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Hoxa9 transforms primary bone marrow cells through specific collaboration with *Meis1a* but not *Pbx1b*

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***Hoxa9*, *Meis1* and *Pbx1* encode homeodomain-containing proteins implicated in leukemic transformation in both mice and humans. Hoxa9, Meis1 and Pbx1 proteins have been shown to physically interact with each other, as Hoxa9 cooperatively binds consensus DNA sequences with Meis1 and with Pbx1, while Meis1 and Pbx1 form heterodimers in both the presence and absence of DNA. In this study, we sought to determine if Hoxa9 could transform hemopoietic cells in collaboration with either Pbx1 or Meis1. Primary bone marrow cells, retrovirally engineered to overexpress *Hoxa9* and *Meis1a* simultaneously, induced growth factor-dependent oligoclonal acute myeloid leukemia in <3 months when transplanted into syngenic mice. In contrast, overexpression of *Hoxa9*, *Meis1a* or *Pbx1b* alone, or the combination of *Hoxa9* and *Pbx1b* failed to transform these cells acutely within 6 months post-transplantation. Similar results were obtained when FDC-P1 cells, engineered to overexpress these genes, were transplanted to syngenic recipients. Thus, these studies demonstrate a selective collaboration between a member of the Hox family and one of its DNA-binding partners in transformation of hemopoietic cells.**

Keywords: *Hox*/leukemia/*Meis*/oncogenes/*Pbx*

Introduction

The *Drosophila HOM-C* family of homeobox genes and their 39 mammalian *Hox* counterparts are best recognized for their role in axial patterning (Krumlauf, 1994). In addition, *Hox* genes appear to play important roles in the control of proliferation and differentiation of several adult tissues. For example, they are expressed in proliferating cells of epidermal origin (C.Largman, personal communication) in breast (Friedmann *et al*., 1994), colon (De Vita *et al*., 1993), kidney (Cillio *et al*., 1992), testis (Watrin and Wolgemuth, 1993) and in primitive hemopoietic cells (Sauvageau *et al*., 1994). Experimentally induced variations in expression levels of specific *Hox* genes alter the proliferation of hemopoietic stem cells (HSC) (Sauvageau *et al*., 1995), as well as that of T cells (Caré *et al*., 1994), B cells (Thorsteinsdottir *et al*., 1997) and natural killer (NK) cells (Quaranta *et al*., 1996).

Hox genes have also been associated with leukemic transformation. The first link between *Hox* gene overexpression and leukemia was obtained from genetic analyses of the WEHI-3B leukemic cell line, which was shown to contain proviral integrations resulting in the transcriptional activation of *Hoxb8* and interleukin-3 (IL-3) expression (Blatt *et al*., 1988). Direct evidence for *Hox* involvement in leukemic transformation came from mice transplanted with bone marrow cells engineered to overexpress *Hoxb8* and IL-3 simultaneously (Perkins *et al*., 1990). These mice succumbed to an aggressive, polyclonal, acute leukemia, whereas no acute disease was detected in recipients of either *Hoxb8*- or IL-3-transduced bone marrow (Perkins *et al*., 1990). More recently, it has been shown that a high proportion of mice transplanted with bone marrow cells which overexpress *Hoxb8*, *Hoxa10* or *Hoxb3*, but not *Hoxb4*, eventually develop acute myeloid leukemia (AML) after a latency of several months (Sauvageau *et al*., 1997; Thorsteinsdottir *et al*., 1997). These long latencies suggest the requirement for secondary genetic events in *Hox*-induced leukemic transformation.

A human *HOX* gene has also been implicated in leukemic transformation. *HOXA9* is overexpressed in a subset of human myeloid leukemias in the form of a fusion with a sub-domain of *NUP98*, as the result of a reciprocal translocation between chromosomes 7 and 11 (Borrow *et al*., 1996). Overexpression of the murine *Hoxa9* (and *Hoxa7*) was also detected in leukemias developing in BXH-2 mice (Nakamura *et al*., 1996). The importance of *Hoxa9* in the regulation of hemopoietic cell proliferation was revealed directly through gene targeting procedures, which showed that *Hoxa9*$^{-/-}$ mice have abnormal B lymphopoiesis and hypoproliferative granulocyte–macrophage progenitors (CFU-GM) (Lawrence *et al*., 1997). In contrast, CFU-GM are hyperproliferative in *Hoxa10*$^{-/-}$ mice (Zhang *et al*., 1996), suggesting that proliferation of granulocyte–macrophage progenitors is at least in part regulated by *Hoxa9* and *Hoxa10*. Despite strong evidence implicating *Hox* genes and specifically *Hoxa9* in leukemia, the molecular mechanisms underlying these *Hox*-induced transformations are poorly understood at present.

In vitro, Hox proteins cooperatively bind DNA with a group of TALE (three amino acid loop extension) homeodomain-containing proteins called PBC (Mann and Chan, 1996). The PBC gene family includes: *Pbx1*, originally cloned as a gene rearranged in a human pre-B leukemia containing a reciprocal translocation between chromosomes 1 (*Pbx1*) and 19 (*E2A*) (Kamps *et al*., 1990), and the highly related *Pbx2* and *3* genes (Monica *et al*., 1991), as well as the *Drosophila exd* (Peifer and Wieschaus, 1990) and the *Caenorhabditis elegans ceh-20* genes (Burglin and Ruvkun, 1992).

Experimental Procedures

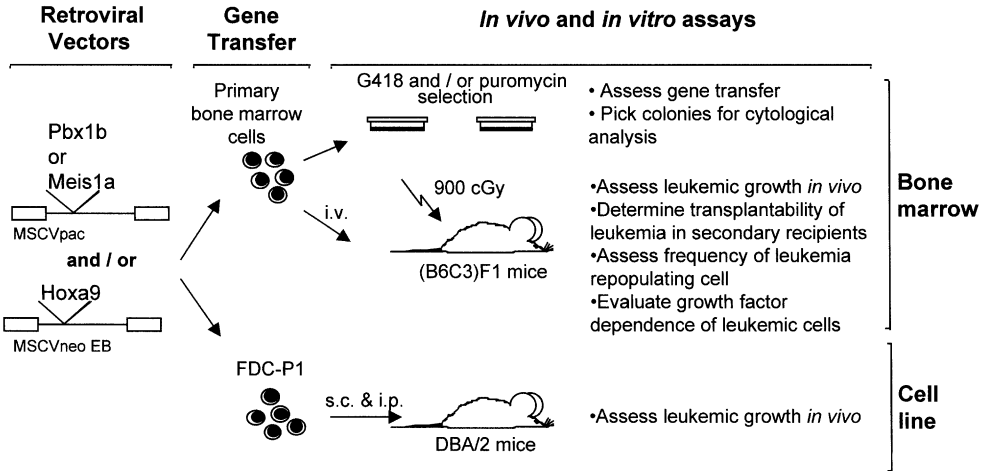

Fig. 1. Overview of the experimental strategies described in these studies.

A prototypical member of a new family of genes encoding TALE homeoproteins, *Meis1*, recently was isolated as a common site of viral integration in 15% of AMLs developing in BXH-2 mice (Moskow *et al*., 1995). This family includes at least two other highly related members, Meis2 and Meis3, and shares ~45% sequence identity with Pbx in the homeodomain (Nakamura *et al*., 1996; Steelman *et al*., 1997). Interestingly, 95% of the BXH-2-derived myeloid leukemias that overexpressed *Meis1* also contained proviral integrations that resulted in the overexpression of *Hoxa7* or *Hoxa9* (Nakamura *et al*., 1996). Together, these data suggested that Meis1 collaborated with either of these Hox proteins in leukemic transformation and that Hox and Meis1 proteins may cooperatively bind DNA similarly to Hox and Pbx.

It is now known that while Hox proteins from paralogs 1–10 can cooperatively bind DNA *in vitro* with Pbx (Chang *et al*., 1996), those from paralogs 9–13 do so with Meis1 (Shen *et al*., 1997a). Thus, Hox members of the 9th and 10th paralogs appear to have the ability to bind DNA as heterodimers with either Meis1 or Pbx1.

