abbreviations: MMLV, murine Moloney leukemia virus; ND, not determined.
[a]Acceleration of tumor onset compared with littermate control animals.
[b]Reference Bijl *et al*. (2005).
[c]Mean of two lines of E2A-PBX1 transgenic mice (see Figure 3a).
[d]Reference Thorsteinsdottir *et al*. (2002).

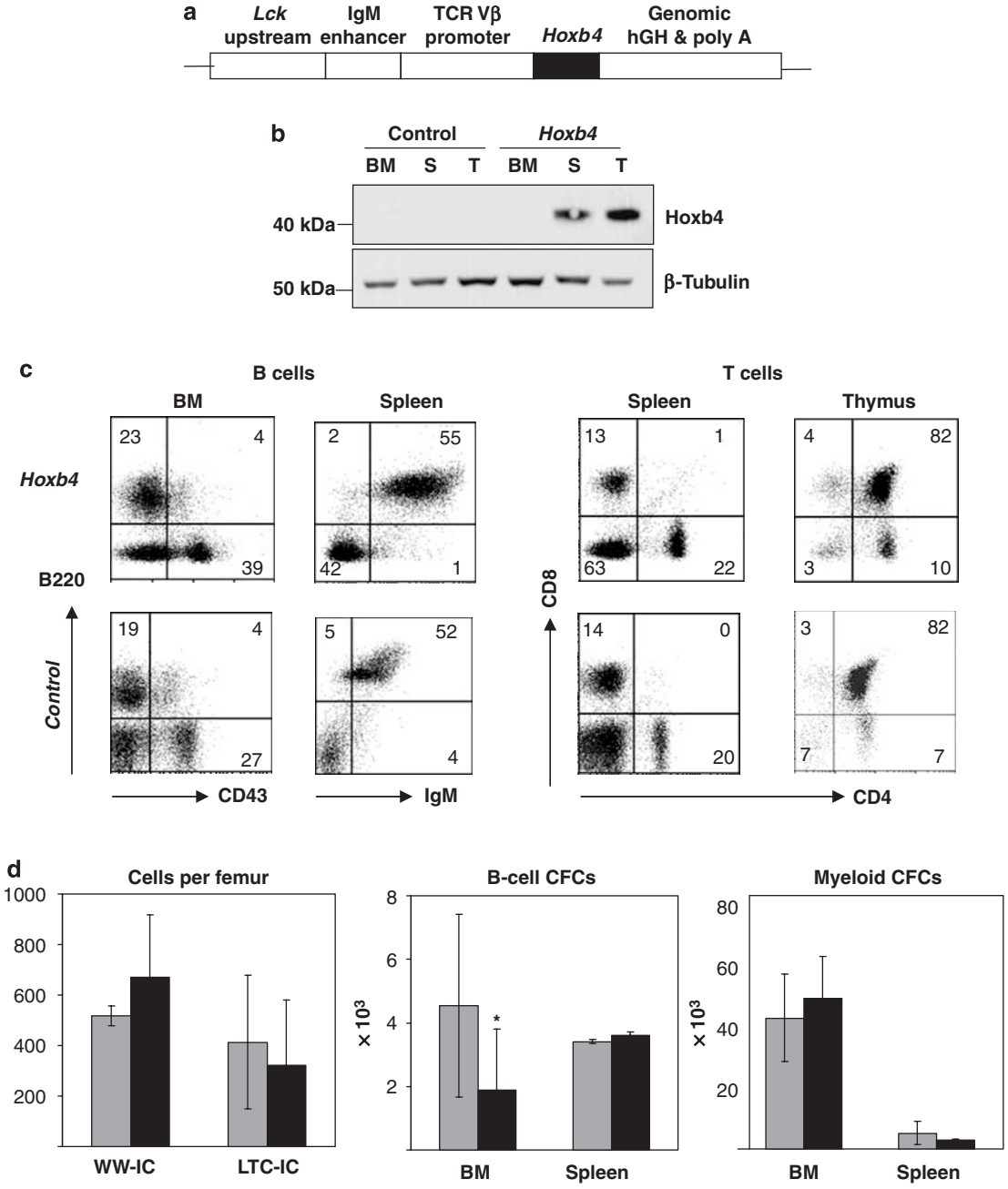


Figure 1 *Hoxb4* transgenic mice. (**a**) Scheme of the transgenic construct with lymphoid promoter and enhancer elements from the TCR Vβ, lck and immunoglobin-μ genes. (**b**) Western blot analysis of Hoxb4 protein levels in bone marrow (BM), spleen (S) and thymus (T) of *Hoxb4* transgenics and control littermates. Tubulin levels are shown as a loading control. (**c**) Fluorescence-activated cell sorting (FACS) profiles of B cells (B220/CD43/IgM) and T cells (CD4/CD8) in the BM, spleen and thymus of *Hoxb4* animals. (**d**) Left panel: determination of early B (Withlock–Witte culture-initiating cells, WW-ICs, $n=3$) and myeloid long-term culture-initiating cells (LTC-ICs, $n=6$) in *Hoxb4* animals and littermate controls; center panel: B-lymphoid clonogenic progenitor numbers in *Hoxb4* animals and littermate controls; right panel: myeloid clonogenic progenitors (colony-forming cells, CFCs, $n=8$) in the BM and spleen of *Hoxb4* animals and littermate controls. *Note nonsignificant reduction in B-cell clonogenic progenitors (two-tail Student's *t*-test, $P=0.064$).

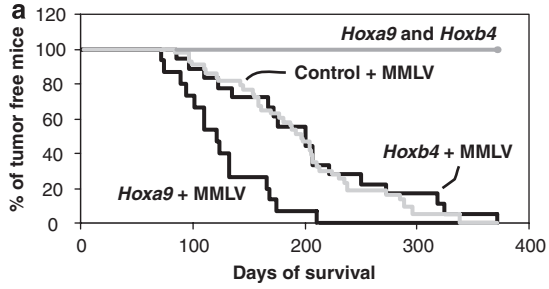
E2A–PBX1 transgenic mice. Using a similar approach, we investigated whether MMLV infection could accelerate the occurrence of lymphoid leukemias in cohorts of *Hoxa9* and *Hoxb4* transgenic mice when compared with control animals. To achieve this, newborn *Hoxa9* ($n=15$) or *Hoxb4* ($n=18$) transgenic mice and their control littermates ($n=43$) were