

PBX3 and MEIS1 Cooperate in Hematopoietic Cells to Drive Acute Myeloid Leukemias Characterized by a Core Transcriptome of the MLL-Rearranged Disease

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Abstract

Overexpression of HOXA/MEIS1/PBX3 homeobox genes is the hallmark of mixed lineage leukemia (MLL)-rearranged acute myeloid leukemia (AML). HOXA9 and MEIS1 are considered to be the most critical targets of MLL fusions and their coexpression rapidly induces AML. MEIS1 and PBX3 are not individually able to transform cells and were therefore hypothesized to function as cofactors of HOXA9. However, in this study, we demonstrate that coexpression of PBX3 and MEIS1 (PBX3/MEIS1), without ectopic expression of a HOX gene, is sufficient for transformation of normal mouse hematopoietic stem/progenitor cells *in vitro*. Moreover, PBX3/MEIS1 overexpression also caused AML *in vivo*, with a leukemic latency similar to that caused by forced expression of MLL-AF9, the most common form of MLL fusions. Furthermore, gene expres-

sion profiling of hematopoietic cells demonstrated that PBX3/MEIS1 overexpression, but not HOXA9/MEIS1, HOXA9/PBX3, or HOXA9 overexpression, recapitulated the MLL-fusion-mediated core transcriptome, particularly upregulation of the endogenous *Hoxa* genes. Disruption of the binding between MEIS1 and PBX3 diminished PBX3/MEIS1-mediated cell transformation and HOX gene upregulation. Collectively, our studies strongly implicate the PBX3/MEIS1 interaction as a driver of cell transformation and leukemogenesis, and suggest that this axis may play a critical role in the regulation of the core transcriptional programs activated in MLL-rearranged and HOX-overexpressing AML. Therefore, targeting the MEIS1/PBX3 interaction may represent a promising therapeutic strategy to treat these AML subtypes. *Cancer Res*; 76(3); 619–29. ©2016 AACR.

Introduction

The mixed lineage leukemia (MLL) gene, located on human chromosome 11 band q23 (11q23), is a common target of chromosomal translocations in acute leukemia. MLL-rearranged

leukemia accounts for 5% to 10% of patients with acute myeloid leukemia (AML) and 7% to 10% of patients with acute lymphoblastic leukemia (ALL), approximately 80% of infant acute leukemia, and the majority of patients with therapy-related AML/ALL secondary to therapy that targets topoisomerase II (like etoposide; refs. 1–7). The critical feature of MLL rearrangements is the generation of a chimeric transcript consisting of 5' MLL and 3' sequences of one of more than 60 different partner genes (2, 3, 6, 7). MLL-AF9, resulting from t(9;11)(p22;q23), is the most common form of MLL-fusion genes in AML (2, 6).

Aberrant expression of a group of homeobox genes including the Cluster A HOX genes and genes encoding HOX cofactors, e.g., MEIS1 (but not MEIS2 or MEIS3) and PBX3 (but not PBX1 or PBX2), is the hallmark of MLL-rearranged leukemias (8–16). HOXA9 and MEIS1 are the two most well-studied downstream target genes of MLL-fusion proteins; their aberrant overexpression has been considered to be required for the induction and maintenance of MLL-rearranged leukemia (11, 17–19), and their coexpression is sufficient to transform cells and induce rapid leukemia (12, 20–23). In contrast, although PBX3 is also significantly upregulated in MLL-rearranged AML (11–15), the role of PBX3 in leukemogenesis was largely unappreciated. Pbx proteins have been shown to be required for linking Hoxa and Meis1 proteins together (24, 25). However, the previous studies of *Pbx* genes were focusing on *Pbx1*, the founding member of the *Pbx* family, which was shown to have no synergistic effect with *Hoxa9* in cell transformation and leukemogenesis (12, 20, 23). Instead,

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we recently showed that *PBX3* and three *HOXA* genes (*HOXA7*, *HOXA9*, and *HOXA11*) composed of an independent predictor of unfavorable survival of patients with cytogenetically abnormal AML (CA-AML; ref. 16). We showed further that *PBX3*, rather than *PBX1* or *PBX2*, is an important cofactor of *HOXA9* in cytogenetically abnormal AML and their coexpression can cause development of rapid AML in mice (26). The prognostic impact and essential oncogenic role of *PBX3* have also been observed in cytogenetic normal AML (27).

Thus far, both *MEIS1* and *PBX3* have been shown to be essential cofactors of *HOXA9* and coexpression of *HOXA9* with either *MEIS1* or *PBX3* can induce rapid AML (12, 20–23, 26). However, unlike *HOXA9*, neither *MEIS1* nor *PBX3* alone can transform normal hematopoietic stem/progenitor cells (HSPC; refs. 20, 21, 23, 26). Thus, *MEIS1* and *PBX3* were simply recognized as cofactors of HOX proteins (especially *HOXA9*) and it was believed that they exert their function solely or mainly through cooperating with HOX proteins (18, 24–28). Surprisingly, here we show that without ectopic expression of a HOX gene, coexpression of *PBX3* and *MEIS1* is sufficient to transform normal HSPCs and induce rapid AML in mice. More interestingly, our genome-wide gene expression profiling analysis shows that forced expression of *PBX3/MEIS1*, but not that of *HOXA9/MEIS1*, *HOXA9/PBX3*, or *HOXA9* alone, can induce the core transcriptome (especially, the upregulation of endogenous *Hoxa* genes, *Pbx3* and *Meis1*) of *MLL*-rearranged AML in mice.

Materials and Methods

Retroviral constructs

MSCVneo-HOXA9 and *MSCV-PIG-PBX3* plasmids with human gene *HOXA9* and *PBX3* coding regions, respectively, were constructed previously by us (26). *MSCVneo-MLL-AF9* was a kind gift from Dr. Scott Armstrong, Memorial Sloan Kettering Cancer Center, New York, NY (29). *MSCV-PIG* vector containing a PGK-puromycin-IRES-GFP (PIG) cassette was kindly provided by Drs. G. Hannan and L. He, Cold Spring Harbor Laboratory, Spring Harbor, NY (30). *MEIS1* coding region sequence was PCR amplified from human normal bone marrow mononuclear cells with primers, forward 5'- ATAGAATTCTATGGCCCAAAGGTAC-3', and reverse 5'- GGCTCTCGAGTAGATGAAGGTTACA -3', was then cloned into *MSCVneo* (Clontech) as *MSCVneo-MEIS1*, or cloned into *MSCV-PIG* as *MSCV-PIG-MEIS1*. Mouse *Pbx3* coding region sequence was PCR amplified from mouse normal bone marrow mononuclear cells with primers, forward 5'- AATAGATCTACCACCATGGAC-GATCAATCCAGGATG-3', and reverse 5'- ACTCTCGAGTTAGT-TAGGAG GTATCCGAGTGC-3', and was then cloned into *MSCV-PIG*, as *MSCV-PIG-Pbx3*. Wild-type and mutant mouse *Meis1*-coding regions were PCR amplified from *MSCVpuro-VP16-Meis1* (WT), *MSCVpuro-VP16-Meis1-M2ΔLRF/ΔLLEL*, and *MSCVpuro-VP16-Meis1-Δ64-202*, which were kindly provided by Dr. M.P. Kamps, University of California, San Diego, La Jolla, CA (24, 25), and then subcloned into *MSCVneo*, as *MSCVneo-Meis1*, *MSCVneo-Meis1-M2ΔLRF/ΔLLEL*, and *MSCVneo-Meis1-Δ64-202*, respectively. All inserts have been confirmed by Sanger sequencing.

