miR-495 is a tumor-suppressor microRNA down-regulated in MLL-rearranged leukemia

Xi Jiang1,2, Hao Huang1,2, Zejuan Li1, Chunjiang He1, Yuanyuan Li3, Ping Chen4, Sandeep Gurbuxanib, Stephen Arnovitz2, Gia-Ming Hong4, Colles Price6, Haomin Ren5, Rejani B. Kunjammaa, Mary Beth Neillya, Justin Salata, Mark Wunderlichc, Robert K. Slanyd, Yanming Zhange, Richard A. Larsona, Michelle M. Le Beaua, James C. Mulloyc, Hematology and Cancer Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229; bDepartment of Genetics, University of Erlangen, 91058 Erlangen, Germany; and cDepartment of Pathology, Northwestern University, Chicago, IL 60611

Contributed by Janet D. Rowley, October 8, 2012 (sent for review July 27, 2012)

Acute myeloid leukemia (AML) is a heterogeneous group of hematopoietic malignancies with variable response to treatment. AMLs bearing MLL (mixed lineage leukemia) rearrangements are associated with intermediate or poor survival. MicroRNAs (miRNAs), a class of small noncoding RNAs, have been postulated to be important gene expression regulators virtually in all biological processes, including leukemogenesis. Through a large-scale, genome-wide miRNA expression profiling assay of 85 human AML and 15 normal control samples, we show that among 48 miRNAs that are significantly differentially expressed between MLL- and non–MLL-rearranged AML samples, only one (miR-495) is expressed at a lower level in MLL-rearranged AML than in non–MLL-rearranged AML; meanwhile, miR-495 is also significantly down-regulated in MLL-rearranged AML samples compared with normal control samples. Through in vitro colony-forming/replating assays and in vivo bone marrow transplantation studies, we show that forced expression of miR-495 significantly inhibits MLL-fusion-mediated cell transformation in vitro and leukemogenesis in vivo. In human leukemic cells carrying MLL rearrangements, ectopic expression of miR-495 greatly inhibits cell viability and increases cell apoptosis. Furthermore, our studies demonstrate that PBX3 and MEIS1 are two direct target genes of miR-495, and forced expression of either of them can reverse the effects of miR-495 overexpression on inhibiting cell viability and promoting apoptosis of human MLL-rearranged leukemic cells. Thus, our data indicate that miR-495 likely functions as a tumor suppressor in AML with MLL rearrangements by targeting essential leukemia-related genes.

Leukemia arises as a result of genetic lesions that cause uncontrolled proliferation in cells of the hematopoietic lineage (1, 2). Chromosome translocations are frequently observed in both acute myeloid leukemia (AML) (2–4) and other hematologic malignancies. The MLL (mixed lineage leukemia; HRX, ALL-1, Hixr) gene, located at 11q23, is frequently involved in chromosome translocations with more than 60 different partner genes (5–8). The critical feature of these chromosomal rearrangements is the generation of an in-frame fusion transcript consisting of 5′ MLL and 3′ sequences of the gene on the partner chromosome (7, 8). MLL-rearranged leukemia occurs in approximately 10% of patients with de novo or treatment-related acute leukemia (9–11). MLL-rearranged leukemia is classified as a disease with an intermediate or poor risk of prognosis (1, 12).

The most well-studied downstream target genes of MLL fusion proteins are HOX9A9 and MEIS1 (13–15), and their aberrant overexpression has been shown to be required for the induction and maintenance of MLL-rearranged leukemia (16–21). HOX proteins can form heterodimers or heterotrimers with members of the three-amino-acid loop extension family of cofactors, including PBX and MEIS proteins, to regulate the transcription of multiple downstream targets directly (22–24). Similar to HOX9A9 and MEIS1, overexpression of PBX3 has also been frequently observed in various subtypes of AML with unfavorable prognosis, particularly in MLL-rearranged leukemia (13, 18, 25–28). Our recent study showed that PBX3 also functions as an oncogene in MLL-rearranged leukemia (29). Thus, both MEIS1 and PBX3 likely function as essential cofactors of HOX9A9 and play critical oncogenic roles in the pathogenesis of MLL-rearranged leukemia.

MicroRNAs (miRNAs) are a class of small, noncoding RNAs that are important for posttranscriptional gene regulation in both health and disease (30, 31). Although aberrant expression of many miRNAs has been observed in various subtypes of AML (8, 29, 32–40), the biological functions of most of them in leukemogenesis have not been characterized.

In the present study, we show first that a miRNA, namely miR-495, is the only miRNA that is significantly down-regulated in MLL-rearranged AML compared with both non–MLL-rearranged AML and normal controls. We then used both in vitro and in vivo models to study the biological function of miR-495 and to identify its critical target genes in MLL-rearranged leukemia.

Results

Expression of miR-495 Is Significantly Down-Regulated in MLL-Rearranged AML

We and others previously showed that a number of miRNAs, such as miR-196b and the miR-17-92 cluster, were aberrantly overexpressed in MLL-rearranged AML compared with normal controls, and these miRNAs likely play important oncogenic roles in MLL-fusion-mediated cell transformation and leukemogenesis (32, 39, 41, 42). To identify potential tumor-suppressor miRNAs in MLL-rearranged AML, we performed a large-scale, genome-wide miRNA expression profiling assay of 85 AML (including 10 MLL- and 75 non–MLL-rearranged AML) and 15 normal control bone marrow (BM) [including 6 CD34+ hematopoietic stem/progenitor cell, 5 CD34+ myeloid progenitor cell, and 4 mononuclear cell (MNC)] samples by use of Exiqon miRNA arrays (Methods).

Using significance analysis of microarrays (SAM) (43), we identified 48 miRNAs that were significantly differentially expressed between the MLL-rearranged (n = 10) and non–MLL-rearranged (n = 75) AML samples; very strikingly, 47 of them (including miR-196b and the individual miRNAs of the miR-17-92 cluster) had a significantly higher expression level, whereas only one (miR-495) had a significantly lower expression level in MLL-rearranged AML than in non–MLL-rearranged AML (q < 0.05, false discover rate [FDR] < 0.05; Fig. L1 and Fig. S1). We next compared miRNA expression between MLL-rearranged AML samples.


