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SPECIAL ISSUE RESEARCH ARTICLE

Hoxa9 Collaborates with E2A-PBX1 in Mouse B Cell Leukemia in Association With Flt3 Activation and Decrease of B Cell Gene Expression

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Background: **The fusion protein E2A-PBX1 induces pediatric B cell leukemia in human. Previously, we reported oncogenic interactions between homeobox (*Hox*) genes and *E2A-PBX1* in murine T cell leukemia. A proviral insertional mutagenesis screen with our *E2A-PBX1* B cell leukemia mouse model identified *Hoxa* genes as potential collaborators to *E2A-PBX1*. Here we studied whether *Hoxa9* could enhance *E2A-PBX1* leukemogenesis. Results: We show that *Hoxa9* confers a proliferative advantage to *E2A-PBX1* B cells. Transplantation experiments with *E2A-PBX1* transgenic B cells overexpressing *Hoxa9* isolated from bone marrow chimeras showed that *Hoxa9* accelerates the generation of *E2A-PBX1* B cell leukemia, but *Hoxa9* is unable to transform B cells alone. Quantitative-reverse transcriptase polymerase chain reaction analysis demonstrated a strong repression of B cell specific genes in these *E2A-PBX1*/*Hoxa9* leukemias in addition to *Flt3* activation, indicating inhibition of B cell differentiation in combination with enhanced proliferation. Overexpression of *Hoxa9* in established *E2A-PBX1* mouse leukemic B cells resulted in a growth advantage *in vitro*, which was also characterized by an enhanced expression of *Flt3*. Conclusions: we show for the first time that *Hoxa9* collaborates with *E2A-PBX1* in the oncogenic transformation of B cells in a mouse model that involves *Flt3* signaling, which is potentially relevant to human disease. *Developmental Dynamics 243:145–158, 2014*.**

Key words: Hox genes; oncogenes; transgenic mouse model; transcription factors

Key findings:

- Critical B cell genes are down-regulated in leukemias induced by *E2A-PBX1* and *Hoxa9*.
- *Hoxa9* mediated block in B cell differentiation in *E2A-PBX1* leukemias involves *Flt3*.

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INTRODUCTION

The fusion protein E2A-PBX1 is generated through the translocation between chromosome 1 and 19 and is prevalent in 25% of pre-B cell leukemias in young children (Kamps et al., 1990; Nourse et al., 1990). *E2A-PBX1* is a potent oncogene with transcriptional activating properties, which can transform a range of cell types, including fibroblasts, myeloid and lymphoid cells (Kamps et al., 1991; Kamps and Baltimore, 1993; Sykes and Kamps, 2004). Structure function analysis revealed critical elements of both genes for transformation of cells by the E2A-PBX1 fusion protein (Monica et al., 1994; Chang et al., 1997). The strong transcriptional activator properties of *E2A-PBX1* are provided by the activation domains (AD) of E2A (van Dijk et al., 1993). Oncogenesis by *E2A-PBX1* is dependent on AD1, which has been shown to interact directly with the KIX domain of transcriptional co-regulator CBP/p300 (Bayly et al., 2004). Disruption of the CBP binding to E2A-PBX1 by substitution of a single leucine residue in the LXXLL motif of AD1 impaired *E2A-PBX1* induced immortalization of primary hematopoietic cells in vitro and development of myeloproliferative disease in bone marrow (BM) recipients (Bayly et al., 2004, 2006).

The *E2A* gene codes for basic helix-loop-helix proteins E12 and E47, which are essential for lymphoid development, inducing the expression of lymphoid specific genes such as *recombinase activating genes, early B cell factor1* (*Ebf1*) and *surrogate light chain λ5* (Bain et al., 1994; Quong et al., 2002). Activation of these genes results in the immunoglobulin rearrangement of B cells at the pre-B cell stage accompanied with the inhibition of proliferation (Quong et al., 2002; Mandal et al., 2009). Mice targeted at the *E2A* locus exhibit a complete block in B cell development beyond the pro-B cell stage and develop T cell leukemias indicating antiproliferative and tumor suppressor properties of E2A (Bain et al., 1997). Thus heterozygosity of the *E2A* gene in the human disease may contribute to the development of leukemia.

The PBX1 fusion partner is a member of the TALE homeodomain transcription factors. These proteins can form complexes with pentapeptide containing homeobox proteins (paralog 1-10), increasing the DNA binding specificity of HOX proteins (Chang et al., 1995, 1996; Lu and Kamps, 1997). The transcriptional repressor/activation function of HOX/PBX1 heterodimers is determined by cell signaling events (Saleh et al., 2000). However, the fusion of the E2A activation domains to the C-terminal of the transcription factor PBX1 converts PBX1 to a constitutive transcriptional activator (van Dijk et al., 1993; Lu et al., 1994). Indeed, it has been demonstrated that E2A-PBX1 can bind to sequences recognized by HOX/PBX1 complexes (Chang et al., 1995; Lu et al., 1995; Phelan et al., 1995).

Domain requirements of *E2A-PBX1* for oncogenic transformation appear to be cell type specific. Transformation of fibroblasts requires both AD1 and AD2 domains plus the Hox cooperating motif (HCM) of PBX1 (Chang et al., 1997), whereas transformation of myeloid cells requires, in addition, the homeodomain of PBX1 (Kamps et al., 1996). Requirement of the homeodomain for transactivation in myeloid transformation (Monica et al., 1994), suggests that deregulation of HOX/PBX1 elements or target genes plays a critical role in *E2A-PBX1* induced myeloid leukemia. Moreover, retroviral overexpression of single *Hox* genes (e.g., *Hoxa9*, *Hoxa10*, *Hoxb3*, *Hoxb6*, and *Hoxb8*) is sufficient to induce leukemia in BM (Perkins and Cory, 1993; Thorsteinsdottir et al., 1997, 2001; Sauvageau et al., 1997; Kroon et al., 1998; Fischbach et al., 2005). It has recently been demonstrated that all *Hoxa* genes except *Hoxa2* and *Hoxa5* have oncogenic potential and could transform BM cells in culture (Bach et al., 2010). Co-overexpression of *Hoxa9* and *E2A-PBX1* results in a shorter latency to myeloid leukemia onset compared with *Hoxa9* alone (Thorsteinsdottir et al., 1999), but interestingly *Hoxa9* did not collaborate with *E2A-PBX1* in mouse T cell leukemia (Bijl et al., 2008).

So far, it is unclear whether oncogenic interactions between *Hox* genes and *E2A-PBX1* occur in development of clinically relevant B cell leukemia. We have previously generated a lymphoid specific transgenic mouse model for *E2A-PBX1* that generates pre B-acute lymphoblastic leukemia (B-ALL), phenotypically similar to human disease, in the absence of T cell maturation (Bijl et al., 2005). Evidence for a potential role of *Hox* genes in *E2A-PBX1* induced B cell leukemia was obtained from a proviral insertional mutagenesis screen using this model. A hot spot for viral integrations in the *Hoxa* locus in association with the presence of *E2A-PBX1* that resulted in an overall activation of the *Hoxa* cluster genes in leukemic cells strongly suggest collaboration of *Hoxa* genes with *E2A-PBX1* in B cell leukemogenesis.

To further investigate the importance of *Hox* collaboration with *E2A-PBX1* in the development of B-ALL, *Hoxa9* was overexpressed in nonmalignant and malignant B cells derived from *E2A-PBX1*/CD3ε$^{-/-}$ transgenic mice. In this study we provide evidence that *Hoxa9* collaborates with *E2A-PBX1* in oncogenic transformation of B cells, which might be mediated through efficient repression of B cell specific genes and activation of *Flt3*, a direct target of Hoxa9 (Wang et al., 2006). Moreover, *Hoxa9* also promoted the growth kinetics and leukemia regeneration potential of primary *E2A-PBX1* induced B cell leukemias that involved changes in expression of genes implicated in hematopoietic differentiation, in particular *Flt3*, *Pdgfδ*, and *Lmo1*. Here we show for the first time the oncogenic transformation of B cells through interactions between *Hox* genes and *E2A-PBX1* involving deregulation of developmental programs.

