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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 co-expression 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 co-expression 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 expression

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AUTHOR CONTRIBUTIONS

Z.L. and J.C. conceived the project and designed the research. Z.L., P.C., R.S., C.H., Y.L., A.G.E., Z.Z., S.G., S.A., H.W., Y.W., S.L., H.H., M.B.N., X.J. and J.C. performed experiments and/or data analyses; Z.L., A.G.E., H.H., G.G.W., X.J., P.P.L., J.J. and J.C. contributed reagents/analytic tools and/or grant support; Z.L. and J.C. wrote the paper. All authors discussed the results and commented on the manuscript.

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.

Keywords

PBX3; MEIS1; HOXA; homeobox gene; *MLL*-rearranged leukemia; core transcriptome; gene expression profiles; cell transformation; leukemogenesis

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 5-10% of patients with acute myeloid leukemia (AML) and 7-10% of patients with acute lymphoblastic leukemia (ALL), ~80% of infant acute leukemia, and the majority of patients with therapy-related AML/ALL secondary to therapy that targets topoisomerase II (like etoposide) (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 up-regulated 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, 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) (16). We showed further that *PBX3*, rather than *PBX1* or *PBX2*, is an important cofactor of *HOXA9* in cytogenetically abnormal AML and their co-expression 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 co-factors of *HOXA9* and co-expression 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 (HSPCs) (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, co-expression 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 up-regulation 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* is a kind gift from Dr. Scott Armstrong (29). MSCV-PIG vector containing a PGK-puromycin-IRES-GFP (PIG) cassette was kindly provided by Drs. Hannon and He (30). *MEIS1* coding region sequence was PCR amplified from human normal BM mononuclear cells with primers, forward 5'- ATAGAATTCATGGCGCAAAGGTAC-3', and reverse 5'-GGCCTCGAGTAGATGAAGTTACA-3', was then cloned into MSCVneo (Clontech, Mountain View, CA) as MSCVneo-*MEIS1*, or cloned into MSCV-PIG as MSCV-PIG-*MEIS1*. Mouse *Pbx3* coding region sequence was PCR amplified from mouse normal BM mononuclear cells with primers, forward 5'-AATAGATCTACCACCATGGACGATCAATCCAGGATG-3', and reverse 5'-ACTCTCGAGTTAGTTAGAG GTATCCGAGTGC-3', 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*-M2 LRF/ LLEL, and MSCVpuro-VP16-*Meis1*- 64-202, which were kindly provided by Dr. Kamps (24, 25), and then subcloned into MSCVneo, as MSCVneo-*Meis1*, MSCVneo-*Meis1*-M2 LRF/ LLEL, and MSCVneo-*Meis1*- 64-202, respectively. All inserts have been confirmed by Sanger sequencing.

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

For primary BMT assays, mouse BM progenitor cells from 4- to 6-week-old wild-type (C57BL/6J CD45.2) mice were co-transduced with different retroviral combinations, and were then cultured in methylcellulose 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 BM cells (1×10^6 ; freshly harvested from a B6.SJL mouse) per recipient mouse.

For secondary BMT assays, primary leukemic BM 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 BM 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 BM cells (from a B6.SJL mouse) per recipient mouse. Leukemic BM cells from two independent primary leukemic mice were used as the donors for the secondary BMT of each group, with 5-6 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 BM cells (CD45.2) per recipient mouse.

Affymetrix gene arrays of mouse samples

A total of 20 mouse BM 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⁺ BM cells (i.e., transplanted donor cells) were sorted with flow cytometry from whole BM cells collected from BMT recipients at the end point. 1 μ g total RNA was used for each sample. The QC 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 (RMA) (31), after hybridization and background correction according to the standard protocols. All genes with a standard deviation of expression values over 0.3 across the 20 samples were used for the unsupervised hierarchical clustering and followed statistic analyses. The microarray data has 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) (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) (32) were used as the gene set input for GSEA. The Database for Annotation, Visualization and Integrated Discovery (DAVID; v6.7) (34, 35) was used to analyze gene sets enriched in genes significantly differentially expressed between different groups of samples.

RNA extraction, quantitative RT-PCR (qPCR), flow cytometry, cytospin, histological hematoxylin and eosin (H&E) 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 (Dallas, TX). Anti-Hoxa5 antibody

(ab140636) was purchased from Abcam (Cambridge, MA). Anti-Hoxa4 antibody (PA1603) was purchased from Boster Biological Technology (Pleasanton, CA). Anti- β -Actin antibody (#3700) was purchased from Cell Signaling Technology, LTD (Danvers, MA).