injected intraperitoneally with MMLV, and monitored for disease development. Although no spontaneous lymphoid malignancies were ever observed in *Hoxb4* or *Hoxa9* transgenic animals that had been kept under observation for over 2 years ($n=15$ for *Hoxb4* and $n=13$ for *Hoxa9*), we detected acute leukemia in 100% of the mice injected with MMLV whether they were transgenic (*Hoxa9* and

Hoxb4) or not (Figure 2a). Leukemia occurrence was significantly shorter in *Hoxa9* mice than in *Hoxb4* transgenics with mean survivals of 125 ± 40 versus 195 ± 65 days, respectively ($P < 0.01$, Figures 2a and b). The shortest latency for disease development in *Hoxa9* mice was 72 days, whereas the first *Hoxb4* and control mice succumbed after 86 and 97 days, respectively (Figure 2a). Interestingly, the average onset of MMLV-induced leukemia was similar between the *Hoxb4* transgenic mice and the control group ($P = 0.76$, Figures 2a and b). Diseased animals presented with enlarged thymi and spleens compared with healthy controls that were not injected with MMLV, and showed infiltration of leukemic blasts in their bone marrow, livers, lungs and kidneys (Figure 2b and data not shown). FACS analyses indicated that both MMLV-infected transgenic and control mice succumbed to T-cell leukemias of $CD4^+$, $CD8^+$ or $CD4^+/CD8^+$ phenotypes. Representative FACS profiles from a $CD4^+$ (*Hoxb4*) and a $CD8^+$ (*Hoxa9*) leukemia are presented in Figure 2c.

From this analysis, it appears that MMLV-induced T-cell tumors were accelerated in *Hoxa9* transgenics and not in the *Hoxb4* animals. Southern blot analyses of DNA isolated from MMLV-induced tumors showed that up to 32% of them had rearrangement in the *c-myc* gene. Few rearrangements were also detected in *Pim1*, *Bmi1* and *Notch1* (Supplementary Table 3).

