miR-9 is an essential oncogenic microRNA specifically overexpressed in *mixed lineage leukemia*–rearranged leukemia

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MicroRNAs (miRNAs), small noncoding RNAs that regulate target gene mRNAs, are known to contribute to pathogenesis of cancers. Acute myeloid leukemia (AML) is a group of heterogeneous hematopoietic malignancies with various chromosomal and/or molecular abnormalities. AML with chromosomal translocations involving the *mixed lineage leukemia* (*MLL*) gene are usually associated with poor survival. In the present study, through a large-scale, genomewide miRNA expression assay, we show that microRNA-9 (miR-9) is the most specifically up-regulated miRNA in *MLL*-rearranged AML compared with both normal control and non–*MLL*-rearranged AML. We demonstrate that miR-9 is a direct target of MLL fusion proteins and can be significantly up-regulated in expression by the latter in human and mouse hematopoietic stem/progenitor cells. Depletion of endogenous miR-9 expression by an appropriate antagomiR can significantly inhibit cell growth/viability and promote apoptosis in human *MLL*-rearranged AML cells, and the opposite is true when expression of miR-9 is forced. Blocking endogenous miR-9 function by anti-miRNA sponge can significantly inhibit, whereas forced expression of miR-9 can significantly promote, MLL fusion–induced immortalization/transformation of normal mouse bone marrow progenitor cells in vitro. Furthermore, forced expression of miR-9 can significantly promote MLL fusion–mediated leukemogenesis in vivo. In addition, a group of putative target genes of miR-9 exhibited a significant inverse correlation of expression with miR-9 in a series of leukemia sample sets, suggesting that they are potential targets of miR-9 in *MLL*-rearranged AML. Collectively, our data demonstrate that miR-9 is a critical oncomiR in *MLL*-rearranged AML and can serve as a potential therapeutic target to treat this dismal disease.

MicroRNAs (miRNAs) are regulatory RNAs, ~22 nucleotides in length, which serve to posttranscriptionally regulate expression of target genes (1). Binding of target genes most frequently happens at the 3′-UTRs and induces mRNA translational repression and, particularly, degradation of the target mRNAs (1–3). Evidence is emerging that miRNAs play critical regulatory roles in virtually all bioprocesses in both normal development and pathogenesis of disease (1–4).

Leukemia is a cancer arisen from uncontrolled proliferation in the hematopoietic lineage. Acute myeloid leukemia (AML) accounts for roughly 70–80% of all adult acute leukemias and 20% of all childhood acute leukemias (5). Approximately 40–60% of AML patients contain chromosomal abnormalities with varying prognosis. Specifically, translocations involving the *mixed lineage leukemia* (*MLL*) gene account for roughly 10% of AML (2, 6, 7). Patients with *MLL*-rearranged AML are often associated with poor prognosis, and effective targeted therapies are not available (2, 6, 7). Dysregulation of miRNAs has been frequently observed in AML, including those carrying *MLL* rearrangements (2). We and others have shown that *MLL* translocations induce aberrant overexpression of a set of miRNAs such as individual miRNAs in the miR-17–92 cluster and miR-196b (8–13).

In the present study, through a large-scale, genomewide miRNA expression profiling assay of 85 human AML cases (including 10 *MLL* rearranged and 75 others), along with 15 normal controls, we show that besides the aforementioned individual miRNAs, a number of other miRNAs are also significantly overexpressed in *MLL*-rearranged AML relative to normal controls and non–*MLL*-rearranged AML. In particular, miR-9 appears to be the most specifically and consistently overexpressed miRNA in *MLL*-rearranged AML. We then show that miR-9 is a direct target of MLL fusions and plays an essential oncogenic role in *MLL*-rearranged leukemia.

