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# Hoxa9 Immortalizes a Granulocyte-Macrophage Colony-Stimulating Factor-Dependent Promyelocyte Capable of Biphenotypic Differentiation to Neutrophils or Macrophages, Independent of Enforced Meis Expression

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**The genes encoding Hoxa9 and Meis1 are transcriptionally coactivated in a subset of acute myeloid leukemia (AML) in mice. In marrow reconstitution experiments, coexpression of both genes produces rapid AML, while neither gene alone generates overt leukemia. Although Hoxa9 and Meis1 can bind DNA as heterodimers, both can also heterodimerize with Pbx proteins. Thus, while their coactivation may result from the necessity to bind promoters as heterodimers, it may also result from the necessity of altering independent biochemical pathways that cooperate to generate AML, either as monomers or as heterodimers with Pbx proteins. Here we demonstrate that constitutive expression of Hoxa9 in primary murine marrow immortalizes a late myelomonocytic progenitor, preventing it from executing terminal differentiation to granulocytes or monocytes in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) or interleukin-3. This immortalized phenotype is achieved in the absence of endogenous or exogenous *Meis* gene expression. The Hoxa9-immortalized progenitor exhibited a promyelocytic transcriptional profile, expressing PU.1, AML1, c-Myb, C/EBP alpha, and C/EBP epsilon as well as their target genes, the receptors for GM-CSF, G-CSF, and M-CSF and the primary granule proteins myeloperoxidase and neutrophil elastase. G-CSF obviated the differentiation block of Hoxa9, inducing neutrophilic differentiation with accompanying expression of neutrophil gelatinase B and upregulation of gp91phox. M-CSF also obviated the differentiation block, inducing monocytic differentiation with accompanying expression of the macrophage acetyl-low-density lipoprotein scavenger receptor and F4/80 antigen. Versions of Hoxa9 lacking the ANWL Pbx interaction motif (PIM) also immortalized a promyelocytic progenitor with intrinsic biphenotypic differentiation potential. Therefore, Hoxa9 evokes a cytokine-selective block in differentiation by a mechanism that does not require *Meis* gene expression or interaction with Pbx through the PIM.**

Mammalian class I *Hox* genes are homologs of the *Drosophila* homeobox genes located within the homeotic complex (*HOM-C* genes). Class I *Hox* genes encode transcription factors that orchestrate anterior-posterior pattern formation (40), determine segmental identity during embryogenesis (28, 37), and contribute to lineage-specific proliferation and/or differentiation of hematopoietic progenitors (30). *Hox* monomers are capable of binding DNA, though their specificity of DNA sequence recognition is increased through heterodimerization with the three-amino-acid loop extension (TALE) family of transcription factors, which include the products of the *Pbx* and *Meis/Prep* genes (7, 8, 23, 24). *Hox* proteins encoded by paralogs 1 to 10 bind DNA as heterodimers with *Pbx* proteins (7) and contact the *Pbx* homeodomain (HD) using a tryptophan consensus motif (YYPWMR or ANWL) designated the *Pbx* interaction motif (PIM), which lies N terminal to the *Hox* HD. *Hox* proteins encoded by paralogs 9 to 13 heterodimerize with *Meis/Prep1* proteins (54) using an interaction surface different from the PIM, which has yet to be characterized. Paralog 9 and 10 *Hox* proteins, such as *Hoxa9* and *Hoxa10*, appear distinct in that they can dimerize with both *Pbx* and *Meis/Prep* proteins (54). *Pbx* and *Meis/Prep* proteins also bind DNA coopera-

tively, using an interaction surface N terminal to their HDs (23). *Meis/Prep* can also bind both *Hoxa9* and *Pbx* in the absence of DNA (8, 55). Trimeric complexes containing *Pbx*, *Meis*, and *Hox* proteins bind *Pbx-Hox* DNA elements and have been isolated from nuclear extracts (e.g., *Hoxa9*, *Pbx2*, and *Meis1* on TGATTTAT motif [55]), demonstrating that members of all three HD families may have the ability to regulate the transcription of a single promoter.

The expression of a number of *Hox* genes is tightly regulated during normal hematopoiesis, and elimination or enforced expression of certain *Hox* genes has been demonstrated to alter hematopoietic differentiation profoundly. For example, *Hoxa9* knockout mice are deficient in the production of mature granulocytes and B lymphocytes (29) and show impaired T-cell differentiation with increased apoptosis (16). Constitutive expression of *Hoxb4* results in selective expansion of CD34<sup>+</sup> stem cells in marrow without adversely affecting the differentiation profiles of hematopoietic lineages in the peripheral blood (52), whereas enforced *Hoxb3* expression increases myeloid cell proliferation and reduces populations of B and T lymphocytes (53). Retroviral transduction of *HOXB7* in human hematopoietic progenitors followed by plating in methylcellulose results in suppressed differentiation and persistent proliferation of a large pool of myeloid precursors (6).

In both mice and humans, the persistent expression of normal or mutant homeodomain proteins in hematopoietic progenitors can result in leukemia. The t(10;14) chromosomal translocation of human T-cell leukemia places transcription of

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the *HOX11* gene under the control of the T-cell receptor delta enhancer (13). In murine marrow cultures, Hox11 immortalizes factor-dependent early myeloid progenitors that induce acute myeloid leukemia (AML) in mice after a long latency, suggesting that other mutational events are required to achieve a fully transformed phenotype (14). Similarly, Hoxb8 blocks differentiation of an interleukin-3 (IL-3)-dependent myeloid progenitor but requires autocrine expression of IL-3 to produce rapid AML (4, 49). Hoxa10 expression suppresses generation of macrophage and pre-B-cell progenitors and produced AML with a long latency of 5 to 10 months (60).

The dysregulated expression of HoxA9 contributes to the genesis of both murine and human AML. BXH-2 mice acquire spontaneous AML in which both *Meis1* and either *Hoxa9* or *Hoxa7* are constitutively activated via proviral integration (44), and expression of endogenous *HOXA9* is a poor prognostic indicator in human AML (11). In a small fraction of human AML, the t(7;11) translocation fuses the N-terminal sequences of the *Nucleoporin 98* (*NUP98*) gene to *HOXA9* sequences just upstream of the PIM and HD (5, 43). This Nup98-HoxA9 protein transforms NIH 3T3 fibroblasts, and its oncogenic function requires *NUP98* sequences that activate transcription through their interaction with CREB binding protein/p300 (19), as well as *HOXA9* sequences that supply the DNA-binding HD.

A growing body of evidence suggests that Hox proteins cooperate with TALE HD proteins in regulating the properties of proliferation, differentiation, and apoptosis in normal cells as well as in dysregulating these same properties in transformed cells. Transformation of Rat-1 fibroblasts by *Hoxb3* or *Hoxb4* requires coexpression of *Pbx1* (27). The chimeric oncoprotein E2a-Pbx1, which fuses the transactivation domains of E2A to the majority of Pbx1 including its HD, immortalizes factor-dependent murine myeloid progenitors in vitro (18) and, when expressed in marrow used to reconstitute lethally irradiated mice, produces AML with a latency of 3 to 8 months (17). By contrast, Hoxa9 cooperates with E2a-Pbx1 to evoke factor independence in immortalized myeloid progenitors, and these progenitors induce AML in less than 2 months (61). Transformation of NIH 3T3 fibroblasts by Nup98-HoxA9 is dependent on the integrity of the HoxA9 PIM, suggesting that interaction with Pbx proteins is essential (19). In mouse marrow reconstitution experiments, expression of *Hoxa9* or *Meis1* alone does not result in an overt leukemia, while their coexpression produces rapid AML (26). While cooperativity between Hox and TALE HD proteins may reflect their necessity to bind promoters as heterodimers in some cases, it may also reflect the need for each factor to alter independent biochemical pathways that cooperate to generate AML. Specifically, in the paradigm of leukemogenesis by coexpression of Hoxa9 and Meis1, it is possible that each factor cooperates with Pbx proteins to evoke independent transformation properties. Here, we address the independent abilities of *Hox9* and *Meis* genes to alter myeloid differentiation. We demonstrate that Hoxa9 blocks differentiation in factor-dependent myeloid progenitors without required coexpression of either endogenous or exogenous *Meis* genes, and we demonstrate that the PIM of Hoxa9 is dispensable for its immortalization potential.

