

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

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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*, *Htrx*) 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 *HOXA9* 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 *HOXA9* 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 *HOXA9* 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 CD33⁺ 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. 1*A* and Fig. S1]. We next compared miRNA expression between *MLL*-rearranged

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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 stem 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 expressional repression 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/*t* (9, 11) and THP-1/*t* (9, 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 or MSCVneo-*MLL-AF9* into normal 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 target genes of miR-495 in both human and mouse genomes (Table S1). Through Gene Ontology analysis, we found that these 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). Through gene set enrichment analysis (48), we show that these genes are significantly enriched in gene sets that are up-regulated in leukemic stem cells or normal hematopoietic stem cells, in those that are up-regulated in *MLL/t*(11q23)-associated AML, and in those that are potential direct targets of MAZ, LEF1, and SP1, three stem-cell-self-renewal-related genes (Table S2).

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., *ACTR1A*, *ANGEL1*, *BAK1*, *BM11*, *C10ORF140*, *CAB39L*, *CRADD*, *GPR126*, *HOXA10*, *KPNB1*, *MEIS1*, *MMRN1*, *MRI*, *MYO6*, *PBX3*, *PTP4A3*, *REEP3*, *RNF2*, *SEN6*, *SET*, *SPRYD4*, *SYT17*, *TMEM97*, and *UBE2Z*), because these genes are expressed at a significantly higher level in the 9 human *MLL*-rearranged leukemia samples compared with both the 9 normal controls 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*,

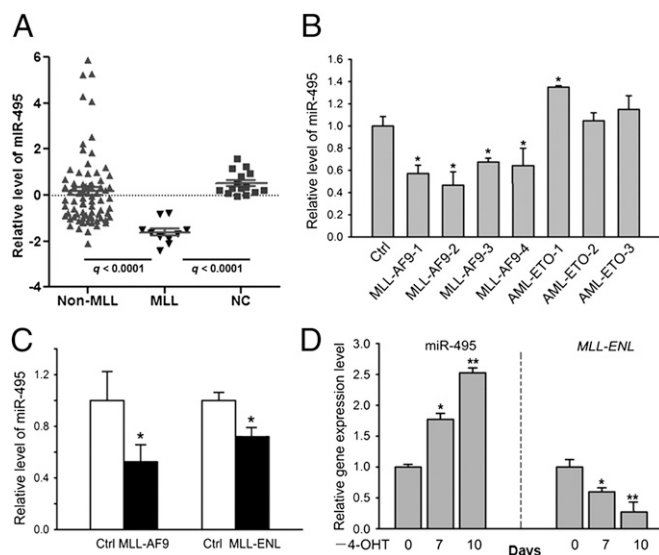


Fig. 1. miR-495 is significantly down-regulated in human *MLL*-rearranged AML compared with non-*MLL*-rearranged AML or normal controls. (A) Relative expression levels of miR-495 in 10 human *MLL*-rearranged AML (MLL), 75 non-*MLL*-rearranged AML (non-*MLL*), and 15 normal control (NC, including 6 CD34⁺ hematopoietic stem/progenitor cell, 5 CD33⁺ myeloid progenitor cell, and 4 MNC) samples, as detected by Exiqon miRNA microarray assays. (B) Quantitative PCR (qPCR) analysis of miR-495 expression level in human cord blood CD34⁺ cells that were retrovirally transduced with MSCV-*MLL-AF9*, MSCV-*AML-ETO*, or empty vector (Ctrl). (C) Down-regulation of miR-495 in *MLL-AF9* or *MLL-ENL*-transduced mouse BM progenitor cells. (D) Withdrawal of 4-OHT (days 0, 7, and 10 are shown) results in a significant increase of miR-495 expression but a significant decrease of *MLL-ENL* expression in *MLL-ENL*-ERTm cells. $*P < 0.05$; $**P < 0.01$, two-tailed *t* test.