T-cell leukemia development in compound Hox/E2A–PBX1 transgenic mice

To examine if *Hoxa9* or *Hoxb4* can accelerate the development of *E2A–PBX1*-induced lymphoid leukemia, *Hox*(*b4* or *a9*) transgenic mice (1 line of each) were crossed with *E2A–PBX1* transgenic animals (2 lines: 19 and 23) and their progeny were monitored for disease development. Spontaneous T-cell leukemias developed in the two lines of compound *Hoxb4/E2A–PBX1* mice



b Survival and tumor characteristics of transgenic and control mice with leukemia induced by MMLV

Genotype	*n*	Survival (days)	Spleen *P*-value	(g)	Thymus/LN (g)	Tissue infiltration	Tumor phenotype
***Hoxb4* + MMLV**	18	202 ± 81	0.76	0.7 ± 0.4	0.7 ± 0.4	Yes	T-ALL
***Hoxa9* + MMLV**	15	125 ± 40	<0.01	0.6 ± 0.4	0.3 ± 0.4	Yes	T-ALL
Control + MMLV	43	195 ± 65		0.9 ± 0.5	0.3 ± 0.4	Yes	T-ALL
Control or *Hox* tg	30	>400		0.1 ± 0.01	<0.02	NA	NA

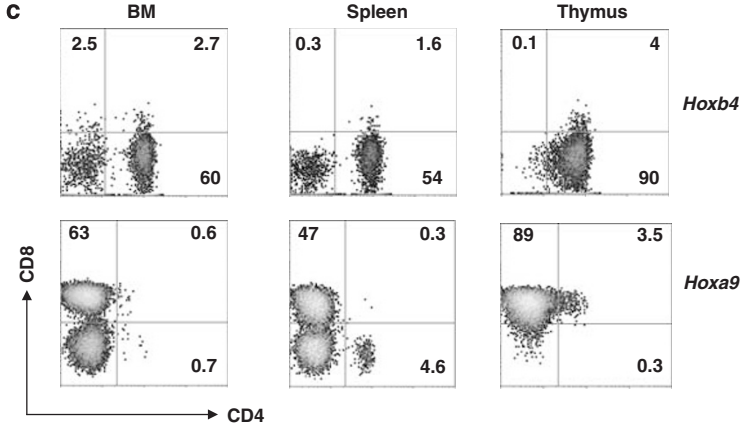
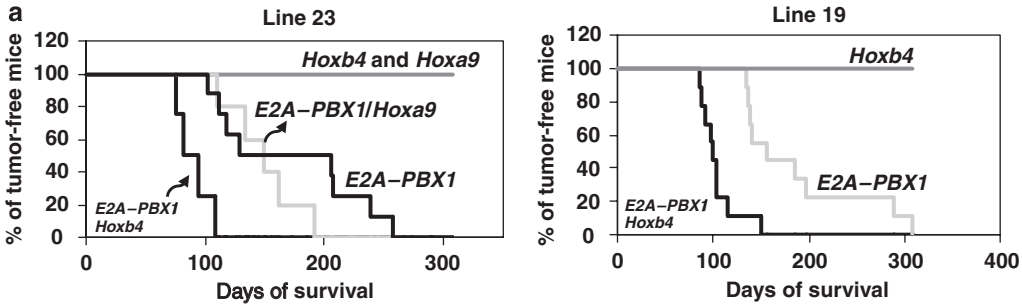


Figure 2 (**a**) Proviral insertional mutagenesis. Survival curves of *Hoxb4* and *Hoxa9* transgenic and their control littermates injected intraperitoneally with murine Moloney leukemia virus (MMLV) 1 day after birth. *Hoxa9* significantly accelerates the onset of the MMLV-induced T-cell leukemia ($P < 0.01$), whereas MMLV-induced leukemia is not accelerated in the *Hoxb4* background. (**b**) Tumor characteristics of MMLV-induced leukemia's in transgenic and normal context. T, thymus; LN, lymph nodes. (**c**). Fluorescence-activated cell sorting (FACS) profiles for bone marrow (BM), spleen and thymus of leukemic mice. Examples of a CD4- and CD8-positive leukemia induced by MMLV in either *Hoxa9* or *Hoxb4* transgenic background are given. Note that the leukemia infiltrated all three organs.



b *Survival and tumor characteristics of transgenic lines for E2A–PBX and compound transgenics*

Genotype	*n*	Survival (days)	*P*-value	Spleen (g)	Thymus/LN (g)	Tissue infiltration	Tumor phenotype
E2A–PBX.19	9	187 ± 66		0.6 ± 0.3	0.3 ± 0.3	Yes	T-ALL
E2A–PBX.19/*Hoxb4*	9	103 ± 20	0.02	0.3 ± 0.3	0.7 ± 0.5	Yes	T-ALL
E2A–PBX.23	8	189 ± 89		0.5 ± 0.5	0.4 ± 0.5	Yes	T-ALL
E2A–PBX.23/*Hoxb4*	4	90 ± 15	0.01	0.4 ± 0.5	0.6 ± 0.3	Yes	T-ALL
E2A–PBX.23/*Hoxa9*	5	149 ± 30	0.36	0.6 ± 0.2	0.2 ± 0.2	Yes	T-ALL