In addition to its reported interactions with selected Hox proteins, Meis1 also appears to be a major intracellular binding partner with Pbx1 (Chang *et al*., 1997). Recent studies have shown that Meis1 and its *Drosophila* homolog Hth have the ability to translocate Exd from the cytoplasm to the nucleus (Rieckhof *et al*., 1997; Pai *et al*., 1998). The control of nuclear translocation of Pbx (or Exd) by Meis (or Hth) is physiologically relevant because functional studies in *Drosophila* indicate that *exd* is active in regions where its product is located to the nucleus (Gonzalez-Crespo and Morata, 1995; Rauskolb *et al*., 1995).

Both genetic and molecular studies have shown that *Pbx* and *Pbx*-like genes are required for some of the biological functions regulated by Hox proteins (Chan *et al*., 1994; Sun *et al*., 1995; Maconochie *et al*., 1997; Rocco *et al*., 1997). We have shown recently that Pbx1b is required for Hoxb4- and Hoxb3-induced transformation of Rat-1 fibroblasts (Krosl *et al*., 1998). In order to test whether the *in vitro* DNA-binding partners of Hoxa9 (i.e. Pbx1 and Meis1) can modulate its effect on differentiation and transformation of hemopoietic cells, we induced, by retroviral gene transfer, the overexpression of *Hoxa9* together with *Meis1a* or with *Pbx1b* in primary murine bone marrow and FDC-P1 cells and analyzed the effects of these manipulations *in vitro* and *in vivo*.

Results

Overview of experimental strategies and retroviral constructs used in this study

The *Hoxa9*, *Meis1a* and *Pbx1b* cDNAs were introduced downstream of the long terminal repeat (LTR) of the MSCVneoEB (*Hoxa9*) or MSCVpac (*Meis1a* and *Pbx1b*) retroviral vectors in order to confer high expression levels of these genes in transduced cells. The experimental strategy used to study the effects of the overexpression of these genes on differentiation and transformation of primary mouse bone marrow cells and the growth factor-dependent FDC-P1 cell line is depicted schematically in Figure 1.

Retroviral gene transfer of Hoxa9, Meis1a and Pbx1b to primary bone marrow cells

Murine primary bone marrow cells were infected with *Hoxa9*-, *Meis1a*- or *Pbx1b*-bearing recombinant retroviruses, or with a combination of *Hoxa9* and *Meis1a*, or *Hoxa9* and *Pbx1b* retroviruses. The gene transfer efficiencies, assessed by *in vitro* colony formation of G418-resistant (i.e. containing *Hoxa9*-bearing proviruses) or puromycin-resistant (i.e. *Meis1a* or *Pbx1b*) clonogenic progenitors, were 50, 35 and 15% for *Hoxa9*, *Meis1a* and *Pbx1b*, respectively. The efficiencies of double infection of progenitor cells were 10% for *Hoxa9* and *Meis1a*, and 5% for *Hoxa9* and *Pbx1b*, as assessed by co-resistance to G418 and puromycin. The ability of the transduced (i.e. drug-resistant) progenitor cells to generate the various colony types normally observed in methylcellulose cultures (i.e. CFU-GM, CFU-GEMM and BFU-E) was not grossly altered by any of these manipulations as assessed by microscopic evaluation using standard criteria

Table I. Absolute numbers of colony-forming cells resistant to G418 (overexpressing *Hoxa9*) and/or puromycin (overexpressing *Meis1a or Pbx1b*) transplanted per mouse

Virus	No. of mice transplanted	No. of G418^r CFC injected/mouse[a]	No. of puro^r CFC injected/mouse[a]	No. of G418^r + puro^r CFC injected/mouse[a]
Experiment 1				
Hoxa9	9	705	n.t.	n.t.
Meis1a	4	n.t.	1380	n.t.
Pbx1b	5	n.t.	300	n.t.
Hoxa9 + Meis1a	10	1140	970	340
Hoxa9 + Pbx1b	10	330	320	80
Experiment 2				
Hoxa9 + Meis1a	5	1530	2300	230
Hoxa9 + Pbx1b	5	2120	2270	260

[a]The number of CFC injected was determined as follows: (cell dose)×(CFC frequency in bone marrow inoculum)×[% CFC resistant to puromycin (*Meis1a* and *Pbx1b*) and/or G418 (*Hoxa9*)].
n.t., not tested.

(Humphries *et al.*, 1981) (n = 88, 45, 19, 27 and 7 colonies evaluated for *Hoxa9*-, *Meis1a*-, *Pbx1b*-, *Hoxa9*- and *Meis1a*-, and *Hoxa9*- and *Pbx1b*-transduced progenitors respectively).

Acute myeloid leukemia arises in recipients of bone marrow cells overexpressing Hoxa9 and Meis1a

To determine whether *Hoxa9* collaborates with either *Meis1a* or *Pbx1b* in leukemic transformation, mice were reconstituted with bone marrow cells infected as described above. Table I shows the numbers of transduced progenitors, obtained from two different infection protocols, that were injected per mouse.

All recipients of bone marrow cells transduced with *Hoxa9*, *Meis1a*, *Pbx1b*, or *Hoxa9* and *Pbx1b* thrived normally for >170 days following transplantation (except for one *Hoxa9* animal, which died 56 days post-transplantation of intestinal obstruction). In contrast, all 15 recipients of cells transduced with *Hoxa9* and *Meis1a* developed AML as early as 49 days post-transplantation (67 ± 16 days; mean ± SD), although one animal survived for 118 days before succumbing to AML (Figure 2). Northern blot analysis of total RNA isolated from spleen cells of leukemic mice confirmed the high expression levels of the retrovirally derived mRNA for both *Hoxa9* and *Meis1a* in these cells (Figure 3). These results demonstrate that by themselves *Hoxa9*, *Meis1a* and *Pbx1b* are not acutely transforming when overexpressed in primary bone marrow cells, but that co-overexpression of *Hoxa9* and *Meis1a* rapidly induces leukemic transformation.

Characterization of leukemic cells overexpressing Hoxa9 and Meis1a

Morphologically, all leukemias developing in the recipients of *Hoxa9*- and *Meis1a*-transduced cells were characterized by partial myeloid differentiation (Figure 4B and C). The bone marrow from the leukemic mice contained 77 ± 12% blast cells with 23 ± 2% more mature myeloid cells such as neutrophils and monocytes. Histochemical analyses showed that although >50% of these cells stained strongly with Sudan black (Figure 4D), they were all negative for periodic acid–Schiff, peroxidase, butyrate and chloroacetate esterase staining, as often seen in poorly differentiated myeloid leukemias (data not shown).

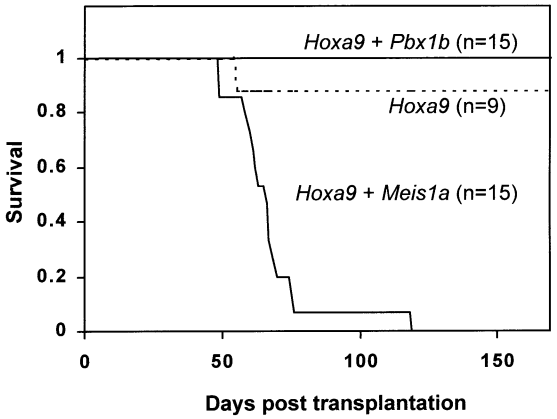

Fig. 2. Survival curve of mice transplanted with transduced bone marrow cells. The fraction of mice surviving up to 170 days post-transplantation of transduced bone marrow cells are shown for *Hoxa9*, *Hoxa9 + Meis1a* and *Hoxa9 + Pbx1b*. Mice transplanted with bone marrow cells transduced with *Meis1a* (n = 4 mice) or *Pbx1b* (n = 5 mice) were healthy for >170 days post-transplantation, and thus their survival curves are identical to *Hoxa9 + Pbx1b*.

Furthermore, the leukemic cells were 100% Mac-1, and 70% Gr-1 positive but were not recognized by antibodies to CD4, CD8 and CD45R (B220) as determined by flow cytometry (data not shown). Based on these data, the *Hoxa9*- and *Meis1a*-induced leukemias would be categorized as M2 (>10% bone marrow differentiated elements) by the French–American–British (FAB) classification.

Although the peripheral white blood cell counts were very high in these mice, the platelet and red blood cell numbers were only marginally decreased (Table II). In addition, all mice analyzed had infiltrated spleen, lymph nodes, bone marrow and thymus at the time they were sacrificed, as determined by Southern blot analyses (shown for mouse No. 11 in Figure 5A) and morphological evaluation (data not shown).