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, St. Louis, MI), 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 previously (36-38). Continued testing to authenticate this cell line was done using qPCR and Western blot 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 Bio-Sciences, Pittsburgh, PA), and transfected into MonoMac-6 cells Amaxa® Nucleofector® Technology (Amaxa Biosystems, Berlin, Germany) at a concentration of 100 nM as described previously (16, 36-38).

RESULTS

Co-expression of *PBX3* and *MEIS1* is sufficient to transform normal HSPCs

Based on previous studies from us and others showing crucial roles of both *PBX3* and *MEIS1* in AML (16, 24-28), we query whether co-expression 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 Figure 1A, *PBX3/MEIS1* co-expression (*PBX3+MEIS1*) was sufficient to transform and immortalize normal mouse bone marrow (BM) progenitor cells, although with relatively fewer colonies compared to co-expression 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 BM progenitor cells (Figure 1A).

Co-expression of *PBX3* and *MEIS1* induces rapid AML in mice

To determine whether co-expression of *PBX3/MEIS1* is also sufficient to induce AML in mice, we conducted *in vivo* mouse BM transplantation (BMT) assays. As shown in Figure 1B and Supplemental Figure 1, similar to the co-expression of *HOXA9* with *MEIS1* or *PBX3*, co-expression of *PBX3* and *MEIS1* also caused a rapid AML in mice. The leukemic latency of the *PBX3+MEIS1* group was comparable to 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 (Figure 1B). Notably, mice of the *PBX3+MEIS1* group exhibited similar, if not more aggressive,

leukemic phenotypes in peripheral blood (PB), BM, spleen and liver tissues compared to the *HOXA9+MEIS1*, *HOXA9+PBX3* and *MLL-AF9* groups (Figure 1C). Our flow cytometry analysis showed that leukemic BM 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 to those of the other AML groups (Supplemental Figure 1).

PBX3/MEIS1-induced AML is transplantable

To determine whether PBX3/MEIS1-induced AML is transplantable, we conducted secondary transplantation assays. First, we collected leukemic BM 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 BM 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 (Figures 2A and B). The two independent donors of each group caused full-blown leukemia with similar latency in secondary recipients (Supplemental Figure 2). 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 (Figure 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 BM 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 Figure 3A). Thus, there are three separate groups, including Group 1/G1 (normal controls; NC); Group 2/G2 (*HOXA9+MEIS1*, *HOXA9+PBX3* and *HOXA9*) and Group 3/G3 (*MLL-AF9* and *PBX3+MEIS1*). We then conducted gene set enrichment analysis (GSEA) (32). No surprise, as both Groups 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 up-regulated by *HOXA9* and *MEIS1*, and genes related to DNA replication, mRNA processing, microRNA 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 (Figure 3B and Supplemental Figure 3A). In contrast, target genes down-regulated by *HOXA9* and *MEIS1*, genes down-regulated in leukemic stem cells or hematopoietic stem cells, genes related to hematopoiesis, hematopoietic cell lineage, apoptosis or p53 signaling pathway, and genes up-regulated in myeloid cell development or in hematopoietic mature cells are enriched in genes expressed at a higher level in Group 1 samples than in Groups 2 and 3 samples (Figure 3B and Supplemental Figure 3A).

On the other hand, Groups 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 with *NPM1* mutations, and target genes up-regulated by *NUP98-HOXA9* fusion gene, are enriched in genes expressed at a higher level in Group 3 samples than in Group 2 samples (Figure 3C and Supplemental Figure 3B). 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 down-regulated 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 (Figure 3C and Supplemental Figure 3B). 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 up-regulated 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 Supplemental Figure 4), 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 discover rate (FDR) <0.01) between the three groups of samples (Supplemental Figure 5A). Through Significance Analysis of Microarrays (SAM) (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 to both Groups 1 and 2 (Figure 4; Supplemental Figures 5B and 6). 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 to both Groups 1 and 3 (Supplemental Figures 5B, 7 and 8). 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 to both Groups 2 and 3 (Supplemental Figure 5B; Supplemental Tables 1 and 2).

Through searching for the Database for Annotation, Visualization and Integrated Discovery (DAVID; v6.7) (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 Supplemental Figure 5C). Gene sets associated with ‘transferase activity’, ‘G-protein modulator’, ‘lysosome’ and ‘cell surface’ are significantly enriched in genes highly expressed in Group 2 (Supplemental Figure 5C). 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 (Supplemental Figure 5C). Consistent with our GSEA analysis results (Figure 3 B and Supplemental Figure 3A), genes associated with cell death/apoptosis and hematopoietic cell differentiation are significantly down-regulated in both Groups 2 and 3 AML samples relative to normal control (Group 1) samples (see Supplemental Figure 9).