#### MATERIALS AND METHODS

**Construction of recombinant plasmids and retroviral constructs.** cDNAs encoding murine *Meis1c*, *Meis2*, *Meis3*, and wild-type and mutant versions of Hoxa9 were subcloned into the polylinker of the proviral vector MSCV2.1. Helper-free virus was produced by cotransfection of 293T cells with MSCV vectors and a packaging-deficient murine leukemia virus provirus (18) and was used for subsequent infection of primary murine marrow. cDNAs encoding E2a-Pbx1, VP16-Pbx<sub>1-343</sub>, and VP16-Pbx<sub>89-343</sub> were subcloned into MSCV2.1

and used for assessment of transcriptional cooperation with wild-type and mutant Hoxa9 proteins by cotransfection in Nalm-6 cells. cDNAs encoding all proteins used in electrophoretic mobility shift assays (EMSA) were cloned into pGEM3zf- (Promega) under direction of the SP6 promoter except for *Meis1a*, which was cloned into pBSK- under the T3 promoter. Wild-type and mutant Hoxa9 proteins used for EMSA following transcription and translation were tagged at their amino termini with a shortened EE tag (EEYMPEA [12]) which was inserted after the initiating methionine.

**Marrow infections.** Primary murine progenitors were isolated from the femurs and tibias of BALB/c mice and separated from mature phagocytic and lymphocytic cells on Ficoll-Paque gradients; 10<sup>6</sup> progenitors were transferred to each well of a six-well tissue culture plate and incubated for 1.0 h with 1 ml of designated helper-free retrovirus containing 5 × 10<sup>5</sup> G418 resistance units and 1 ml of marrow culture medium (MCM; RPMI 1640, 10% fetal bovine serum [FBS], 1× antibiotics [penicillin and streptomycin {pen/strep}], 1× glutamine, 16 U of granulocyte-macrophage colony-stimulating factor [GM-CSF] [18]). Polybrene was added to 8 μg/ml. After 1 h, 6 ml of MCM was added to dilute the Polybrene to 2 μg/ml, and the cells were incubated under conditions of 37°C and 5% CO<sub>2</sub>. After 3 days, 4 ml of medium was removed and replaced with 4 ml of fresh MCM. Nonadherent cells were transferred every 7 days to new plates.

**Antiserum and immunoblots.** A fragment of *Hoxa9* encoding the HD and C terminus was cloned downstream of glutathione S-transferase (GST) sequences in the vector pGEX2T (Pharmacia). The resulting 30-kDa GST-Hoxa9 fusion protein was purified by glutathione affinity column chromatography as specified by the manufacturer (Pharmacia) and dialyzed against phosphate-buffered saline (PBS). Antiserum against the purified GST-Hoxa9 fusion protein was raised in rabbits (Covance Research Products, Berkeley, Calif.). Guinea pig antisera against the mouse macrophage acetyl-low-density lipoprotein (LDL) scavenger receptor was a kind gift from Christopher Glass (65). Proteins in primary myeloblast populations were dissolved in Laemmli sample buffer and boiled for 5 min. Proteins in 5 × 10<sup>4</sup> cells were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) through 12.5% gels, transferred to a PVDF-Plus membrane, detected using polyclonal Hoxa9 antisera at a 1:1,000 dilution, and visualized using a chemiluminescent Phototope-HRP detection kit (New England Biolabs).

**Northern blots.** Cytoplasmic RNA was purified from 5 × 10<sup>8</sup> myeloid progenitors (RNEasy; Qiagen), and a portion was subjected to poly(A) selection (Oligotex; Qiagen); 15 μg of cytoplasmic RNA or 5 μg of poly(A)-selected RNA was resolved by formaldehyde-agarose gel electrophoresis. RNA was transferred to positively charged nylon membrane (GeneScreen Plus; NEN). [<sup>32</sup>P]dCTP-labeled DNA probes were prepared from 100 ng of DNA subjected to random hexamer oligolabeling (Pharmacia). Hybridization in Ultrahyb (Ambion) and washing were carried out at 42°C according to the manufacturer's protocols. In general, probes for transcription factors were hybridized to blots containing polyadenylated mRNA, and probes for all other gene products were hybridized to blots containing total mRNA.

**Mutagenesis.** Site-directed mutations in the Hoxa9 PIM and HD were created using a QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol and verified by sequencing. VP16-Pbx fusions were generated by creating an *MluI* site corresponding to the location of amino acid 2 or 89 of Pbx1a and inserting sequences encoding transactivation domain of VP16 with flanking *MluI* sites in frame generated by PCR.

**EMSA.** Oligonucleotides containing consensus binding sites for Pbx1 and Hoxa9 (tcacggTGATTTATgagcactcctcgg) and for Meis1 and Hoxa9 (ccagatcTGACAGTTTTTACgacagcagcactcctcgg) (nucleotides in the actual DNA-binding sites within the oligonucleotide are shown in capital letters) were synthesized (Genosys Biotechnologies, Inc., The Woodlands, Tex.) and labeled with [<sup>32</sup>P]ATP to equal specific activities by phosphorylation of a common reverse oligonucleotide that was annealed to the 3' portion of the single-stranded oligonucleotides, filled in using deoxynucleoside triphosphates, and Klenow polymerase, and isolated after separation through 17% polyacrylamide gels run in 0.5× TBE (27 mM Tris, 27 mM boric acid, 0.6 mM EDTA). Coupled SP6- or T3-driven transcription-translation was performed in vitro using the Promega TNT coupled reticulocyte lysate system in accordance with manufacturer's protocol. EE-tagged Hoxa9 constructs in pGEM3Zf- were used for EMSA because they produced significantly larger quantities of protein in coupled transcription-translation than their untagged counterparts. Nuclear extracts of immortalized cells were prepared as previously described (25). For EMSA, 40,000 cpm of probe was incubated with 3 to 6 μl of in vitro-translated proteins or nuclear extracts in the presence of 1 μg of poly(dI-dC) in a buffer containing 10 mM Tris (pH 7.5), 1 mM dithiothreitol, 0.1% NP-40, and 5% glycerol for 30 min at room temperature. For supershifting or ablation of complex formation, 2 μl of polyclonal antibody against Pbx1 or Hoxa9 was added and incubated for an additional 20 min. Complexes were separated by electrophoresis in 6% polyacrylamide gels and run in 0.5× TBE for 2 h at 160 V. Protein-DNA complexes were visualized by autoradiography. Equal molar amounts of wild-type and mutant Hoxa9, VP16Pbx1, and Meis proteins were added as assayed by parallel transcription-translation reactions performed in the presence of [<sup>35</sup>S]methionine followed by quantitation of proteins resolved by SDS-PAGE.

**Cooperative transactivation assays.** Nalm-6 cells were grown to log phase in RPMI-10% FBS-1% pen/strep and electroporated at a concentration of 7 × 10<sup>6</sup> cells per sample with 6 μg of each appropriate expression plasmid (e.g., Hoxa9

Hoxa9 Panel	Biochemical Analysis						Primary Murine Marrow: Response to Cytokines					
	Mono	DNA Binding:			Transactivation		Immortalization	G-CSF			M-CSF	
		Hetero with:	with:		GM-CSF	Neutrophil Diff.		Granulocytic Genes Upregulated:		Macrophage Diff.	Scavenger Receptor Upregulated	
	Pbx1	VP16-Pbx1 89-343	VP16-Pbx1 1-343	VP16-Pbx1 89-343	VP16-Pbx1 1-343			gp91 HOX	NGB	LF		
Tag Hoxa9	+	+	+	+	+	+	+	n/d	n/d	n/d	+	n/d
Hoxa9	+	+	+	+	+	+	+	+	+	-	+	+
Hoxa9-WA	+	-	-	-	-	+	+	n/d	n/d	n/d	n/d	n/d
Hoxa9-WF	+	-	-	-	+/-	+	+	-	+	-	+	+
Hoxa9-Atet	+	-	-	-	-	+	+	+	+	-	+	+
Hoxa9-Gtet	+	-	-	-	-	+	+	+	+	-	+	+
Hoxa9-N51S	-	-	-	-	-	-	-	n/a	n/a	n/a	n/a	n/a

FIG. 1. Summary of the phenotypes of wild-type *Hoxa9*, PIM mutant panel, and DNA-binding mutant. Hoxa9 PIM consists of the amino acid sequence ANWL. Amino acid sequences of PIM mutants are indicated. Tag Hoxa9 is an N-terminal EE-tagged Hoxa9 with amino acid sequence EEYMPEA. Hoxa9-N51S is a DNA-binding mutant. DNA binding studies were performed with EE-tagged versions of wild-type Hoxa9, PIM, and N51S mutants due to the greater abundance of protein production in coupled transcription-translation; + or - refers to positive or negative scoring in an assay. For assay details and quantitative comparison of wild-type versus mutant performance, refer to the text and the following figures. n/d, not determined; n/a, not applicable.

wild type or mutants and VP16-Pbx1 fusions) and reporter plasmids. The luciferase reporter contains six TGATTTAT motifs upstream of a minimal *fox* promoter 5' to the luciferase gene (pGL3-basic luciferase vector; Promega). Renilla reporter plasmid was cotransfected for internal normalization of luciferase expression. Cells were harvested 60 to 65 h postelectroporation, and luciferase assays were performed using the Dual-Luciferase reporter assay system (Promega) according to the manufacturer's specifications.