To characterize the *Hoxa9*- and *Meis1a*-induced leukemias further, bone marrow cells from leukemic animals were analyzed in clonogenic progenitor assays. In methylcellulose cultures supplemented with 10% serum, WEHI-3-conditioned medium and erythropoietin, the bone marrow leukemic progenitors grew with a plating efficiency of 1.9 ± 0.6% (n = 3 different mice, 83 ± 26%

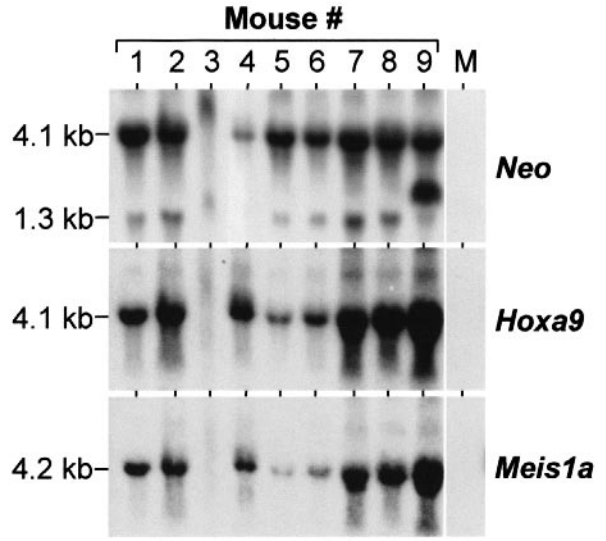

Fig. 3. Expression of *Hoxa9* and *Meis1a* in spleen cells isolated from leukemic recipients of *Hoxa9*- and *Meis1a*-transduced bone marrow cells. Total spleen RNAs from nine leukemic mice (experiment 1) and one normal mouse (M) were analyzed by Northern hybridization to determine the expression of the retrovirally derived *Hoxa9* (4.1 kb) and *Meis1a* (4.2 kb) transcripts. Autoradiographs are shown for membranes hybridized to probes specific for: *Neo*^{r} (top panel) showing both the expression of the 4.1 kb LTR-driven *Hoxa9*-bearing mRNA, as well as the internal *pgk* promoter-driven 1.3 kb *Neo*^{r} mRNA; *Hoxa9* (middle panel); and *Meis1a* (bottom panel). Mouse numbers are identified above the top panel. Exposure times were 18–24 h. The abnormal signals in lane 3 are secondary to poorly run RNA. The *pgk* promoter-driven *Neo*^{r} in lane 9 is of abnormal size. This probably represents a splice variant as the integrated provirus is not rearranged in these cells (Figure 5C, lane 9). Each lane was loaded equally as determined by ethidium bromide staining of the membrane and by hybridization to an 18S rRNA probe (not shown).

being G418 and puromycin resistant) compared with 0.2% for normal bone marrow cells (Table II). Cytospin preparations of these colonies analyzed by Wright staining showed that they contained >70% blasts with up to 25% mature myeloid elements (mostly promyelocytes). In an attempt to derive a cell line from these leukemic cells, 12 drug-resistant colonies (from two mice) were picked after 7 days of growth and plated in liquid cultures containing identical components to those present in the primary semi-solid cultures. These secondary cultures were incapable of supporting the growth and survival of the leukemic cells for more than 10 days. In addition, colony formation by the bone marrow leukemic progenitor cells was dependent on the presence of exogenous growth factors (i.e. 0 versus 800–1300 colonies per 50 000 bone marrow cells in the absence or presence of IL-3, respectively, two mice analyzed). In summary, the *Hoxa9*- and *Meis1a*-induced leukemias are characterized by poorly differentiated, growth factor-dependent cells of the myeloid lineage.

Clonal analysis of Hoxa9- and Meis1a-induced leukemias

To verify the presence and integrity of the *Hoxa9* and *Meis1a* proviruses in the leukemic samples, Southern blot analyses were performed on genomic DNAs extracted from various hemopoietic tissues and digested with *Kpn*I to release the proviruses. Figure 5 shows that all samples analyzed contained the intact integrated proviruses of both *Meis1a* (4.2 kb, Figure 5B) and *Hoxa9* (4.1 kb, Figure 5C). Some of these leukemic samples also contained rearranged *Hoxa9* or *Meis1a* proviruses (e.g. mice Nos 5, 7, 9, 12 and 14, Figure 5B and C), as previously reported for the MSCV retroviral vectors (Hawley *et al*., 1997).

To determine the number of leukemic clones present in each recipient of *Hoxa9*- and *Meis1a*-transduced cells, genomic DNA was also digested with restriction enzymes which cleave only once in the integrated proviruses (i.e. *Bgl*II or *Eco*RI), thus releasing fragments the size of which depend on the integration site of each provirus (Figure 5D). Based on the autoradiographic intensities of the different fragments, several leukemic samples consisted of at least two clones. For example, while the restriction fragments from mouse No. 11 all have similar intensities, probably indicating a single clone, bands of clearly different intensities identify two separate clones in mice Nos 2, 4, 5 and 9 (Figure 5D). The presence of more than a single clone in several of the leukemic samples suggested that the overexpression of *Hoxa9* and *Meis1a* in primary bone marrow cells directly leads to AML.

Determination of the frequency of the leukemia-repopulating cell in recipients of Hoxa9- and Meis1a-induced leukemias

The leukemias that developed in recipients of *Hoxa9*- and *Meis1a*-transduced bone marrow cells were readily transplantable to both irradiated and non-irradiated secondary recipients. The time required for leukemia to develop in secondary recipients was independent of whether the mice were irradiated or not, but varied between 23 and 42 days depending on the transplanted cell dose (Table III).

For two different primary leukemias (mice Nos 5 and 6), we attempted to determine the frequency of the biologically relevant cell that can generate leukemia in a secondary recipient when transplanted, or the leukemia-repopulating cell (LRC). Using principles of limiting dilution, a wide range of cell numbers (10^6 to 50) were injected together with a radioprotective dose of normal cells (i.e. 10^6) in lethally irradiated secondary recipients. All secondary recipients, including those receiving just 50 leukemic bone marrow cells, developed AMLs (Table III). Northern blot analyses of RNA extracted from the spleens of secondary recipients confirmed that the leukemic cells overexpressed *Hoxa9* and *Meis1a* from the retroviral LTRs (data not shown).

In order to follow the 'transplantability' of the various leukemic clones present in some of the primary recipients of *Hoxa9*- and *Meis1a*-transduced cells, Southern blot analysis was performed on genomic DNA isolated from the leukemic cells that grew in secondary recipients. For example, when the biclonal leukemia from primary mouse No. 5 was transplanted, three of the four secondary recipients of 500 or 50 leukemic bone marrow cells appeared to be repopulated with only one clone (clone 'a', animals 5.5, 5.6 and 5.8, Figure 6), whereas the leukemias which arose in the secondary recipients of 10^6 to 3000 leukemic bone marrow cells contained both clones 'a' and 'b' (mice Nos 5.1–5.4, Figure 6). These data suggested that limiting dilution of the LRC was reached for clone 'b' (but not for clone 'a') in secondary recipients of 50 or 500 leukemic bone marrow cells. Thus, for the two clones analyzed, the frequency of the LRC is in the