Results

miR-9 Is the Most Specifically and Consistently Overexpressed miRNA in *MLL*-Rearranged AML. We performed a large-scale, genomewide miRNA expression profiling assay of 85 AML primary patient samples (including 10 *MLL* rearranged and 75 others; Table S1), along with 15 normal control [6 $CD34^+$ hematopoietic stem/progenitor cell, 5 $CD33^+$ myeloid progenitor cell, and 4 mononuclear cell (MNC)] samples, by use of Exiqon miRCURY LNA arrays (v10.0; covering 757 human miRNAs). As shown in Fig. 1*A*, 29 miRNAs are expressed at a significantly higher level [significant analysis of microarrays (SAM), $q < 0.05$; false discovery rate (FDR) < 0.05] in *MLL*-rearranged AML than in both normal controls and non–*MLL*-rearranged AML; in contrast, the opposite pattern was observed for only 1 miRNA (i.e., miR-495) (14). Consistent with our previous miRNA expression profiling assay using a bead-based method (8, 13), we found in this new profiling assay that individual miRNAs in the miR-17–92 cluster (miR-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92) or its

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Data deposition: The microarray data sets have been submitted to the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession nos. GSE30258, GSE34184, and GSE34185).

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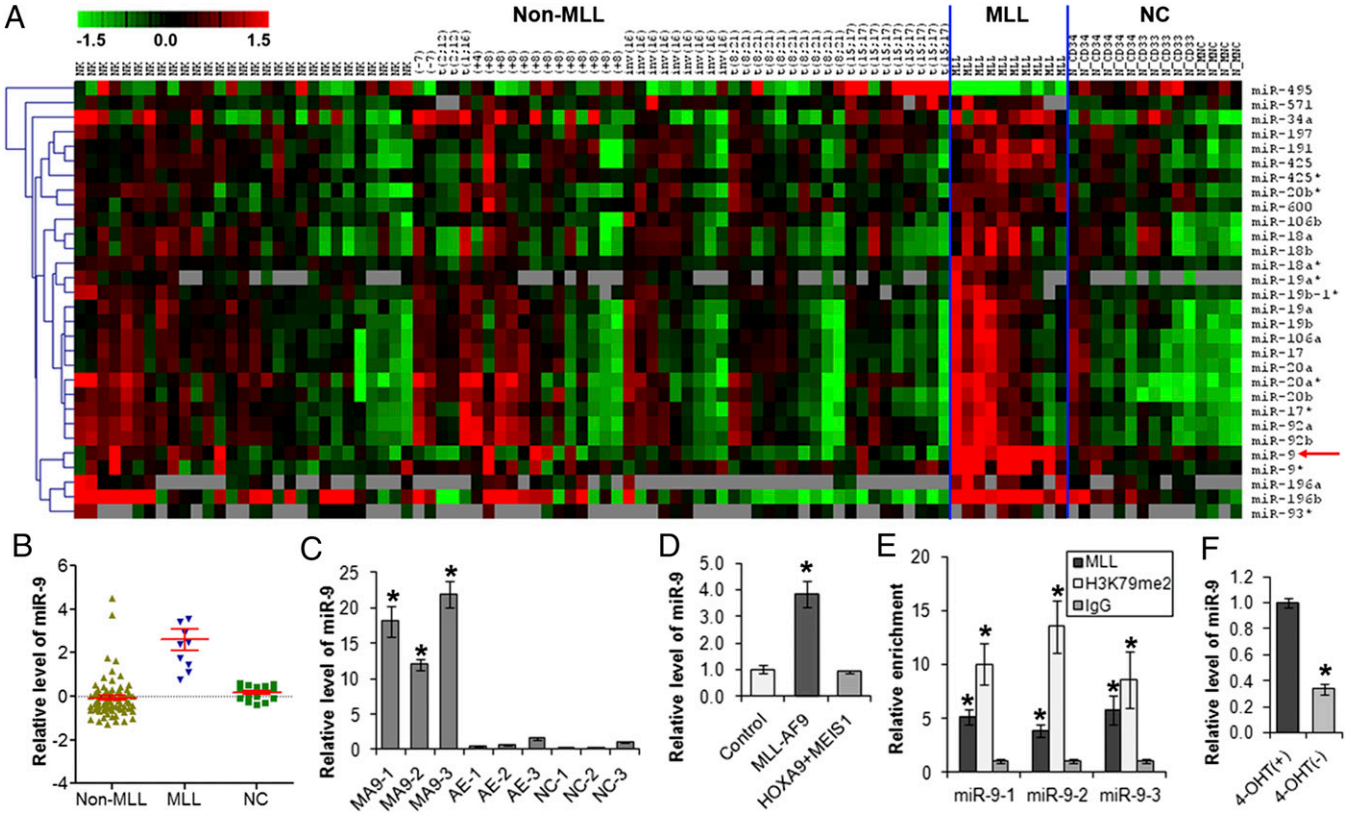