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 (Supplemental Figure 5C); 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 Supplemental Figures 10).

Notably, 22 (i.e., *Ash2l*, *Baz2b*, *Chd9*, *Dcun1d1*, *Erb2ip*, *Eya1*, *Hoxa2*, *Hoxa3*, *Hoxa4*, *Hoxa5*, *Hoxa6*, *Hoxa7*, *Hoxa9*, *Hoxa10*, *Ikzf2*, *Meis1*, *Pbx3*, *Rnf220*, *Runx2*, *Sgk3*, *Tnpo1* and *Tsc22d2*; see Figure 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 (Supplemental Table 3). In contrast, none of the 211 genes under-expressed in Group 3 and none of the 1,190 genes highly expressed in Group 1 is potential direct target of MLL-fusions (Supplemental Table 3). These data indicate that MLL-fusion proteins often up-regulate, rather than down-regulate, 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 Figure 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 (Supplemental Figure 11). 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 down-regulation 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 down-regulation of expression of *HOXA* genes (Figure 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 up-regulation 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 biological effects caused by co-expression 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*-M2 LRF/ LLEL and *Meis1*- 64-202) based on the constructs reported previously (24, 25). We then conducted colony-forming/replating assays with mouse BM progenitor cells retrovirally transduced with *Pbx3*+*Meis1* (wild-type), *Pbx3*+*Meis1*-M2 LRF/ LLEL, *Pbx3*+*Meis1*- 64-202, along with *PBX3*+*MEIS1*, *HOXA9*+*MEIS1*, *MLL-AF9*, and control vectors. As shown in Figure 6A, co-expression of mouse *Pbx3* and *Meis1* genes had a similar effect to co-expression of human *PBX3* and *MEIS1* genes on cell transformation/immortalization, with more than 200 colonies per dish after series of replating. In contrast, co-expression of *Pbx3* with mutated *Meis1* (*Meis1*-M2 LRF/ LLEL or *Meis1*- 64-202) lost the capacity to form a significant number of colonies after series of replating (Figure 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, co-expression of *Pbx3* with *Meis1* mutants (*Meis1*-M2 LRF/ LLEL or *Meis1*- 64-202) could not up-regulate expression of endogenous *Hoxa* genes to a level as high as that induced by co-expression of *Pbx3* with wild-type *Meis1*, a phenomenon verified by immunoblotting (Figures 6B and C). Thus, disruption of the binding between Meis1 and Pbx3 also substantially abrogates their synergistic effect on up-regulation 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 co-expression of *PBX3* and *MEIS1*, not co-expression of *HOXA9* and *MEIS1* (or *HOXA9* and *PBX3*), that can recapitulate the core transcriptome of *MLL*-rearranged AML, especially the significant up-regulation 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 up-regulated by both co-expression of *PBX3* and *MEIS1* and forced expression of *MLL-AF9*. Notably, forced expression of *HOXA9* alone or together with *MEIS1* or *PBX3* cannot up-regulate 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 up-regulate expression of endogenous *Pbx3* and *Hoxa* genes (25, 48). In

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AML	acute myeloid leukemia
BMT	bone marrow transplantation
HSPC	hematopoietic stem/progenitor cell
GSEA	Gene Set Enrichment Analysis
KEGG	Kyoto Encyclopedia of Genes and Genomes Pathway Database
DAVID	The Database for Annotation, Visualization and Integrated Discovery

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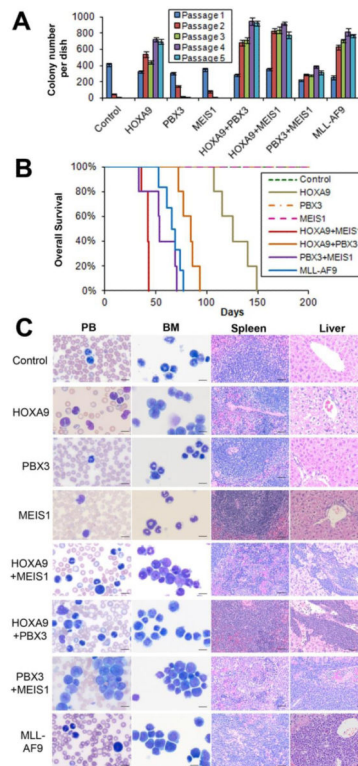


Figure 1. Co-expression of *PBX3* and *MEIS1* can transform normal mouse bone marrow (BM) progenitor cells *in vitro* and induce rapid AML *in vivo*

(A) *In vitro* colony-forming/replating assays. Briefly, mouse normal BM progenitor (lineage negative; Lin⁻) cells collected from 4- to 6-week-old B6.SJL (CD45.1) mice were retrovirally co-transduced 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±SD values of colony counts are shown. (B) Mouse BM transplantation (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 8 groups. Peripheral blood (PB) and BM cells were stained with Wright-Giemsa. The spleen and liver tissues were stained with hematoxylin and eosin (H&E). The length of bars represents 10 μm for PB and BM, and 100 μm for spleen and liver.