The authors declare no conflict of interest.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession nos. GSE30258 and GSE43185).

1X.J., H.H., and Z.L. contributed equally to this work.
2To whom correspondence may be addressed. E-mail: jrowley@bsd.uchicago.edu or jchen@bsd.uchicago.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1217519109/-/DCSupplemental.
AML and normal controls and found that 44 miRNAs (including miR-196b and the individual miRNAs of the miR-17-92 cluster) had a significantly higher expression level, whereas 32 miRNAs (including miR-495) had a significantly lower level of expression in MLL-rearranged AML than in the normal controls (q < 0.05, FDR < 0.05; Fig. 1A and Fig. S2). Thus, miR-495 is the only miRNA that is expressed at a significantly lower level in MLL-rearranged AML compared with both non-MLL-rearranged AML and normal controls.

miR-495 Is Down-Regulated by MLL-Fusion Proteins. To determine whether MLL fusions directly regulate the level of miR-495, we retrovirally transduced MLL-AF9, a fusion gene resulting from a common translocation between chromosome 9 and 11 (44), into human normal CD34+ cord blood progenitor cells (45) and observed that the expression level of miR-495 was significantly (P < 0.05) down-regulated by forced expression of MLL-AF9 (Fig. 1B). Similarly, a significant down-regulation (P < 0.05) of miR-495 was also observed in mouse normal BM progenitor cells after retroviral transduction of MSCVneo-MLL-AF9 or MSCVneo-MLL-ENL (Fig. 1C). We also used the MLL-ENL-ERTm cell line, a cell line stably expressing the conditional MLL-ENL derivative (13, 46), to investigate the dependence of miR-495 expression on the presence of MLL fusion proteins further. As shown in Fig. 1D, after withdrawal of 4-hydroxytamoxifen (4-OHT) for 7–10 d, miR-495 expression was significantly increased (P < 0.05), along with a diminishing of MLL-ENL expression.

miR-495 Functions as a Tumor-Suppressor miRNA in Vitro. To investigate the biological function of miR-495, we performed a colony-forming/replating assay. Normal mouse BM progenitor cells were transduced with MSCV-PIG [empty vector bearing a phosphoglycerate kinase (PGK)-puromycin/internal ribosome entry site (IRES)-GFP cassette (47) as a negative control] or MSCV-PIG-miR-495, together with MSCVneo or MSCVneo-MLL-AF9, and were then plated onto methylcellulose medium. The colonies were replated every 7 d under the same conditions. As shown in Fig. 2A, forced expression of miR-495 significantly inhibited (P < 0.05) the colony-forming capacity induced by MLL-AF9 after replating (i.e., in the second and third rounds of plating). Notably, forced expression of miR-495 substantially promoted cell differentiation as shown by cytospin analysis (Fig. 2B). We next investigated the biological function of miR-495 in human MLL-rearranged leukemia cells. As shown in Fig. 2C, overexpression of miR-495 consistently inhibited the growth/proliferation of MONOMAC-6 (∼0.1, 11) and THP-1 (∼0.1, 11) cells from day 3 after transfection. Forced expression of miR-495 also significantly inhibited cell viability and promoted apoptosis in MONOMAC-6 and THP-1 cells (Fig. 2D). These results indicate that miR-495 has an inhibitory effect on leukemia cell growth in vitro.

miR-495 Inhibits MLL-Fusion-Mediated Leukemogenesis in Vivo. To assess the in vivo function of miR-495 in leukemogenesis, we retrovirally cotransduced MSCV-PIG or MSCV-PIG-miR-495 together with MSCVneo-MLL-AF9 or MSCVneo-MLL-ENL into mouse BM progenitor cells isolated from B6.SJL donor mice (CD45.1; the mice were killed 5 d after 5-fluorouracil treatment) and then plated onto methylcellulose medium. After 7 d of selection of double-transduction-positive cells with puromycin and G418, we collected and washed the colony cells and then transplanted the cells into lethally irradiated C57BL/6 recipient mice. We found that forced expression of miR-495 significantly delayed leukemogenesis mediated by MLL-AF9 (median overall survival, 67 d vs. 52 d; P = 0.002) (Fig. 3A). Forced expression of miR-495 remarkably reduced the proportion of immature blast cells in both peripheral blood and BM (Fig. 3B). These findings suggest that miR-495 does play a tumor-suppressor role in MLL-rearranged leukemia.

Identification of Potential Target Genes of miR-495. To identify potential target genes of miR-495, we have also performed (protein-coding) gene expression profiling of 79 of the above 100 human samples, including 70 AML (composed of 9 MLL- and 61 non-MLL-rearranged AML cases) and 9 normal controls (composed of 3 CD34+, 2 CD33+, and 4 MNC samples) by use of an Agilent custom-design microarray platform (Methods). Through correlation of expression of miR-495 with that of genes across the 79 samples, we found that 471 genes exhibited a significantly inverse correlation of expression with miR-495 (r < −0.2, P < 0.05, Pearson correlation). Of the 471 genes, 128 are predicted potential target genes of miR-495 in both human and mouse genomes (Table S1). Through Gene Ontology analysis, we found that the candidate target genes are significantly enriched in biological process categories such as “response to stimulus,” “signal transduction,” “cell activation,” “chromatin modification,” and “hemopoietic or lymphoid organ development” (Fig. S3).