Mouse bone marrow transplantation (*in vivo* reconstitution) assays

For primary bone marrow transplantation (BMT) assays, mouse bone marrow progenitor cells from 4- to 6-week-old wild-type (C57BL/6J CD45.2) mice were cotransduced with different retroviral combinations, and were then cultured in methylcellulose

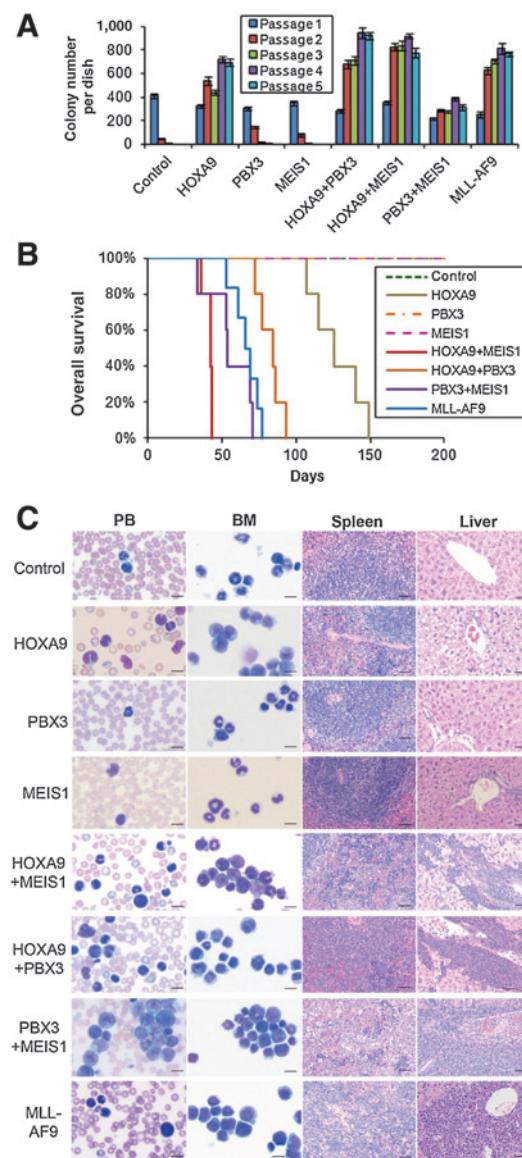


Figure 1. Coexpression of *PBX3* and *MEIS1* can transform normal mouse bone marrow progenitor cells *in vitro* and induce rapid AML *in vivo*. A, *in vitro* colony-forming/replating assays. Briefly, mouse normal bone marrow progenitor (lineage negative; Lin-) cells collected from 4- to 6-week-old B6.SJL (CD45.1) mice were retrovirally cotransduced with *MSCVneo*+*MSCV-PIG* (control), *MSCVneo-HOXA9*+*MSCV-PIG* (*HOXA9*), *MSCVneo*+*MSCV-PIG-MEIS1* (*MEIS1*), *MSCVneo*+*MSCV-PIG-PBX3* (*PBX3*), *MSCVneo-HOXA9*+*MSCV-PIG-MEIS1* (*HOXA9+MEIS1*), *MSCVneo-HOXA9*+*MSCV-PIG-PBX3* (*HOXA9+PBX3*), *MSCVneo-MEIS1*+*MSCV-PIG-PBX3* (*PBX3+MEIS1*), or *MSCVneo-MLL-AF9*+*MSCV-PIG* (*MLL-AF9*). The colony cells were replated every 7 days for up to 5 passages and colony numbers were counted for each passage. Mean \pm SD values of colony counts are shown. B, mouse BMT assays were conducted for the above 8 groups with the first-passage colony cells (CD45.1) as donors, which were transplanted into lethally irradiated 8- to 10-week-old C57BL/6 (CD45.2) recipient mice. Kaplan-Meier curves are shown. Five mice were studied in each group, except for the *MLL-AF9* group, in which 6 mice were studied. C, cell/tissue morphologies of the eight groups. Peripheral blood (PB) and bone marrow (BM) cells were stained with Wright-Giemsa. The spleen and liver tissues were stained with hematoxylin and eosin. The length of bars represents 10 μ m for peripheral blood and bone marrow, and 100 μ m for spleen and liver.

medium to select double-transduction-positive cells. Seven days later, colony cells were collected and washed, and were then injected by tail vein into lethally irradiated (960 rads) 8- to 10-week-old B6.SJL (CD45.1) recipient mice with 0.5×10^6 donor cells plus a radioprotective dose of whole bone marrow cells (1×10^6 ; freshly harvested from a B6.SJL mouse) per recipient mouse.

For secondary BMT assays, primary leukemic bone marrow cells were obtained from the primary BMT recipients of the HOXA9+MEIS1, HOXA9+PBX3, PBX3+MEIS1, and MLL-AF9 groups, when the mice developed with full-blown AML. The CD45.1 $^{+}$ leukemic bone marrow cells were sorted by flow cytometry and then injected through tail vein into lethally irradiated (850 rads) 8- to 10-week-old B6.SJL (CD45.1) secondary recipient mice with 1×10^5 donor cells plus 1.8×10^6 whole bone marrow cells (from a B6.SJL mouse) per recipient mouse. Leukemic bone marrow cells from two independent primary leukemic mice were used as the donors for the secondary BMT of each group, with 5 to 6 recipients per donor. Another secondary transplantation assay was conducted with leukemic spleen cells (CD45.1 $^{+}$) obtained from the spleen of primary HOXA9+MEIS1, PBX3+MEIS1, and MLL-AF9 AML mice, with 1×10^5 donor cells (CD45.1) plus 1.8×10^6 radioprotective whole bone marrow cells (CD45.2) per recipient mouse.

Affymetrix gene arrays of mouse samples

A total of 20 mouse bone marrow samples from the control (NC; $n = 5$), HOXA9+MEIS1 ($n = 3$), HOXA9+PBX3 ($n = 3$), HOXA9 ($n = 3$), PBX3+MEIS1 ($n = 3$), and MLL-AF9 ($n = 3$) AML groups were analyzed with Affymetrix GeneChip Mouse Gene 1.0 ST Array (Affymetrix). For each sample, the CD45.1 $^{+}$ bone marrow cells (i.e., transplanted donor cells) were sorted with flow cytometry from whole bone marrow cells collected from BMT recipients at the end point. One microgram total RNA was used for each sample. The quality control test and array assays were done in the core facility of National Human Genome Research Institute, NIH (Bethesda, MD). The quantified signals were normalized using Robust Multi-array Average (31), after hybridization and background correction according to the standard protocols. All genes with a SD of expression values over 0.3 across the 20 samples were used for the unsupervised hierarchical clustering and followed statistical analyses. The microarray data have been deposited into the GEO database (GSE68643; Reviewer access: <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=clorwkcodjuxzyl&acc=GSE68643>).