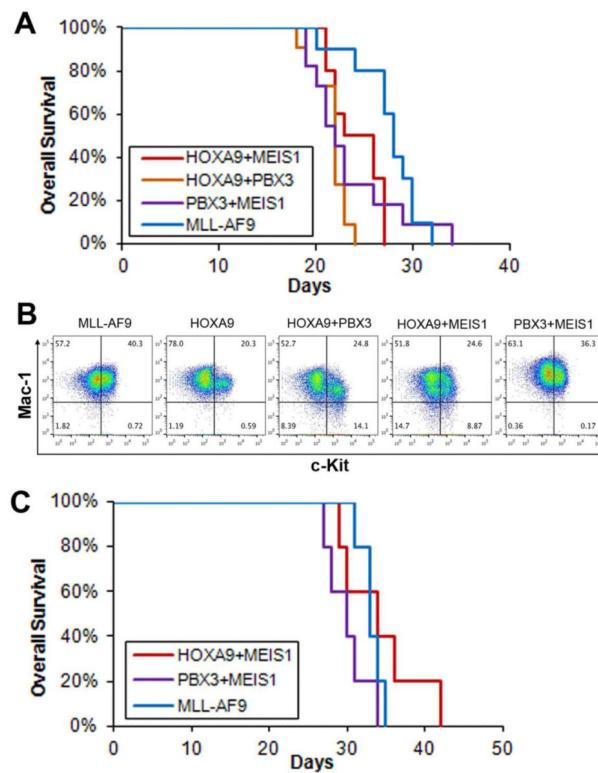


Figure 2. PBX3/MEIS1-induced AML is transmissible in secondary transplantation recipients (A) Kaplan-Meier survival curves of secondary transplantation recipient (CD45.2+) mice transplanted with primary leukemic BM cells (CD45.1+) of the *HOXA9+MEIS1* (recipient mouse number: n=10), *HOXA9+PBX3* (n=11), *PBX3+MEIS1* (n=11) and *MLL-AF9* (n=10) groups. Primary AML BM 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 BM cells from the above secondary BMT recipient mice. Antibodies against Mac-1 and c-Kit were used. Flow data of leukemic BM samples from one recipient mouse is 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.

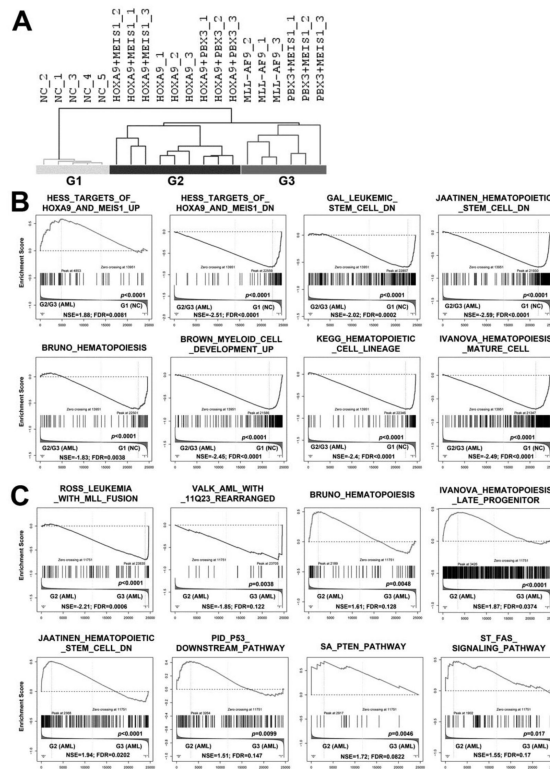


Figure 3. Comparison of gene expression profiles between different groups of leukemic or normal control cells

(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; FDR, false discovery rate.