RESULTS

Hoxa Gene Expression in Malignant and Nonmalignant *E2A-PBX1* B Cells

The expression of all eleven *HOXA* genes was measured in four human B cell leukemia samples harboring the fusion *E2A-PBX1* and two without *E2A-PBX1* by quantitative-reverse transcriptase polymerase chain reaction (Q-RT-PCR). In addition, RNA-Seq data from 23 pre-B ALLs without known major translocations were

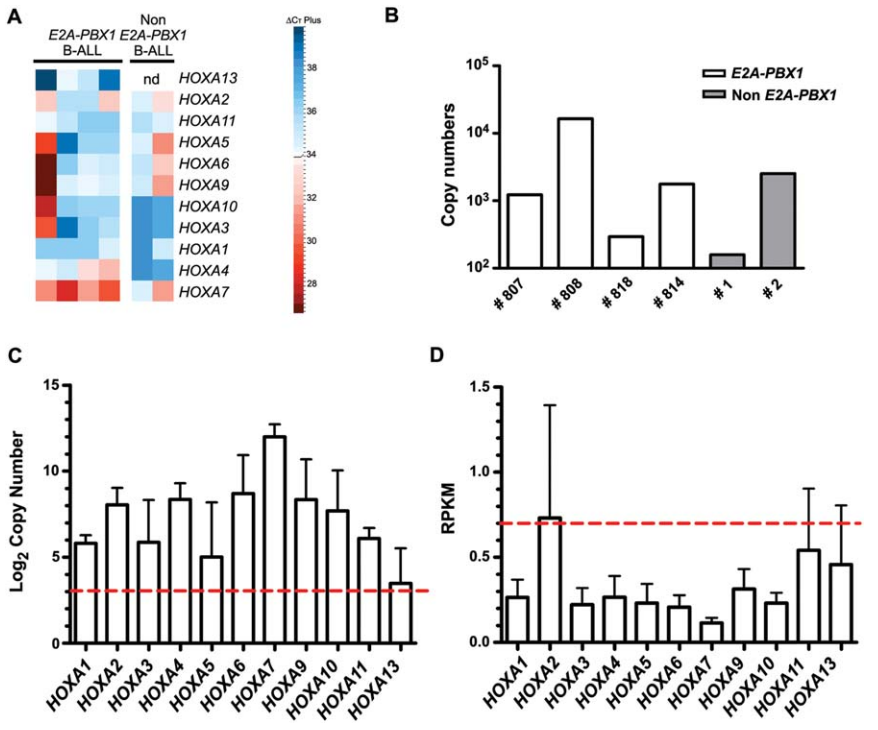


Fig. 1. *Hoxa* gene expression in human and mouse *E2A-PBX1* leukemias. **A:** Heatmap of *HOXA* genes expression in human pediatric *E2A-PBX1*+ (n = 4) and *E2A-PBX1*- (n = 2) B-ALLs. **B:** Cumulated *HOXA* genes expression in human B-ALLs. Standard curve generated copy numbers for each group are calculated from the average C_T values normalized for *GAPDH* (C_T = 23) in each group according to the following formula $2^{(38\text{-}CT)}$. **C,D:** Average *HOXA* gene expression in human pre-B ALLs positive for *E2A-PBX1* (n = 4) obtained by Q-RT-PCR (C) and without known major translocations (n = 23) obtained by RNASeq (D). The C_T values (Q-RT-PCR), corrected for *GAPDH* expression at C_T = 20, were related to the RPKM values (RNASeq) according to the following formula $\log RPKM = -0.17*(\Delta C_T) + 2.4$. This formula has been empirically determined based on comparison of Q-RT-PCR data and RPKM data from the same sample (Sauvageau's Lab, personal communication). The threshold of *HOX* gene expression is considered around C_T = 35, which corresponds to a RPKM value of 0.7 (indicated by dotted line). Note that *E2A-PBX1* pre-B ALLs express all *HOXA* genes. The only pre-B ALL that showed expression of several *HOX* genes had an amplification of *RUNX1*. RPKM = Reads per kilobase of transcript per million mapped reads. B-ALL = B cell acute lymphoblastic leukemia.

available. All *E2A-PBX1* samples expressed the majority of *HOXA* genes, but the expression levels for the individual genes were very variable (Fig. 1A). The expression of *HOXA* genes in non-*E2A-PBX1* B-ALL was in general negligible to low/medium (Fig. 1A, C, D). Contrarily to the non-*E2A-PBX1* B-ALLs, at least one *HOXA* gene was expressed at high levels in individual *E2A-PBX1* leukemias, and notably *HOXA7* expression was constantly high across the *E2A-PBX1* samples. Intriguingly, one *E2A-PBX1* leukemia sample showed high expression levels of almost the entire *HOXA* cluster, resulting in one log higher accumulated *HOXA* gene expression (Fig. 1B).

The *Hoxa* gene expression levels were also measured in B cell leukemias derived from spleen of *E2A-PBX1*/*CD3*$\varepsilon^{-/-}$ transgenic mice and compared with the expression in Moloney Murine Leukemia Virus (MMLV) induced B cell leukemias. *Hoxa* gene expression was low to medium/high in nine *E2A-PBX1* positive leukemias and severely deregulated in two (~ one log higher) (Fig. 2A). The expression levels for *Hoxa* genes were also low to medium/high in MMLV induced B cell leukemias, but extreme high expressions were not observed. Expression of *Hoxa* genes in *E2A-PBX1* leukemic cells tended to be higher than in B cells from healthy preleukemic *E2A-PBX1* transgenic mice (Fig. 2B). Of interest, the levels of *Hoxa* gene expression were also occasionally very high in B cells sorted from BM of preleukemic *E2A-PBX1* transgenic mice (Fig. 2C). Overall the expression of *Hoxa* genes is clearly present in *E2A-PBX1* transgenic malignant B cells and reflects those in immature B cells found in the BM. The aberrant high *Hoxa* gene expression in a subset of *E2A-PBX1* leukemias suggests that the *Hox* pathway might contribute to *E2A-PBX1* leukemogenesis.

Ex Vivo Cultures of *Hoxa9* Transduced *E2A-PBX1* B Cells

To directly test the effect of high *Hox* gene expression on the growth response to IL-7 of *E2A-PBX1* B cells, B220+ cells were sorted from BM of three to five month old healthy / preleukemic transgenic mice. Q-RT-PCR analyses verified that the basal *Hoxa* gene expression levels in these B cells were low to moderate (data not shown). Isolated cells were transfected with *Hoxa9* or green fluorescent protein (GFP) retroviral vectors and subsequently grown in B cell supporting medium (Fig. 3A). In each of three independent experiments *Hoxa9* transduced *E2A-PBX1* B cells grew faster than control, reaching expansions up to 800-fold over the initial population within 20 days (Fig. 3B and data not shown). Maximum expansion in these cultures, observed at 11 days, was only eight-fold. Doubling times, calculated based on the exponential growth of the cultures, were consistently one to five days shorter for *Hoxa9* transduced *E2A-PBX1* B cell cultures than its corresponding controls (Fig. 3C; $P = 0.03$). The expansion of the *E2A-PBX1*/*Hoxa9* B cell cultures was supported by an increasing number of clonogenic progenitors, while in control cultures progenitors numbers were significantly lower after day 9 (Fig. 3D). Immunophenotyping by flow cytometry showed that the *E2A-PBX1* cells from both control and *Hoxa9* transduced cultures were B220+/CD43+/IgM-/BP1+ (Fig. 3E). To exclude whether *Hoxa9* alone could confer such a growth expansion within the same magnitude, similar cultures were initiated with CD3$\varepsilon^{-/-}$ control B cells. Although *Hoxa9* cultures grew faster than control, only a maximum of four-fold expansion was observed (at day 11) followed by a decline (data