Gene set analyses

Gene Set Enrichment Analysis (GSEA; ref. 32) was used to analyze gene sets enriched in different groups of samples. KEGG (Kyoto Encyclopedia of Genes and Genomes Pathway Database) gene set (33) and "chemical and genetic perturbation" gene set obtained from MsigDB (The Molecular Signatures Database; ref. 32) were used as the gene set input for GSEA. The Database for Annotation, Visualization and Integrated Discovery (DAVID; v6.7; refs. 34, 35) was used to analyze gene sets enriched in genes significantly differentially expressed between different groups of samples.

RNA extraction, quantitative RT-PCR, flow cytometry, cytochrome c, histologic hematoxylin and eosin staining, and Western blotting

These assays were conducted as described previously (16, 36–39) with some modifications. Antibodies for Pbx3 (sc-891), Hoxa9 (sc-17155), Hoxa7 (sc-17152), and Meis1 (sc-10599) were purchased from Santa Cruz Biotechnology, Inc. Anti-Hoxa5

antibody (ab140636) was purchased from Abcam. Anti-Hoxa4 antibody (PA1603) was purchased from Boster Biological Technology. Anti- β -actin antibody (#3700) was purchased from Cell Signaling Technology, Ltd.

Statistical analyses

Overall survival was estimated according to the method of Kaplan and Meier, and the log-rank test was used to assess statistical significance. The Kaplan-Meier method, log-rank test, and *t* test were performed with Partek Genomics Suite (Partek Inc), WinSTAT (R. Fitch Software), and/or Bioconductor R packages. Any *P* values less than 0.05 were considered statistically significant.

Human AML cell line and siRNA transfection

MonoMac-6 AML cell line, carrying t(9;11)/MLL-AF9, was maintained in the Chen laboratory and cultured as described

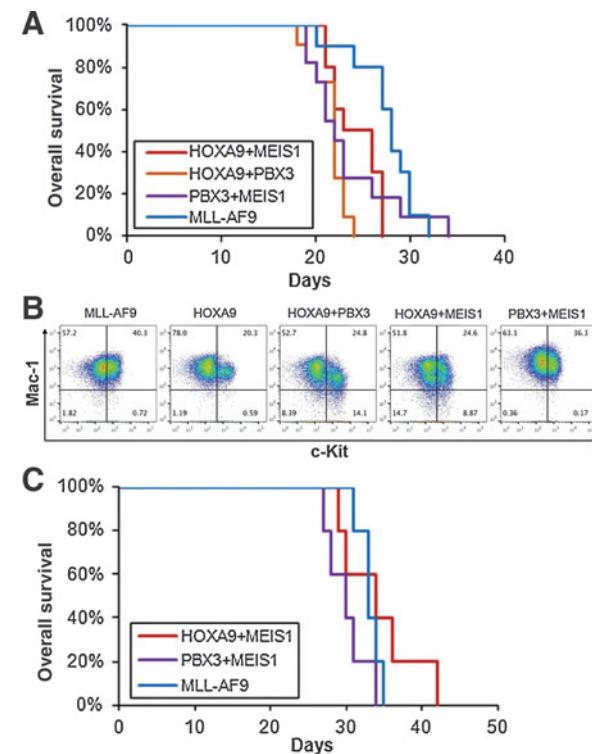
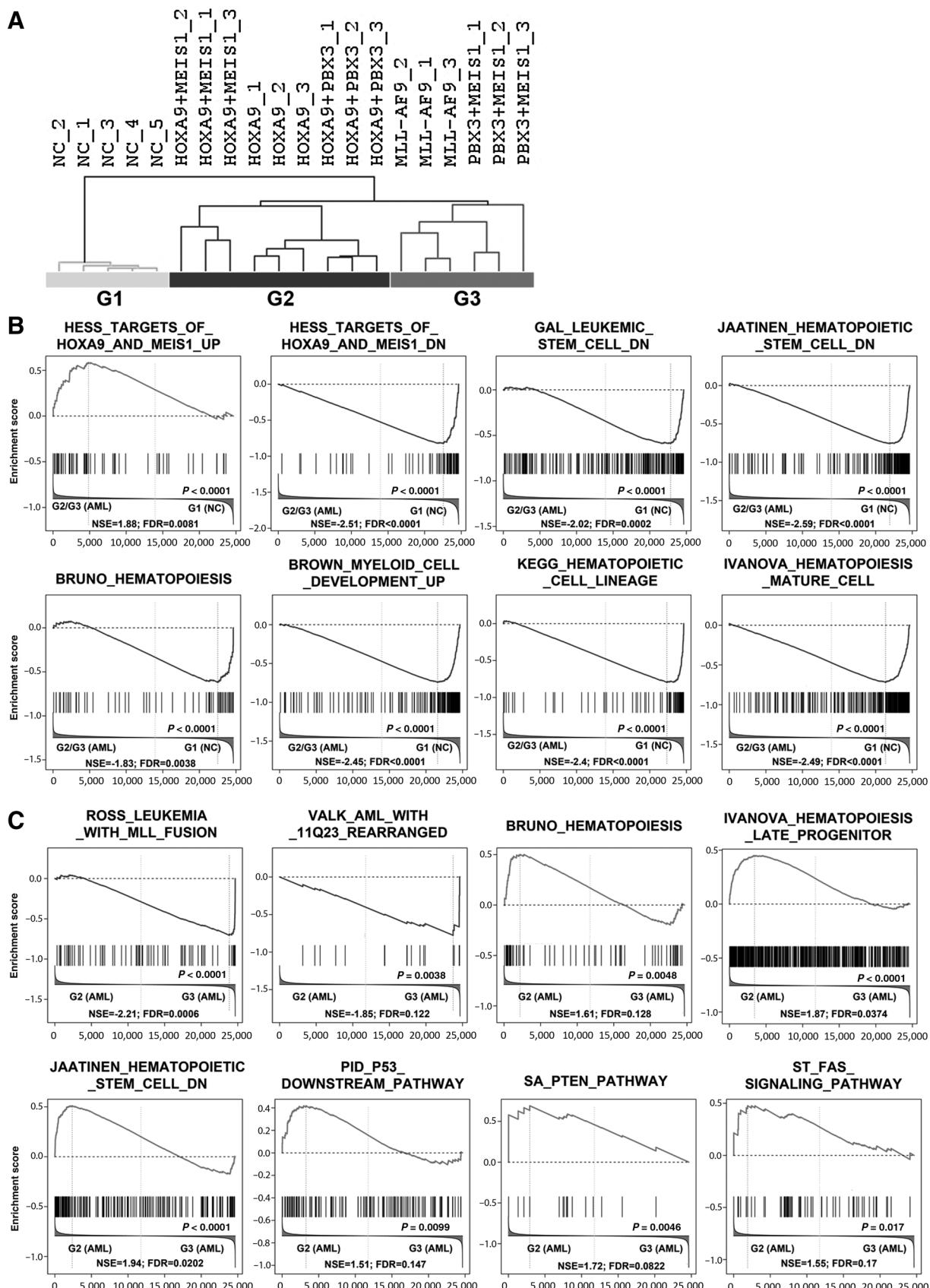