**Myeloid differentiation assays.** Myeloid progenitors ( $10^6$  per sample) were washed twice in PBS to remove GM-CSF from the medium. Cells were resuspended in RPMI 1640–10% FBS–1% pen/strep with addition of the appropriate cytokine (0.5 ng of G-CSF per ml; 10 ng of M-CSF per ml; 1  $\mu$ M all-*trans* retinoic acid [ATRA]; 1 ng of IL-3 per ml). To assay neutrophil differentiation, cells were harvested after 48 h of culture in G-CSF, cytospun onto a coverslip, stained with Wright-Giemsa stain, and visualized and photographed under light microscopy. A second sample was boiled in Laemmli sample buffer for immunoblot analysis. To assay macrophage differentiation, cells were cultured in M-CSF for 7 to 10 days, with removal of two-thirds of old media by careful aspiration and replenishment with fresh M-CSF medium every 3 days. Monocytes and macrophages were harvested and stained with Wright-Giemsa stain or boiled in Laemmli sample buffer for immunoblot analysis.

**Fluorescence-activated cell sorting (FACS) analysis of GRI, Mac1, or F4/80 expression.** Fluorescein isothiocyanate-coupled monoclonal antibodies directed against GR1 and Mac1 (CD11b) were purchased from Pharmingen, and phycoerythrin-Cy5-coupled F4/80 was obtained from Serotec. As specified by the manufacturer (Pharmingen), 1 million cells were labeled for 30 min at 4°C in PBS–1% FBS–0.1% NaN<sub>3</sub>. Flow cytometry data were acquired with the program CELLQuest on either a FACScan (Becton Dickinson, San Jose, Calif.) or FACSCalibur benchtop flow cytometer attached to either a Macintosh Quadra 650 or G3 computer. At least 20,000 events were collected, and live cells were gated for analysis by forward and side scatter signals and lack of propidium iodide staining. Thresholds for positive antibody staining were set to include approximately 2% of the cells incubated without antibody.

**Quantitation of NADPH oxidase activity.** Nitroblue tetrazolium (NBT) reduction activity was assayed as a marker for cellular differentiation. Cells ( $2.5 \times 10^5$  in 200  $\mu$ l of growth medium) were added to 800  $\mu$ l of 0.125% NBT (Sigma) in the presence of  $2 \times 10^{-7}$  M 12-*O*-tetradecanoylphorbol 13-acetate. The cells were incubated for 25 min at 37°C, pelleted, resuspended in 200  $\mu$ l of PBS, and cytospun onto a coverslip. A Safranin-O (Sigma) counterstain (5 min, 0.5% Safranin-O in 20% ethanol) was performed, and the cells were visualized by light microscopy to assay for the dark purple deposits indicative of a positive NBT reduction.

## RESULTS

**Persistent expression of Hoxa9 blocks differentiation of a GM-CSF-dependent progenitor.** cDNAs encoding Hoxa9 (Fig. 1), Meis1, Meis2, and Meis3 were subcloned in the retroviral

vector MSCV. Helper-free viruses of similar titers were generated and used to infect  $5 \times 10^4$  marrow progenitors cultured in GM-CSF (see Materials and Methods). Cultures infected by virus encoding Meis1, Meis2, and Meis3 yielded the same growth and differentiation kinetics as did controls, exhibiting rapid proliferation over the first 7 days accompanied by the production of monocytes and neutrophils. After 14 days, proliferation ceased, and remaining cells exhibited macrophage morphology. By contrast, 19 of 21 cultures infected with virus expressing *Hoxa9* yielded outgrowths of immortalized progenitors that were first visible after 10 days, overgrew the cultures by day 21 (Fig. 2A and E), and have been cultured continuously for 18 months. Ten to 20% of cells in each population exhibited neutrophilic differentiation, and 1 to 3% showed monocytic differentiation (Table 1). Immunoblotting with Hoxa9 antisera identified an abundant protein in *Hoxa9* virus-infected cells (Fig. 3, lanes 5 to 7) that comigrated with recombinant Hoxa9 protein (lane 1) and was not present in cells immortalized by Hoxb8 or E2a-Pbx1 (lanes 3 and 4). A version of *Hoxa9* encoding an N-terminal EE tag (which was used for subsequent EMSA studies due to its enhanced protein production in coupled transcription-translation [Fig. 1]) also immortalized primary marrow (five of six cultures) in the same manner as the wild-type *Hoxa9*. Hoxa9 antisera revealed a major antigen in these cells (Fig. 3, lanes 8 to 10) that was 2 kDa larger than that in cells immortalized by wild-type *Hoxa9* virus. Immortalization by Hoxa9 did not abrogate factor dependence, as all 24 cultures indicated above died in the absence of GM-CSF.

**Clonal myeloid progenitors immortalized by Hoxa9 differentiate morphologically to neutrophils or macrophages in response to G-CSF, M-CSF, or ATRA.** The ability of Hoxa9-immortalized cultures to exhibit lineage-specific myeloid differentiation was tested by shifting cells from GM-CSF to medium containing G-CSF or M-CSF or by the addition of ATRA to cells maintained in GM-CSF. In G-CSF, proliferation ceased within 24 h and cells differentiated to neutrophils within 72 h as evidenced by Wright-Giemsa staining (Fig. 2B and F). Differentiation induced by G-CSF did not result from