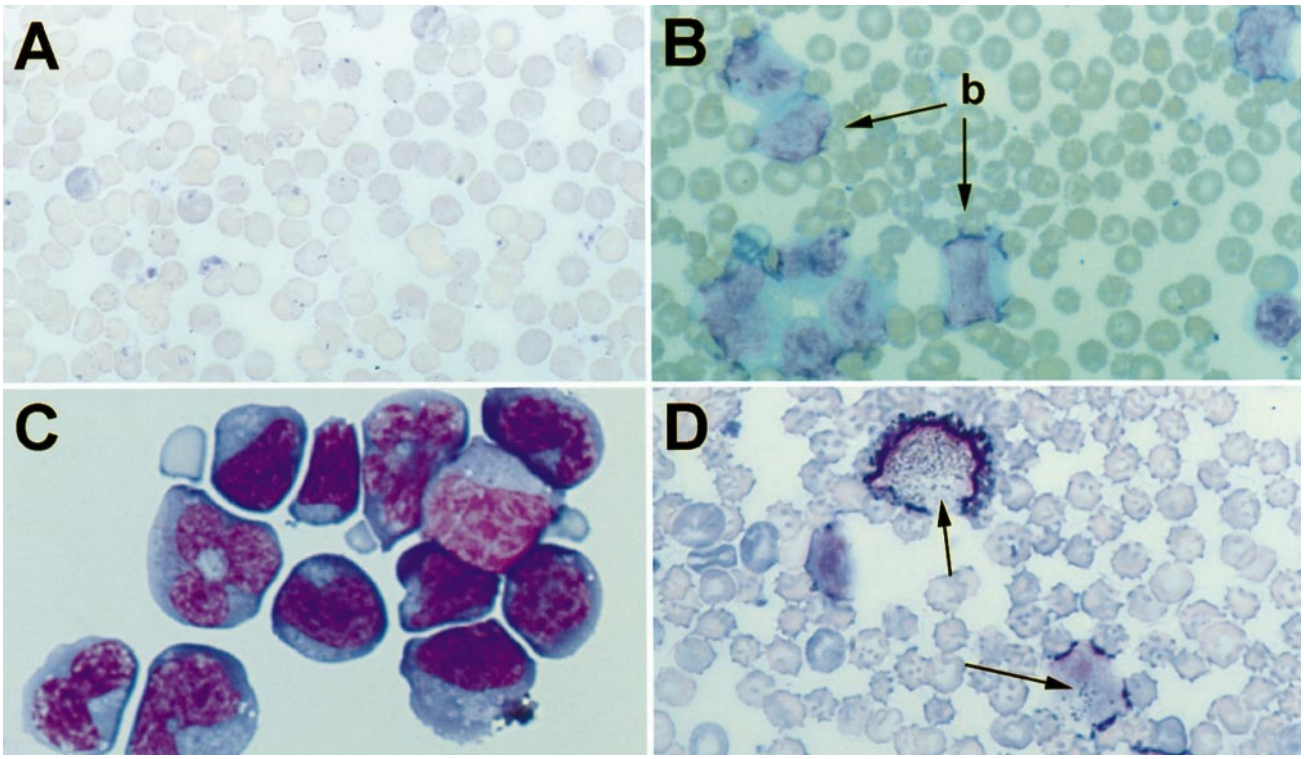

Fig. 4. Morphological analyses of peripheral blood and bone marrow cells isolated from leukemic mice. Wright–Giemsa-stained material is shown for: peripheral blood from a normal B6C3 mouse (**A**); peripheral blood (**B**) and bone marrow cells (**C**) from a leukemic mouse that was transplanted with *Hoxa9*- and *Meis1a*-transduced primary bone marrow cells; leukemic blasts [as in (B)] with Sudan black-staining granules indicated by arrows (**D**). b = blasts.

Table II. Hemopoietic parameters of recipients of transduced bone marrow cells analyzed at <12 weeks post-transplantation

Mice[a]	Peripheral blood values				Spleen size (g)	CFC (% resistant to G418 + puromycin)	
	WBC (10^9/l)	RBC (10^{12}/l)	Plat.[b] (10^9/l)	% blast		per femur ($\times 10^3$)	per spleen ($\times 10^3$)
Non-leukemic mice							
Control mice[c] ($n = 4$)	3.6 ± 0.7	10.6 ± 0.5	672 ± 38[d]	0	0.1	36 ± 15 (0)	6 (0)
Hoxa9 ($n = 3$)	7.6 ± 1.2	9.4 ± 0.8	716[e,g]	0	n.a.	n.a.	n.a.
Meis1 ($n = 4$)	7.3 ± 1.1	9.6 ± 0.2	686 ± 311[d]	0	n.a.	n.a.	n.a.
Pbx1 ($n = 4$)	9.2 ± 0.8	9.8 ± 0.1	511 ± 79	0	n.a.	n.a.	n.a.
Hoxa9 + *Pbx1* ($n = 4$)	7.7 ± 2.0	9.1 ± 0.2	n.a.[g,h]	0	0.09	50 ± 7[f] (3.3 ± 2.5)	n.a.
Leukemic mice							
Hoxa9 + *Meis1* ($n = 8$)	146 ± 76	7.0 ± 1.5	201 ± 68	81 ± 9	0.27 ± 0.03	380 ± 120 (83 ± 26)	109 ± 161[d]

[a]All age matched.
[b]Platelet values may vary due to aggregation.
[c]These mice have never been irradiated or transplanted.
[d]$n = 2$; [e]$n = 1$; [f]$n = 3$.
[g]Morphological evaluation of blood smears showed normal numbers in all mice examined.
[h]Not assessed.
n.a., not available.

range of 1 in 50 to 1 in 500 bone marrow cells for one clone and more frequent than 1 in 50 bone marrow cells for the other clone. Based on transplantation of human leukemias into SCID mice, the LRC frequency was estimated at ~1 in 250 000 leukemic bone marrow cells (Lapidot *et al.*, 1994). Our results thus demonstrate that the frequency of the LRC is much higher than estimated in a xenogenic transplantation model (i.e. human→mouse).

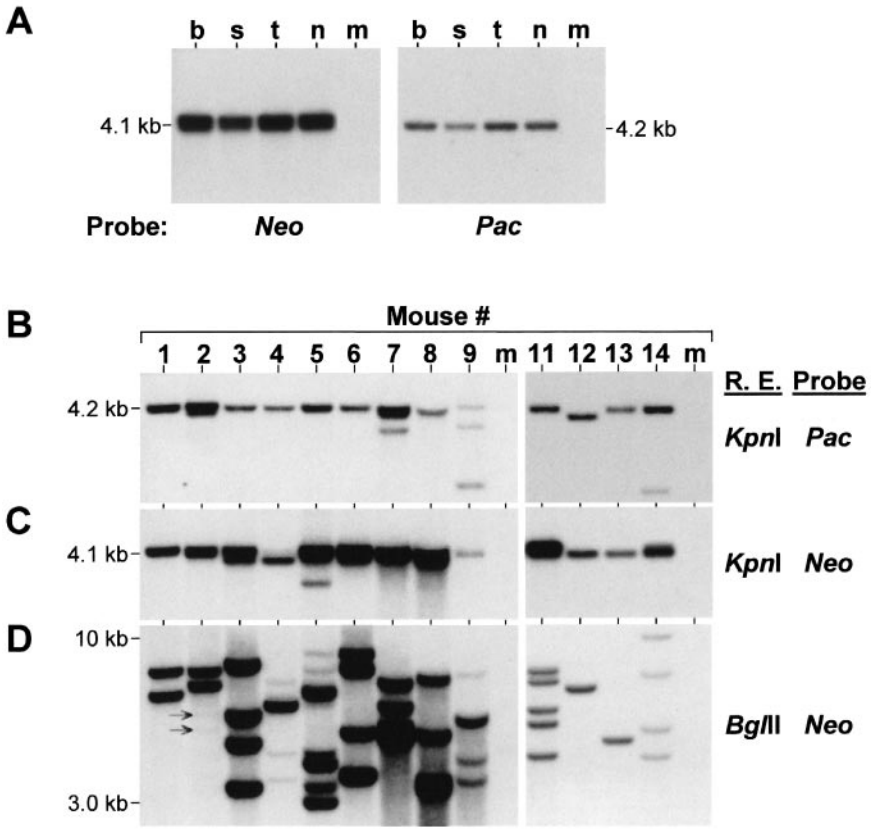

Fig. 5. Southern blot analysis of DNA isolated from the bone marrow of primary recipients of *Hoxa9*- and *Meis1a*-transduced cells. Ten μg of genomic DNAs isolated from the bone marrow (or other hemopoietic tissues where indicated) of leukemic mice 1–9 (experiment 1), and 11–14 (experiment 2), and from the bone marrow of a normal mouse (m) were digested with *Kpn*I and *Bgl*II, resolved on agarose gels, blotted and hybridized to a probe specific for *Neo*^{r} (identifying *Hoxa9*-bearing proviruses) or *Pac* (identifying *Meis1a*-bearing proviruses) as indicated. The MSCV viral LTRs contain *Kpn*I sites, and digestion with *Kpn*I releases the provirus. Both *Hoxa9* and *Meis1a* proviruses (4.1 and 4.2 kb, respectively, as indicated by size markers) were detected in the bone marrow (b), spleen (s), thymus (t) and lymph nodes (n) as shown for mouse No. 11 (**A**), and shown for the bone marrow of all mice analyzed (**B** and **C**). Fragments of different intensities in the digestions with *Bgl*II, which cleaves once in the provirus, identify two leukemic clones in mice 2, 4, 5 and 9, while leukemias from the other mice appear to contain one clone each (**D**). Exposure times were 24–48 h. R.E., restriction enzyme used in digestion. The arrows in (D) show the faint bands presumably representing a minor leukemic clone.