Figure 2. PBX3/MEIS1-induced AML is transmissible in secondary transplantation recipients. A, Kaplan-Meier survival curves of secondary transplantation recipient (CD45.2 $^{+}$) mice transplanted with primary leukemic bone marrow cells (CD45.1 $^{+}$) of the HOXA9+MEIS1 (recipient mouse number: $n = 10$), HOXA9+PBX3 ($n = 11$), PBX3+MEIS1 ($n = 11$), and MLL-AF9 ($n = 10$) groups. Primary AML bone marrow cells from two donor mice were used for each group. There is no significant difference ($P > 0.1$) between survival of the PBX3+MEIS1 group and that of any other three groups. B, flow cytometry analysis of leukemic bone marrow cells from the above secondary BMT recipient mice. Antibodies against Mac-1 and c-Kit were used. Flow data of leukemic bone marrow samples from one recipient mouse are shown as representative for each group. C, Kaplan-Meier survival curves of secondary transplantation recipient (CD45.2 $^{+}$) mice transplanted with primary leukemic spleen cells (CD45.1 $^{+}$) of the HOXA9+MEIS1 ($n = 5$), PBX3+MEIS1 ($n = 5$), and MLL-AF9 ($n = 5$) groups. Primary AML spleen cells from one donor mouse were used for each group. There is no significant difference ($P > 0.1$) between survival of the PBX3+MEIS1 group and that of any other two groups.



previously (36–38). Continued testing to authenticate this cell line was done using qPCR and Western blot analysis to validate the existence of MLL-AF9 when this line was used in this project. Anti-PBX3 or anti-MEIS1 siRNA oligos and control siRNA oligos were purchased from Dharmacon (Catalog #: L-020121-00-0005, L-011726-00-0005 and D-001810-10-05; GE Healthcare Biosciences), and transfected into MonoMac-6 cells Amaxa Nucleofector Technology (Amaxa Biosystems) at a concentration of 100 nmol/L as described previously (16, 36–38).

Results

Coexpression of PBX3 and MEIS1 is sufficient to transform normal HSPCs

On the basis of previous studies from us and others showing crucial roles of both PBX3 and MEIS1 in AML (16, 24–28), we query whether coexpression of *PBX3/MEIS1* can mimic that of *HOXA9/MEIS1* or *HOXA9/PBX3* in inducing cell transformation. To this end, we conducted *in vitro* colony-forming/replating assays. As shown in Fig. 1A, *PBX3/MEIS1* coexpression (*PBX3+MEIS1*) was sufficient to transform and immortalize normal mouse bone marrow progenitor cells, although with relatively fewer colonies compared with coexpression of *HOXA9/MEIS1* (*HOXA9+MEIS1*) or *HOXA9/PBX3* (*HOXA9+PBX3*), or forced expression of MLL-AF9. As expected, *HOXA9* alone, but not *MEIS1* or *PBX3* alone, also transformed mouse bone marrow progenitor cells (Fig. 1A).

Coexpression of PBX3 and MEIS1 induces rapid AML in mice

To determine whether coexpression of *PBX3/MEIS1* is also sufficient to induce AML in mice, we conducted *in vivo* mouse BMT assays. As shown in Fig. 1B and Supplementary Fig. S1, similar to the coexpression of *HOXA9* with *MEIS1* or *PBX3*, coexpression of *PBX3* and *MEIS1* also caused a rapid AML in mice. The leukemic latency of the *PBX3+MEIS1* group was comparable with that of the *MLL-AF9* group ($P = 0.24$; log-rank test) and slightly longer than that of the *HOXA9+MEIS1* group ($P = 0.054$), but significantly shorter than that of the *HOXA9+PBX3* group ($P = 0.021$) or *HOXA9* alone group ($P = 0.0018$). Neither *MEIS1* nor *PBX3* alone caused leukemia within 200 days (Fig. 1B). Notably, mice of the *PBX3+MEIS1* group exhibited similar, if not more aggressive, leukemic phenotypes in peripheral blood, bone marrow, spleen, and liver tissues compared with the *HOXA9+MEIS1*, *HOXA9+PBX3*, and *MLL-AF9* groups (Fig. 1C). Our flow cytometry analysis showed that leukemic bone marrow cells of the *PBX3+MEIS1* group had largely similar proportions of Mac-1⁺ (a myeloid lineage cell marker) and/or c-Kit⁺ (a stem/progenitor cell marker) cells compared with those of the other AML groups (Supplementary Fig. S1).

PBX3/MEIS1-induced AML is transplantable

To determine whether *PBX3/MEIS1*-induced AML is transplantable, we conducted secondary transplantation assays.

First, we collected leukemic bone marrow cells from primary BMT recipient mice of the *HOXA9+MEIS1*, *HOXA9+PBX3*, *PBX3+MEIS1*, and *MLL-AF9* groups and then transplanted into lethally irradiated secondary recipient mice. Leukemic bone marrow cells from two independent primary recipient mice were used as donor cells for the secondary BMT of each group. All secondary BMT recipient mice transplanted with *PBX3+MEIS1* primary AML cells developed full-blown AML within 35 days, with similar leukemic latency to those transplanted with *HOXA9+MEIS1*, *HOXA9+PBX3*, or *MLL-AF9* AML cells (Fig. 2A and B). The two independent donors of each group caused full-blown leukemia with similar latency in secondary recipients (Supplementary Fig. S2). In addition, we also conducted secondary transplantation assays using primary leukemic spleen cells as donor cells, and also found that *PBX3+MEIS1* leukemic spleen cells caused full-blown AML in all secondary BMT recipients within 35 days, in a manner similar to *HOXA9+MEIS1* or *MLL-AF9* leukemic spleen cells (Fig. 2C).

Genome-wide gene expression profiles of *PBX3/MEIS1* AML are more similar to those of *MLL-AF9* AML than to those of *HOXA9/MEIS1*, *HOXA9/PBX3*, or *HOXA9* AML

We then conducted microarray-based genome-wide gene expression profiling assays with leukemic or control bone marrow cells collected from primary BMT recipients. Surprisingly, in an unsupervised hierarchical clustering analysis, we found that *PBX3+MEIS1* AML samples clustered together with *MLL-AF9* AML samples, whereas *HOXA9+MEIS1*, *HOXA9+PBX3*, and *HOXA9* AML samples formed a separate cluster and so did the normal control samples (see Fig. 3A). Thus, there are three separate groups, including group 1 (normal controls; NC); group 2 (*HOXA9+MEIS1*, *HOXA9+PBX3*, and *HOXA9*), and group 3 (*MLL-AF9* and *PBX3+MEIS1*). We then conducted GSEA (32). No surprise, as both group 2 and 3 samples are leukemia samples with ectopic and/or induced endogenous expression of homeobox genes, they share a series of gene sets that distinguish them from normal control samples. Notably, target genes upregulated by *HOXA9* and *MEIS1*, and genes related to DNA replication, mRNA processing, miRNA biogenesis, RNA metabolism, or translation, are enriched in genes expressed at a higher level in groups 2 and 3 samples than in group 1 samples (Fig. 3B and Supplementary Fig. S3A). In contrast, target genes downregulated by *HOXA9* and *MEIS1*, genes downregulated in leukemic stem cells or hematopoietic stem cells, genes related to hematopoiesis, hematopoietic cell lineage, apoptosis or p53 signaling pathway, and genes upregulated in myeloid cell development or in hematopoietic mature cells are enriched in genes expressed at a higher level in group 1 samples than in group 2 and 3 samples (Fig. 3B and Supplementary Fig. S3A).