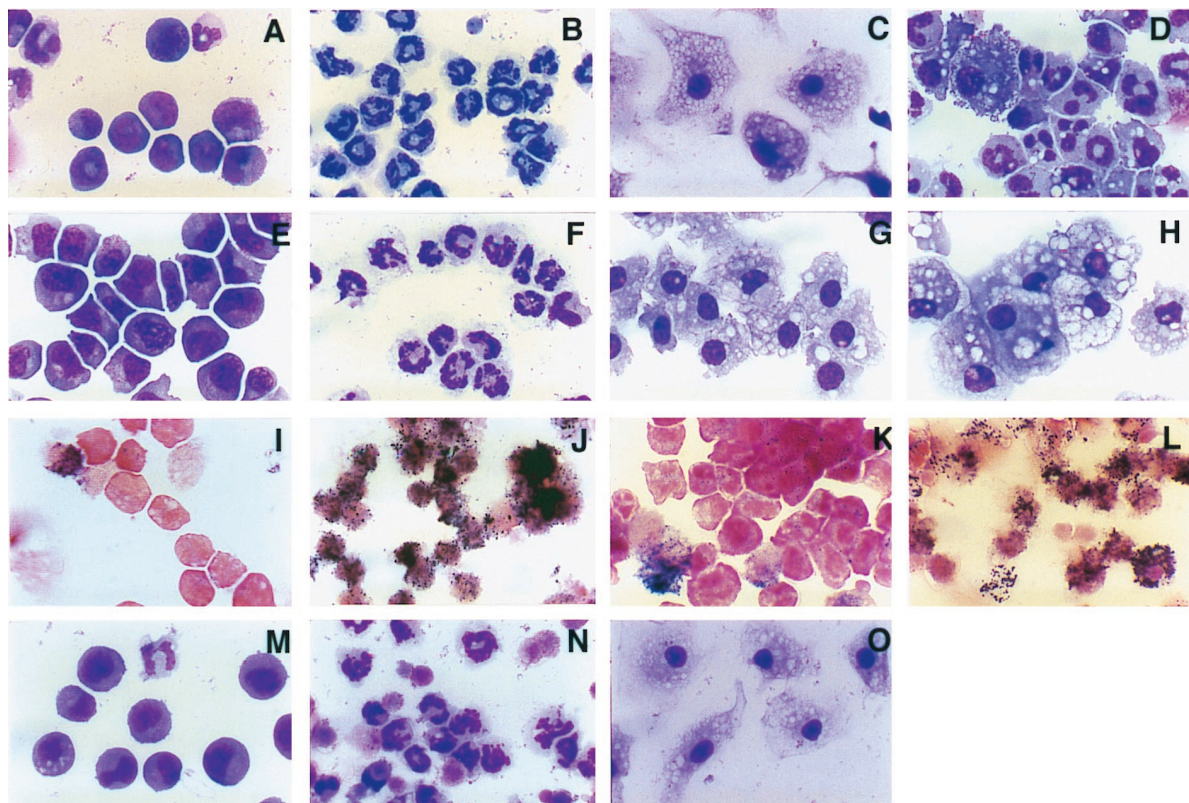


FIG. 2. Hoxa9-immortalized cells differentiate morphologically into neutrophils or macrophages in response to G-CSF, M-CSF, or ATRA. Myeloid progenitors immortalized by Hoxa9 (clones HF1 and HF2) were cultured in GM-CSF (A and E), G-CSF for 72 h (B and F), M-CSF for 120 h (C and G), or GM-CSF plus ATRA for 5 days (D and H) and stained with Wright-Giemsa stain. Cells immortalized by wild-type Hoxa9 (clone HF1) were cultured in GM-CSF (I) or in G-CSF for 72 h (J) and stained for NADPH oxidase activity. Cells immortalized by PIM mutant Hoxa9-Atet were cultured in GM-CSF (K) or in G-CSF for 48 h (L) and stained for NADPH oxidase activity. Myeloid progenitors immortalized by PIM mutant Hoxa9-Atet were cultured in GM-CSF (M), G-CSF for 72 h (N), or M-CSF for 120 h (O) and stained with Wright-Giemsa stain.

reductions in Hoxa9, as both its abundance (immunoblot analysis [not shown]) and its monomeric DNA binding and heterodimerization with endogenous Pbx proteins (see Fig. 6B, lane 7 versus lane 1) remained unchanged. M-CSF induced differentiation to macrophages within 5 days (Fig. 2C and G). Consistent with the ability of GM-CSF signaling to block activation and nuclear translocation of STAT3 through the G-CSF receptor (62, 66), neither G-CSF nor M-CSF altered

proliferation or differentiation of cells maintained in GM-CSF. This effect is consistent with observations that IL-3 is able to suppress G-CSF-mediated signaling in 32Dcl3 murine myeloblasts (57). Biphenotypic differentiation was confirmed for nine cultures, with 90 to 96% of cells exhibiting neutrophilic differentiation in response to G-CSF, and 40 to 95% of cells exhibiting monocytic differentiation in response to M-CSF (Table 1). In the presence of GM-CSF, ATRA induced growth

TABLE 1. Morphology and NADPH oxidase expression of myeloid progenitor cell lines immortalized by HoxA9 and HoxA9 mutant proteins grown in the presence of GM-CSF and assayed in the presence of GM-CSF or after a shift to G-CSF for 48 h

HoxA9 form	Morphology <sup>a</sup> in designated lymphokine (%)						% NADPH oxidase positive in designated lymphokine	
	GM-CSF			G-CSF, 48 h			GM-CSF	G-CSF, 48 h
	Blasts	Neut	Mac	Blasts	Neut	Mono		
HoxA9 HF1	89	10	1	6	88	6	15	87
HoxA9 HF1.1	76	22	2	0	99	0.6	7	91
HoxA9 HF1.4	82	17	1	0.7	99	0.3	5	95
Tag A9 HF1	75	22	3	4	91	5	11	89
Tag A9 HF2	85	14	1	8	88	4	15	85
HoxA9-Atet	86	14	0	6	92	2	19	97
HoxA9-Gtet	93	7	0	9	90	1	14	93
HoxA9-WF-1	99	1	<1	93	2	5	1.4	32
HoxA9-WF-2	88	0	12	0	0	100	0.8	8
HoxA9-WF-5	92	0	8	50	0	50	1	30
HoxA9-WF-6	75	0	25	0	0	100	8.4	41

<sup>a</sup> Neut, neutrophil; Mac, macrophage; Mono, monocyte.

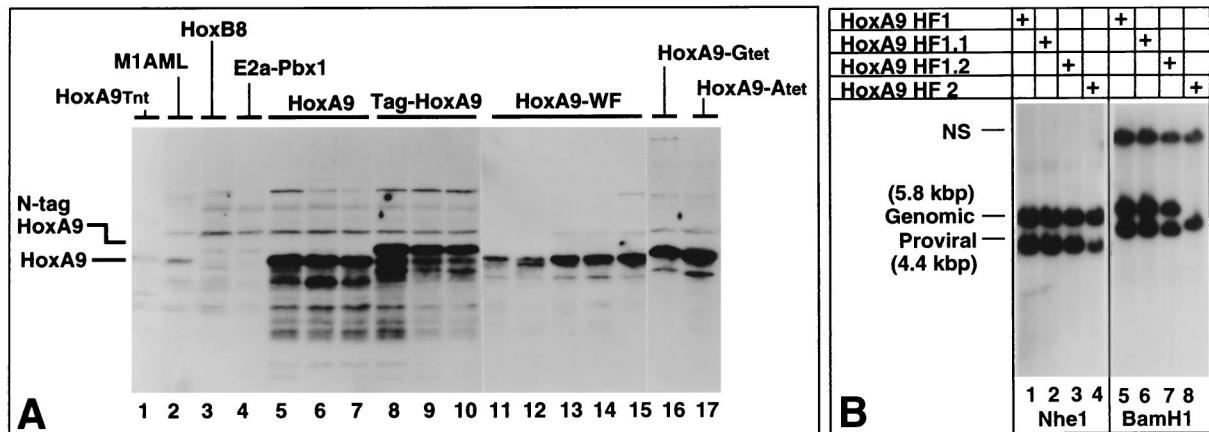


FIG. 3. Progenitors having biphenotypic differentiation potential show strong expression of Hoxa9 and are clonal. (A) Identification of wild-type and mutant Hoxa9 proteins in immortalized myeloblasts from primary marrow. The abundance of Hoxa9 in total cell lysate was quantitated by immunoblot analysis using an antiserum raised against the HD C terminus of murine Hoxa9. The mobility of recombinant Hoxa9 equaled that of Hoxa9 expressed in myeloblast cell lines and is designated at the left as Hoxa9. The higher mobility of the N-terminal EE-tagged Hoxa9 is designated at left as N-tag Hoxa9. Lanes: 1, recombinant Hoxa9 produced by coupled transcription-translation; 2, M1AML cells containing coactivation of *Hoxa9* and *Meis1*; 3 to 17, myeloid progenitors immortalized by Hoxb8 (lane 3), E2a-Pbx1 (lane 4); Hoxa9 (lanes 5 to 7), EE-tagged Hoxa9 (lanes 8 to 10), Hoxa9-WF (lanes 11 to 15), Hoxa9-Gtet (lane 16), and Hoxa9-Atet (lane 17). (B) Hoxa9-immortalized progenitors are clonal and thus exhibit biphenotypic potential. Shown is Southern blot analysis of DNA derived from Hoxa9-immortalized populations HF1 (lanes 1 and 5), HF1.1 (lanes 2 and 6), HF1.2 (lanes 3 and 7), and HF2 (lanes 4 and 8). DNA was cleaved with *NheI* and probed with *Hoxa9* cDNA (lanes 1 to 4) or cleaved with *BamHI* and probed with *neo* sequences (lanes 5 to 8). The location of the genomic and proviral fragments containing Hoxa9 are indicated at left as NS. A background band present in all samples probed with *neo* sequences (lanes 5 to 8) is indicated at left as NS. The relative ratio of genomic to proviral Hoxa9 signal in lanes 1 to 3 of panel A was 1:1, confirming the presence of two proviral integrations, and was 2:1 in the sample of DNA in lane 4, confirming a single integration.

arrest and either monocytic (Fig. 2H) or mixed monocytic and neutrophilic (Fig. 2D) differentiation. In contrast, E2a-Pbx1- or Hoxb8-immortalized GM-CSF-dependent progenitors failed to differentiate in response to G-CSF, M-CSF, or ATRA.