Because we are interested in MLL-rearranged AML, we next narrowed down the number of candidate target genes of miR-495 from 128 to 24 (i.e., ACTRIA, ANGELI, BAK1, BMI1, CI00RF140, CAB39L, CRADD, GPR126, HOXA10, KPNB1, MEIS1, MRRN1, MR1, MYO6, PBX3, PTP4A3, REEP3, RN2F, SENP6, SET, SPRYD4, SYT17, TEMEM97, and UBEZ2). Because these genes are expressed at a significantly higher level in the 9 human MLL-rearranged leukemia samples compared with the 9 normal control samples and the 61 non-MLL-rearranged AML samples, in an inverse relationship to miR-495 (Fig. 4A). Furthermore, in the analysis of Affymetrix gene arrays of 9 MLL-AF9–mediated mouse leukemia samples and 6 control samples (39), we observed that 7 (i.e., Bmi1, Hoxa10, Meis1, Pbx3, Rnf2,
Both PBX3 and MEIS1 Are Direct Targets of miR-495. As shown in Fig. 4B, PBX3 and MEIS1 are the two potential target genes of miR-495 that exhibited the most significant up-regulation in MLL-AF9 leukemic BM cells relative to the normal control BM cells. Moreover, these two genes have proven to be broadly involved in multiple processes of hematopoiesis and leukemogenesis, as cofactors of HOX genes (13, 16, 18, 22, 23, 49, 50). Therefore, we sought to determine whether PBX3 and MEIS1 are genuine direct target genes of miR-495. As expected, we observed that forced expression of miR-495 significantly reduced (P < 0.05) the endogenous expression of PBX3 and MEIS1 in human MLL-rearranged leukemic cell lines, including MONOMAC-6, KOC-48/t (4, 11), and THP-1 (Fig. 5A). Similarly, MLL-AF9 transduction resulted in a six- to eightfold increase in endogenous expression of PBX3 and MEIS1 in mouse BM progenitor cells, whereas coexpression of miR-495 reduced their levels to approximately 50% (Fig. 5B). Finally, our luciferase reporter and mutagenesis assays showed that miR-495 targeted the 3’ UTR of both PBX3 and MEIS1 directly (Fig. 5C and D). Thus, several lines of evidence indicate that both PBX3 and MEIS1 are direct target genes of miR-495.

Forced Expression of Either PBX3 or MEIS1 Can Reverse the Effects of miR-495 in Human MLL-Rearranged Leukemic Cells. We then investigated whether PBX3 and MEIS1 are truly functionally important target genes of miR-495 in MLL-rearranged leukemic cells. As shown in Fig. 6A, forced expression of PBX3 alone could significantly (P < 0.05) increase cell viability and promote cell growth/proliferation, whereas decreasing apoptosis of MONOMAC-6 cells, in a manner opposite to miR-495; forced expression of MEIS1 alone could also significantly promote cell growth/proliferation, although its forced expression showed no significant effect on cell viability and apoptosis (Fig. 6). More importantly, cotransfection of PBX3 or MEIS1 with miR-495 could completely reverse the effects of miR-495 on cell viability, apoptosis, and cell growth in MONOMAC-6 cells (Fig. 6). A similar phenomenon was also observed in THP-1, a different human leukemia cell line with MLL rearrangements (Fig. 5F). Therefore, our data suggest that both PBX3 and MEIS1 are functionally important target genes of miR-495 in MLL-rearranged leukemic cells.

Discussion

In the present study, we identified only one miRNA (miR-495) that was expressed at a significantly lower level in human MLL-rearranged AML (n = 10) than in non–MLL-rearranged AML (n = 75); in contrast, 47 miRNAs were expressed at a significantly higher level in the former than in the latter (Fig. 1). This observation is in accordance with the notion that MLL fusion proteins usually promote expression of downstream target genes at the transcriptional level (13–15, 32, 39, 41, 42). Compared with normal hematopoietic cell controls, miR-495 (along with 31 other miRNAs) is significantly down-regulated in MLL-rearranged AML (Fig. 2). We then showed that ectopic expression of MLL fusion genes in both human and mouse normal hematopoietic stem/progenitor cells could significantly down-regulate endogenous expression of miR-495 and that the depletion of MLL fusions resulted in the up-regulation of miR-495 (Fig. 1). Thus, our data suggest that there is an MLL-fusion-mediated negative regulation of the production of miR-495 in hematopoietic cells.

Thus far, although the research on the role of miR-495 in cancer is still in its infancy, miR-495 has been implicated in both oncogenic (in breast cancer) and tumor suppressor (in gastric cancer) roles in solid tumors (51, 52). Here we show that miR-495 functions as a critical tumor suppressor in MLL-rearranged leukemia. First of all, we demonstrated that forced expression of miR-495 could significantly inhibit colony-forming capacity of mouse normal BM progenitor cells mediated by MLL fusions (Fig. 2A) and induce cell differentiation (Fig. 2B). In human
leukemic cells with MLL rearrangements (e.g., MONOMAC-6 and THP-1 cells), we found that ectopic expression of miR-495 could significantly inhibit cell growth/proliferation (Fig. 2C) and increase apoptosis while decreasing cell viability (Fig. 2D). Furthermore, we also performed mouse BM transplantation assays and showed that forced expression of miR-495 could significantly inhibit MLL-AF9-mediated leukemogenesis in transplanted mice (Fig. 3).