On the other hand, group 2 and 3 samples also have significantly different enrichments of a series of gene sets. In particular, genes are highly expressed in *MLL*-rearranged leukemia or AML

Figure 3.

Comparison of gene expression profiles between different groups of leukemic or normal control cells. A, unsupervised hierarchical clustering analysis of the control ($n = 5$), *HOXA9+MEIS1* ($n = 3$), *HOXA9+PBX3* ($n = 3$), *HOXA9* ($n = 3$), *PBX3+MEIS1* ($n = 3$), and *MLL-AF9* ($n = 3$) groups. The hierarchical clustering tree is shown. B, gene sets that are shared by group 2 (G2; including *HOXA9+MEIS1*, *HOXA9+PBX3*, and *HOXA9*) and group 3 [G3; including *PBX3+MEIS1* and *MLL-AF9* (MA9)] samples, with a significantly different pattern in group 1 [G1; normal control (NC)] samples, as detected by GSEA (32). C, gene sets that are differentially enriched between group 2 and group 3 samples. NSE, normalized enrichment score.

with *NPM1* mutations, and target genes upregulated by *NUP98-HOXA9* fusion gene, are enriched in genes expressed at a higher level in group 3 samples than in group 2 samples (Fig. 3C and Supplementary Fig. S3B). Indeed, besides *MLL*-rearranged leukemia, previous studies have reported that the *HOXA/MEIS1/PBX3* genes are also highly expressed in AML with *NPM1* mutations or *NUP98-HOXA9* fusion (40–43). In contrast, genes related to hematopoiesis, p53 pathway, PTEN pathway, FAS signaling pathway, genes highly expressed in hematopoietic late progenitor cells, and genes downregulated in hematopoietic stem cells or in *NPM1*-mutated AML, are enriched in genes expressed at a higher level in group 2 samples than in group 3 samples (Fig. 3C and Supplementary Fig. S3B). Thus, genes highly expressed in group 2 are more enriched with genes belonging to the pathways related to apoptosis and cell differentiation, while genes highly expressed in group 3 are enriched with gene sets upregulated in *MLL*-rearranged, *NPM1* mutated, or *NUP98-HOXA9* leukemic cells. Most of these gene sets are also significantly ($P < 0.05$) enriched in the group of *PBX3+MEIS1* samples relative to group 2 samples (see Supplementary Fig. S4), further suggesting that the *PBX3+MEIS1* samples share the core gene sets with the *MLL-AF9* samples.

***PBX3/MEIS1* AML recapitulates the core transcriptome of *MLL-AF9* AML**

We then performed ANOVA analysis and identified 2,562 genes that are significantly differentially expressed [false discovery rate (FDR) < 0.01] between the three groups of samples (Supplementary Fig. S5A). Through significance analysis of microarrays (44), we found that 166 and 211 genes are expressed at a significantly higher and lower level ($q < 0.05$; FDR < 0.01), respectively, in group 3 compared with both groups 1 and 2 (Fig. 4 and Supplementary Figs. S5B and S6). A total of 279 and 85 genes are expressed at a significantly higher and lower level ($q < 0.05$; FDR < 0.05), respectively, in group 2 compared with both groups 1 and 3 (Supplementary Figs. S5B, S7, and S8). A total of 1,190 and 1,022 genes are expressed at a significantly higher and lower level ($q < 0.05$; FDR < 0.05), respectively, in group 1 compared with both groups 2 and 3 (Supplementary Fig. S5B; Supplementary Tables S1 and S2).

Through searching for the DAVID (34, 35), we found that gene sets associated with "homobox transcription factor," "embryonic development," "DNA binding" and "transcription/transcription regulator activity" are significantly enriched (FDR < 0.05) in genes highly expressed in group 3 (see Supplementary Fig. S5C). Gene sets associated with "transferase activity," "G-protein modulator," "lysosome" and "cell surface" are significantly enriched in genes highly expressed in group 2 (Supplementary Fig. S5C). Gene sets associated with "immune response," "hematopoiesis," "leukocyte/lymphocyte/erythrocyte/myeloid cell differentiation" and "regulation of cell death/apoptosis" are significantly enriched in genes highly expressed in group 1 (Supplementary Fig. S5C). Consistent with our GSEA analysis results (Fig. 3B and Supplementary Fig. S3A), genes associated with cell death/apoptosis and hematopoietic cell differentiation are significantly downregulated in both groups 2 and 3 AML samples relative to normal control (group 1) samples (see Supplementary Fig. S9). Such data suggests that the repression of those genes is highly likely critical for the development of leukemia of both groups 2 and 3. In genes that are expressed at a significantly lower level in group 3 than in both groups 1 and 2, gene sets related to "hematopoiesis," "regulation

of cell differentiation," "stem cell differentiation," "apoptosis," "immune system process," and "positive regulation of cell activation" are significantly enriched (Supplementary Fig. S5C); notably, those genes are expressed at the lowest level in group 3 and at the highest level in group 1, while at the middle level in group 2 samples (see Supplementary Figs. S10).

Notably, 22 (i.e., *Ash2l*, *Baz2b*, *Chd9*, *Dcun1d1*, *Erbb2ip*, *Eya1*, *Hoxa2*, *Hoxa3*, *Hoxa4*, *Hoxa5*, *Hoxa6*, *Hoxa7*, *Hoxa9*, *Hoxa10*, *Ikzf2*, *Meis1*, *Pbx3*, *Rnf220*, *Runx2*, *Sgk3*, *Tnpo1*, and *Tsc22d2*; see Fig. 4) out of the 166 highly expressed genes in group 3 (13.3%) are potential direct targets of *MLL*-fusion proteins as detected by at least one of the three published genome-wide ChIPseq or ChIP-chip assays of *MLL*-fusion targets (45–47). Such proportion (13.3%) is significantly greater ($P < 0.0001$; χ^2 test) than that (4/279; 1.4%) of the genes highly expressed in group 2 (Supplementary Table S3). In contrast, none of the 211 genes underexpressed in group 3 and none of the 1,190 genes highly expressed in group 1 is potential direct target of *MLL* fusions (Supplementary Table S3). These data indicate that *MLL*-fusion proteins often upregulate, rather than downregulate, expression of their target genes, and that the *PBX3/MEIS1* combination sufficiently resembles *MLL*-fusion proteins in promoting expression of a cohort of core target genes of *MLL* fusions.