In fact, the LRC frequency is at least comparable with (or higher than) the plating efficiency of these cells in methylcellulose cultures (1.9%, see above).

The transforming potential of Hoxa9 is not accelerated by the overexpression of Pbx1b

In order to determine which of the three genes analyzed in this study on its own has a leukemogenic potential when overexpressed in bone marrow cells, recipients of cells transduced with *Hoxa9* ($n = 5$), *Meis1a* ($n = 4$) or *Pbx1b* ($n = 4$) were followed for up to a year post-transplantation (Table IV). Three of the five recipients of *Hoxa9*-transduced cells developed AML within 7 months post-transplantation, and in the other two mice, a pre-leukemic disease was present as evidenced by increased spleen size (0.4 g) and by a noticeable increase in peripheral blood immature myeloid cells of the granulocytic lineage. In contrast, none of the recipients of *Meis1a*- or *Pbx1b*-transduced cells developed leukemia or pre-leukemia, even after 10–14 months of observation (Table IV). AML also developed in recipients of bone marrow cells transduced with both *Hoxa9* and *Pbx1b*, but at no faster rate than in recipients of *Hoxa9*-transduced cells. Importantly, the viral expression of both *Hoxa9* and *Pbx1b* was documented in two of these leukemias (data not shown). These data thus indicate that of the three genes analyzed in this study, only *Hoxa9* is capable of inducing

Table III. Occurrence of acute myeloid leukemia in secondary and tertiary recipients of bone marrow-derived leukemic cells overexpressing *Hoxa9* and *Meis1a*

Mouse ID[a]	No. of bone marrow cells injected per recipient	Time to AML (days)
Secondary recipients		
1.1, 2.1, 5.1, 7.1, 8.1 ($n = 9$)	10^6	23 ± 3
5.2, 6.1 ($n = 3$)	10^5	28 ± 0
5.3, 6.2 ($n = 7$)	10^4	30 ± 1
5.4, 6.3 ($n = 6$)	3000	34 ± 1
5.5, 5.6	500	42
5.7, 5.8	50	42
Tertiary recipients		
1.1.1, 2.1.1 ($n = 2$)	10^6	25

[a]The first digit in the mouse identification (ID) number corresponds to the origin of bone marrow graft (i.e. primary recipient), the second and third ID digits are specific to each secondary and tertiary recipient respectively.

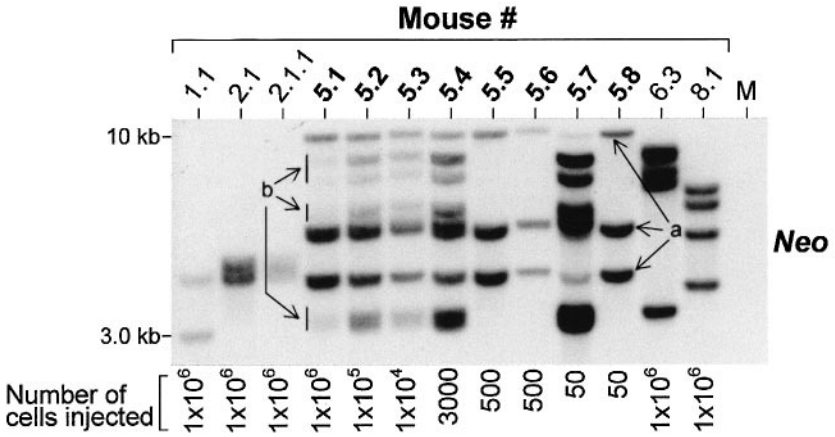

Fig. 6. Clonal analysis of leukemias developing in secondary and tertiary recipients. Southern blots analysis was performed on 10 μg of genomic DNA prepared from bone marrow cells obtained from secondary and tertiary recipients of leukemic cells isolated from the bone marrow of primary leukemic mice (see Table III). The DNA was digested with *Eco*RI, which cleaves the integrated provirus once, and membranes were hybridized to a probe specific for *Neo*^{r}. Lanes are identified based on the origin of the bone marrow injected in the secondary recipients. For example, lane number 5.1 represents the secondary recipient No. 1 which received bone marrow cells from primary donor No. 5. The number of cells injected per mouse is indicated below each lane and size markers are shown on the left. Exposure time was 24 h. The two leukemic clones derived from mouse No. 5 are identified by sets of restriction fragments labeled 'a' and 'b'. While clone 'a' is present in all secondary recipients of bone marrow cells from mouse No. 5, mice 5.5, 5.6 and 5.8 do not appear to contain clone 'b'.

monoclonal AMLs when overexpressed in bone marrow cells, while *Meis1a*, but not *Pbx1b*, dramatically accelerates *Hoxa9*-induced leukemias.

To confirm that *Pbx1b* does not accelerate *Hoxa9*-induced leukemias, we performed a modified bone marrow infection procedure aimed at transplanting a high number of *Hoxa9*- and *Pbx1b*-transduced colony-forming cells (CFC) per mouse (see Table I, experiment 2, and Materials and methods). In this experiment, 260 *Hoxa9*- and *Pbx1b*-transduced CFC were transplanted per mouse versus 230 for *Hoxa9* and *Meis1a* (compared with 80 and 340, respectively, in the first experiment, see Table I). The results from this second experiment were similar to those of the first in that AML developed in all five recipients of *Hoxa9*- and *Meis1a*-transduced cells in 63 ± 7 days, while mice transplanted with *Hoxa9*- and *Pbx1b*-infected cells remained healthy for >6 months post-transplantation.

To verify that mice transplanted with *Hoxa9*- and *Pbx1b*-transduced cells were indeed reconstituted with long-term repopulating cells (LTRC) that contained both proviruses, three mice were sacrificed 6 months after transplantation and their bone marrow cells were plated in methylcellulose under G418 (*Hoxa9*) and puromycin (*Pbx1b*) selection. The proportion of clonogenic progenitors resistant to both drugs varied between 1 and 6%, or up to 2400 *Hoxa9*- and *Pbx1b*-transduced CFC per femur (i.e. 6%×40 000 CFC per femur). The presence of both *Hoxa9* and *Pbx1b* proviruses in the same progenitor cell was confirmed by Southern blot analysis of genomic DNA isolated from two G418- and puromycin-resistant colonies that were obtained from one of these mice (Figure 7).

Together, these experiments confirmed that, in contrast to *Meis1a*, *Pbx1b* overexpression does not enhance the rate of leukemic transformation induced by *Hoxa9*.

Hoxa9 and Meis1a also collaborate to transform the FDC-P1 hemopoietic cell line

The ability of *Hoxa9* to collaborate specifically with *Meis1a*, but not *Pbx1b* in acute leukemic transformation was also observed in FDC-P1 cells. In these experiments, FDC-P1 cells were transduced, selected and then transplanted to syngenic DBA/2 mice. All mice that received FDC-P1 cells overexpressing *Hoxa9* and *Meis1a* developed AML within 14 days, while recipients of cells overexpressing *Hoxa9* and *Pbx1b*, or any of these three genes alone, remained healthy for at least 90 days after transplantation.