Through a series of correlation analyses, we identified 24 candidate target genes of miR-495 that exhibited a significantly inverse correlation of expression with miR-495 in 79 human samples (including 9 MLL-rearranged and 61 non-MLL-rearranged AML samples, along with 9 normal control samples) and were expressed at a significantly higher level in the MLL-rearranged AML samples compared with both the non-MLL-rearranged AML and normal control samples, in a manner completely opposite to miR-495 (Fig. 4A). Of the 24 candidate target genes, 7 (including Bmi1, Hoxa10, Meis1, Phx3, Rnf2, Set, and Spry4) were also significantly over-expressed in MLL-AF9 mouse leukemia samples relative to normal controls (Fig. 4B). Previous studies from us and others indicated that both MEIS1 and PBX3 are important downstream target genes of MLL fusion proteins and play a critical oncogenic role (likely through cooperating with HOXA4 genes, e.g., HOXA9) in the development and maintenance of MLL-rearranged leukemia (13, 14, 16, 17, 21, 29). Thus, we focused on MEIS1 and PBX3 for further studies. We showed that forced expression of miR-495 could significantly repress endogenous expression of MEIS1 and PBX3 (Fig. 5A and B), and our luciferase reporter/mutagenesis assays confirmed that both genes are genuine direct targets of miR-495 (Fig. 5C and D). Furthermore, we showed that coexpressing MEIS1 or PBX3 (the expression construct contains only coding region, with no 3′ UTR of the gene) could reverse the effects of ectopically expressed miR-495 in human leukemia cells with MLL rearrangements (Fig. 6 and Fig. S4). Therefore, PBX3 and MEIS1 are also functionally important direct target genes of miR-495 in MLL-rearranged leukemia. In the future, it would also be important to determine whether some other potential targets we identified in Fig. 4 are essential direct target genes of miR-495 in MLL-associated leukemia, and indeed some of those genes such as BMI1 and HOXA10 have been shown to play critical oncogenic roles in MLL-rearranged leukemia (15, 21, 53).

We and others have shown previously that MLL fusion proteins could up-regulate expression of the miR-17-92 cluster and miR-196b (28, 39, 41, 42, 54), and here we show that MLL fusions could also repress expression of miR-495. Tumor suppressor genes such as RASSF2 and CDKN1A (i.e., p21) have been identified as direct target genes of the miR-17-92 cluster in MLL-rearranged leukemia, and it is possible that some other well-known tumor-suppressor target genes (e.g., PTEN and BIM) (55–57) may also be genuine targets of this miRNA cluster in MLL-rearranged leukemia. Interestingly, we have shown that miR-196b targets both oncogenes (e.g., HOXA9 and MEIS1) and tumor-suppressor genes (e.g., PAI5) in MLL-rearranged leukemia, although its repression of expression of the tumor-suppressor target genes likely plays a predominant role in leukemogenesis, and thereby overall miR-196b functions as an oncogenic miRNA (39). In the present study,
Fig. 5. PBX3 and MEIS1 are direct target genes of miR-495. (A) Ectopic expression of miR-495 significantly (P < 0.05) represses endogenous expression of PBX3 and MEIS1 in MLL-rearranged AML cells. The cells were transfected with MSCV-PIG (control) or MSCV-PIG-miR-495, and then the effect of miR-495 overexpression was analyzed 48 h after transfection at both mRNA (Left; detected by qPCR, and the GAPDH expression level was used for normalization) and the protein (Right; detected by Western blot) levels. (B) Inhibitory effect of miR-495 on the endogenous expression of PBX3 and MEIS1 in BM cells of the BM transplantation recipient mice shown in Fig. 3A. Gene levels were normalized to the level of endogenous Gapdh. (C and D) miR-495 directly targets PBX3 (C) and MEIS1 (D) as detected by luciferase reporter and mutagenesis assays. In HEK293T cells, plasmids encoding the wild-type 3′ UTR of PBX3 or MEIS1 (namely, PBX3/MEIS1-3′ UTR) or the mutant 3′ UTR in which the predicted miR-495 binding site was mutated (namely, PBX3/MEIS1-3′ UTRmut), together with MSCV-PIG or PSCV-Pig-miR-495, were cotransfected with β-gal reporter control vector. Luciferase reporter assays were done 48 h after transfection. Forced expression of miR-495 could significantly repress luciferase activity of the reporter gene bearing the 3′ UTR of PBX3 or MEIS1 in human 293T cells, whereas mutation at the predicted target site in the 3′ UTR abrogated the repression. The normalized luciferase activities represent the firefly: β-gal ratios normalized to the control sample. Error bars present SD obtained from three independent experiments. *P < 0.05; **P < 0.01, two-tailed t test.

we show that as a tumor suppressor miRNA, miR-495 directly targets critical oncogenic target genes such as MEIS1 and PBX3, both of which are transcriptionally up-regulated by MLL fusions and play essential roles in the development and maintenance of MLL-rearranged leukemia.

Collectively, results from our present study together with those from previous studies (28, 39, 41, 42, 54) delineate a complex signaling network mediated by MLL fusions in MLL-rearranged leukemia, in which miRNAs contributed as essential gene expression modulators (a model is shown in Fig. S5). Thus, these studies not only substantially broaden our understanding of the complex mechanisms underlying the pathogenesis of MLL-rearranged leukemia but also implicate potential new therapeutic strategies to treat MLL-rearranged leukemia, a type of disease with resistance to present therapy. For example, in the future, we can treat MLL-rearranged leukemia with clinically applicable nanoparticles that packaged with both mimic oligos for miR-495 and the antagoniR oligos for the miR-17-92 cluster, so that we cannot only deplete expression of critical oncogenes (e.g., MEIS1 and PBX3) but also restore expression of essential tumor suppressor genes (e.g., RASSF2 and p21, and probably also PTEN and BIM) (55–57) to reach effective antitumor effects.

Materials and Methods

miRNA and miRNA Expression Profiling Assays. The miRNA expression profiling assays of the 100 human samples (including 10 MLL-rearranged, 75 nonMLL-rearranged AML, and 15 normal controls) were conducted by use of Exiqon miRCURY LNA arrays (v10.0; covering 757 human miRNAs). The miRNA microarrays of the 79 human samples (including 9 MLL-rearranged AML, 61 non-MLL-rearranged AML, and 9 normal control) and the 15 mouse BM samples (including 9 MLL-AF9-induced AML samples and 6 negative control samples) were conducted by use of Agilent’s custom-design microarrays and Affymetrix GeneChip Mouse Gene 1.0 ST arrays, respectively. Part of the array data have been reported elsewhere recently (29, 39, 58). The microarray data sets have been deposited in the Gene Expression Omnibus (GEO) database (accession nos. GSE30258 and GSE34185).

Cell Culture and Transfection. MONOMAC-6, THP-1, and KOCL-48 cells were grown in RPMI medium 1640 and transfected using the Amaxa Nucleofector Technology (Amaza Biosystems). More details are provided in SI Materials and Methods.