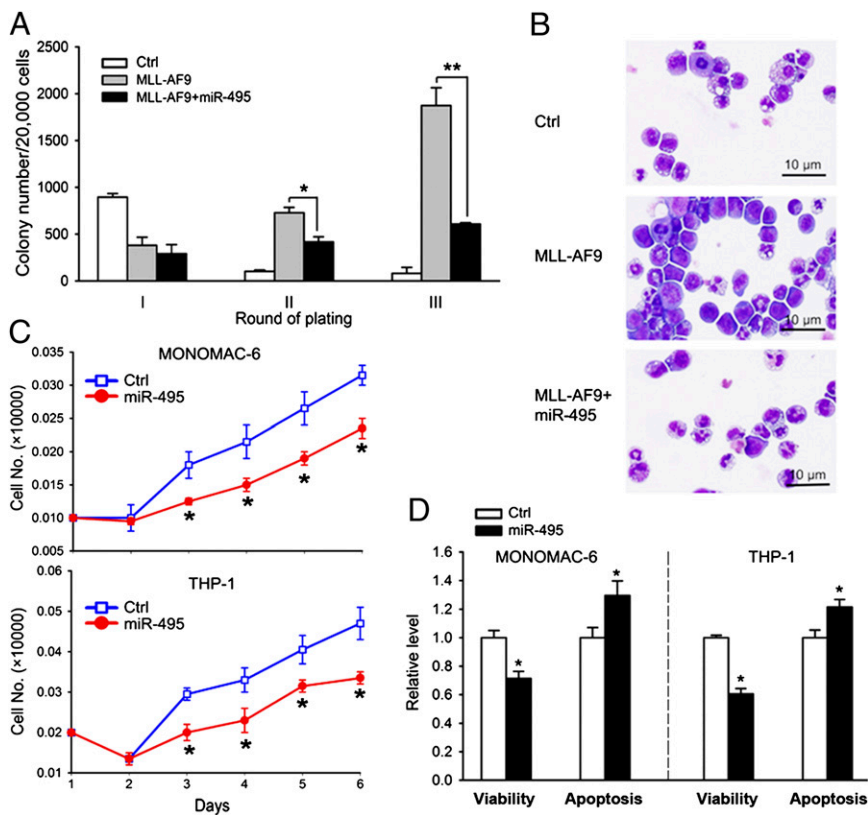


Fig. 2. miR-495 functions as a tumor-suppressor miRNA in vitro. (A) miR-495 inhibits *MLL-AF9*-induced cell transformation of normal mouse BM progenitor cells. Normal mouse BM cells were retrovirally transduced with MSCVneo-MSCV-PiG (as control), MSCVneo-*MLL-AF9*+MSCV-PiG (*MLL-AF9*), or MSCVneo-*MLL-AF9*+MSCV-PiG-miR-495 (*MLL-AF9*+miR-495), and colony forming/replating assays were done thereafter. (B) miR-495 promotes the differentiation of *MLL-AF9*-transduced mouse BM progenitor cells. Colony-forming cells were collected for cytospin analysis from the secondary round of replating. (C) Ectopic expression of miR-495 inhibits cell growth/proliferation of MONOMAC-6 and THP-1 cells. Cells were transfected with MSCV-PiG (Ctrl) or MSCV-PiG-miR-495 (miR-495). Cell numbers were counted every day after transfection for 6 d. (D) Forced expression of miR-495 decreases cell viability and promotes apoptosis of MONOMAC-6 and THP-1 cells. Cells were transfected with MSCV-PiG (Ctrl) or MSCV-PiG-miR-495 (miR-495), and cell viability and apoptosis were assessed 48 h after transfection. * $P < 0.05$; ** $P < 0.01$, two-tailed t test.

Set, and *Spyd4*) of the above 24 candidate target genes were significantly overexpressed ($q < 0.05$; FDR < 0.01 ; SAM) in *MLL-AF9* mouse leukemia samples relative to normal controls (Fig. 4B). Thus, these seven genes are the highly possible target genes of miR-495 in *MLL*-rearranged leukemia.