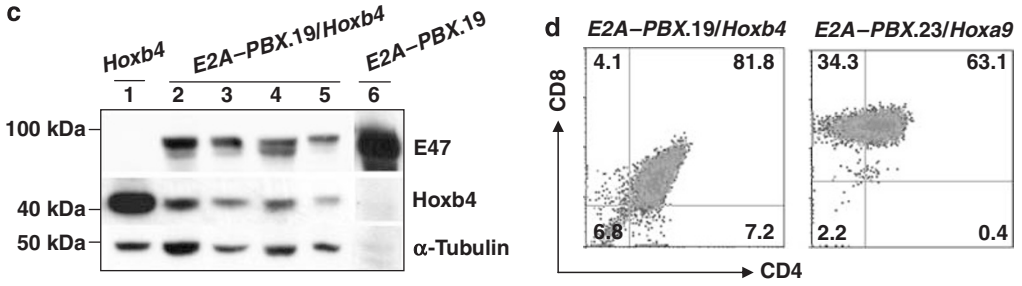


Figure 3 (**a**) Survival curves of compound *E2A–PBX1*/*Hoxb4* and *E2A–PBX1*/*Hoxa9* transgenic mice and single *E2A–PBX1* transgenic mice (lines19 and 23). For single *E2A–PBX1* transgenic mice, only mice that succumbed to T-cell leukemia were included. (**b**) Survival and tumor characteristics of compound and single *E2A–PBX1* transgenic mice with T-cell leukemia. (**c**) Western blot analysis of E2A–PBX1, and Hoxb4 protein levels in total cell lysates derived from thymoctes from a healthy *Hoxb4* transgenic mouse (lane 1), leukemia cells from compound (lanes 2–5) and single transgenic mice (lane 6). Lanes 2 and 3 represent different organs from the same mouse. T, thymus; M, mediastinal, including thymus and lymph nodes; LN_{mes}, mesenterial lymph node. (**d**) Fluorescence-activated cell sorting (FACS) profiles of the T-cell tumors derived from compound transgenic mice. Cells were stained with antibodies directed to CD4 and CD8 T-cell markers. Examples of $CD4^+/CD8^+$ and $CD4^{low}/CD8^+$ leukemias are given for E2A–PBX1-induced leukemias in either *Hoxb4* or *Hoxa9* background, respectively.

with a much shorter latency than observed in both lines of *E2A–PBX1* mice (Figures 3a and b). As a result, compound *Hoxb4*/*E2A–PBX1*.19 and *Hoxb4*/*E2A–PBX1*.23 transgenic mice survived 103 ± 20 and 90 ± 15 days compared with 187 ± 66 and 189 ± 89 days determined for the cohorts of *E2A–PBX1*.19 and *E2A–PBX1*.23 animals, respectively ($P < 0.02$, Figure 3b). The T-cell leukemias that were observed in compound *Hoxb4*/*E2A–PBX1* mice expressed both the E2A–PBX1 (detected by the E47 antibody, Figure 3c) and the HOXB4 proteins (Figure 3c), and they shared similar phenotypes to those isolated from the two lines of *E2A–PBX1* transgenic animals. The majority of these leukemias were $CD4^+CD8^-$, $CD4^-CD8^+$ or $CD4^+CD8^+$ (data not shown, see Figure 3d for two representative profiles and Supplementary Table 4).

T-cell leukemias were also observed in *Hoxa9*/*E2A–PBX1*.23 compound transgenic mice, but the acceleration of disease latency was not as impressive as that determined for *Hoxb4*/*E2A–PBX1*.23 (Figure 3a). Although the collaboration with *Hoxa9* did not reach statistical significance, there was a trend for disease acceleration as indicated by a reduction of 40 days in mean survival time between *Hoxa9*/*E2A–PBX1*.23 (149 days) and *E2A–PBX1*.23 (189 days, Figure 3c) and a noticeable acceleration in lethality for the longest survivors of both cohorts (compare the right portion of the survival curves in Figure 3a for *Hoxa9*/*E2A–PBX1*.23 (gray line) versus *E2A–PBX1*.23). Together, these results indicated that *Hoxb4*, and possibly *Hoxa9*, genetically interact with *E2A–PBX1* in T-cell leukemia in mice.