Cell Apoptosis and Viability Assay. Cell apoptosis and viability were assessed 48 h after transfection using the ApoLive-Glo Multiplex Assay Kit (Promega) according to the manufacturer’s manuals.

Luciferase Reporter and Mutagenesis Assays. Luciferase reporter and mutagenesis assays were conducted as described previously (39), with some modifications (SI Materials and Methods).

Colony-Forming/Replating Assay. These experiments were conducted as described previously (32) with some modifications (SI Materials and Methods).

Fig. 6. Both PBX3 and MEIS1 are functionally important target genes of miR-495 in MLL-rearranged leukemic cells. (A) Analysis of the effects of forced expression of miR-495 (MSCV-PIG-miR-495–MSCVneo), PBX3 (MSCV-PIG+mSCVneo-PBX3), MEIS1 (MSCV-PIG+mSCVneo-MEIS1), and MEIS1+miR-495 (MSCV-PIG-miR-495+MSCVneo-MEIS1), respectively, on cell viability (Upper) and apoptosis (Lower) of MONOMAC-6 cells. Cell viability and apoptosis were detected 48 h after transfection. (B) Analysis of their effects on cell growth/proliferation of MONOMAC-6 cells. Cell numbers were counted every day after transfection for 6 d. The coding regions (CDS) of PBX3 and MEIS1 were cloned into MSCVneo, and thus their ectopic expression would not be repressed by endogenous or cotransfected miR-495. The cells transfected with MSCV-Pig+MSCVneo (Ctrl) were used as controls. *P < 0.05; **P < 0.01, two-tailed t test.
BM Transplantation. Normal BM cells of B6.SJL (CD45.1) mice were retrovirally transduced with corresponding constructs, through two rounds of spinoculation (39), and then injected by tail vein into lethally irradiated (960 rads) B- to 10-fold wild-type C57BL/6 (CD45.2) recipient mice with 3 × 10^5 donor cells per mouse plus a radioprotective dose of 1 x 10^5 whole BM cells. All experiments on mice were approved by the Institutional Animal Care and Use Committee of the University of Chicago.

Histopathology and Immunohistochemistry. Tissue samples were fixed in formalin, embedded in paraffin, sectioned, and stained with H&E. Cytospins of peripheral blood and BM were stained with Wright-Giemsa.

ACKNOWLEDGMENTS. We thank Gregory Hannon, Scott Hammond, Lin He, and Scott Armstrong for providing retroviral constructs. This work was supported in part by National Institutes of Health (NIH) Grant R01 CA127277 (to J.C.), a Leukemia and Lymphoma Society Translational Research Grant (to J.D.R. and J.C.), an American Cancer Society Research Scholar grant (to J.C.), the G. Harold and Leila Y. Mathers Charitable Foundation (J.C.), the Fidelity Foundation (J.D.R. and J.C.), the University of Chicago Committee on Cancer Biology Fellowship Program (X.J.), a Leukemia and Lymphoma Society Special Fellowship (to Z.L.), Gabrielle’s Angel Foundation for Cancer Research (X.J., H.H., Z.L., and J.C.), NIH R01 CA118319 Sub-Award (to J.C.M., J.C., and NIH Grant CA040046 (to M.M.B.)) and P30 CA145499 Cancer Center Support Grant (CCSG) (to M.M.B.). J.C.M. is a Leukemia and Lymphoma Society Scholar.
Supporting Information

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SI Materials and Methods

Leukemic Samples and Treatment Protocols. All of the acute myeloid leukemia (AML) patient samples were obtained at the time of diagnosis or relapse and with informed consent at the University of Chicago Hospital or other collaborative hospitals and were approved by the institutional review board of the institutes/hospitals. All patients were treated according to the protocols of the corresponding institutes/hospitals. The samples were stored in liquid nitrogen until used. Blasts and mononuclear cells (MNCs) were purified by use of NycoPrep 1.077A (Axis-Shield) according to the manufacturer’s manual. All cell lines were maintained in the laboratory.

Normal Control Samples. The 15 normal bone marrow (BM) controls included 6 CD34+ hematopoietic stem/progenitor cell, 5 CD33+ myeloid progenitor cell, and 4 MNC samples. Three CD34+ samples were purchased from AllCells directly. The remaining three CD34+, five CD33+, and four MNC normal control cell samples were isolated from normal BM cells purchased from AllCells. MNCs were isolated using NycoPrep 1.077A (Axis-Shield) according to the manufacturer’s manual. The CD34+ and CD33+ cell samples were sorted by flow cytometry using corresponding monoclonal antibodies (BD Biosciences). The purity of the sorted CD34+ or CD33+ cell samples was assessed by flow cytometry as being over 97%.

Cell Culture and Transfection. THP-1 and KOCL-48 cells were grown in RPMI medium 1640 (Invitrogen) containing 10% (vol/vol) FBS, 1% Heps, and 1% penicillin-streptomycin. MONOMAC-6 cells were maintained in RPMI 1640 supplemented with 10% (vol/vol) FBS, 1% Heps, 2 mM l-glutamine, 100X non-essential amino acid, 1 mM sodium pyruvate, 9 µg/mL insulin, and 1% penicillin-streptomycin. siRNAs (Thermo Scientific) and/or plasmids were transfected into MONOMAC-6 cells with Cell Line Nucleofector Kit V following program T-037, and the other cells (THP-1 and KOCL-48) following program U-001, using the Amaza Nucleofector Technology (Amaza Biosystems). Experiments were performed 48 h after transfection.

The MLL-ENL-ERtm cell line was kept in RPMI 1640 supplemented with IL-3, IL-6, and GM-CSF, 10 ng/mL; stem cell factor (SCF), 100 ng/mL; and 10% (vol/vol) FBS, and 1% penicillin-streptomycin. 4-Hydroxytamoxifen (Sigma-Aldrich) was added at a final concentration as a 1-mM stock solution in ethanol. Cells were collected for experiments at the indicated days after drug withdrawal.