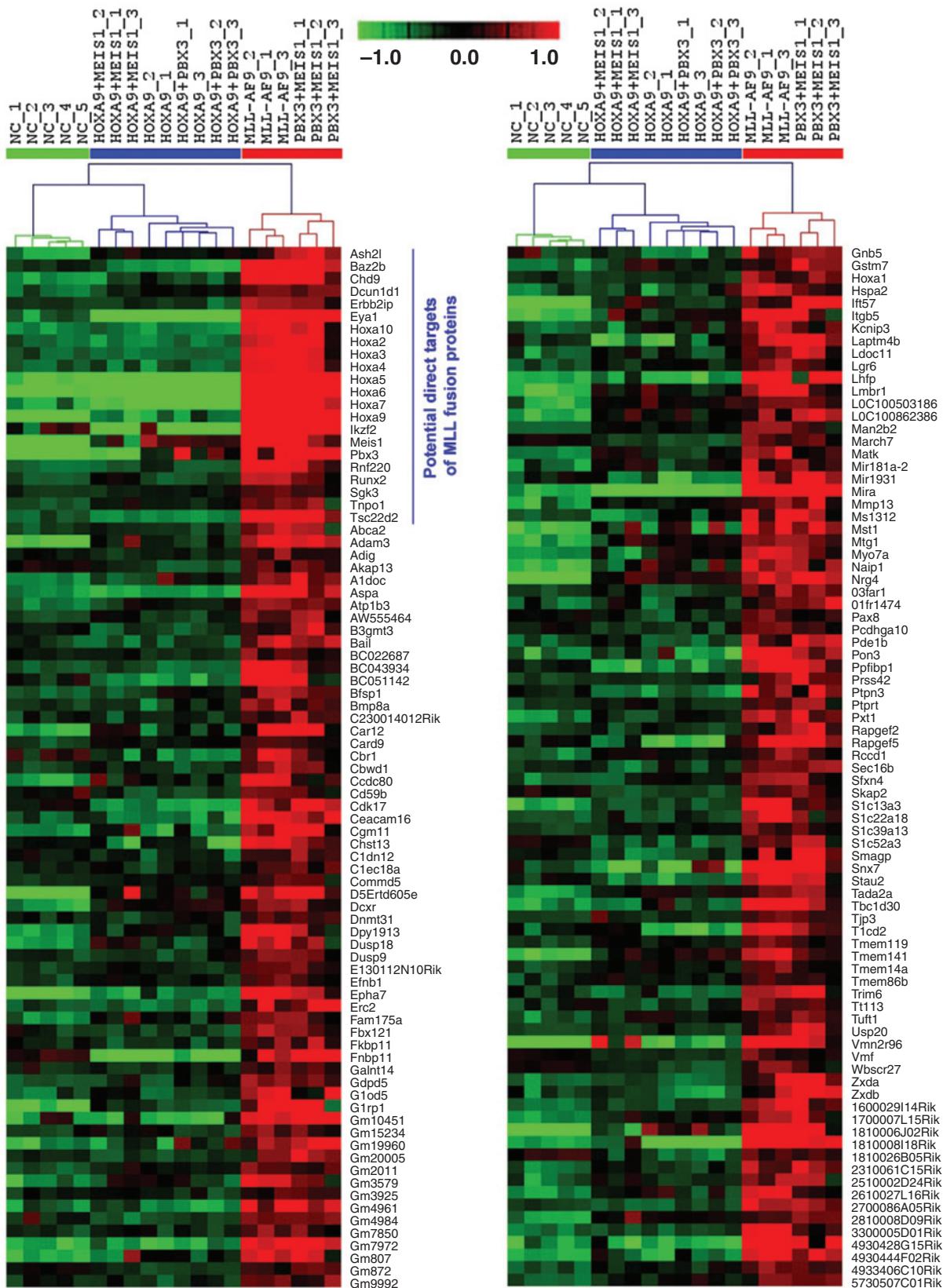
Unlike the *PBX3+MEIS1* AML cells, the AML cells with *HOXA9+PBX3* or *HOXA9+MEIS1* exhibited a very low level of endogenous expression of the *Hoxa* genes (see Fig. 4). To investigate whether ectopic *HOXA9* exerted a suppressive effect on the expression of endogenous *Hoxa* genes, we ectopically expressed *HOXA9* in *PBX3+MEIS1* AML cells. As expected, forced expression of *HOXA9* did not suppress endogenous *Hoxa* expression (Supplementary Fig. S11). Thus, our data indicate that the incapability of *HOXA9+PBX3* or *HOXA9+MEIS1* to upregulate endogenous expression of *Hoxa* genes is not due to a transcriptional suppression mediated by exogenous expression of *HOXA9*.

Knockdown of *PBX3* and/or *MEIS1* leads to a significant downregulation of endogenous *HOXA* expression in *MLL*-rearranged AML cells

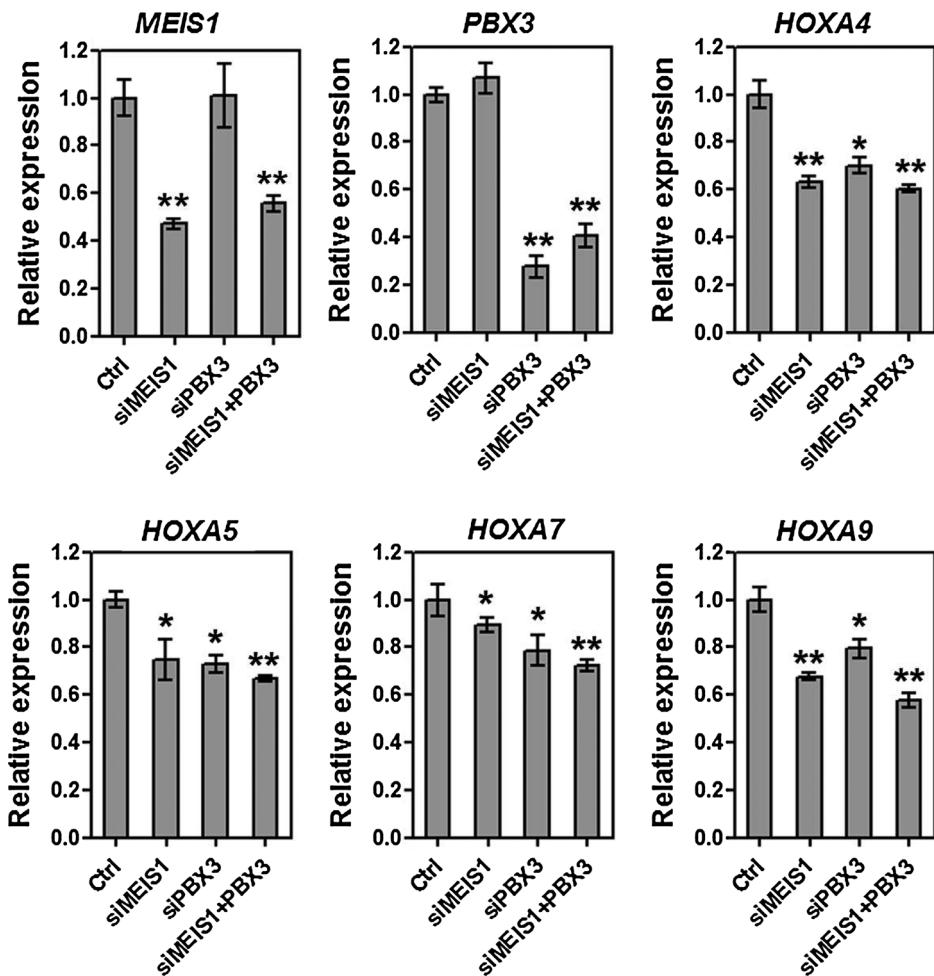
To investigate whether a high level of *PBX3* or *MEIS1* expression is also required to maintain the high level of endogenous *HOXA* expression in *MLL*-rearranged AML cells, we transfected anti-*PBX3* and/or anti-*MEIS1* siRNA oligos into MonoMac-6 cells. As expected, knockdown of *PBX3* and *MEIS1* each alone or together resulted in a significant downregulation of expression of *HOXA* genes (Fig. 5), further demonstrating the importance of *PBX3/MEIS1* in regulating expression of the *HOXA* genes.