Table IV. Long-term follow-up and incidence of late occurring (i.e. >6 months) leukemia or pre-leukemia in mice reconstituted with bone marrow cells overexpressing *Hoxa9*, *Meis1a* or *Pbx1b*

Mice	Observation periods (months)	Cause of death	Incidence of late-occurring AML or pre-AML
Hoxa9			
A9.1	6	AML	5/5
A9.2	6.5	AML	
A9.3	6.5	AML	
A9.4	8	sacrificed, pre-leukemic[a]	
A9.5	8	sacrificed, pre-leukemic	
Meis1a			
M.1	7	undetermined, NOD	0/4
M.2	10.5	sacrificed, NOD	
M.3	13.5	sacrificed, enlarged cervical node (~5 mm)[b]	
M.4	14	sacrificed, ovarian tumor	
Pbx1b			
P.1	7.5	sacrificed, NOD	0/4
P.2	10.5	sacrificed, enlarged cervical node (~5 mm)[b]	
P.3	10.5	sacrificed, NOD	
P.4	14	sacrificed, NOD	

[a]Pre-leukemic mice had enlarged spleens and pale bone marrow which contained an increase in both granulocytic cells and myeloid CFC.
[b]These mice had no evidence of transduced cells in their bone marrow as evaluated by Southern or Northern blot analyses. In addition, the proportion of bone marrow progenitor cells resistant to puromycin was <1% in these mice. All other mice were repopulated by transduced cells when sacrificed.
NOD, no evidence of disease.

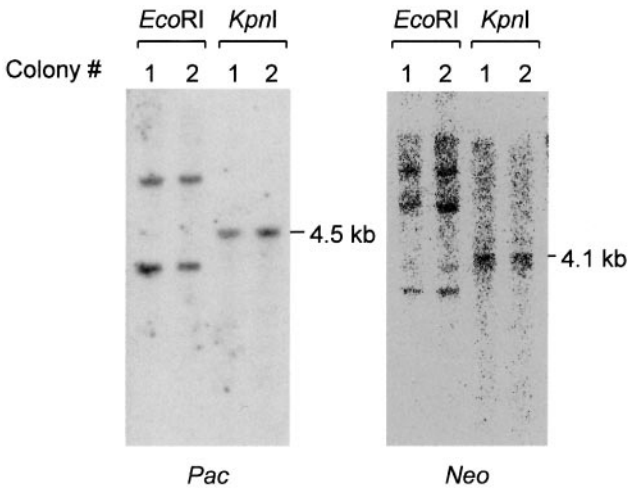

Fig. 7. *Hoxa9* and *Pbx1b* proviruses are present in the same clonogenic progenitor cells isolated from the bone marrow of a transplanted mouse. Six months post-transplantation, bone marrow cells were isolated from a mouse transplanted with *Hoxa9*- and *Pbx1b*-transduced bone marrow cells and were plated in methylcellulose cultures under G418 and puromycin selection. Colonies were expanded in liquid cultures, two of which yielded sufficient cells to perform Southern hybridization analyses. Genomic DNAs were digested with *Eco*RI or *Kpn*I, resolved on an agarose gel, transferred onto a nylon membrane, hybridized to a *Pac* probe (identifying *Pbx1b*-bearing proviruses), then stripped and hybridized to a *Neo*^{r} probe (identifying *Hoxa9*-bearing proviruses). *Kpn*I digests reveal the presence of both full-length proviruses containing *Hoxa9* (4.1 kb) and *Pbx1b (*4.5 kb), while the identical *Eco*RI patterns indicate that the two colonies originated from the same clone containing three *Hoxa9*-bearing and two *Pbx1b*-bearing viral integrants. Exposure times were 24 and 72 h for *Pac* and *Neo*^{r}, respectively.

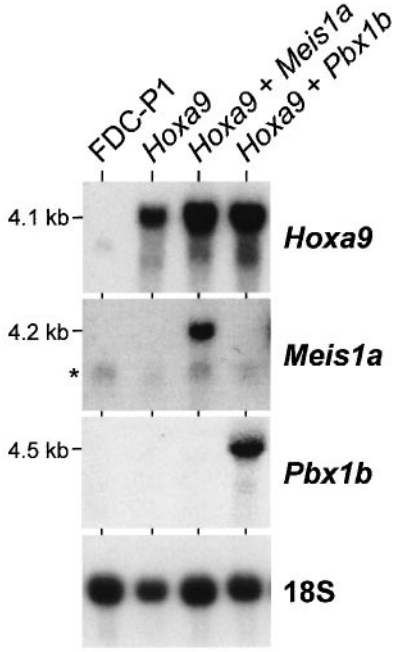

Fig. 8. Northern blot analyses documenting the expression of *Hoxa9*, *Meis1a* and *Pbx1b* in FDC-P1 cells. Ten μg per lane of total cellular RNAs from untransduced (FDC-P1) cells and from cells transduced with *Hoxa9*, *Hoxa9* and *Meis1a*, and *Hoxa9* and *Pbx1b* were separated in formaldehyde gels, blotted and hybridized to probes for *Hoxa9* (upper panel), *Meis1a* (second), *Pbx1b* (third) or 18S rRNA (bottom). The asterisk indicates that in addition to the retroviral LTR-derived transcript, the *Meis1a* probe hybridized to an endogenously expressed RNA the size of which is consistent with the 3.8 kb transcript of *Meis1* (Moskow *et al*., 1995). Exposure times were 18–24 h for *Hoxa9*, *Meis1a* and *Pbx1b*, and 1 h for 18S.

These observations were important because it was demonstrated recently that in *Drosophila* the activity of the Pbx1 homolog Exd is dependent on the presence of Hth, or its functional homolog Meis1 (Rieckhof *et al*., 1997; Pai *et al*., 1998). This suggested that the lack of acute leukemic transformation in cells overexpressing *Hoxa9* and *Pbx1b* could be due to the lack of Meis1 expression. However, as FDC-P1 cells express readily detectable levels of *Meis1* (Figure 8, asterisk), these experiments suggest that it is not the absence of endogenous *Meis1* that explains the inability of the co-overexpression of *Hoxa9* and *Pbx1b* to transform hemopoietic cells, and confirm that leukemic transformation of primary bone marrow cells resulting from the overexpression of *Hoxa9* and *Meis1a* is sustained in FDC-P1 cells as well.

Discussion

The studies presented here document that only the co-overexpression of *Hoxa9* and *Meis1a* results in rapid leukemic transformation of primary bone marrow cells. Interestingly, we recently showed that Pbx1b collaborates with Hoxb3 and with Hoxb4 to transform Rat-1 fibroblasts when overexpressed (Krosl *et al*., 1998). Together, our studies suggest that *Meis1a* and *Pbx1b* can induce cellular transformation when co-overexpressed with distinct *Hox* partners. It will be interesting to determine if these observations can be generalized to other Hox proteins, such as Hoxb8 or Hoxa10, which are known to participate in leukemic transformation.

Does overexpression of Hoxa9 and Meis1a lead to spontaneous leukemic transformation of hemopoietic cells?

Fourteen of 15 mice that received bone marrow cells overexpressing both *Hoxa9* and *Meis1a* developed overt leukemia in 49–75 days post-transplantation (Figure 2). The early onset and the small variation in the time of onset of the disease suggested that the overexpression of *Hoxa9* and *Meis1a* might be sufficient for acute leukemic transformation. Several other lines of evidence further support this possibility. First, leukemias that developed in several of these mice were biclonal, not monoclonal. Second, there was no significant difference for leukemia onset between certain primary recipients of *Hoxa9*- and *Meis1a*-transduced bone marrow cells (i.e. 49 days) versus secondary recipients injected with limited numbers of LRC (i.e. 42 days).

Overall, the relatively short time required for AML to occur in recipients of transduced primary bone marrow cells indicates that the collaborating leukemia-inducing proto-oncogenes *Hoxa9* and *Meis1a* are among the most potent known to date. The average of 67 ± 16 days after transplantation for *Hoxa9* and *Meis1a* was at least twice as fast as the time required for the E2A–Pbx fusion protein to induce leukemia when overexpressed in primary bone marrow (Kamps and Baltimore, 1993) and, as summarized in Figure 9, only the combination of IL-3 and Hoxb8 appears more potent (Perkins *et al*., 1990).