Hoxa gene expression levels in E2A–PBX1 and MMLV-induced T-cell leukemias

We previously observed *Hoxa* genes deregulation in a mouse model of *E2A–PBX1*-induced leukemia (Bijl *et al*., 2005). Recent evidence further supports a role for *Hoxa* genes deregulation in several subtypes of human and mouse T-cell leukemias (Soulier *et al*., 2005; Speleman *et al*., 2005; Su *et al*., 2006; Van Vlierberghe

et al., 2008), (Dik *et al*., 2005; Bergeron *et al*., 2006; Caudell *et al*., 2007; Cauwelier *et al*., 2007). To gain further insight into the expression levels of all 11 *Hoxa* genes in our T-cell leukemia specimens, we used quantitative reverse transcription–PCR analyses as previously described (Thompson *et al*., 2003). For this analysis, absolute *Hoxa* gene expression levels were first determined in normal unpurified thymocytes ($n=4$ specimens: two wild-type and two *Hoxb4* transgenic mice). This analysis showed that normal thymocytes express high levels of certain *Hox* genes, namely *Hoxa7*, *Hoxa4* and *Hoxa5* all with copy numbers above 500. Other 3′ *Hox* genes, such as *Hoxa1*, *a2*, *a3* and *a6*, are moderately expressed. 5′ *Hox* genes including *Hoxa9*, *a10*, *a11* and *a13* are expressed at very low levels (from 5 to 65 copies, Figure 4a). Although there was a tendency to decreased expression of 3′ *Hoxa* genes (*Hoxa2* to *Hoxa7*) in the presence of the *Hoxb4* transgene, *Hoxb4* overexpression did not significantly change the profile of individual *Hoxa* gene expression in thymocytes except for *Hoxb4* itself as copy numbers differ less than two-fold (thus <1 cycle time; Figures 4a and c).

We then compared *Hoxa* genes and *Hoxb4* expression profiles between T-ALL from two subgroups: MMLV versus *E2A–PBX1* (see Figure 4b). These results showed an important difference between the expression levels of several 3′ *Hox* genes (that is, *Hoxa1* to *Hoxa9*) that are expressed at much higher levels in E2A–PBX1 tumors when compared with MMLV-induced tumors. This result cannot be attributed to phenotypal differences between the specimens as the expression levels of *Hoxa* genes in individual leukemia samples with different T-cell phenotype induced by MMLV ($CD4^+/CD8^+$ or $CD4^+$, $n=2$ each) or by *E2A–PBX1* ($CD4^+/CD8^+$ or CD8, $n=2$ each) were comparable in each group (data not shown). Although the number of tumors that could be analysed remains low, it is important to note that neither *Hoxb4* nor *Hoxa9* overexpression changed this trend except for a noticeable reduction in *Hoxa6* and *Hoxa7* expression levels in *E2A–PBX1* × *Hoxb4* leukemias (see *Hoxb4* genotype in Figure 4b). 5′ *Hoxa* genes, such as *Hoxa9*, *a10* and *a11*, are also more expressed in *E2A–PBX1* leukemias than in MMLV-induced T-ALL. This difference was also blunted by the overexpression of *Hoxb4* in the *E2A–PBX1* tumors (compare fourth to third column in Figure 4b). Thus, MMLV-induced diseases, either from *Hoxb4* transgenic mice or from littermate controls, showed the lowest values for *Hoxa* gene expression with the exception of *Hoxa7* at 128 copies (Figure 4b). Similar to the expression in normal thymocytes, the tendency toward lower expression of *Hoxa3* to *Hoxa7* persisted in *Hoxb4* transgenic compared with control MMLV-induced leukemias. These leukemias expressed 10–100-fold lower levels of *Hoxa1* to *Hoxa7* genes than detected in normal thymocytes (Figure 4c). Remarkably, *Hoxb4* is expressed at moderate levels in all leukemias from both subgroups (Figure 4b). Interestingly, *Hoxa7* and *a6* expression