Retroviral integration analysis was performed to demonstrate the clonality of cultures exhibiting biphenotypic differentiation. Cell lines derived from marrow infected with helper free *Hoxa9* virus were designated HF followed by a number to distinguish the populations. Subclones HF1.1 and HF1.2 were derived from the population Hoxa9 HF1. Integration sites were determined by cleavage of genomic DNA with *BamHI* followed by Southern blot analysis using neomycin phosphotransferase (*neo*) cDNA as a probe (Fig. 3B). Both parental Hoxa9 HF1 cells, as well as subclones HF1.1 and HF1.2, contained two bands (lanes 5 to 7), indicating that the original HF1 population was clonal and contained two integrated proviruses. Like the parental cells, each subclone differentiated into neutrophils after 3 days in G-CSF (Table 1) or macrophages after 5 days in M-CSF. The Hoxa9 HF2 population contained a single integrated provirus (Fig. 3B, lane 8). When cleaved in the long terminal repeat with *NheI* and probed with *Hoxa9* cDNA (lanes 1 to 4), all clones contained the 4.4-kb proviral fragment, indicating that no rearrangements had occurred, as well as the 5.8-kb genomic *Hoxa9* fragment. This analysis demonstrated that biphenotypic differentiation is an intrinsic property of Hoxa9-immortalized progenitors.

In addition to morphological characterization, expression of the lineage-specific and differentiation-associated surface markers Ly6G (GR1) and Mac1 (Cd11b) was examined in the Hoxa9-immortalized progenitors cultured in GM-CSF, G-CSF, or M-CSF. FACS analysis demonstrated that both surface antigens were expressed at low levels in progenitors cultured in GM-CSF and were upregulated to high levels in the majority of progenitors induced to differentiate by culture in G-CSF or M-CSF (Table 2). Expression of the mouse macrophage acetyl-LDL scavenger receptor, which is upregulated in the terminal

stages of macrophage differentiation (65), was induced specifically by M-CSF (Fig. 4, lane 4 versus lanes 2 and 3). Additionally, FACS analysis indicated that the macrophage-specific antigen recognized by the F4/80 antibody increased from a ratio of 60% dim:27% bright in GM-CSF-immortalized populations to a ratio of 13% dim:75% bright in cells induced to undergo differentiation in the presence of M-CSF. Therefore, Hoxa9-immortalized progenitors exhibited normal upregulation of lineage- and stage-specific differentiation antigens, consistent with their biphenotypic differentiation potential.

**The Hoxa9-immortalized myeloid progenitor exhibits most but not all of the biochemical and genetic events accompanying differentiation to neutrophils or macrophages.** Pu.1, c-Myb, AML1, C/EBP alpha, and C/EBP epsilon are transcrip-

TABLE 2. Cell surface abundance of antigens Ly6-G and Mac1, using FACS analysis

Hoxa9 protein	Lymphokine	Intensity with designated FACS marker <sup>a</sup>			
		Ly6G (GR1)		Mac1	
		% Dim	% Bright	% Dim	% Bright
Hoxa9 HF1	GM-CSF	74	3	92	5
	G-CSF	50	48	40	59
	M-CSF	61	20	55	40
Hoxa9-WF	GM-CSF	82	2	76	12
	G-CSF	60	39	28	72
	M-CSF	69	20	34	57
Hoxa9-Atet	GM-CSF	55	6	73	24
	G-CSF	38	48	17	78
	M-CSF	ND	ND	ND	ND
Hoxa9-Gtet	GM-CSF	61	11	56	41
	G-CSF	30	55	20	77
	M-CSF	ND	ND	ND	ND

<sup>a</sup> Bright represents at least 1-log difference in signal intensity versus dim. ND, not determined.

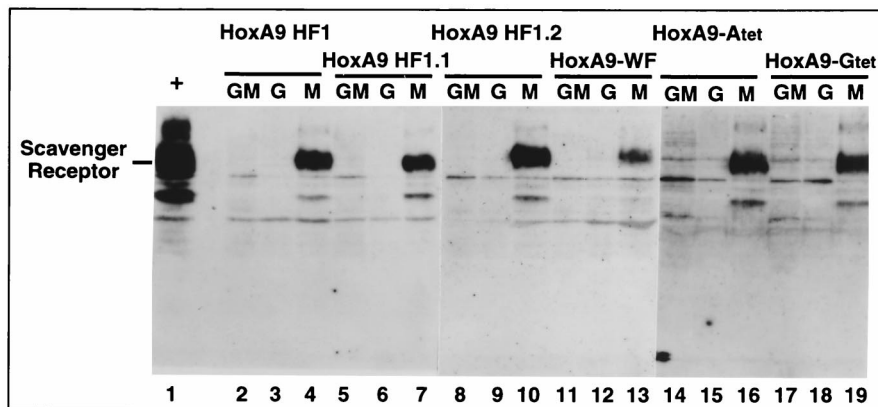


FIG. 4. M-CSF induces expression of the macrophage scavenger receptor in Hoxa9-immortalized promyelocytes. Shown is Western blot detection of the macrophage-specific scavenger receptor in promyelocytes immortalized by Hoxa9 (clone HF1 [lanes 2 to 4], subclone HF1.1 [lanes 5 to 7], and subclone HF1.2 [lanes 8 to 10]), Hoxa9-WF (clone 1; lanes 11 to 13), Hoxa9-Atet (lanes 14 to 16), and Hoxa9-Gtet (lanes 17 to 19), each grown in GM-CSF (lanes 2, 5, 8, 11, 14, and 17), G-CSF for 72 h (lanes 3, 6, 9, 12, 15, and 18), or M-CSF for 120 h (lanes 4, 7, 10, 13, 16, and 19). Lane 1, mouse peritoneal macrophages (as a positive control).

tion factors that cooperate in the concerted activation of myeloid gene transcription (3, 15, 36, 45–48, 50, 56, 58, 63, 67). In promyelocytes, Pu.1 and C/EBP alpha contribute to the transcriptional activation of genes encoding the receptors for G-CSF, M-CSF, and GM-CSF (68), C/EBP epsilon contributes to the activation of the G-CSF receptor (56, 63), and AML1 contributes to the activation of the M-CSF receptor (67). Mice lacking C/EBP alpha fail to produce neutrophils, in part, because they fail to express receptors for the myeloid cytokines G-CSF (69) and IL-6 (70). Mice lacking C/EBP epsilon or Pu.1 produce differentiated neutrophils that fail to synthesize secondary granule proteins or gp91phox (2), a subunit of the phagocyte respiratory burst oxidase. Consistent with their expression of the receptors for G-CSF, M-CSF, and GM-CSF, cells immortalized by Hoxa9 expressed Pu.1, AML1, c-Myb, C/EBP alpha, and C/EBP epsilon (Fig. 5A, lane 5).

Neutrophil elastase (NE) and myeloperoxidase (MPO) are primary granule proteins produced in promyelocytes. Transcription of NE is also activated by Pu.1, C/EBP alpha, C/EBP epsilon, AML1, and c-Myb (45, 46), while that of MPO is activated by AML1 and MZF1 (3). NE and MPO were also expressed strongly in Hoxa9-immortalized cells (Fig. 5A, lane 5), reiterating that normal levels of their transcriptional activators were present.

The neutrophil respiratory burst oxidase (NADPH oxidase) catalyzes the transfer of electrons from NADPH to molecular oxygen to form superoxide, which is required for the microbicidal and cytotoxic activities of neutrophils and macrophages. NADPH oxidase is normally upregulated during differentiation of GM-CSF-responsive progenitors (Fig. 5A, lanes 1 to 3) and is a marker of the late promyelocytic stage. Ten percent of Hoxa9-immortalized cells grown in GM-CSF contained NADPH oxidase activity (Fig. 2I), which correlated with weak expression of gp91phox (Fig. 5A, lane 5). Switching cells to G-CSF resulted in a significant increase in NADPH oxidase activity (Fig. 2J) and upregulation of gp91phox transcription (Fig. 5A, lane 7). The observation that G-CSF signaling can overcome the block in gp91phox transcription suggests that Hoxa9 prevents the expression or activity of a transcriptional activator associated with differentiation in response to GM-CSF rather than establishing a dominant block in gp91phox transcription.