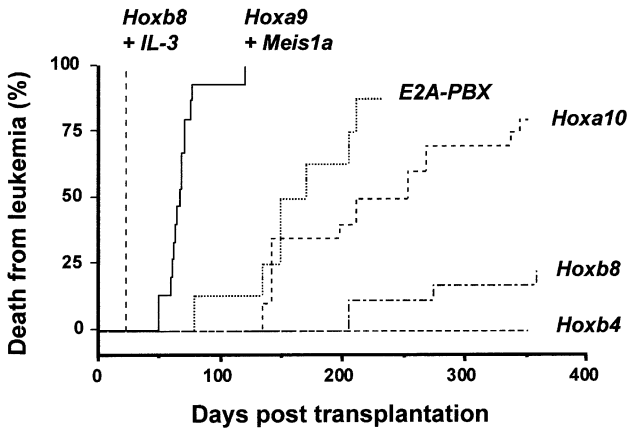

Fig. 9. Incidence of leukemia in recipients of bone marrow cells retrovirally transduced with various homeobox-containing cDNAs. Survival curves are shown for mice transplanted with primary bone marrow cells overexpressing *Hoxa9* and *Meis1a* (this study), IL-3 and *Hoxb8* (Perkins *et al*., 1990), *E2A–Pbx* (Kamps and Baltimore, 1993), *Hoxa10* (Thorsteinsdottir *et al*., 1997), *Hoxb8* (Perkins and Cory, 1993) and *Hoxb4* (Sauvageau *et al*., 1995). Note that *Hoxb4* does not induce leukemic transformation of bone marrow cells when overexpressed from the 5′ LTR of the MSCV retroviral vector. Based on our most recent data, the incidence of leukemia in mice reconstituted with bone marrow cells overexpressing *Meis1a*, *Pbx1b*, *Hoxa9*, or *Hoxa9* and *Pbx1b* is lower than observed for *Hoxa10*.

Primitive hemopoietic cells are the targets for transformation by Hoxa9 and Meis1a

An altered balance between self-renewal divisions and differentiation characterizes leukemias. In acute leukemia, differentiation and maturation are blocked such that undifferentiated cells accumulate and perturb the function of several organs including the bone marrow. The morphology of G418- and puromycin-resistant colonies (i.e. containing cells overexpressing both *Hoxa9* and *Meis1a*) obtained in methylcellulose cultures showed that freshly transduced primary bone marrow cells did not lose the capacity to differentiate. In contrast, when these transduced primary bone marrow cells were transplanted, few differentiated cells (>70% blasts) were observed in the bone marrow and blood of the leukemic recipient mice of such cells. This suggests that the overexpression of *Hoxa9* and *Meis1a* may have an inhibitory effect on differentiation of a hemopoietic cell type more primitive than a clonogenic progenitor.

Considering the short period of time required for clinical leukemia to manifest in primary recipients of *Hoxa9*- and *Meis1a*-transduced cells and the large number of transduced clonogenic progenitors injected per mouse [i.e. 340 and 230 per mouse in two different experiments (Table I)], the few leukemic clones observed per mouse suggest that leukemic transformation occurred in a cell type which is less abundant than the majority of the clonogenic progenitors. Since a large proportion (40–50%) of colony-forming cells obtained from bone marrow pretreated as described are multipotent (i.e. CFU-GEMM) (Thorsteinsdottir *et al*., 1997), then it would appear that the cell type transformed by the combined action of these two genes might be more primitive than a CFU-GEMM.

Based on previous results that estimated the frequency of HSC at 1 in 6000 bone marrow cells treated as detailed in Materials and methods (Sauvageau *et al*., 1995), two or three *Hoxa9*- and *Meis1a*-transduced HSC were injected per primary recipient. This is similar to the number of leukemic clones detected per mouse (one or two, see Figure 5D), and thus would be compatible with the HSC being the target for transformation. However, all leukemias analyzed in primary recipients had characteristics of myeloid, but not lymphoid cells. Since hemopoietic stem cells have both myeloid and lymphoid potential, these findings suggest that the HSC may not be the target for transformation by *Hoxa9* and *Meis1a*. Other possible explanations for the absence of lymphoid leukemia are the retroviral promoter specificity, or a functional bias of *Hoxa9*, which has been associated with myeloid leukemias in both mice (Nakamura *et al*., 1996) and humans (Borrow *et al*., 1996).

Together, these findings suggest that the target cell transformed by *Hoxa9* and *Meis1a* is best represented by a rare cell type which by its frequency and lineage is between a totipotent HSC and a multipotent clonogenic progenitor.

Hoxa9- and Meis1a-overexpressing leukemic cells are dependent on exogenous growth factors for their growth ex vivo

Although most leukemia-derived cell lines grow autonomously (i.e. without exogenous growth factors), many myeloid leukemic cell lines from patients have now been identified that do remain dependent on hemopoietic growth factors (Hassan and Drexler, 1995). In semi-solid cultures, the growth of *Hoxa9*- and *Meis1a*-overexpressing leukemic cells required, in addition to 10% fetal calf serum (FCS), the presence of IL-3. The necessity for exogenous growth factors for the '*ex vivo*' growth of the *Hoxa9*/*Meis1a*-overexpressing leukemic cells differs from that reported with the overexpression *Hoxb8* or *Hoxa10* where the majority of the leukemic cells had the capacity for growth *in vitro* in the absence of growth factors (Perkins and Cory, 1993; Thorsteinsdottir *et al*., 1997). Interestingly, *Hoxb8*-overexpressing leukemic cells were found to produce IL-3 (Perkins and Cory, 1993) while there was no evidence that *Hoxa10*-overexpressing leukemic cells produced this cytokine. Since the vast majority of leukemic cells derived from patients suffering from AML are also dependent on exogenous cytokine for their growth (Rodriguez-Cimadevilla *et al*., 1990), the results presented here suggest that the *Hoxa9*/*Meis1a*-induced leukemia may represent an interesting model to study the biology of human AML.

What is the molecular basis for the acute leukemic transformation of bone marrow cells overexpressing Hoxa9 and Meis1a?

Since *Hoxa9*, *Meis1* and *Pbx1* have all been linked to leukemogenesis, and Hoxa9 cooperatively binds DNA with Pbx1 (Chang *et al*., 1996) and with Meis1 (Shen *et al*., 1997a), it was somewhat surprising to observe that only Hoxa9 and Meis1a co-overexpression resulted in leukemic transformation. One possible explanation might be that target genes regulated by Hoxa9/Meis1 are different from those regulated by Hoxa9/Pbx1. In agreement with this hypothesis, site selection studies have shown that DNA-binding sites of Pbx1 and Hoxb9 (the product of a gene within the same parolog as *Hoxa9*) differ from those identified in similar studies performed with Hoxa9 and Meis1 (Shen *et al*., 1997b). Although the *in vivo* relevance

of the *in vitro* site selection studies remains to be elucidated, it was demonstrated recently that these sites resemble *bona fide* target sites (Peers *et al*., 1995). Thus, the differences in their DNA-binding specificities may account for the fact that *Hoxa9* and *Meis1a*, but not *Hoxa9* and *Pbx1b*, acutely transform hemopoietic cells.

Recent studies have, however, added an additional level of complexity to the Hox–Pbx and Hox–Meis interactions that may offer alternative interpretations of our results. In addition to its interactions with Hox proteins, Pbx1 has also been shown to interact with Meis1, in both the absence (Chang *et al*., 1997) and presence of DNA (Chang *et al*., 1997; Knoepfler *et al*., 1997). It is possible, therefore, that Hoxa9, Meis1 and Pbx1 operate as a heterotrimer to induce cellular transformation. In this model, endogenous Pbx would be a central player in *Hoxa9/Meis1a*-induced leukemia because biochemical studies suggest that in addition to enhancing Hox–DNA binding activity, Pbx may act as a 'linker protein' between Hox and Meis1 or Meis1-like proteins such as Prep1 (Berthelsen *et al*., 1998).

It was shown recently in *Drosophila*, that the nuclear translocation of Exd, the homolog of Pbx1, requires Hth, or its functional homolog Meis1 (Rieckhof *et al*., 1997; Pai *et al*., 1998). These findings suggested that in cells overexpressing *Hoxa9* and *Meis1a*, endogenously expressed Pbx1 could be transported to the nucleus by retrovirally expressed Meis1a and contribute to leukemic transformation through cooperative DNA binding with Hoxa9. Conversely, the lack of transformation of primary bone marrow cells overexpressing *Hoxa9* and *Pbx1b* could be related to the absence of endogenous *Meis1* expression in these cells. This possibility seems attractive because *Meis1* appears not to be expressed in bone marrow cells (Afonja *et al*., 1997).