The binding between *Meis1* and *Pbx3* is essential for their synergistic effects on cell transformation and upregulation of endogenous homeobox genes

Previous studies have identified a functional domain in *Meis1* protein that is critical for its binding with *Pbx* proteins (24, 25). To determine whether the binding between *PBX3* and *MEIS1* is also critical for the biologic effects caused by coexpression of *PBX3* and *MEIS1*, we cloned mouse *Pbx3* gene, and also subcloned the wild-type mouse *Meis1* gene and two mutants with point mutations or a regional deletion in the *Pbx*-binding domain (i.e., *Meis1-M2ΔLRF/ΔLLEL* and *Meis1-Δ64-202*) based

**Figure 4.**

Heatmap of expression profiles of the 116 genes that are expressed at a significantly higher level in group 3 (i.e., *PBX3+MEIS1* and *MLL-AF9* samples) than in both groups 1 and 2. Of them, 22 genes are potential direct target genes of MLL-fusion proteins. Expression data was mean centered. Red represents a high expression (scale shown in the top middle).

**Figure 5.**

Effects of knockdown of *PBX3* and/or *MEIS1* on the expression of endogenous *HOXA* genes in *MLL*-rearranged AML cells. Endogenous expression levels of *MEIS1*, *PBX3*, *HOXA4*, *HOXA5*, *HOXA7*, and *HOXA9* in MonoMac-6/t(9;11) AML cells were detected by qPCR 48 hours after transfection of anti-*MEIS1* siRNA oligos (si*MEIS1*) and/or anti-*PBX3* siRNA oligos (si*PBX3*), or of scrambled control oligos (Ctrl). Mean \pm SD values are shown. *, P < 0.05; **, P < 0.01.

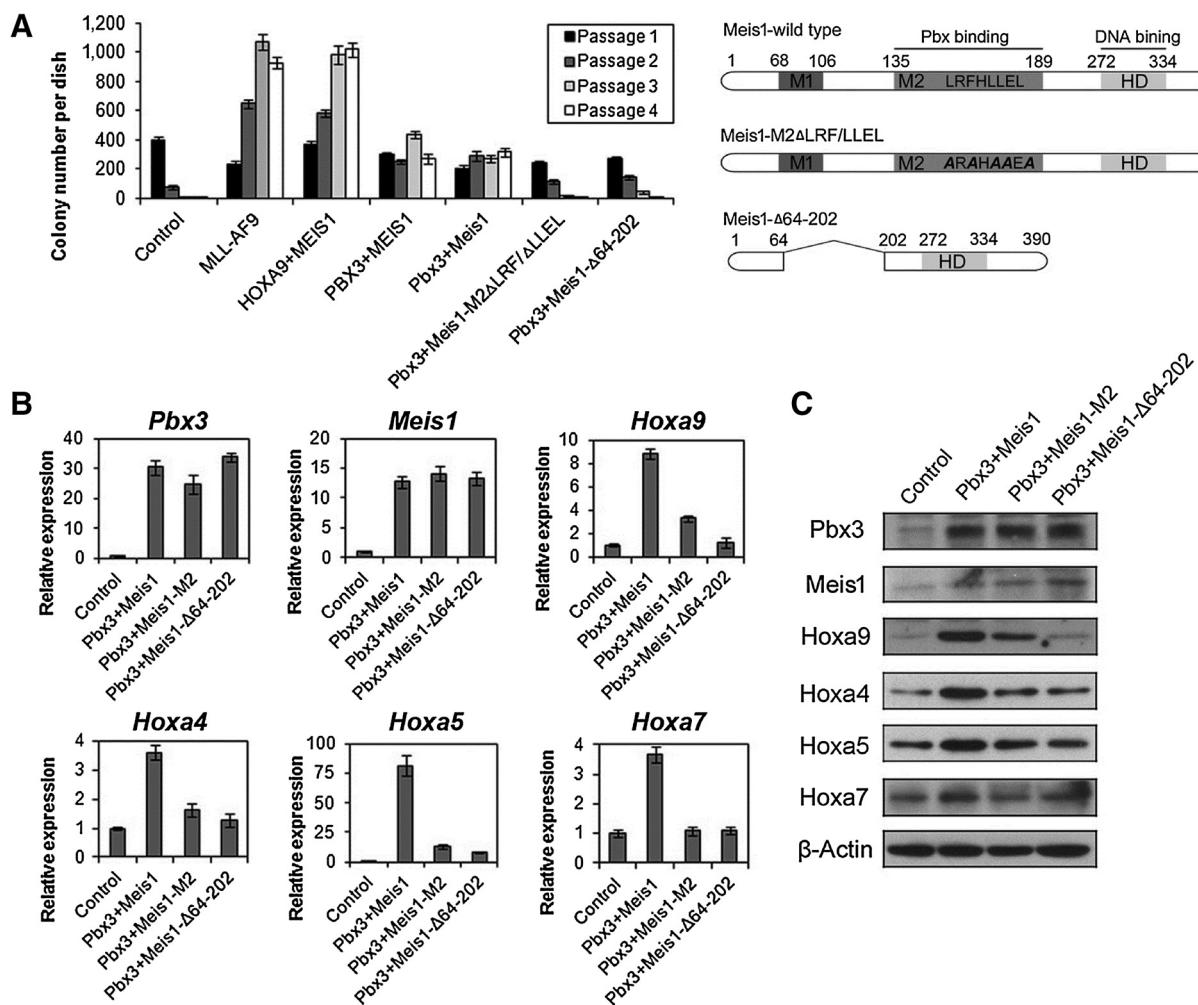
on the constructs reported previously (24, 25). We then conducted colony-forming/replating assays with mouse bone marrow progenitor cells retrovirally transduced with *Pbx3*+*Meis1* (wild-type), *Pbx3*+*Meis1*-M2ΔLRF/ΔLLEL, *Pbx3*+*Meis1*-Δ64-202, along with *PBX3*+*MEIS1*, *HOXA9*+*MEIS1*, *MLL*-AF9, and control vectors. As shown in Fig. 6A, coexpression of mouse *Pbx3* and *Meis1* genes had a similar effect to coexpression of human *PBX3* and *MEIS1* genes on cell transformation/immortalization, with more than 200 colonies per dish after series of replating. In contrast, coexpression of *Pbx3* with mutated *Meis1* (*Meis1*-M2ΔLRF/ΔLLEL or *Meis1*-Δ64-202) lost the capacity to form a significant number of colonies after series of replating (Fig. 6A), indicating that disruption of the binding between *Meis1* and *Pbx3* substantially inhibits their synergistic effect on cell transformation.