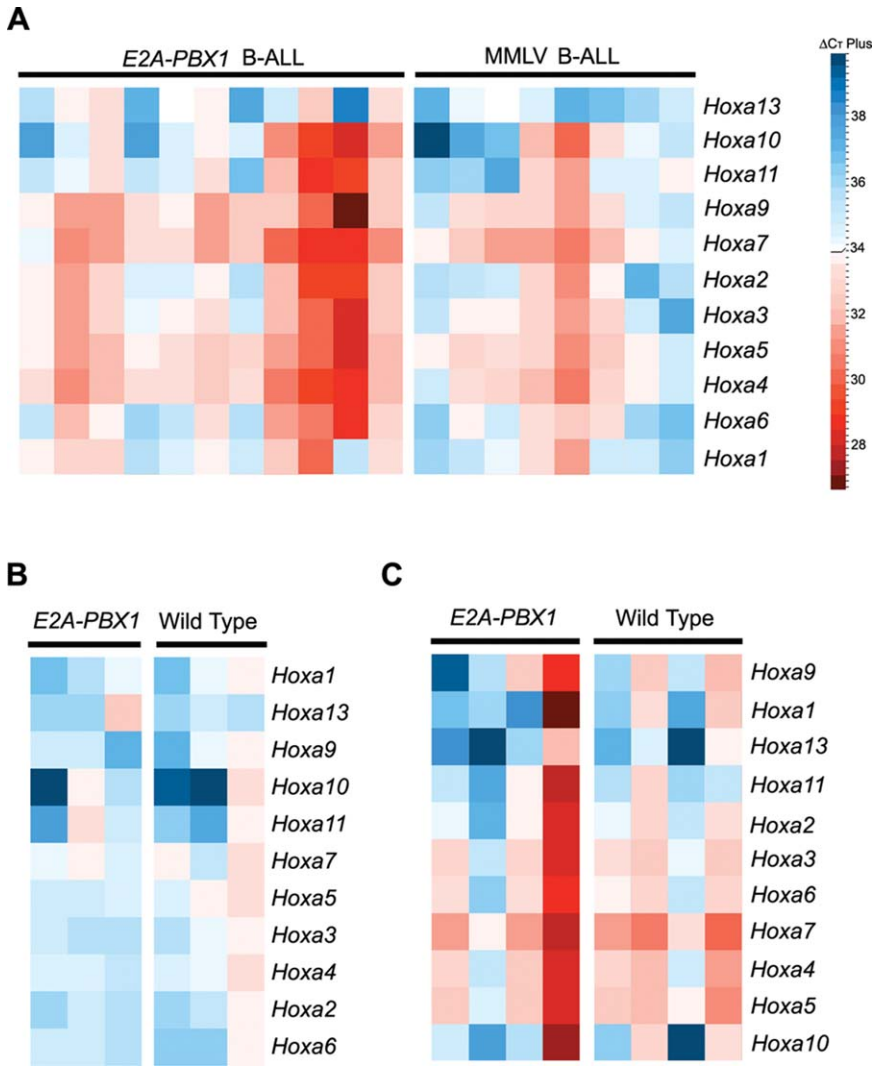


Fig. 2. **A,B:** Heatmaps of *Hoxa* genes expression in leukemias from (A) *E2A-PBX1/CD3ε*$^{-/-}$ transgenic mice (n = 11) and MMLV induced B-ALLs (n = 8) and of (B) Nonmalignant B220+ B cells purified from spleen of healthy 3–5 months old *E2A-PBX1/CD3ε*$^{-/-}$ transgenic and wild-type mice. **C:** Heat map of *Hoxa* gene expression in nonmalignant B cells sorted from bone marrow of 3- to 5-month-old healthy *E2A-PBX1/CD3ε*$^{-/-}$ transgenic (n = 4) and wild-type mice (n = 4). Note the high expression of *Hoxa* genes in one of the *E2A-PBX1* positive samples (lane 4). B-ALL, B-acute lymphoblastic leukemia; MMLV, Moloney Murine Leukemia Virus. nd, not determined.

not shown). Thus *Hoxa9* and *E2A-PBX1* together provide B cells a strong proliferation capacity in vitro, which could be indicative for their collaboration in B cell leukemia induction.

Evaluation of Interaction Between *E2A-PBX1* and *Hoxa9* in B Cell Leukemogenesis

E2A-PBX1 transgenic BM chimeras were generated overexpressing either *Hoxa9* or, GFP. Retroviral overexpression of *Hoxa9* in total BM cells is known to give rise to a myeloid leukemia in transplantation recipient mice within five months (Kroon et al., 1998). Therefore, all chimeras were killed after 1 month and GFP+ donor derived *E2A-PBX1* B cells were purified from the BM and transferred into irradiated recipients together with supporting BM helper cells (Fig. 4A). In a first experiment, mice that received *E2A-PBX1/Hoxa9* B cells (n = 2) developed leukemia within three months (Table 1), while recipients of *E2A-PBX1/GFP* B cells (n = 3) did not show signs of leukemia after four months at which time no GFP+ cells could be detected. FACS analysis showed that *E2A-PBX1/Hoxa9* leukemic cells from both mice expressed CD43 and variable percentages of B220 and Mac-1 (Fig. 4B and data not shown), indicating a potential biphenotypic origin of these leukemias. Transfer of leukemic cells into mice resulted in generation of secondary leukemias with reduced number of B220+ cells (Fig. 4B). As the change in immunophenotype of the leukemias could result from the presence of minor clones that became more prominent, leukemias were analyzed for their clonal composition. Southern Blot analysis showed that leukemias were monoclonal as the same clone was detected in all five transplantation recipients (Fig. 4C). Thus the loss of B220 expression in secondary leukemias was not the result of the selective advantage of a B220 negative clone. To prevent transformation of a primitive B220+/Mac-1+ B cell by *Hoxa9*, a second experiment was conducted using sorted B cells that were negative for Mac-1. Recipients of both *Hoxa9* (n = 7) and control (n = 5) *E2A-PBX1* B cells developed leukemia (Table 1). However, in the context of *Hoxa9* the average latency time to development of disease was significantly reduced compared with control (survival 35.7 ± 17.6 for *E2A-PBX1/Hoxa9* vs. 48.8 ± 5.2 days for *E2A-PBX1/GFP*; $P = 0.04$; Table 1; Fig. 4D). Mice transplanted with *Hoxa9* transduced wild-type B cells did not succumb of disease within the timeframe observed. FACS analysis confirmed a B cell phenotype for all the leukemias as demonstrated by high expression of CD19. However, the pan B cell marker B220 was consistently much lower expressed in the majority of both *E2A-PBX1/Hoxa9* and *E2A-PBX1/GFP* leukemias (Fig. 4E). Interestingly, several leukemias of both groups exhibited Mac-1 expression on a small percentage of the cells (Table 2). To test whether the leukemias could be re-initiated with maintenance of the phenotype, 1-2 × 10^6 cells of leukemias induced by *E2A-PBX1/Hoxa9* (n = 3) or *E2A-PBX1/GFP* (n = 2) were injected into recipients (n = 2 for each leukemia). All mice succumbed to leukemia between two and four weeks post-transplantation (data not shown). Several *E2A-PBX1/Hoxa9* and *E2A-*