Together, the results presented here demonstrate that transformation of primary bone marrow cells and FDC-P1 cells is observed when *Hoxa9* is overexpressed with *Meis1a* but not with *Pbx1b*. Based on these results and on our observations showing that Pbx1b is potently oncogenic in the presence of Hoxb3 and Hoxb4 (Krosl *et al*., 1998), our current hypothesis is that cellular transformation by members of the Hox family depends on specific collaboration with selected members of the TALE homeodomain family.

Materials and methods

Animals

All mice were originally bought from the Jackson Laboratories (Bar Harbor, MA) and then bred and maintained in the specific pathogen-free (SPF) animal facility of the Clinical Research Institute of Montreal (IRCM). Donors of primary bone marrow cells were >12-week-old (C57Bl/6J×C3H/HeJ) F_1 (B6C3) males and recipients were 7- to 12-week-old syngenic females. All animals were housed in ventilated microisolator cages and provided with sterilized food and acidified water. In experiments involving FDC-P1 cells, syngenic DBA/2 recipients of 5–6 weeks old were injected subcutaneously and intraperitoneally with 1.5×10^6 cells.

Generation of recombinant retroviruses

The complete coding region of the mouse *Hoxa9* cDNA (a kind gift of Dr Corey Largman, San Francisco, CA) was introduced in the *Bam*HI–*Xho*I site of the MSCVneoEB retroviral vector (which confers G418 resistance) using standard procedures (Davis *et al*., 1994b). Similarly, the mouse *Meis1a* and human *Pbx1b* (a kind gift of Dr Michael Cleary, Palo Alto, CA) were subcloned into the *Eco*RI and *Hpa*I site of MSCV-PGK-PAC retroviral vector (which confers puromycin resistance and was kindly provided by Dr R.Hawley, Sunnybrook Research Institute, Toronto, Ontario). High-titer helper-free recombinant retroviruses were produced by calcium phosphate precipitation of plasmid DNA into both the ecotropic GP+E-86 (Markowitz *et al*., 1988a) and the amphotropic GP+envAM12 (Markowitz *et al*., 1988b) packaging cell lines as described (Sauvageau *et al*., 1995). Efforts to produce high-titer *Meis1a* or *Pbx1b* viral producer cells were unsuccessful. At best, stable producers of *Meis1a* or *Pbx1b* were generating viral titers in the range of 5×10^4 c.f.u./ml. Viral supernatants obtained from BOSC-23 cells transiently infected with the *Pbx1b* cDNA were used when indicated. Absence of helper virus generation in the various viral producer cells was verified by failure to serially transfer virus conferring antibiotic resistance to Rat-1 fibroblast cells (Cone and Mulligan, 1984).

Cell lines

The ecotropic packaging cell line, GP+E-86, and the amphotropic cell line, GP+envAMP12, used to generate the recombinant retroviruses, were maintained in HXM medium as described (Sauvageau *et al*., 1995). At 24 h prior to harvest of viral supernatant or co-cultivation with bone marrow cells, viral producer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% newborn calf serum. The murine hematopoietic cell line FDC-P1 (Dexter *et al*., 1980) was maintained in RPMI with 10% FCS and supplemented with 5 ng/ml of IL-3 obtained from supernatants of COS cells or from the supernatant of FDC-P1 cells infected with a retrovirus overexpressing the mouse IL-3 cDNA (E.Kroon). All cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air, unless otherwise specified, and all reagents including media and sera were purchased from Gibco Life Technologies.

Retroviral infection of primary bone marrrow cells and FDC-P1 cells

Bone marrow cells were harvested from B6C3 mice 4 days after intravenous injection of 150 mg/kg body weight of 5-fluorouracil (5-FU) (Sigma) by flushing femurs with 2% FCS in phosphate-buffered saline (PBS) using a 21 gauge needle. Single cell suspensions of 5×10^5 cells/ml were then cultured in a Petri dish for 48 h in DMEM containing 15% FCS, 10 ng/ml human IL-6, 6 ng/ml murine IL-3 and 100 ng/ml murine Steel factor. Cells were then harvested, resuspended in the same medium supplemented with 6 μg/ml of polybrene (Sigma), and plated at 1–2×10^5 cells/ml on confluent viral producer cell monolayers irradiated at 155 cGy (cesium source, 179 cGy/min). For double infections with *Hoxa9* and *Meis1a*, or *Hoxa9* and *Pbx1b*, the respective viral producer cells were counted and seeded at equal numbers 24 h prior to the addition of bone marrow cells. Cells were co-cultured for 48 h with a medium change afer 24 h. In experiment No. 2 (Table I), viral supernatant from *Pbx1b*-transfected BOSC-23 cells was added to the culture. Loosely adherent and non-adherent cells were recovered from the co-cultures by agitation and repeated washing of dishes with PBS containing 2% FCS. Recovered bone marrow cells were washed once and then counted. All growth factors were used as diluted supernatants from appropriately transfected COS cells prepared at IRCM.

FDC-P1 cells were infected by exposure to filtered (0.2 μm, low protein binding filter, Millipore, Bedford, MA) viral supernatant from the ecotropic virus-producing cells. The viral supernatants were supplemented with 6 μg/ml polybrene (Sigma), FCS to a final concentration of 20% and IL-3 to 5 ng/ml. Transduced cells were selected and maintained in 1.3 mg/ml of G418 or 1.8 μg/ml puromycin as these concentrations were determined to be toxic to untransduced FDC-P1 cells.

Transplantation of retrovirally infected bone marrow

Lethally irradiated (900 cGy, 179 cGy/min, ^{137}Cs γ-rays, J.L.Shepherd, CA) 7- to 12-week-old (B6C3)F_1 (Ly5.2) mice were injected intravenously with 1×10^5–1×10^6 bone marrow cells harvested from co-cultivation with viral producer cells and 4×10^5 bone marrow cells freshly harvested from a (B6C3)F_1 donor.

In vitro clonogenic progenitor assays

For myeloid clonogenic progenitor assays, cells were plated on 35 mm Petri dishes (Corning, Fisher) in a 1.1 ml culture mixture containing 0.8% methylcellulose in α-medium supplemented with 10% FCS, 5.7% bovine serum albumin (BSA), 10^{-5} M β-mercaptoethanol, 1 U/ml human urinary erythropoietin (Epo), 10% WEHI-conditioned medium, 2 mM glutamine and 200 μg/ml transferrin in the presence or absence of 1.3 mg/ml of G418 and/or 1.3 μg/ml puromycin. Bone marrow cells harvested from the co-cultivation with virus producer cells or recovered

from reconstituted animals were plated at a concentration of $1–5\times10^3$ cells/ml or $2–5\times10^4$ cells/ml, respectively. Colonies were scored on days 12–14 of incubation as derived from CFU-GM, BFU-E or CFU-GEMM according to standard criteria (Humphries *et al*., 1981).

Flow cytometry

Flow cytometry of hemopoietic cells was performed as previously described (Sauvageau *et al*., 1997).

DNA and RNA analyses

To assess proviral integration, Southern hybridization analyses were performed as described (Pawliuk *et al*., 1994) using standard techniques. High molecular weight DNA was digested with *Kpn*I, which cleaves in the LTRs and releases the proviral genome, or with *Eco*RI or *Bgl*II, which cleave the provirus once to release DNA fragments specific to the proviral integration site(s). Total cellular RNA was isolated with the TRIzol reagent (Gibco-BRL), resolved by formaldehyde–agarose gel electrophoresis, and transferred onto nylon membranes (Zeta-Probe; Bio-Rad). Membranes were pre-hybridized, hybridized and washed as described (Davis *et al*., 1994a). Probes were generated from the *Xho*I–*Sal*I fragment of pMC1neo (*Neo*^{r}) (Thomas and Capecchi, 1987), the *Hin*dIII–*Cla*I fragment of MSCV-PGK-PAC (*Pac*), or the full-length cDNAs from *Hoxa9*, *Pbx1b* and *Meis1a*, and labeled with ^{32}P by random primer extension as described (Lawrence *et al*., 1995). To assess the relative amounts of rRNA loaded, Northern blots were probed for 18S RNA using end-labeled oligonucleotide 5′-ACG GTA TCT GAT CGT CCT CGA ACC-3′.

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