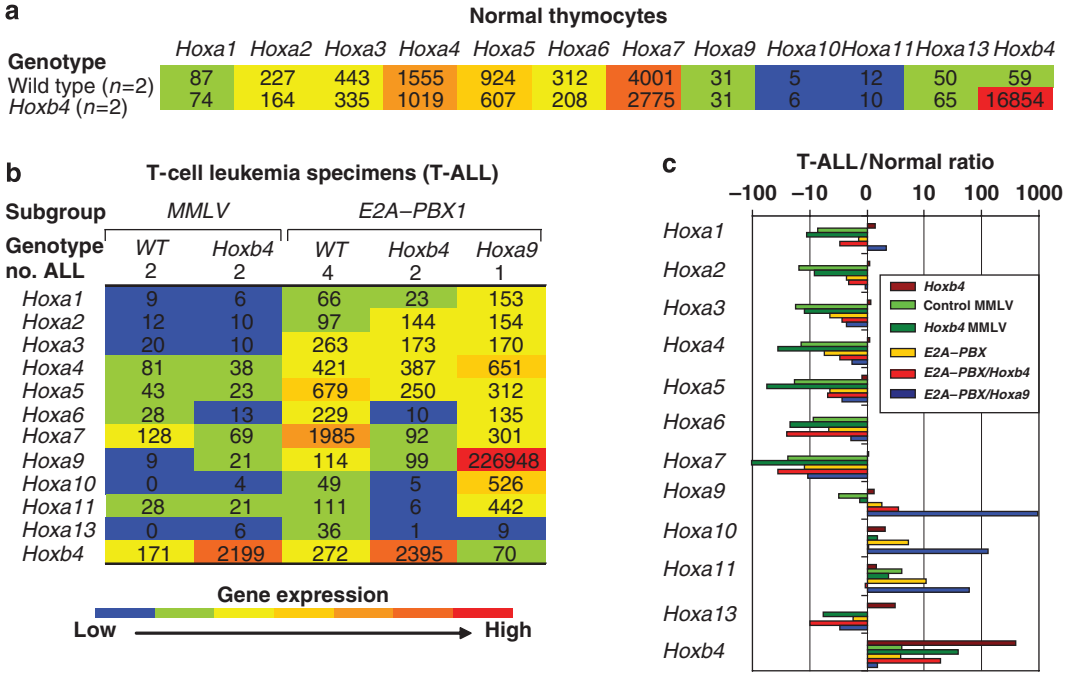


Figure 4 The expression of *Hoxa* cluster genes and *Hoxb4* (in copy numbers) (**a**) in normal thymocytes derived from wild-type and *Hoxb4* transgenic mice and (**b**) in five groups of T-cell acute lymphocytic leukemias (T-ALLs) induced by murine Moloney leukemia virus (MMLV) (columns 1 and 2) or *E2A–PBX* (columns 3–5) in a transgenic or wild-type background. The expression was determined in 50 ng RNA, normalized for *GAPDH* ($C_T=18$). Absolute copy numbers for each group are calculated from the average C_T value in each group according to the following formula $2^{(38-ct)}$. (**c**) Fold difference in *Hoxa* and *Hoxb4* gene expression of *Hoxb4* transgenic thymocytes and control or transgenic T-cell leukemias induced by murine Moloney leukemia virus (MMLV) or *E2A–PBX1* normalized to the expression in wild-type thymocytes. Note that eight-fold difference (2^3), which corresponds to a ΔC_T of 3, is considered significant.

levels are reduced in T-ALL that occurred in *Hoxb4* transgenics (genotype in Figure 4b), possibly indicating some level of cross-regulation or that *Hoxb4* expression compensates for these two genes. Finally, we observed that *Hoxa10* and *Hoxa11* expression is mostly restricted to *E2A–PBX1* or to *E2A–PBX1*/*Hoxa9* leukemias (Figures 4a–c).

Discussion

A role for *HOXA* genes in human acute T-cell leukemia was first suggested by Ferrando *et al*. (2003), who observed increased expression of *HOXA9* and *HOXA10* in MLL rearranged T- and B-ALLs. Since then transcriptional activation of specific *HOXA* genes has been reported for several subsets of T-ALL with different cytogenetic abnormalities (Dik *et al*., 2005; Soulier *et al*., 2005; Speleman *et al*., 2005; Van Vlierberghe *et al*., 2008), identifying the activation of the *HOXA* cluster genes as an important event in T-cell leukemogenesis. Despite these observations, no mouse model has yet demonstrated a role for *Hox* genes in T-cell leukemia. The genetic interaction between *Hoxb4* and *E2A–PBX1* in the pathogenesis of mouse T-ALL as reported herein partly fills this gap. Moreover, this paper shows that *Hoxa* genes are expressed at higher levels in murine *E2A–PBX1* T-cell leukemias than in similar diseases induced by proviral insertional mutagenesis (MMLV), thus supporting a role for the *Hox* network in *E2A–PBX1*-induced T-cell transformation.