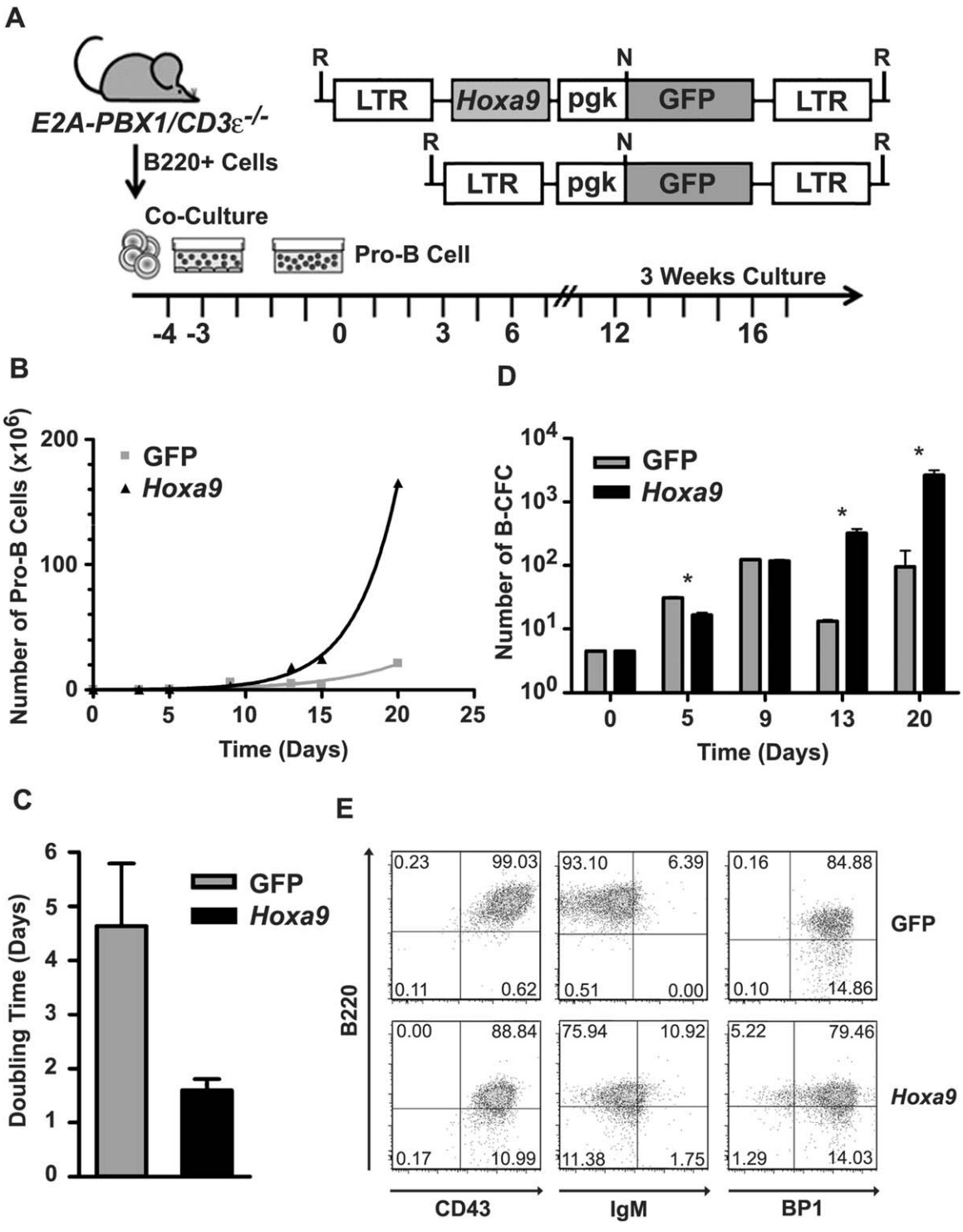


Fig. 3. Analysis of *E2A-PBX1/CD3ε*$^{-/-}$ B cell cultures overexpressing *Hoxa9* or control. **A:** Schematic of the experimental strategy and representation of *Hoxa9*-GFP and control-GFP retroviral vectors. Restriction sites relevant for this study are indicated. R, *Eco*RI; N, *Nco*I. **B:** Representative growth curves of *E2A-PBX1/CD3ε*$^{-/-}$ pro-B cells transduced with *Hoxa9* or GFP (n = 3). **C:** Bar graph presenting the doubling time for *Hoxa9* and control pro-B cell cultures ($P = 0.03$). **D:** Total IL-7 responsive B cell progenitors in the pro-B cell cultures transduced with *Hoxa9* or GFP measured by colony forming cell at indicated time points (n = 2). *$P \leq 0.05$ 1-tailed Student's *t*-test. **E:** FACS profiles for B cells surface markers involved in staging the B cell differentiation confirming the pro-B cell phenotype (B220+/CD43+/IgM-/BP1+) of *Hoxa9* and control *E2A-PBX1/CD3ε*$^{-/-}$ cultured cells. IgM, Immunoglobulin M; BP1, B cell Protein1. B-CFC, B cell colony forming cell.

PBX1/GFP re-initiated leukemias cells showed lower B220 expression compared with primary leukemias. In addition, expression of the B cell marker CD19 was also reduced in most cases (Table 2). Recently it has been reported that *Hoxa9* is inversely expressed during B cell differentiation with *Ebf1* (Gwin et al., 2010).

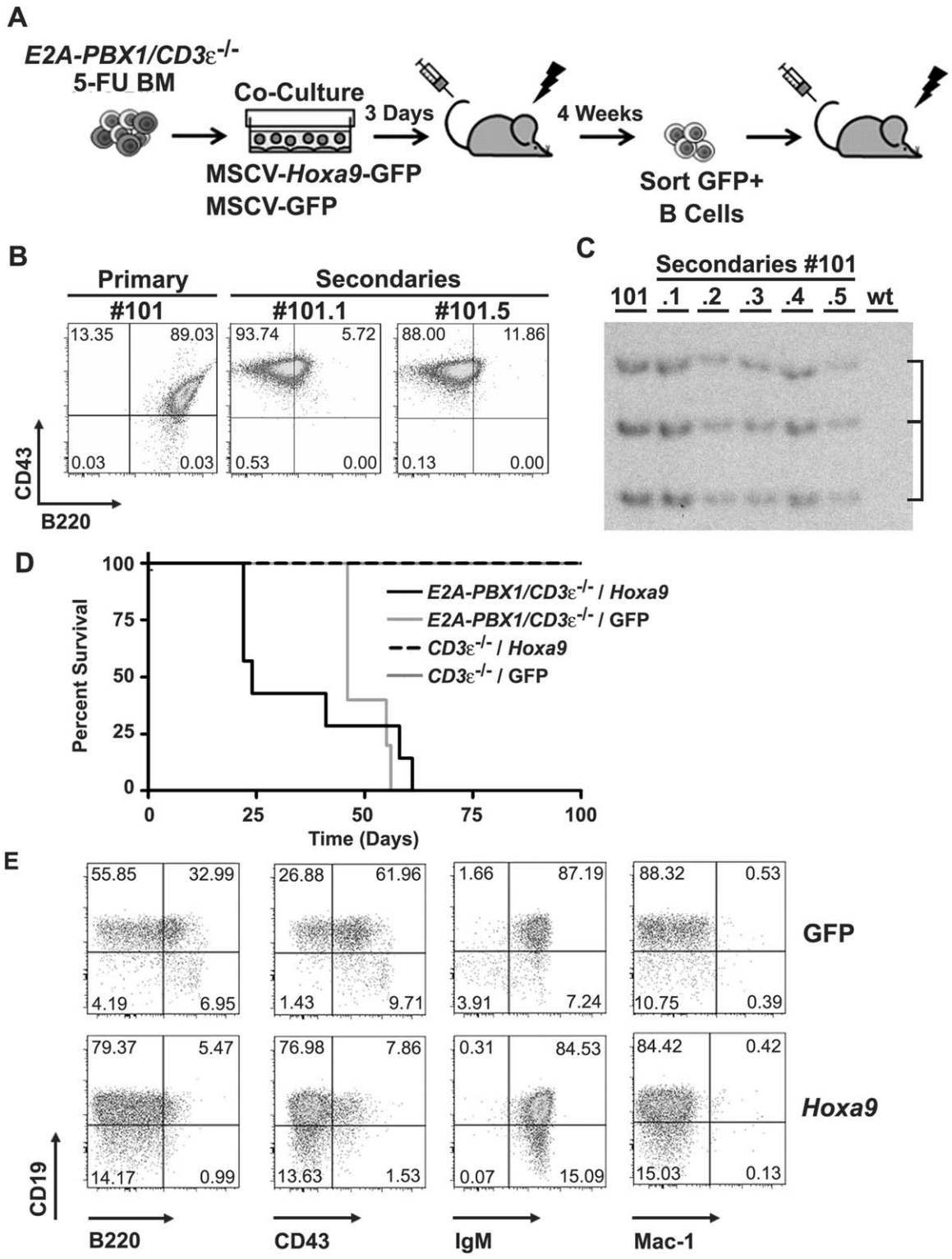


Fig. 4. In vivo analysis of *Hoxa9* or control transduced *E2A-PBX1/CD3ε*$^{-/-}$ B cells. **A:** Schematic of the experimental strategy. **B:** FACS profile of primary leukemia #101 (Experiment 1) and two secondary leukemias regenerated from #101 showing surface markers CD43 and B220. Plots are gated on GFP+ cells. Note that expression of B220 is lower in the secondary leukemias. **C:** Clonal analysis of secondary recipients of *E2A-PBX1/CD3ε*$^{-/-}$ leukemia #101 by Southern blot on analysis on genomic DNA isolated from primary and secondary leukemias. Note that the same sets of fragments of equal intensity for all chimeras indicate their monoclonal origin. **D:** Kaplan-Meier survival curves of mice transplanted with *E2A-PBX1/CD3ε*$^{-/-}$ B cells transduced with *Hoxa9* (n = 7) or GFP (n = 5) and *CD3ε*$^{-/-}$ B cells transduced with *Hoxa9* (n = 2) or GFP (n = 9). *Hoxa9* significantly accelerates mean latency to *E2A-PBX1* leukemia, $P = 0.04$, 1-tailed Student's *t*-test, but curves are not significantly different Chi Square test $P = 0.08$. **E:** FACS analysis of *E2A-PBX1/Hoxa9* and *E2A-PBX1* induced B cell leukemias.