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, KOCL-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. We found that 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. S4). 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. S1). 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 human normal hematopoietic cell controls, miR-495 (along with 31 other miRNAs) is significantly down-regulated in *MLL*-rearranged AML (Fig. S2). 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

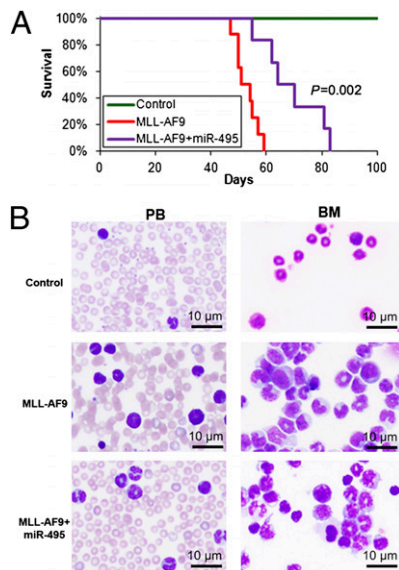


Fig. 3. miR-495 inhibits MLL-fusion-mediated leukemogenesis in vivo. (A) Mouse BM transplantation assay of the control group (Control; MSCVneo+MSCV-PIG; $n = 5$), the MLL-AF9 group (MSCVneo-MLL-AF9+MSCV-PIG; $n = 8$), and the MLL-AF9+miR-495 group (MSCVneo-MLL-AF9+MSCV-PIG-miR495; $n = 6$). The MLL-AF9+miR-495 group developed leukemia significantly ($P = 0.002$, log-rank test) slower than the MLL-AF9 alone group. (B) miR-495 increased cell differentiation in both peripheral blood (PB) and BM of the recipient mice.

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*, *Pbx3*, *Rnf2*, *Set*, and *Spyd4*) 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 *HOXA* 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 leukemic cells with *MLL* rearrangements (Fig. 6 and Fig. S4). Therefore, *PBX3* and *MEIS1* are two 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., *FAS*) 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,

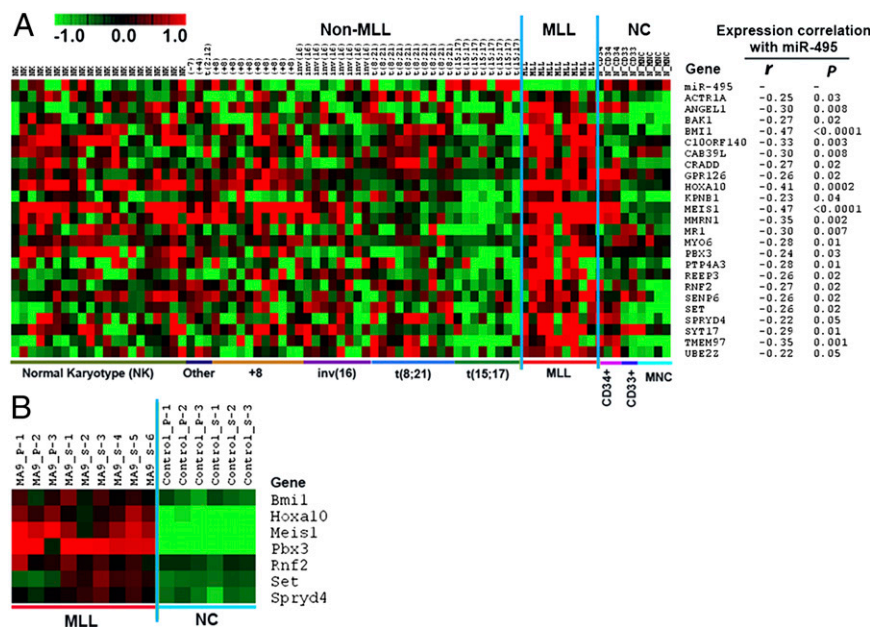
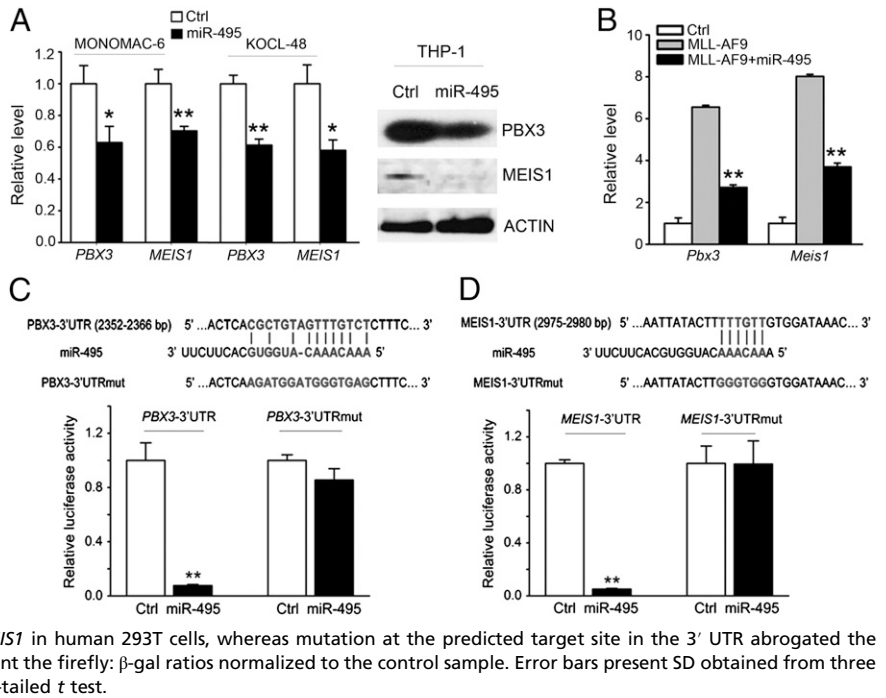