Genes encoding the secondary granule proteins neutrophil lactoferrin (LF) and neutrophil gelatinase B (NGB) are ex-

pressed late in the neutrophilic differentiation of GM-CSF-dependent progenitors (Fig. 5A, lanes 1 to 3). NGB was not expressed in Hoxa9-immortalized promyelocytes though it was significantly upregulated by G-CSF (lanes 5 versus 7). LF transcription was very low in Hoxa9-immortalized cells and was not induced by either G-CSF or M-CSF despite the fact that LF has been shown to be upregulated by G-CSF in murine 32D13 cells (21). Therefore, certain blocks in transcriptional activation established by Hoxa9 cannot be relieved by signaling through the G-CSF receptor.

Normal downregulation of gene transcription also accompanied macrophage differentiation in Hoxa9-immortalized progenitors. Similar to monocytic differentiation in human KG1 and HL60 cells (9, 41, 42), exposure of Hoxa9-immortalized cells to M-CSF downregulated expression of MPO, NE, and C/EBP epsilon (lane 9).

In summary, this analysis indicated that the overall pattern of myeloid-specific gene expression appeared largely normal in Hoxa9-immortalized progenitors in response to G-CSF and M-CSF. However, the failure to upregulate a small subset of genes critical to the execution of the late differentiation program in the presence of GM-CSF is indicative of the principal defect caused by persistent *Hoxa9* expression.

**Hoxa9-immortalized progenitors do not contain detectable levels of endogenous *Meis* transcripts; therefore, it is unlikely that Hoxa9 blocks myeloid differentiation through interaction with endogenous *Meis* proteins.** While Hoxa9 did not require exogenous *Meis* gene expression to block myeloid differentiation, we considered the possibility that it could act in concert with endogenous *Meis* proteins. However, upon Northern blot analysis, we were unable to detect endogenous expression of any of the 3 *Meis* genes in the Hoxa9-immortalized progenitors (Fig. 5A, lane 5) or in normally differentiating myeloid progenitors (lanes 1 to 3), though their expression could be detected easily in NIH 3T3 fibroblasts (lane 4). We conclude that *Meis* genes are not normally expressed during the later stages of myelopoiesis and, therefore, that their products cannot contribute to the differentiation arrest established by Hoxa9.

**The PIM of Hoxa9 is required for cooperative heterodimerization with Pbx but not for transcriptional cooperativity or for immortalization of myeloid progenitors.** The PIM of Hoxa9 consists of a tryptophan motif, ANWL, that lies N terminal to the HD. The requirement of the Hoxa9 PIM for cooperative DNA binding with Pbx, for cooperative transcrip-

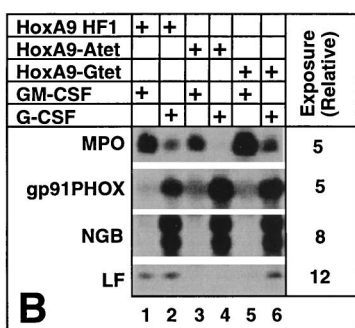
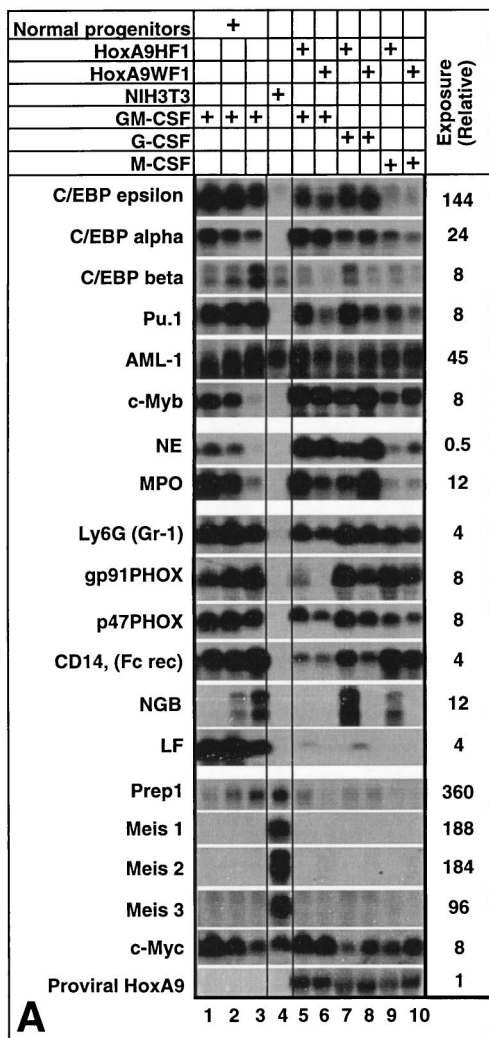


FIG. 5. Hoxa9-immortalized progenitors exhibit a promyelocytic gene expression profile and upregulate expression of terminal differentiation genes in response to G-CSF and M-CSF. (A) Northern blot analysis of genes expressed in promyelocytes immortalized by wild-type Hoxa9 (clone HF1; lanes 5, 7, and 9) or by Hoxa9-WF (clone 1; lanes 6, 8, and 10) compared with gene expression in GM-CSF-responsive progenitors undergoing terminal differentiation to neutrophils and macrophages in the presence of GM-CSF (lanes 1 to 3) and with those expressed in NIH 3T3 fibroblasts (lane 4). By comparison to lane 1, lanes 2 and 3 contain RNA from the same cells cultured for an additional 24 and 48 h, respectively, in medium plus GM-CSF. The identity of each probe is designated at the left, and the origin of the RNA samples is identified above each lane. (B) Transcriptional responses to G-CSF is the same in cells immortalized by wild-type Hoxa9, Hoxa9-Atet, or Hoxa9-Gtet. The identity of the RNA sample is indicated above each lane, and the identity of the transcript is given to the left of each panel. rec, receptor.

tional activation with Pbx, and for myeloid immortalization was tested by converting this ANWL element to AAAA (Atet), to GGGG (Gtet), or to the more subtle point mutant ANFL (WF) or ANAL (WA) (Fig. 1). The abilities of the WF, WA, and Atet mutants of Hoxa9 to heterodimerize with E2a-Pbx1, Pbx1, and VP16-Pbx1 are depicted in Fig. 6A. While Hoxa9 monomeric binding was unaffected, all mutations in the Hoxa9 PIM virtually eliminated heterodimerization with Pbx proteins as assayed by gel shift analysis on the TGATTTAT consensus sequence (lanes 5 to 7 versus 4; lanes 12 to 14 versus 11; lanes 18 to 20 versus 17). The very low abundance of heterodimer complex formation by each PIM mutant with Pbx1 or VP16-Pbx1 was difficult to estimate due to the presence of a comigrating background band (lanes 12 to 14 and 18 to 20). However, when parallel heterodimers were formed with E2a-Pbx1, it was evident that the abundance of complexes with the PIM mutants comprised less than 5% of those formed with the wild-type Hoxa9 protein.

Surprisingly, the WF and WA mutants retain 40 and 56%, and the Atet and Gtet mutants retain 55 and 89%, of the transactivation potential of wild-type Hoxa9 in coexpression with the complete *Pbx1b* coding sequence containing the VP16 transactivation domain at its N terminus (VP16-Pbx<sub>1-343</sub> [Table 3]). By contrast, the Atet and Gtet mutants failed to exhibit significant transcriptional cooperativity with a modified version of VP16-Pbx1 containing only Pbx1 residues 89 to 343 of Pbx1 (VP16-Pbx<sub>189-343</sub>), the same portion of Pbx1 fused with E2a in the E2a-Pbx1. Thus, the N-terminal 89 residues Pbx1b permit a form of transcriptional cooperativity with Hoxa9 in vivo that is not evident in gel shift assays. The transcriptional cooperativity observed between VP16-Pbx<sub>1-343</sub> and the Hoxa9 PIM mutants could be mediated by interaction of the N terminus of Pbx1b with a domain of Hoxa9 outside the ANWL, or alternatively by endogenous Prep1 acting to bridge Pbx1b and Hoxa9 mutants in a trimeric complex.