TABLE 1. Collaboration Between *Hoxa9* and *E2A-PBX1/CD3ε-/-* in B Cell Leukemogenesis

Genotype	MSCV vector	n	Cell population	Cell Dose	Leukemic mice (%)	Survival (days)
Experiment 1						
E2A-PBX1/CD3ε$^{-/-}$	*Hoxa9*-GFP	2	B220+	3×10^5	100	76 ± 14.1
E2A-PBX1/CD3ε$^{-/-}$	GFP	3	B220+	5×10^5	0	na[b]
Experiment 2						
E2A-PBX1/CD3ε$^{-/-}$	*Hoxa9*-GFP	7	B220+/Mac-1-	3×10^5	100	35.7 ± 17.6[a]
E2A-PBX1/CD3ε$^{-/-}$	GFP	5	B220+/Mac-1-	5×10^5	100	48.8 ± 5.2[a]
CD3ε$^{-/-}$	*Hoxa9*-GFP	2	B220+/Mac-1-	5×10^5	0	na[b]
CD3ε$^{-/-}$	GFP	9	B220+/Mac-1-	5×10^5	0	na[b]

[a]Mice were sacrificed after 4 months.
[b]$P = 0.04$ 1-tailed Student's t-test; a one-tailed test was chosen based on the hypothesis that *Hoxa9* accelerates *E2A-PBX1* leukemia.
na = not applicable.

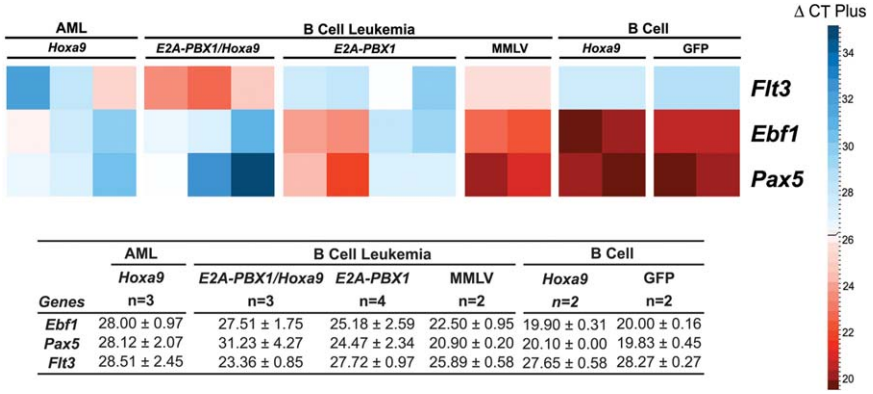
Hoxa9 appeared to be required for the expansion of pro-B cell populations, mediated through direct activation of *Flt3*. Therefore, we sought to analyze whether *Hoxa9* expression in the context of *E2A-PBX1* also induced *Flt3* expression and could affect B cell differentiation genes *Ebf1* and *Pax5*. Q-RT-PCR analysis showed that *Flt3* was 16-fold and 4-fold higher expressed in *E2A-PBX1/Hoxa9* leukemias compared with *E2A-PBX1* ($P = 0.003$) or MMLV induced B cell leukemias ($P = 0.03$; Fig. 5), respectively. A large difference in *Flt3* expression was also observed with *Hoxa9* induced myeloid leukemia. In addition, expression of B cell differentiation genes *Pax5* and *Ebf1* was reduced in *E2A-PBX1/Hoxa9* leukemias reaching significance compared with MMLV ($P = 0.022$ and 0.015, respectively) and close to significance compared with *E2A-PBX1* induced leukemias (P = 0.055 for *Pax5* and P = 0.074 for *Ebf1*). Expression analysis of nonmalignant B cells sorted from *Hoxa9* BM chimeras showed a two-fold increase in *Flt3* expression compared with GFP B cells, but no difference in *Ebf1* and *Pax5* expression was observed. Thus these experiments show that *Hoxa9* can collaborate with *E2A-PBX1* in B cell leukemogenesis, but does not transform B cell alone, and that deregulation of B cell specific genes might be important in this oncogenic process.

Hoxa9 Overexpression in *E2A-PBX1* Primary Leukemias

In addition to its oncogenic interactions with *E2A-PBX1* in leukemia induction, we questioned whether *Hoxa9* could enhance the leukemic properties of established *E2A-PBX1* leukemias. Primary *E2A-PBX1* B cell leukemias generated from our transgenic mouse line were transduced with *Hoxa9*-GFP or control GFP retroviral vectors and grown on S17 feeder cells in the presence of lymphoid specific growth factors (Fig. 6A). Three of nine leukemias transduced with control vector and five out nine transduced with *Hoxa9* were able to grow under these conditions (Table 3), indicating that *Hoxa9* enhances the culturing of *E2A-PBX1* leukemic B cells. Moreover, the expansion of *E2A-PBX1* B cell leukemia cultures was significantly enhanced in the presence of *Hoxa9* for two out of the three leukemias growing under both conditions (#225 and #282, Table 3), exemplified by an increasing ratio of *Hoxa9* vs control cells for leukemia #225

TABLE 2. Immunophenotype of Primary and Their Corresponding Secondary Leukemias Expressing *Hoxa9* or GFP

	Leukemia	Donor cells	B220	CD19	CD43	Mac-1
Primary	#50	*E2A-PBX1/Hoxa9*	6.9	85.4	9.4	0.5
Secondary	#82		10.3	76.7	20.3	17.7
	#85		25.4	69.7	34.4	27.9
Primary	#56	*E2A-PBX1/Hoxa9*	46.9	73.7	50.6	11.4
Secondary	#78		17.0	78.2	53.4	12.9
	#79		14.2	72.0	46.7	11.4
Primary	#54	*E2A-PBX1/Hoxa9*	56.4	89.9	65.9	5.6
Secondary	#76		20.2	59.9	50.3	8.7
	#77		29.5	75.1	58.4	6.6
Primary	#68	*E2A-PBX1*/GFP	41.0	89.2	71.1	0.9
Secondary	#86		37.7	14.3	10.9	1.4
	#87		27.0	12.7	8.3	2.2
Primary	#69	*E2A-PBX1*/GFP	22.0	95.9	41.6	3.3
Secondary	#70		22.7	60.5	51.6	35.9
	#71		21.3	51.6	51.2	33.9



	AML	B Cell Leukemia			B Cell	
	Hoxa9	*E2A-PBX1/Hoxa9*	*E2A-PBX1*	MMLV	*Hoxa9*	GFP
Genes	n=3	n=3	n=4	n=2	n=2	n=2
Ebf1	28.00 ± 0.97	27.51 ± 1.75	25.18 ± 2.59	22.50 ± 0.95	19.90 ± 0.31	20.00 ± 0.16
Pax5	28.12 ± 2.07	31.23 ± 4.27	24.47 ± 2.34	20.90 ± 0.20	20.10 ± 0.00	19.83 ± 0.45
Flt3	28.51 ± 2.45	23.36 ± 0.85	27.72 ± 0.97	25.89 ± 0.58	27.65 ± 0.58	28.27 ± 0.27

Fig. 5. Differential expressions of *Flt3*, *Ebf1*, and *Pax5* in *E2A-PBX1/Hoxa9* leukemias. Heat map for expressions of these genes in B cell leukemias induced by MMLV (n = 2), *E2A-PBX1* (n = 4) or *E2A-PBX1/Hoxa9* (n = 3), and in nonmalignant B cells transduced with *Hoxa9* (n = 2) or control GFP (n = 2) was generated with DataAssist v3.0 (Applied Biosystems). Each row presents the expression of a different gene. Average cycle threshold (C_T)-values for these genes are given below for each of these groups and for *Hoxa9* induced AML as reference. High C_T-values representing low expression levels are presented in green, while low C_T-values indicting high expression levels are indicated in red. MMLV, Moloney Murine Leukemia Virus. AML = acute myeloid leukemia.