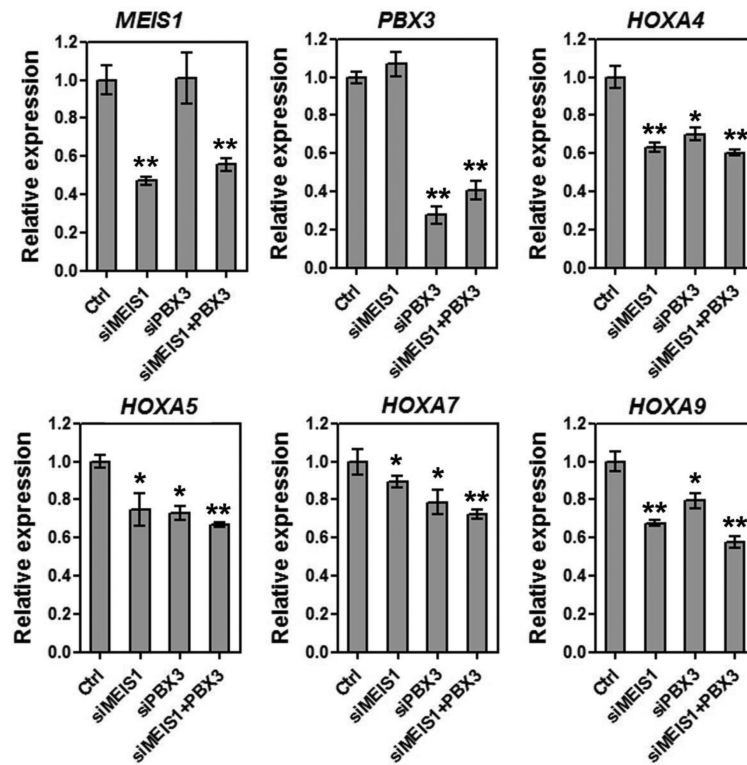


Figure 5. Effects of knockdown of *PBX3* and/or *MEIS1* on the expression of endogenous *HOXA* genes in *MLL*-rearranged AML cells

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 post transfection of anti-*MEIS1* siRNA oligos (si*MEIS1*) and/or anti-*PBX3* siRNA oligos (si*PBX3*), or of scrambled control oligos (Ctrl). Mean±SD values are shown. *, $p < 0.05$; **, $p < 0.01$.

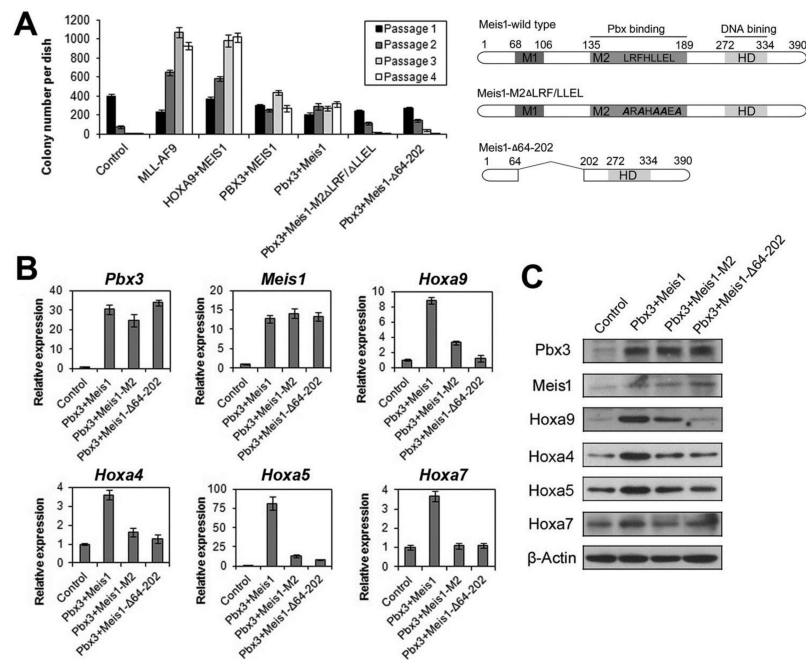


Figure 6. The binding between Meis1 and Pbx3 is critical for their synergistic effects on cell transformation and up-regulation of homeobox genes

(A) *In vitro* colony-forming/replating assays. Briefly, mouse normal BM progenitor (lineage negative; Lin⁻) cells collected from 4- to 6-week-old B6.SJL (CD45.1) mice were retrovirally co-transduced 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*-M2 LRF/ LLEL+MSCV-PIG-*Pbx3* (*Pbx3+Meis1-M2 LRF/ LLEL*) or MSCVneo-*Meis1*-64-202+MSCV-PIG-*Pbx3* (*Pbx3+ Meis1-64-202*). The colony cells were replated every 7 days for up to 4 passages and colony numbers were counted for each passage. Mean±SD values of colony counts are shown (left panel). The domain structure of the wild-type and mutant Meis1 proteins are also shown (right panel). (B,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 Figure 6A). Mean±SD values are shown in Figure 6B.