The Atet and Gtet mutants of Hoxa9 also immortalized GM-CSF-dependent primary myeloid progenitors (Fig. 3, lanes 16 and 17). Compared to cells immortalized by wild-type Hoxa9, the Atet and Gtet populations contained the same proportion of blasts and differentiating cells (Fig. 2M; Table 1), the same morphologic differentiation to neutrophils and macrophages in response to G-CSF and M-CSF (Fig. 2N and O), the same G-CSF-dependent upregulation of NADPH oxidase activity (Fig. 2K and L), the same transcriptional upregulation of gp91phox and NGB (Fig. 5B, lanes 4 and 6 versus 2), the same failure to express significant levels of LF (Fig. 5B, lanes 1, 3, and 5), and the same induction of the macrophage acetyl-LDL scavenger receptor in response to M-CSF (Fig. 4, lanes 14 to 19). Intriguingly, the Hoxa9-WF PIM point mutant yielded a block in differentiation that was more powerful than that effected by wild-type Hoxa9. Myeloid progenitors immortalized by Hoxa9-WF (Fig. 3, lanes 11 to 15) failed to differentiate into neutrophils in response to either GM-CSF or G-CSF (Table 1), and two of six cultures tested exhibited monocytic differentiation in response to G-CSF. Hoxa9-WF-immortalized progenitors also failed to express NGB in response to G-CSF (Fig. 5A, lanes 8 and 10). In other regards, however, Hoxa9-WF-immortalized progenitors were similar to those immortalized by wild-type Hoxa9, expressing Pu.1, C/EBP alpha, cEBP epsilon, and NE, upregulating Ly6G and gp91phox expression in response to G-CSF and M-CSF, upregulating CD14 more strongly in response to M-CSF than to G-CSF, upregulating the macrophage acetyl-LDL scavenger receptor gene in response to M-CSF (Fig. 4, lanes 11 to 13), and downregulating C/EBP epsilon, NE, and MPO in response to M-CSF but not to G-CSF (Fig. 5A, lanes 8 versus 10).



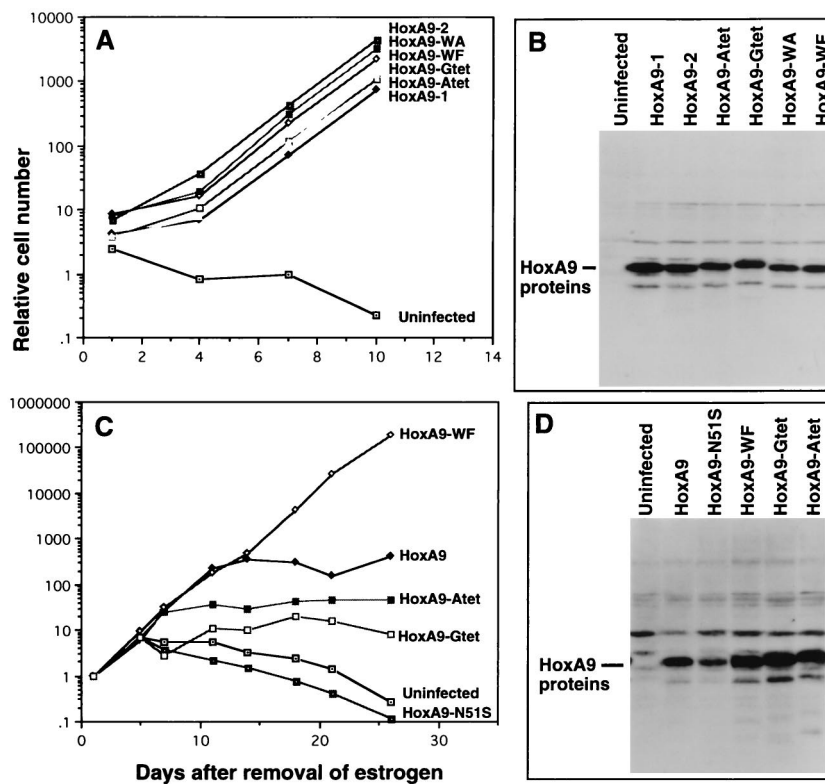


FIG. 7. PIM mutants of *Hoxa9* exhibit the same ability as wild-type *Hoxa9* to reestablish differentiation arrest CM3neo cells but differ from wild-type *Hoxa9* in reestablishing differentiation arrest in CM1puro cells. CM3neo (A) or CM1puro (C) cells were infected with retrovirus expressing wild-type *Hoxa9* or mutants of *Hoxa9* in the PIM or HD. At 48 h after infection of CM3neo cells, estrogen was removed, and the number of live nonadherent cells in the cultures was quantitated over 10 days. Populations whose differentiation arrest was reestablished by *Hoxa9* proteins were analyzed for the level of *Hoxa9* protein expression by immunoblotting using anti-*Hoxa9* sera (B). At 48 h after infection of CM1puro cells, cells were selected for retroviral gene expression by growth in G418, and the abundance of *Hoxa9* proteins was quantitated using anti-*Hoxa9* immunoblotting before estrogen was removed (D). Once estrogen was removed, the abundance of live nonadherent cells was quantitated over 26 days. The identity of the *Hoxa9* protein encoded by retrovirus used to infect each population is designated adjacent to the growth profiles in both panels A and C and above each lane in immunoblots B and D.

Pbx activator proteins (Table 3). Cells were selected in G418, and anti-*Hoxa9* immunoblots demonstrated similar expression of each protein (Fig. 7D). Following the removal of estrogen, both the control cells and the cells expressing the DNA-binding mutant, *Hoxa9*-N51S, proliferated approximately 10-fold and differentiated to macrophages and neutrophils (Fig. 7C). Myeloblasts expressing *Hoxa9* exhibited a marked suppression of differentiation indicated by the production of approximately 50-fold more cells, which assumed a steady-state rate of proliferation and differentiation. *Hoxa9*-Atet and *Hoxa9*-Gtet suppressed differentiation significantly but not as strongly as wild-type *Hoxa9*. In contrast, *Hoxa9*-WF induced a block in differentiation stronger than that induced by wild-type *Hoxa9*, causing cells to expand exponentially. The difference in the response of CM1puro and CM3neo cells toward differentiation arrest by *Hoxa9* PIM mutants suggests that *Hoxa9*-WF may have an expanded target cell profile, while tetrameric PIM mutants of *Hoxa9* are somewhat more limited in target cell profile.

## DISCUSSION

We have demonstrated that *Hoxa9* blocks the differentiation of a promyelocytic cell in the absence of *Meis* gene expression through a mechanism that requires DNA binding but not the *Hoxa9* PIM. Promyelocytes immortalized by *Hoxa9* had no apparent defect in the transcription of early promyelocytic

genes and expressed the transcription factors Pu.1, AML1, c-Myb, C/EBP alpha, C/EBP beta, and C/EBP epsilon, which cooperate (36, 46, 63) to establish myeloid-specific transcription of downstream target genes such as MPO (3), NE (45, 46), and the receptors for M-CSF (50, 67), G-CSF (56, 63), and GM-CSF (15). *Hoxa9* therefore does not inhibit the transcription of genes that establish the early myeloid phenotype nor their genetic targets. However, in the presence of GM-CSF, *Hoxa9* did not permit the expression of the late promyelocytic gene encoding gp91phox or those encoding the secondary granule proteins NGB and LF. Transcription of these genes requires expression of additional transactivators (e.g., MS-1 for the CD11b promoter [10]) as well as decreased activity of CAAT displacement protein (CDP/cut), a transcriptional repressor that competes for CAAT-binding sites in the gp91phox, LF, and NGB promoters (33, 39).