(Fig. 6B), and a slightly but significantly reduced doubling time (30.7 ± 0.9 and 28.2 ± 0.5 hr for control and *Hoxa9*, respectively, P=0.04; Fig. 6C). This growth advantage of *Hoxa9* transduced leukemic cells was supported by an increase in leukemic B cells with clonogenic properties, which are expected to maintain the culture as their normal B progenitor equivalents (Fig. 6D). Compared with B cell colonies induced by nonmalignant progenitors, leukemic colonies were more disorganized and morphologically aberrant (Fig. 6E). However, FACS analysis showed no differences in the common B cell surface markers in the presence of *Hoxa9* (data not shown). These data show that overexpression of *Hoxa9* in established *E2A-PBX1* leukemic B cells could enhance growth capacity and proliferation kinetics of several primary *E2A-PBX1* leukemias.

Gene Expression Profiling of *E2A-PBX1* Primary Leukemias Overexpressing *Hoxa9*

To analyze which genes could underpin the observed changes induced by *Hoxa9*, expression analysis was performed by Q-RT-PCR on *Hoxa9* and GFP cultured *E2A-PBX1* leukemia cells (#111 and #225) using TaqMan primer/probe sets for 110 genes including several genes important for lymphoid and myeloid differentiation (Supp. Table S1, which is available online). Significant increased expression (Log_{10} >0.3) was observed for twelve genes (#225) or 13 genes (#111) in the presence of *Hoxa9* (Table 4). The expression of only two genes, *Flt3* and *Pdgfd*, was significantly and consistently increased in both *Hoxa9* transduced leukemias compared with their respective controls. *Flt3* has been previously reported as a direct target of *Hoxa9* and is known for its involvement in leukemia. In addition a set of fourteen genes was significantly down-regulated (Log_{10} < −0.3) in *Hoxa9* leukemia #225, while only three genes in *Hoxa9* leukemia #111 (Table 4). One of these genes, *Lmo1* a Lim domain protein involved in transcriptional regulation, was common to both leukemias. Surprisingly, the expression of seven genes, including *Pdgfb*, *Col1a1*, *Pparg*, *Timp2*, *Ctnna*, *Gas6*, and *Sox4* were differentially expressed in *Hoxa9* leukemia cells #111 and #225. Of these genes *Sox4* has been recently shown to be regulated by *Hoxa9* (Huang et al., 2012). Up-regulated genes by *Hoxa9* in leukemia #225 include several transcription factors, such as *Runx3* and *Cebpα*, which are genes associated to the myeloid lineage. In *Hoxa9* #111 leukemic cells the up-regulation of two collagen encoding genes (*Col3a1* and *Col1a1*) and two genes related to cell adhesion (*Timp2* and cadherin *Ctnna*) was striking. Noteworthy is that adhesion molecules have been shown to be targets of *Hox* genes and *Col3a1* has been proposed to be a direct target gene for *Hoxa9* in adipocytes. These data show that *Flt3* is a consistent target of *Hoxa9* in malignant B cells and that the expression of several genes associated to hematopoietic differentiation is changed in the presence of *Hoxa9*.

DISCUSSION

Aberrant *Hox* gene expression is associated with leukemias, most notably those that harbor fusion proteins involving MLL (Armstrong et al., 2002; Ferrando et al., 2003). Large expression screens have not yet associated altered *HOX* gene expression with pediatric pre-B cell leukemias positive for the t(1;19) translocation encoding for *E2A-PBX1* (Yeoh et al., 2002). This is attributable to incomplete probe set coverage of the *HOXOME* on these arrays and thus does not exclude abnormal *HOX* gene expression levels in these leukemias. In this study we show that *E2A-PBX1* induced B cell leukemias generated in mice using a transgenic model express most of the *Hoxa* genes, which are at significantly higher levels than B-ALLs induced by MMLV. The inconsistent aberrantly high levels of *Hoxa* gene expression seen in both mouse and human *E2A-PBX1* leukemias indicate that *HOXA* genes might indeed play an important role in the development of this disease. It is not clear why these *HOXA* gene levels are not consistently high in all *E2A-PBX1* leukemias. It is possible that *HOX* genes from other clusters are aberrantly expressed or non-HOX/PBX pathways might be more prominently contribute to *E2A-PBX1* leukemia and do not require high *HOX* expression levels. A decrease in *HOX* gene expression is normally observed with the maturation of hematopoietic cells (Sauvageau et al., 1994; Lebert-Ghali et al., 2010) and thus maintenance of moderate *HOX* gene expression levels might be sufficient to block differentiation as observed for *Hoxa9* overexpression at the pre-B cell stage (Thorsteinsdottir et al., 2002).

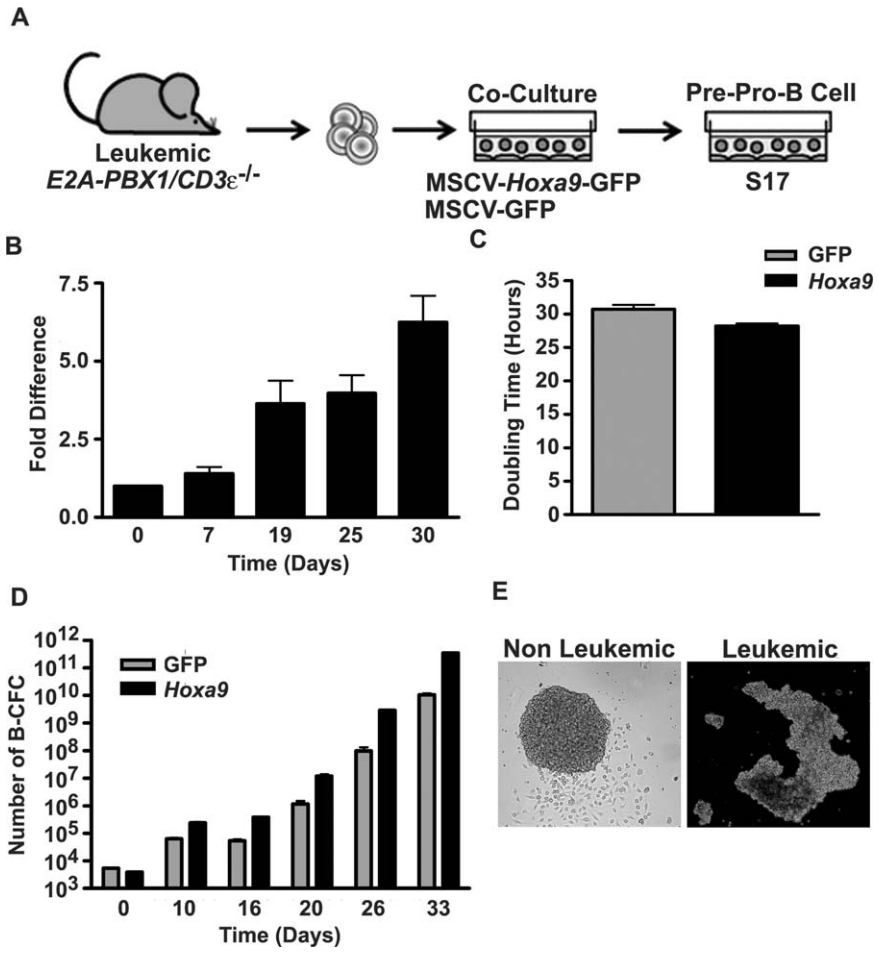


Fig. 6. Analysis of *E2A-PBX1/CD3ε*$^{-/-}$ leukemic cells overexpressing *Hoxa9* or control. **A:** Schematic of the experimental strategy. **B:** Fold difference in growth of *E2A-PBX1/CD3ε*$^{-/-}$ leukemic cells #225 transduced by *Hoxa9* over control at given time points. **C:** Doubling times for *E2A-PBX1* leukemia #225 overexpressing *Hoxa9* or control (n = 2). **D:** Total of clonogenic B cell progenitors in cultures of *E2A-PBX1/CD3ε*$^{-/-}$ leukemia #225 overexpressing *Hoxa9* or control measured by colony forming cell at indicated time points. **E:** Example of B cell colony initiated by *E2A-PBX1* leukemic clonogenic B cell progenitors (left). As a reference a typical round shaped colony initiated by nonmalignant B cell progenitors is shown (right). *$P \leq 0.05$ 1-tailed Student's *t*-test. B-CFC = B cell colony forming cell.