Fig. 4. Expression profiles of candidate target genes of miR-495. (A) Expression profiles of the 24 candidate target genes of miR-495 and their expression correlation with miR-495 in the set of 79 human samples (including 9 *MLL*-rearranged AML, 61 non-*MLL*-rearranged AML, and 9 normal control). r , correlation coefficient; P , P value. Pearson correlation was applied to analyze the correlation. (B) Expression profiles of the seven potential target genes of miR-495 in the set of 15 mouse BM cell samples collected from mouse BM transplantation assays, which include 9 *MLL-AF9* mouse leukemic BM cell samples (3 from primary BM transplantation recipient mice) and 6 from secondary BM transplantation recipient mice) and 6 normal control BM cell samples (3 each from primary and secondary BM transplantation recipient mice). *MA9*, *MLL-AF9*; $_P$, primary transplantation recipient mouse; $_S$, secondary transplantation recipient mouse. 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 left).

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 MSCV-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 antagomiR 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.

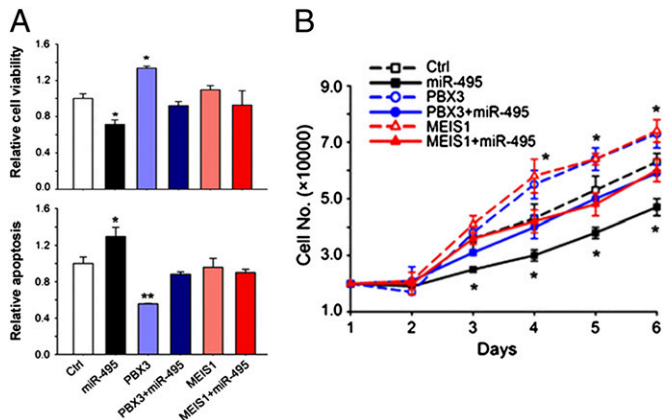


Fig. 6. Both *PBX3* and *MEIS1* are functionally important target genes of miR-495 in *MLL*-rearranged leukemic cells. (A) Analysis of the effects of forced expression of miR-495 (MSCV-PIG-miR-495+MSCVneo), *PBX3* (MSCV-PIG+MSCVneo-*PBX3*), *PBX3*+miR-495 (MSCV-PIG-miR-495+MSCVneo-*PBX3*), *MEIS1* (MSCV-PIG+MSCVneo-*MEIS1*), and *MEIS1*+miR-495 (MSCV-PIG-miR-495+MSCVneo-*MEIS1*), respectively, on cell viability (Upper) and apoptosis (Lower) of 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.

Materials and Methods

miRNA and mRNA Expression Profiling Assays. The miRNA expression profiling assays of the 100 human samples (including 10 *MLL*-rearranged, 75 non-*MLL*-rearranged AML, and 15 normal controls) were conducted by use of Exiqon miRCURY LNA arrays (v10.0; covering 757 human miRNAs). The mRNA 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 (Amaxa 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*).

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) 8- to 10-wk-old C57BL/6 (CD45.2) recipient mice with 3×10^5 donor cells per mouse plus a radioprotective dose of 1×10^6 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.

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