We propose that *Hoxa9* prevents the execution of a genetic program downstream of C/EBP alpha, C/EBP epsilon, c-Myb, and Pu.1, a genetic program that mediates the transcription of genes specific to differentiated cell function as well as cell cycle arrest (64). In the context of cell differentiation, persistent expression of *Hoxa9* has a broader impact than persistent expression of CDP/cut or elimination of Pu.1 or C/EBP epsilon, each of which prevents expression of gp91phox and secondary granule genes while permitting morphologic differentiation and proliferation arrest (1, 2, 33, 34, 35, 37, 39). In other respects, the effect of *Hoxa9* is more restricted, preventing

transcription of the late differentiation genes gp91phox and NGB in response to GM-CSF, yet permitting their transcription in response to G-CSF. This factor-selective immortalization could result from specific Hoxa9 transcriptional targeting, such as the repression of a gene essential for GM-CSF-induced differentiation signaling (e.g., STAT5 [59]).

What is the relationship between the Hoxa9-immortalized myeloid progenitors isolated by this in vitro protocol and the normal hematopoietic progenitors that express Hoxa9 in vivo? In knockout mice, elimination of *Hoxa9* results in reduced populations of mature lymphocytes and granulocytes (29), increased apoptosis, and impaired differentiation of T-cells (16), suggesting that Hoxa9 normally functions in a common progenitor cell. If Hoxa9 is normally expressed in the context of a common progenitor and downregulated during differentiation, then its expression in the immortalized myeloid progenitors that we describe here would be outside its normal context, and it would target genes aberrantly and potentially interfere with the function of other Hox proteins. In this regard, it is important to note in Fig. 6B that (i) Hoxa9 is the only detectable factor in myeloid progenitors capable of binding the TTAT site as a monomer, (ii) Hoxa9-Pbx heterodimers are by far the most abundant Pbx-Hox complex present in these progenitors, and (iii) there exists a low-abundance Pbx complex containing a different partner which is supershifted with Pbx antibodies (lane 5) but not eliminated by antibodies to Hoxa9 (lane 6). Therefore, GM-CSF dependent immortalization may be achieved by persistent Hoxa9 protein expression that alters gene transcription as a monomer or effectively competes for endogenous Pbx partners. Alternatively, if these Hoxa9-immortalized myeloid progenitors represent a stage in differentiation close to that in which Hoxa9 is normally expressed, it may be that a normal function of Hoxa9 is to control lineage commitment of biphenotypic progenitors. The fact that we were unable to detect endogenous *Hoxa9* gene expression in these cells by Northern blotting (data not shown) may indicate that this progenitor could be attempting to exit a window of differentiation by downregulating transcription of endogenous *Hoxa9*. We consider this second scenario less likely, however, as myeloblasts conditionally immortalized by E2a-Pbx1 do not express endogenous Hoxa9 prior to the block reestablished by retroviral *Hoxa9*. We therefore suggest that Hoxa9 acts to prevent myeloid differentiation by establishing an aberrant pattern of gene expression in a biphenotypic progenitor that normally does not express Hoxa9.

Our observation that HoxA9 immortalization is independent of *Meis* gene expression brings into question the role of *Meis* proteins in myeloid leukemogenesis involving coactivation of *Hoxa9* and *Meis* genes. In addition to their de novo coexpression commonly observed in AML derived from BXH-2 mice, coexpression of *Hoxa9* and *Meis1* in either FDCP1 cells or heterologous marrow is required to evoke leukemia in recipients (26). In light of our results, it is understandable that the expression of *Hoxa9* produced no observable effect in factor-dependent FDCP1 cells (26), as these cells already contain an intrinsic block in differentiation during culture in GM-CSF. The effect of Hoxa9 alone may also not have been readily apparent in primary marrow cultures because marrow was not cultured for significant periods, permitting selection for immortalized progenitors (26). Consistent with the results from Kroon et al. (26), when we introduced Hoxa9-immortalized progenitors HF1 and HF2 into sublethally irradiated recipients, no leukemias arose over a period of 6 months, indicating the requirement for cooperating genetic mutations such as *Meis* expression.

We suggest that though Hoxa9 is sufficient to block the

intrinsic myeloid differentiation program in GM-CSF-dependent progenitors in vitro, the ability of this cell to differentiate in response to a variety of other factors (G-CSF, M-CSF, and ATRA) favors its differentiation (rather than self-renewal) in vivo. In support of this hypothesis, our preliminary studies indicate that subsequent enforced expression of *Meis1a* in Hoxa9-immortalized cells suppresses differentiation by G-CSF and M-CSF, suggesting that *Meis* may cooperate with Hoxa9 by establishing blocks in complementary differentiation pathways. Additionally, *Meis* may act to increase the efficiency of Hoxa9 on those same target promoters influenced by Hoxa9 alone. The independent immortalizing potential of Hoxa9 suggests that its activation in vivo may subject a normal progenitor to positive selection independent of *Meis* expression and that mutations in the *Meis* genes are likely to be secondary events in the development of overt leukemia.

The cooperative DNA binding observed between Hoxa9 and Pbx proteins leads one to question whether interaction with Pbx proteins is essential for myeloid immortalization by Hoxa9. Unlike the PIM sequences of Hoxb3 and Hoxb4, which are essential for cooperativity with Pbx in both transcription and transformation assays (27), the Hoxa9 ANWL motif was dispensable for immortalization and was only partially needed for transcriptional cooperativity with VP16-Pbx1<sub>1-343</sub>, despite the fact that it was required for cooperative DNA binding in vitro. In contrast, the fact that the Hoxa9 ANWL motif was essential for cooperative transactivation with VP16-Pbx1<sub>89-343</sub> suggests that residues 1 to 89 of Pbx1 may contact Hoxa9 and stabilize the heterodimer in the absence of interactions between the Pbx1 HD and the Hoxa9 ANWL PIM. As the ANWL motif of Nup98-HoxA9 is also essential for transcriptional cooperativity with Pbx and for transformation of NIH 3T3 fibroblasts (19), the second interaction surface may involve interaction of Pbx1 with the N-terminal 160 residues of Hoxa9, which are absent in Nup98-HoxA9. Nup98-HoxA9 may target the same promoters as Hoxa9 plus *Meis1*, and persistent production of *Meis1* could orchestrate transcriptional activation in a manner similar to translocation with Nup98. Identifying the accessory factors that cooperate with Hoxa9 on target promoters will ultimately result from identifying and characterizing direct HoxA9 target promoters whose gene products mediate differentiation arrest. The importance of this goal is underscored by the fact that HoxA9 is expressed in the majority of human AML (11, 20, 31).

Hoxa9-immortalized progenitors represent a useful tool for the investigation of mechanisms orchestrating granulocytic and macrophage differentiation. Similar to the majority of human AML (categories M1 to M5 [22, 51]), the human promyelocytic HL60 cell line, and the murine 32Dcl3 cell line (45), they express MPO and NE but fail to express secondary granule proteins. Like HL60 and mouse EPRO cells, Hoxa9-immortalized promyelocytes retain bilineage differentiation potential (32) and, like EPRO cells treated with ATRA or 32Dcl3 cells grown in G-CSF, can be induced to undergo neutrophil differentiation accompanied by expression of a subset of secondary granule genes. Among cells with bilineage potential, however, Hoxa9-immortalized progenitors are unique in that they undergo cytokine-inducible morphologic differentiation to neutrophils (G-CSF) or macrophages (M-CSF) accompanied by the expression of selected secondary granule proteins or the F4/80 antigen and the macrophage acetyl-LDL scavenger receptor. Therefore, Hoxa9-immortalized progenitors represent a model system for delineating the basis by which signaling through the G-CSF or M-CSF receptors initiates different genetic cascades leading to granulocytic or monocytic differentiation.

While *Hoxa9*-immortalized cells may be useful for studying downstream events in differentiation, the conditional myelopoiesis cell lines (CM3neo and CM1puro) represent a more powerful model in which one can determine how *Hoxa9* alters the transcription of target genes to prevent differentiation. Understanding how any myeloid oncoprotein prevents differentiation ultimately involves determining how the oncoprotein directly interacts with a promoter or with other chromatin-based regulatory elements to prevent the expression of myeloid-specific differentiation genes. In the case of *Hoxa9*, for example, understanding how it prevents transcription of *gp91phox* or *LF* represents a starting point from which one can backtrack to identify a single *Hoxa9* target gene whose activation or repression is ultimately responsible for the downstream event. The CM cell lines provide a system in which one can identify a specific transcriptional event that occurs during normal differentiation but fails to occur in the presence of the oncoprotein. This general approach provides a rational basis for identifying relevant target genes of *Hoxa9* and other myeloid oncoproteins.

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