Not surprisingly, our further qPCR assays showed that, despite that *Pbx3* and *Meis1*, wild-type or mutants, show the comparable level, coexpression of *Pbx3* with *Meis1* mutants (*Meis1*-M2ΔLRF/ΔLLEL or *Meis1*-Δ64-202) could not upregulate expression of endogenous *Hoxa* genes to a level as high as that induced by coexpression of *Pbx3* with wild-type *Meis1*, a phenomenon verified by immunoblotting (Fig. 6B and C). Thus, disruption of the binding between *Meis1* and *Pbx3* also substantially abrogates their synergistic effect on upregulation of expression of endogenous homeobox genes.

Discussion

Here we show, for the first time, that forced expression of both *PBX3* and *MEIS1* can transform/immortalize normal HSPCs *in vitro* and induce a rapid AML *in vivo*. Although the binding ability of *Meis1* with *Pbx* proteins has been reported previously to be essential for the synergistic effect between *Meis1* and *Hoxa9* and for the function of *MLL*-fusion proteins (18, 24, 25), no efforts have been exerted to investigate whether forced expression of both *MEIS1* and *PBX3* is sufficient to transform cells and induce leukemia, without forced expression of a *HOXA* gene. Indeed, because neither *Meis1* nor *Pbx3* alone can induce cell transformation and leukemogenesis (20, 21, 23, 26), they have been thought to mainly play supportive roles in facilitating *HOXA* proteins in regulating their downstream targets (18, 24–28). Therefore, our new finding reveals the functional importance of *PBX3* and *MEIS1* in cell transformation and leukemogenesis.

More strikingly, we further show that it is the coexpression of *PBX3* and *MEIS1*, not coexpression of *HOXA9* and *MEIS1* (or *HOXA9* and *PBX3*), that can recapitulate the core transcriptome of *MLL*-rearranged AML, especially the significant upregulation of a set of homeobox transcriptional factors (in particular, the *Hoxa* genes, *Meis1* and *Pbx3*). In fact, a total of more than 160 genes (including the Homeobox genes) are significantly upregulated by both coexpression of *PBX3* and *MEIS1* and forced expression of

**Figure 6.**

The binding between Meis1 and Pbx3 is critical for their synergistic effects on cell transformation and upregulation of homeobox genes. A, *in vitro* colony-forming/replating assays. Briefly, mouse normal bone marrow progenitor (lineage negative; Lin⁻) cells collected from 4- to 6-week-old B6.SJL (CD45.1) mice were retrovirally cotransduced with MSCVneo+MSCV-PIG (control), MSCVneo-MLL-AF9+MSCV-PIG (MLL-AF9), MSCVneo-HOXA9+MSCV-PIG-MEIS1 (HOXA9+MEIS1), MSCVneo-MEIS1+MSCV-PIG-PBX3 (PBX3+MEIS1), MSCVneo-Meis1+MSCV-PIG-Pbx3 (Pbx3+Meis1), MSCVneo-Meis1-M2ΔLRF/ΔLLEL+MSCV-PIG-Pbx3 (Pbx3+Meis1-M2ΔLRF/ΔLLEL), or MSCVneo-Meis1-Δ64-202+MSCV-PIG-Pbx3 (Pbx3+Meis1-Δ64-202). The colony cells were replated every 7 days for up to four passages and colony numbers were counted for each passage. Mean ± SD values of colony counts are shown (left). The domain structure of the wild-type and mutant Meis1 proteins are also shown (right). B and C, qPCR (B) and Western blotting (C) analyses of expression levels of a series of representative Homeobox genes in colony cells from the first passage of four groups (samples generated from colony-forming/replating assays shown in Fig. 6A). Mean ± SD values are shown in Fig. 6B.

MLL-AF9. Notably, forced expression of HOXA9 alone or together with MEIS1 or PBX3 cannot upregulate expression of most of those genes at all. Indeed, consistent with our observation, previous studies also showed that forced expression of *Hoxa9* and *Meis1* each alone or together cannot significantly upregulate expression of endogenous *Pbx3* and *Hoxa* genes (25, 48). In addition, more than 210 genes are significantly repressed by both MLL-fusion proteins and the PBX3/MEIS1 combination; although these genes are also repressed by HOXA9 alone or together with MEIS1 or PBX3, the repression degrees are much more moderate. Therefore, despite the close relationship between HOXA9, MEIS1, and PBX3 (18, 24–27), only the PBX3/MEIS1 combination, but not the HOXA9/MEIS1 or HOXA9/PBX3 combination (or HOXA9 alone), can resemble MLL-fusion proteins in inducing critical transcriptional programs. Further systematic studies are war-

ranted in the future to decipher the underlying molecular mechanism(s). It is possible that a certain threshold of abundance of both PBX3 and MEIS1 is required for them to initiate their cooperation in inducing expression of endogenous HOXA genes, and the induced HOXA proteins can further cooperate with PBX3 and MEIS1 and enhance their downstream transcriptional programs, which in turn leads to the full establishment of the core transcriptome of MLL-rearranged AML. In contrast, because forced expression of HOXA9 alone or together with MEIS1 or PBX3 cannot achieve sufficiently high abundance of both PBX3 and MEIS1, and thereby cannot induce the core transcriptome of MLL-rearranged AML.

Besides MLL-rearranged AML, AMLs with *NPM1* mutations or *NUP98-HOXA9* fusion, which together account for more than 30% of total AML cases, are also featured with aberrant

overexpression of a set of homeobox genes, including the HOXA/MEIS1/PBX3 genes (40–43, 49). No surprise, gene sets upregulated in AML with *NPM1* mutations or *NUP98-HOXA9* fusion are significantly enriched in genes highly expressed in both *PBX3/MEIS1* and *MLL-AF9* AML cells. Thus, it is highly likely that the cooperation between PBX3 and MEIS1 is critical for the establishment of the core transcriptomes of a wide range of AMLs, not limited to *MLL*-rearranged AML.

Furthermore, our current study together with previously studies (18, 24, 25) suggest that the binding between MEIS1 and PBX proteins is critical for the biologic functions of the PBX3/MEIS1 combination and MLL-fusion proteins in cell immortalization, leukemogenesis, and regulation of transcriptional programs. Therefore, targeting the binding between MEIS1 and PBX3 may represent a promising therapeutic strategy to cure leukemia with aberrant overexpression of homeobox genes, such as AMLs with *MLL*-rearrangements, *NPM1* mutations, or *NUP98-HOXA9* fusion. We propose that small-molecule compounds or small peptides can be developed to specifically inhibit the binding between MEIS1 and PBX3 and they can be applied in clinic, alone or together with other therapeutic agents, to treat leukemia.

In summary, we demonstrate that forced expression of PBX3 and MEIS1 is sufficient to induce cell transformation and leukemogenesis, and to resemble MLL-fusion proteins in inducing the core transcriptional programs, especially the upregulation of a set of homeobox genes. The biologic functions of the PBX3/MEIS1 combination are dependent on their binding ability. Thus, our finding is of great significance as it shows that two previously assumed "supporting actors" (i.e., PBX3 and MEIS1) highly likely act as "leading actors" in the establishment of the core transcriptome of AML with *MLL* rearrangements, and probably also in that of AML with *NPM1* mutations or *NUP98-HOXA9* fusion. Targeting the binding between PBX3 and MEIS1 represents an attractive

therapeutic strategy to treat a wide-range of AML with aberrant overexpression of homeobox genes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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