Our data also show an impressive expansion of *E2A-PBX1* pro-B cells by *Hoxa9* in vitro at a magnitude that was not observed for B cells overexpressing either *E2A-PBX1* or *Hoxa9* individually. The synergistic actions of *E2A-PBX1* and *Hoxa9* in proliferation suggest that they activate complementing pathways. Also this is the first time that *Hoxa9* has been shown to give a proliferative advantage to primary pro-B cells. These data are consistent with earlier reports that *Hoxa9* is normally expressed in pro-B cells and is required for the generation of B cell progenitors (Lawrence et al., 1997; Gwin et al., 2010).

Retroviral and transgenic mouse models have shown genetic interactions between *E2A-PBX1* and *Hoxa9* or *Hoxb4* in myeloid and T cell leukemia development, respectively (Thorsteinsdottir et al., 1999; Bijl et al., 2008). In the present study we show for the first time collaboration between a *Hox* gene and *E2A-PBX1* in the transformation of B cells. Despite the fact that *Hoxa9* is a potent oncogene, inducing myeloid leukemia (Kroon et al., 1998; Thorsteinsdottir et al., 2001), we showed that *Hoxa9* induced transformation of B cells is dependent on the presence of *E2A-PBX1*. This is in accordance with the absence of lymphoid leukemia development in lymphoid specific *Hoxa9* transgenic mice (Thorsteinsdottir et al., 2002). Of interest, our data show that *Hoxa9* can transform advanced hematopoietic progenitors in the presence of the right collaborating oncogene, in this case *E2A-PBX1*. This is in contrast to findings in the myeloid environment where potent oncogenic combination of *Hoxa9* and *Meis1* efficiently transformed cells from the stem cell fraction LKS, but did very poorly in transformation of c-kit^{lo} granulocyte macrophage progenitors (Wang et al., 2010). This study also reported that the *Hoxa9*/*Meis1* or *MLL*/*AF9* transformation events are dependent on the presence of active β-catenin. Of interest, a member of the Wnt family, *Wnt16b*, has been previously identified as a putative target of E2A-PBX1 (McWhirter et al., 1999), which expression appeared essential for the survival of human cell lines with the rearrangement t(1;19) (Mazieres et al., 2005). Also it has been shown that pro-B cells are dependent on Wnt signaling (Reya et al., 2000, Staal and Clevers, 2005). It is, therefore, not surprising that our *E2A-PBX1*/*Hoxa9* leukemias displayed predominantly a pro-B cells (B220+/CD43+/IgM-) phenotype. The presence of Mac-1 on a low percentage of leukemic cells, which increased in secondary leukemias of some *E2A-PBX1*/*Hoxa9* or *E2A-PBX1*/*GFP* primary leukemias is likely the result of granulocyte-colony stimulating factor receptor expression, which is a target of E2A-PBX1 (de Lau et al., 1998).

The Q-RT-PCR results suggest that signaling through *Flt3* may play a role in the acceleration of *E2A-PBX1* leukemia onset by *Hoxa9* as well as in the enhanced proliferative properties of *Hoxa9* transduced established *E2A-PBX1* induced leukemias. However, increased *Flt3* expression in *Hoxa9* transduced control B cells that fail to develop leukemia indicates that activation of this pathway alone is not sufficient to transform B cells. The strong down-regulation of *Ebf1* and *Pax5* expression suggests that repression of B cell differentiation in combination with enhanced proliferative signals provided by *Flt3* together might mediate the oncogenic interactions between *Hoxa9* and *E2A-PBX1* in leukemogenesis. In the *Flt3*

TABLE 3. Hematopoietic Characterization and In Vitro Growth Potential of Primary *E2A-PBX1* B-ALL

		Weight	Phenotype leukemic B cells					Growth	
Mouse	Age (days)	Spleen (g)	B220	CD19	CD43	IgM	Mac-1	GFP	*Hoxa9*
#225	167	2.4	100.0	100.0	73.1	99.8	5.6	+	+
#231	238	0.5	100.0	100.0	7.3	97.7	8.1	−	+
#111	211	nd	100.0	100.0	49.6	99.2	13.5	+	+
#272	260	0.2	100.0	100.0	93.5	20.5	0.0	−	−
#252	230	0.7	100.0	100.0	99.0	0.0	0.0	−	−
#173	302	nd	100.0	100.0	14.2	nd	0.0	−	−
#211	248	1.0	100.0	100.0	nd	nd	nd	−	−
#282[a]	165	0.2	100.0	100.0	99.1	1.7	2.1	+	+
#286[a]	104	0.6	100.0	100.0	7.0	99.9	0.0	−	+

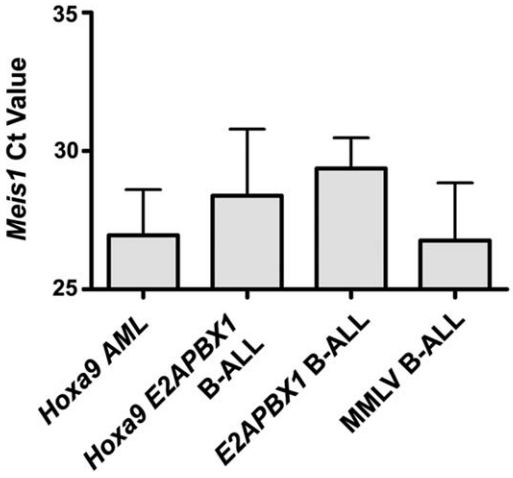
[a]At the moment of sacrifice mouse did not show signs of disease.
nd = not determined.

promoter binding regions for Hoxa9 and Pbx/Meis1 have been determined and Hoxa9 has been shown to activate transcription of *Flt3* (Wang et al., 2006; Gwin et al., 2010; Volpe et al., 2013). Although no evidence exist regarding promoter binding by Pbx or E2A-PBX1 it is not excluded that Hoxa9 and E2A-PBX1 both bind as a complex to the *Flt3* promoter, resulting in a strong activation of transcription. We do not expect that *Meis1*, which is a collaborator oncogene to *Hoxa9* in myeloid leukemia, plays a critical role in *E2A-PBX1* B-ALL. *Meis1* is expressed in *E2A-PBX1* and MMLV mouse leukemias, but its expression is not significantly

TABLE 4. Differential Expressed Genes in Hoxa9 Transduced Leukemias #225 and #111

Leukemic cells #225			Leukemic cells #111		
Gene	Function	Log_{10}(RQ)	Gene	Function	Log_{10}(RQ)
Up-regulated genes					
Ager	Development, inflammation	0.83	*Col3a1*	Extracellular matrix	1.84
Flt3	Hematopoietic differentiation	0.79	*Flt3*	Hematopoietic differentiation	0.49
Id3	Repression lymphocyte differentiation	0.64	*Pparg*	Transcriptional regulation	1.41
Pdgfd	Proliferation and differentiation	0.51	*Pdgfd*	Proliferation and differentiation	0.34
Runx3	Myeloid differentiation	0.51	*Gas6*	Cell growth and survival	0.72
Cebpa	Myeloid differentiation	0.50	*Mme*	Cleavage	0.64
Cdkn2a	Cell cycle regulation	0.48	*pdgfb*	Proliferation and differentiation	0.59
Gata3	T-cell differentiation	0.44	*Timp2*	Cell adhesion	0.48
Ebf1	B-cell differentiation	0.44	*Col1a1*	Extracellular matrix	0.40
Cybb	Neutrophil function	0.38	*Sox4*	B-cell differentiation	0.38
BLK	B-cell differentiation	0.35	*Mta2*	Transcriptional regulation	0.32
Cdkn1b	Cell cycle regulation	0.35	*Ctnna*	Cell adhesion	0.31
			Cdk4	Cell cycle regulation	0.31
Down-regulated genes					
Ets2	Differentiation	−2.22	*Ephb4*	Development	−0.44
Lmo1	Transcriptional regulation	−0.40	*Lmo1*	Transcription regulation	−0.52
Fn1	Cell adhesion	−1.36	*rpl3a*	Translation	−0.32
Pdgfb	Proliferation and differentiation	−1.31			
Ssp1	Differentiation/immune response	−0.95			
Cola1a1	Extracellular matrix	−0.85			
Pparg	Transcription regulation	−0.80			
Timp2	Cell adhesion	−0.74			
Ctnna	Cell adhesion	−0.58			
Gas6	Cell growth and survival	−0.53			
Sox4	B-cell differentiation	−0.49			
Mdm2	p53 degradation/cell survival	−0.36			
Pik3r1	Signalling	−0.36			
Itgam	Myeloid cell function/adhesion	−0.32			

Note that gene expression is considered to be up-regulated in context with Hoxa9 when Log_{10}(RQ) values are > 0.3 and down-regulated when values are < -0.3.