The profound perturbations in T-cell development in bone marrow chimeras generated from the retroviral overexpression of *Hoxb3* or *Hoxa9* or *Hoxa10* (Sauvageau *et al*., 1997; Thorsteinsdottir *et al*., 1997; Kroon *et al*., 1998), together with the reduction in lymphocyte numbers in *Hoxa9*$^{-/-}$ mice, suggest that some of these genes might play an important role in T-cell development. Indeed, the expression of *HOXA7*, *HOXA9*, *HOXA10* and *HOXA11* was demonstrated in early thymic T progenitors, which was progressively lost with the differentiation collinear with their 3′ to 5′ localization in the cluster (Taghon *et al*., 2003). The required downregulation of *HOXA* genes for T-cell differentiation was illustrated by enforced expression of *HOXA10* in human cord blood progenitors in fetal thymic organ cultures (Taghon *et al*., 2002). Thus, the expression of *Hoxa10* and *Hoxa11*, and the relative high expression of the 3′ located genes, including *Hoxa5* and *Hoxa7*, might contribute to disease development in our *E2A–PBX1* transgenic mice possibly by preventing T-cell maturation.

The observed difference in collaboration of *Hoxa9* versus *Hoxb4* with *E2A–PBX1* remains intriguing. Although our small screen could not detect a significant contribution of *Hoxa9* to the *E2a-PBX1*-dependent leukemogenesis, we cannot exclude that *Hoxa9* and *E2A–PBX1* do collaborate. It is possible that T-cell progenitors at stages before transgene expression are sensitive to high levels of *Hoxa9* (Taghon *et al*., 2003). The collaboration between *Hoxa9* and MMLV-induced leukemogenesis dampens—but does not eliminate—this possibility.

This screen also highlighted the low probability for the creation of conditions required to uncover the *Hoxb4* transformation ability. In contrast to *Hoxa9*, overexpression of *Hoxb4* alone has never resulted in hematological malignancies. However, some recipients of bone marrow cells co-infected with *Hoxb4* and antisense *Pbx1* cDNA carrying recombinant retroviruses presented with AML (Cellot *et al*., 2007). Leukemic clones in all cases harbored high numbers of the integrated proviruses, suggesting that retroviral insertions perturbed the activity of oncogenes that promoted transformation in the *Hoxb4*^{high}*Pbx1*^{low} cellular context. Recently, a study in large animals showed that overexpression of *Hoxb4* alone can eventually lead to myeloid leukemia. The leukemic cells contained a high number of retroviral integrations near oncogenes such as *LMO2* and *prdm16* after extended ($\sim$2 years) latency (Zhang *et al*., 2008). Finally, co-overexpression of *Hoxb4* and *Meis1* was reported to result in the development of AML (Pineault *et al*., 2004 and N Mayotte and GS, data not shown). *Hoxb4* thus appears to posses a weak leukemogenic potential that can be activated with a limited subset of oncogenes (*Meis1*, *LMO2* and *prdm16*), which now includes *E2A–PBX1*.

Our data suggest that the cellular context restricts the oncogenic activity of *Hox* genes to specific oncogenes that are activated by insertional mutagenesis (MMLV) for *Hoxa9* and by *E2A–PBX* for *Hoxb4*. Interestingly, our expression data presented in Figures 4b and c suggest that *Hoxb4* expression (provided as a transgene) may overcome the need for certain *Hoxa* gene activation in *E2A–PBX* T-ALL, implying that interactions between *E2A–PBX* and *Hox* genes may be critical and deterministic.

Materials and methods

Transgenic animals

Transgenic mice for *Hoxb4* and *Hoxa9* were generated using the same vector (Thorsteinsdottir *et al*., 2002) and methodology as that described for the recently reported *E2A–PBX1* lines (Bijl *et al*., 2005). The backbone of the pLIT3 vector has been described elsewhere (Hough *et al*., 1994). In short, cDNA was cloned downstream of a T-cell receptor Vβ promoter, immunoglobulin (Ig) enhancer elements and sequences from the proximal promoter of the *lck* gene. The human growth hormone (*hGH*) gene containing a frameshift mutation in the coding region was inserted 3′ of the transgene to provide introns for enhanced expression. Transgenic animals were generated using standard techniques (Hogan, 1983). Basically, the transgene was injected into pronuclei of (C57Bl/6J $\times$ C3H) F2 hybrid zygotes. A total of 2–4 h after injection, surviving eggs were transplanted into oviducts of pseudopregnant CD-1 host females. Transgenic progeny were identified by dot blot analysis of tail genomic DNA using an *hGH* probe.