Cell Apoptosis, Viability, and Proliferation Assays. For apoptosis and viability assays, 48 h after transfection, cells were collected and seeded at requested concentration. Cell apoptosis and viability were assessed using the ApoLive-Glo Multiplex Assay Kit (Promega) according to the corresponding manufacturer’s manuals.

For cell proliferation assays, one million cells were electroporated with 100 µM siRNA, or 1.0 µg plasmid. Twenty-four hours after transfection, cells were seeded in 96-well plates at the concentration of 10,000 cells per well. Cell numbers were counted for the indicated number of days.

Target Gene Analysis. Four major miRNA–target prediction programs/databases, including TargetScan (www.targetscan.org) (1), PITA (http://genie.weizmann.ac.il/pubs/mir07/) (2), miRanda (http://www.microrna.org) (3), and miRBase Targets (http://microrna.sanger.ac.uk) (4), were used for the identification of putative miRNA–target pairs.

Plasmid Construction. The expression vector of miR-495 was amplified by PCR using the following primers: forward 5'-AAT CAT GGT TCT CIG CCT CTG GG-3' and reverse 5'-GGC AAG CTT TCC TGT GTT GAA TTA AGA CC-3'. MEIS1 3' UTR: forward 5'-AAT AAT ACT AGT CTA CTT CCT CTG GG-3' and reverse 5'-GGC AAG CTT TCC TGT GTT GAA TTA AGA CC-3'. MEIS1 3' UTR: forward 5'-AAT ACT AGT CAT CGG TCA TGT GTG TAT-3' and reverse 5'-AAT AAT AGG CTT CAA CTG GGC TGC TGG TT-3', and then were cloned into pMIR-REPORT Luciferase miRNA Expression Reporter Vector (Ambion). Seven mutations were induced by PCR according to the sequence shown previously for the miR-495 binding site mutants of 3' UTR of PBX3 and MEIS1. All of the insertions were confirmed by DNA sequencing.

RNA Extraction and Quantitative RT-PCR. Total RNA was extracted with the miRNeasy extraction kit (Qiagen) and was used as template to synthesize cDNA for quantitative RT-PCR (qPCR) analysis in a 7900HT real-time PCR system (Applied Biosystems). TaqMan qPCR assay was performed to validate the differential expression patterns of miRNAs using kits from Applied Biosystems. qPCR with SYBR Green dye (Qiagen) was used to determine expression of mRNA genes. snoRNA202, GAPDH, or Gapdh was used as endogenous controls for qPCR of miRNA and mRNA, respectively. Each sample was run in triplicate. qPCR primers are available upon request.

Immunoblotting. Transiently transfected THP-1 cells were harvested and lysed with RIPA buffer (Thermo Scientific). Proteins from the lysate were fractionated by electrophoresis through 4–15% polyacrylamide gels (Bio-Rad) and transferred to polyvinylidene fluoride membranes using Tris-Glycine Transfer buffer (Thermo Scientific). Blots were incubated with IRDye 800CW-conjugated or 700CW-conjugated antibody, and infrared fluorescence images were obtained with the Odyssey infrared imaging system (Li-Cor Bioscience). Anti-PBX3, anti-MEIS1 (Abcam), and anti-ACTIN (Santa Cruz Biotechnology) antibodies were used to detect corresponding proteins.

Exiqon microRNA Array Assay. Our microRNA (miRNA) expression profiling assay of 85 (10 MLL-rearranged and 75 non-MLL-rearranged) AML samples and 15 human normal BM samples was performed by Exiqon using the miRCURY LNA arrays (v1.0; covering 757 human miRNAs). Briefly, after passing sample quality control (QC) on the Bioanalyser2100 and RNA measurement on the Nanodrop instrument, the samples were labeled using the miRCURY Hy3/Hy5 power labeling kit and hybridized on the miRCURY LNA Array (v.10.0; containing 757 human miRNAs). Each chip/slide contained two arrays. One individual sample (0.5 µg) labeled with Hy3 and an aliquot
(0.5 μg) of the common reference pool (a mixture of individual samples, allowing normalization across a set of arrays and direct comparison of all samples) labeled with Hy5 were put into the two arrays of a given chip. The quantified signals were then normalized using the global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm (7). When calling a particular miRNA failed on an array, its expression value was indicated by the acronym “NA.” The criterion for deciding that a miRNA had failed on a particular array was that three or more of the four replicated measures of this miRNA were flagged (i.e., the signal is below background) by the image analysis software. In addition, if the SD of signals of the replicated probes of a given miRNA in a particular array (i.e., an individual sample) was greater than 0.4, this miRNA also failed on this array. The expression values are log2 (Hy3/Hy5) ratios, which were obtained on the basis of the normalized data for which replicated measurements on the same slide have been averaged. Median-centering genes for each array and median-centering each gene across all arrays were conducted then for heatmap illustration.

**Agilent Custom-Design Gene Arrays of Human Samples.** Gene expression profiling of 79 human samples (9 MLL-rearranged AML, 61 non-MLL-rearranged AML, and 9 normal controls) was analyzed by use of Agilent’s custom-design microarrays (Agilent Technologies) as described previously (8). RNA quality control, cRNA amplification, hybridization, and image scan were conducted in the Functional Genomics Facility of University of Chicago. Briefly, RNA quantity and integrity was assessed using NanoDrop ND-1000 spectrophotometers and followed by analysis using an Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA (0.5 μg per sample) was reverse transcribed, amplified, and labeled with Cy3 using Agilent one-color amplification protocol (Agilent Quick-Amp Labeling Kit, One-color; One-Color Microarray-Based Gene Expression Analysis). The amplified cRNA samples were hybridized overnight and then washed according to the protocol of Agilent Oligo Microarray (One-Color Microarray-Based Gene Expression Analysis V5.7, GE 8x15K, Gene Expression Hybridization Kit). Array slides were scanned on a GenPix 4000B scanner at 570 PMT and 100% Power standard setting according to the manufacturer’s instruction (Molecular Devices). Array slide images were then analyzed using Agilent Feature Extraction (9.5.1.1) to obtain gene expression signals and array QC reports. The Partek Genomics Suite was used for the data normalization. Briefly, background adjustment, quantile normalization, and-log transformation were used to normalize and treat gene expression intensities, and then median-centering genes for each array and median-centering each gene across all arrays were conducted for heatmap illustration. Pearson correlation was used to assess expression correlation between miR-150 and predicted target genes by use of the Partek Genomics Suite. The complete microarray data set have been deposited in the Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo/, accession no. GSE30285).