	AML	B Cell Leukemia		
	Hoxa9 n=3	*E2A-PBX1/Hoxa9* n=3	*E2A-PBX1* n=4	MMLV n=3
Meis1	26.95 ± 2.86	28.39 ± 4.15	29.37 ± 2.19	26.76 ± 3.61

Fig. 7. Expression of *Meis1* in mouse B cell and myeloid leukemias obtained by Q-RT-PCR. Values are corrected for GAPDH $C_T = 19$.

different in the context with *Hoxa9* (Fig. 7). Also contrarily to *Pbx1*, *Meis1* is normally associated with activation of transcription (Sagerstrom, 2004; Choe et al., 2009) like E2A-PBX1 and, therefore, its presence might be redundant.

Finally, we show that the proliferation of primary *E2A-PBX1* B cell leukemias was enhanced in the presence of *Hoxa9*. Our targeted expression array showed that *Flt3* and *Pdgfδ* could contribute to these biological functions. In addition, genes associated with myeloid differentiation, such as *Cebpα* and *Runx3*, were higher expressed in the context of *Hoxa9* in these leukemic cells, which concurs with the reported requirement for *Hoxa9* in myeloid differentiation (Lawrence et al., 1997) and its ability to induce myeloid leukemia in mice (Kroon et al., 1998; Thorsteinsdottir et al., 2001). Furthermore, *Hoxa9* induced up-regulation of *Id3*, a gene associated with repression of lymphoid differentiation, fits the current view that *Hoxa9* needs to be down-regulated for B cell specification by *Ebf1* and *Pax5*. The fact that lymphoid specific transgenic mice for *Hoxa9* have a partial block at the pre-B cell stage corresponds with this view (Thorsteinsdottir et al., 2002). It remains puzzling why a set of seven genes, including *Sox4*, demonstrates differential expression in two primary *E2A-PBX1* leukemias. It is known that HOX-PBX complexes might act both as transcriptional activators as well as repressors, which are dependent on recruitment of additional factors, and thus suggest that the molecular profile of these two leukemias might be different. It is of note that the major site (and potentially the site of origin) of leukemia #111 was located in the liver in contrast to leukemia #225 that had principal involvement in the spleen, which might explain the differences in expression of the extracellular matrix components and adhesion molecules. In line with such a context dependent transcriptional regulation is that *Pim1*, a gene regulated by *Hoxa9* in myeloid cells is not differentially expressed in *Hoxa9* overexpressing leukemic B cells.

In conclusion our data show oncogenic interactions between *Hoxa9* and *E2A-PBX1* in transformation of B cells that may be mediated through complementing pathways involving proliferation conferred by *Flt3* and *Pdgfδ* signaling and inhibition of B cell developmental programs. The importance of such *HOX* associated pathways for maintenance of *E2A-PBX1* B cell leukemias remains to be determined.

EXPERIMENTAL PROCEDURES

Material

Trizol (Invitrogen Corporation, Carlsbad, CA), samples of human pediatric ALL harboring the t(1;19) translocation were obtained from the Biobank of the Sainte-Justine Hospital (Montreal). Two control ALL samples for Q-RT-PCR were obtained from the Banque de cellules de Leucemiques du Quebec (BCLQ; www.bclq.org), while RNASeq data were obtained from 23 pre-B ALL samples (FAB L1). No translocations were detected in control ALLs. A standard Ficoll-Pacque gradient was performed on human samples to isolate mononuclear cells. All human samples contained over 70% of blasts. C57Bl6 inbred wild-type mice were purchased from Jackson Laboratories (Bar Harbor, ME). Transgenic mice for the E2APBX1 fusion protein were kept on a $CD3\varepsilon^{-/-}$ background as previously described (Bijl et al., 2005). Mice were bred and maintained in a specific pathogen free animal facility of the HMR Research Center. For the induction of B cell leukemias, 1-day-old newborn $CD3\varepsilon^{-/-}$ mutant mice were intraperitoneally injected with 10 infectious units of MMLV (van Lohuizen et al., 1991; Bijl et al., 2005). All animal protocols were approved by the Animal Care Committee of the HMR Research Center.

Quantitative RT-PCR

Total RNA was isolated by Trizol reagent (Invitrogen) in combination with RNeasy® clean up columns (Qiagen, Toronto, ON), DNase-I-treated and cDNA was prepared using MMLV-Reverse Transcriptase and random primers (all Invitrogen) according to the manufacturer's protocols. Q-PCR was carried out using SYBR Green chemistry (Applied Biosystems, Toronto, Canada) on an ABI 7500 thermal cycler (Applied Biosystems). Oligonucleotides for all 11 murine and human *Hoxa* genes were used according to previously described and validated sequences (Thompson et al.,

2003; Dickson et al., 2009), and were tested to be used with SYBR Green (Lebert-Ghali et al., 2010). Validated TaqMan GEx assays for the other genes of interest were obtained by Applied Biosystems. Only replicate cycle threshold (C_T)-values within 0.5 C_T are accepted for *Hox* genes and 0.2 C_T for the endogenous control *GAPDH*. Copy numbers for *Hoxa* genes are calculated from the average C_T values in each group according to the following formula $2^{(38\text{-}CT)}$. Values less than 10 ($\sim C_T = 35$) are considered not expressed.

Flow Cytometry and Cell Sorting

Phenotypic characterizations of B cell cultures were performed using the following conjugated antibodies: CD45R/B220-APCCy7, CD43-APC, IgM-biotin, CD11b (Mac-1)-PE (BioLegend, San Diego, CA). Biotinylated antibodies were detected with PerCP5.5 conjugated streptavidin (BioLegend). Mortality levels were determined using Dapi (Invitrogen). FACS analyses were performed on a FACS LSRII with FACSDiva software (BD Bioscience, Mississauga, ON). Data were further analyzed using FlowJo software (Tree Star Inc., Ashland, OR). All cell sortings have been performed on a FACS Aria II with FACSDiva software (BD Bioscience).

B Cell Cultures and Retroviral Infections

E2A-PBX1 preleukemic and leukemic B cells were prestimulated overnight in the presence of IL-7 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 three day co-cultivation on packaging cell line (GP +E-86) (Markowitz et al., 1990) engineered to stably express MSCV-*Hoxa9*-GFP or control GFP retroviruses. Preleukemic cells were then cultured in OptiMem (Invitrogen) media supplemented with IL-7 (10 ng/ml; Invitrogen), 10% B cell tested Fetal Bovine Serum (Stem Cell Technologies, Vancouver, CA), 5×10^{-5} M 2-Mercaptoethanol (Mallinckrodt Baker Inc., Phillipsburg, NJ), 1× Penicillin-Streptomycin and 50 μg/ml Gentamycin (both Wisent Inc., St-Bruno, QC). Leukemic cells were grown on S17 feeder cells in Iscove's (Invitrogen) with its corresponding cytokines and supplements as for healthy B cells. Clonogenic progenitor assays were performed as has been described by us previously (Fournier et al., 2012; Lebert-Ghali et al., 2010).

Generation of Chimeras

BM chimeras were generated as described before by us (Fournier et al., 2012), with the difference that BM cells from *E2A-PBX1*/*CD3ε*$^{-/-}$ transgenic mouse were used in retroviral infections with *Hoxa9*-GFP or control MSCV-GFP retroviruses. One month posttransplantation mice were killed and GFP positive B cells were sorted from the BM. Transduced B cells were re-transplanted into lethally irradiated C57BL/6 mice together with 2×10^5 total BM cells from congenic B6SJL mice. Peripheral blood analyses were performed bi-weekly following transplantation to monitor leukemia development.

Statistical Analysis

To compare survival curves a logrank Chi-square test was performed using the curve comparison option in GraphPad Prism v.4. All other statistical analysis was done using a Student *t*-test and *F*-test.

Clonality Analysis

Genomic DNA was isolated from fresh BM cells using DNAzol (Invitrogen), then digested with *Nco*I or *Eco*RI (New England Biolabs, Unit 6 Pickering, ON) and analyzed for retroviral integrations by Southern blotting using a probe against the *GFP* gene.

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