Statistical analysis

Evaluation of significant acceleration of disease in the *Hox* transgenic background for MMLV-induced or *E2A–PBX1*-induced lymphomagenesis was obtained by performing a two-tailed Student's *t*-test. The rate of acceleration is considered significant when *P*-value <0.05.

Flow cytometry
Bone marrow, spleen and thymus cells were isolated from leukemic mice and incubated with the following antibody sets to detect T cell: CD4-FITC and CD8-PE (BD Bioscience Pharmingen, San Diego, CA, USA); B cell: B220-PE, B220-PE-cy5, CD19-APC, BP1-PE, CD43-FITC, IgM-bio and IgD-FITC (all from BD Bioscience Pharmingen), myeloid cell populations: Mac1-bio and Gr1-FITC (both from BD Bioscience Pharmingen). Biotinylated antibodies were detected with phycoerythrin (PE), allophycoerythrin (APC) (BD Bioscience Pharmingen) or PE-cy7 (e-Bioscience, San Diego, CA, USA)-conjugated streptavidin. Fluorescence was analysed using the FACS Calibur (BD Bioscience, San Jose, CA, USA) or the LSR II (BD Bioscience), using DIVA software. Multicolor FACS data were analysed with WinMDI or FCS Express (De Novo Software, Los Angeles, CA, USA) software.

Quantitative RT–PCR
Total RNA was isolated from fresh tumor cells (BM, spleen, thymus and enlarged lymph nodes,) by Trizol (Invitrogen, Life Technologies, Carlsbad, CA, ISA), and Dnase I treated. cDNA was generated from 5 μg of RNA using MMLV reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Quantitative PCR was performed in triplicate for each sample as described before (Bijl *et al*., 2005), using SYBR Green (Applied Biosystems, Toronto, Canada) and run on a thermal cycler ABI 7500 (Applied Biosystems). Primers for the detection of *Hox* gene expression were used as designed by Thompson *et al*., and were validated for use with SYBR green. Triplicates were accepted in a 0.5 C_T range.

Protein analysis
Preparation of cellular extracts derived from control, single or compound transgenic mice and western blot analyses were performed as described (Krosl and Sauvageau, 2000). Briefly, proteins were separated by gel electrophoresis using 10% polyacrylamide–SDS and transferred to Immobilon P membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% non-fat milk in TBST (20 mM Tris-Cl, pH 7.6, 140 mM NaCl and 0.05% Tween 20) and incubated with E47 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) to detect E2A–PBX1 or an antibody detecting Hoxb4 (Krosl *et al*., 2003). Bound antibodies were detected using horseradish peroxidase-conjugated anti-rabbit antibodies (Sigma, St Louis, MO, USA) followed by enhanced chemiluminescence (ECL; Amersham, Buckinghamshire, UK).

In vitro *clonogenic progenitor assays*
For myeloid clonogenic progenitor assays, cells were plated in 35 mm dishes in 1% methylcellulose in DMEM (Dulbecco's modified Eagle media) supplemented with 10% fetal calf serum (FCS), 5.7% bovine serum albumin, 10^{-5} β-mercaptoethanol (β-ME), 5 U of erythropoietin (Epo) per milliliter, IL-3 10 ng/ml, IL-6 10 ng/ml, steel 50 ng/ml, 2 mM glutamine and 200 mg transferrin per milliliter. Fetal liver (FL) cells of mutant and control embryos were plated at concentrations of 0.5×10^5 cells per milliliter. BM and spleen from *Hoxb4* mice were plated at 3×10^4 and 1×10^6 cells per milliliter, respectively. Colonies were scored on days 12–14 of incubation and identified according to standard criteria. Pre-B-cell clonogenic progenitor assays were only performed using cells isolated from *Hoxb4* and control mice. For this assay, 3×10^5 bone marrow cells or 1×10^6 spleen cells were plated in 1% methylcellulose in DMEM supplemented with 30% FCS (selected for B cells), 10^{-4} β-ME, 2 mM glutamine and 0.2 ng/ml IL-7. Pre-B-cell colonies were scored on day 8.

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