**Affymetrix Gene Arrays of Mouse Samples.** As described previously (8), a total of 15 mouse BM samples, including 6 primary (including 3 each of negative control and MLL-AF9) and 9 secondary (including 3 negative control and 6 MLL-AF9) obtained from the in vivo mouse BM reconstitution assays were analyzed by use of Affymetrix GeneChip Mouse Chip Gene 1.0 ST Array. Each array contains 28,853 mouse genes being represented on the array by ~27 probes spread across the full length of a given gene, providing a more complete and more accurate picture of gene expression than 3′-based expression array designs. The RNA quality control, cDNA amplification, hybridization, and image scan were conducted in the Functional Genomics Facility of University of Chicago. After hybridization and background correction according to the standard protocols, the quantified signals were then normalized using Robust Multiarray Average, which is a robust algorithm of background adjustment, quantile normalization, and log transformation (9). Then median-centering genes for each array and median-centering each gene across all arrays were also conducted for heatmap illustration. The complete-microarray data set is already available at the GEO database, and the accession number is GSE34185.

**Luciferase Reporter and Mutagenesis Assays.** Luciferase reporter and mutagenesis assays were conducted as described previously, with some modifications (10, 11). Briefly, for transfection, HEK293T cells were plated in 96-well plates at a concentration of 6,000 cells per well in triplicate for each condition. After overnight incubation, cells were transfected with 20 ng of the pMIR-REPORT bearing the PBX3 or MEIS1 3′ UTR or the 3′ UTR with mir-495 binding site mutations, and 20 ng of MSCV-mir-495 or an empty MSCV vector using Effectene Transfection Reagent (Qiagen) according to the manufacturer’s protocol. pMIR-REPORT Beta-galactosidase Reporter Control Vector (Ambion) (1 ng) was cotransfected for transfection efficiency control in all transfections. Cells were lysed, and firefly luciferase and β-galactosidase activities were detected using the Dual-Light Combined Reporter Gene Assay System (Applied Biosystems) 48 h after transfection. Firefly luciferase activity was normalized to β-galactosidase activity for each transfected well. Each experiment was performed in triplicate and repeated three times.

**Packaging of Recombinant Retroviruses, Transduction of Cells, and in Vitro Colony-Forming/Replating Assays.** Those experiments were conducted as described previously (10, 12), with some modifications. Briefly, retrovirus vectors were cotransfected with pCL-Eco packaging vector (IMGENEX) into 293T cells using Effectene Transfection Reagent (Qiagen) to produce the retroviruses. BM cells were harvested from a cohort of 4- to 6-wk-old B6.SJL (CD45.1) donor mice after 5 d of 5-fluorouracil treatment, and primitive hematopoietic progenitor cells were enriched with the Mouse Lineage Cell Depletion Kit (Miltenyi Biotec). An aliquot of enriched hematopoietic progenitor cells was added to retroviral supernatant together with polybrene in a plate, which were centrifuged at 2,000 × g for 2 h at 32 °C (i.e., “spinoculation” (8, 10, 13)), and then the media was replaced with fresh media and incubated for 20 h at 37 °C. Next day, the same procedure was repeated once.

Then, on the day after the second spinoculation, an equivalent of 2.0 × 10^6 cells were plated onto a 35-mm Petri dish in 1.1 mL of Methocult M3230 methylcellulose medium (Stem Cell Technologies) containing 10 ng/mL each of murine recombinant IL-3, IL-6, and GM-CSF, and 30 ng/mL of murine recombinant SCF (R&D Systems), along with 1.0 mg/mL of G418 and/or 2 μg/mL of puromycin. For each transduction, there were two duplicate dishes. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO2 in air. The colonies were replated every 7 d under the same conditions. The colony-forming/replating assays were repeated three times.

**BM Transplantation Assays.** C57BL/6 (CD45.2) and B6.SJL (CD45.1) were purchased from Jackson Laboratories or Harlan Laboratories. Both male and female mice were used for the experiments. All laboratory mice were maintained in the animal facility at the University of Chicago. All experiments on mice in our research protocol were approved by Institutional Animal Care and Use Committee of the University of Chicago.

Normal BM cells of B6.SJL (CD45.1) mice were retrovirally transduced with MSCVneo + MSCV-PIG (as control), MSCVneo-MLL-AF9 + MSCV-PIG (i.e., MLL-AF9), and MSCVneo-MLL-AF9 + MSCV-PIG-miR-495 (i.e., MLL-AF9 + miR-495), respectively, through two rounds of “spinoculation.” Then, retrovirally transduced donor cells were injected by tail vein into lethally irradiated (960 rads) 8- to 10-wk-old C57BL/6 (CD45.2) recipient mice
with $3 \times 10^5$ donor cells plus a radioprotective dose of whole BM cells ($1 \times 10^6$; freshly harvested from a C57BL/6 mouse) per recipient mouse.

**Statistical Software.** The miRNA and gene/exon array data analyses were conducted by use of the Partek Genomics Suite, TIGR Multiple Array Viewer software package (TMeV version 4.6; TIGR) (14), and/or Bioconductor R packages. The miRNA–gene expression correlation was analyzed by use of the Partek Genomics Suite. The heatmaps were constructed by use of TIGR Multiple Array Viewer software package. The t test, Kaplan-Meier method, and log-rank test, etc. were performed with WinSTAT (R. Fitch Software) and/or the Partek Genomics Suite. Gene Ontology analysis was done with the R package (15).

Fig. S2. Expression profiles of the 76 miRNAs that are significantly differentially expressed (q < 0.05, FDR < 0.05) between MLL-rearranged AML (n = 10) and normal controls (n = 15). Expression data were mean centered, and the relative value for each sample is represented by a color, with red representing a high expression and green representing a low expression (scale shown at upper right).
Fig. S3. Gene Ontology analysis of the 128 candidate target genes of miR-495. The number of genes among the 128 candidate targets enriched in each Biological Process (BP) category is shown. The P value scale is shown as a bar on the middle right.
Both PBX3 and MEIS1 are functionally important target genes of miR-495 in THP-1 cells. The coding regions (CDS) of PBX3 and MEIS1 were cloned into MSCVneo, and thus their ectopic expression would not be repressed by endogenous or cotransfected miR-495. *P < 0.05; **P < 0.01, two-tailed t test. The cells transfected with MSCV-PIG+MSCVneo (Ctrl) were used as controls. (A) Analysis of the effects of forced expression of miR-495 (MSCV-PIG-miR-495+MSCVneo), PBX3 (MSCV-PIG+MSCVneo-PBX3), PBX3+miR-495 (MSCV-PIG-miR-495+MSCVneo-PBX3), MEIS1 (MSCV-PIG+MSCVneo-MEIS1), and MEIS1+miR-495 (MSCV-PIG-miR-495+MSCVneo-MEIS1), respectively, on cell viability (Upper) and apoptosis (Lower) of THP-1 cells. Cell viability and apoptosis were detected 48 h after transfection. (B) Analysis of their effects on cell growth/proliferation of THP-1 cells. Cell numbers were counted every day after transfection for 6 d.
**Fig. S5.** Model to illustrate the miRNAs deregulated by MLL fusions and the genes targeted by those miRNAs. The miRNAs and genes shown in red are oncogenic miRNAs or genes, whereas those shown in green are tumor-suppressor ones. The arrows in red represent positive regulation, whereas the lines in black represent negative regulation.

**Table S1.** List of the 128 candidate target genes of miR-495 that exhibited a significantly inverse correlation of expression with miR-495 in 79 human samples (including 70 AML and 9 normal controls)

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Table S2. Enrichment of the 128 candidate target genes of miR-495 in gene sets in MSigDB

<table>
<thead>
<tr>
<th>Gene set name</th>
<th>Description</th>
<th>No. genes in overlap</th>
<th>P value</th>
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<tbody>
<tr>
<td>Enriched in genes up-regulated in leukemic or normal hematopoietic stem cells</td>
<td>Genes up-regulated in CD34+ [Gene ID = 947] cells isolated from BM of CML (chronic myelogenous leukemia) patients, compared with those from normal donors.</td>
<td>24</td>
<td>2.57E-09</td>
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<td><strong>DIAZ_CHRONIC_MEYLOGENOUS_LEU.K.EMIA_UP</strong></td>
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<tr>
<td><strong>BYSTYKH_HEMATOPOIESIS_STEM_CELL_QTL_TRANS</strong></td>
<td>Transcripts in hematopoietic stem cells (HSC) which are transregulated (i.e., modulated by a QTL (quantitative trait locus) not in a close proximity to the gene).</td>
<td>17</td>
<td>6.37E-07</td>
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<tr>
<td>Enriched in genes up-regulated in MLL-associated leukemia</td>
<td>The ‘MLL signature 1’: genes up-regulated in pediatric AML (acute myeloid leukemia) with rearranged MLL [Gene ID = 4297] compared with all AML cases with the intact gene.</td>
<td>11</td>
<td>6.08E-07</td>
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<td><strong>MULLIGHAN_MLL_SIGNATURE_1_UP</strong></td>
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<td><strong>MULLIGHAN_MLL_SIGNATURE_2_UP</strong></td>
<td>The ‘MLL signature 2’: genes up-regulated in pediatric AML (acute myeloid leukemia) with rearranged MLL [Gene ID = 4297] compared with the AML cases with intact MLL and NPM1 [Gene ID = 4869].</td>
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<td><strong>YAGI_AML_WITH_11Q23_REARRANGED</strong></td>
<td>Genes specifically expressed in samples from patients with pediatric acute myeloid leukemia (AML) bearing 11q23 rearrangements.</td>
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<tr>
<td><strong>YAGI_AML_WITH_T_9_11_TRANSLOCATION</strong></td>
<td>Genes specifically expressed in samples from patients with pediatric acute myeloid leukemia (AML) bearing (9;11) translocation.</td>
<td>9</td>
<td>4.84E-09</td>
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<td><strong>ROSS_LEU.K.EMIA_WITH_MLL_FUSIONS</strong></td>
<td>Top 100 probe sets associated with MLL fusions [Gene ID = 4297] irrespective of the lineage of the pediatric acute leukemia.</td>
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<td>1.35E-09</td>
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<tr>
<td>Enriched in potential targets of MAZ, LEF1 and SP1</td>
<td>Genes with promoter regions [-2kb,2kb] around transcription start site containing the motif GGAGGGR which matches annotation for MAZ: MYC-associated zinc finger protein (purine-binding transcription factor)</td>
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<td>3.98E-10</td>
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<td><strong>GGGAGGR_V$MAZ_Q6</strong></td>
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<td><strong>CTTTGT_V$LEF1_Q2</strong></td>
<td>Genes with promoter regions [-2kb,2kb] around transcription start site containing the motif CTTTGT which matches annotation for LEF1: lymphoid enhancer-binding factor 1</td>
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<td>7.99E-15</td>
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<tr>
<td><strong>GGGCGR_V$SP1_Q6</strong></td>
<td>Genes with promoter regions [-2kb,2kb] around transcription start site containing the motif GGGCGGR which matches annotation for SP1: Sp1 transcription factor</td>
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<td>1.74E-10</td>
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