Fig. 1. MLL fusion proteins directly up-regulate expression of miR-9. (*A*) Expression profiles of 30 miRNAs that are significantly ($q < 0.05$; FDR < 0.05; SAM) up-regulated (29 miRNAs) or down-regulated (1 miRNA, i.e., miR-495) in *MLL*-rearranged AML ($n = 10$) compared with both normal controls [$n = 15$, including 6 $CD34^+$ hematopoietic stem/progenitor, 5 $CD33^+$ myeloid progenitor, and 4 MNC cells] and non–*MLL*-rearranged AML ($n = 75$). Expression data are mean centered, and the relative value for each sample is represented by a color: high expression is represented with red and low expression is represented with green (scale shown in the upper left). (*B*) Relative expression levels of miR-9 in 10 human *MLL*-rearranged AML (i.e., MLL), 75 non–*MLL*-rearranged AML (i.e., Non-MLL), and 15 normal control (i.e., NC) samples, as detected by Exiqon miRNA microarray assays. (*C*) qPCR analysis of miR-9 expression level in human cord blood $CD34^+$ cells that were retrovirally transduced with MSCV-*MLL-AF9* (MA9-1, -2, and -3), MSCV-*AML-ETO* (AE-1, -2, and -3), or empty vector (NC-1, -2, and -3). (*D*) qPCR analysis of miR-9 expression level in colony cells derived from mouse BM progenitor cells retrovirally transduced with *MLL-AF9 (MLL-AF9*-MSCVneo+MSCVpig), HOXA9+MEIS1 (HOXA9-MSCVpig+MEIS1-MSCVneo), or empty vector (MSCVneo+MSCVpig). (*E*) ChIP assay of miR-9 promoter regions using anti-MLL (N-terminal), anti-IgG, and anti-H3K79me2 (H3K79 di-methylation) antibodies in MONOMAC-6 cell line. (*F*) Withdrawal of 4-hydroxy-tamoxifen (4-OHT) (day 7 is shown) results in a significant decrease of miR-9 expression in *MLL-ENL*-ERtm cell line. *$P < 0.05$, two-tailed *t* test.

paralogous clusters (i.e., miR-106a-363 and miR-106b-25) are highly expressed in *MLL*-rearranged AML (Fig. 1*A*). Similarly, overexpression of miR-196a/b (8–10) was also confirmed in this new assay (Fig. 1*A*). However, among the 29 overexpressed miRNAs, miR-9 appears to be the most specifically and consistently overexpressed one in *MLL*-rearranged AML (Fig. 1 *A* and *B*). The significant up-regulation of miR-9 was also observed in various leukemia cell lines (including MONOMAC-6, THP-1, ML-2, and KOCL-48) carrying *MLL* rearrangements but not in those without *MLL* translocations (e.g., HL-60 and U937), compared with normal controls (Fig. S1).

miR-9 Is a Direct Downstream Target of MLL Fusion Proteins. We then investigated whether increased expression of miR-9 is directly attributed to *MLL* translocations. We found that miR-9 could be significantly up-regulated by retrovirally transduced *MLL-AF9* [resulting from *t*(9;11)(p22;q23), the most frequent *MLL* rearrangement observed in AML], but not by *AML1-ETO* [resulting from *t*(8;21)], in human hematopoietic stem/progenitor cells (15) (Fig. 1*C*). Similarly, *MLL-AF9* could also significantly up-regulate miR-9 expression in normal mouse bone marrow (BM) progenitor cells (Fig. 1*D*). In contrast, we could not observe a significant up-regulation of miR-9 expression in mouse BM progenitor cells transduced with *HOAX9* and *MEIS1* (Fig. 1*D*), which are two critical downstream targets of MLL fusion proteins, and their aberrant overexpression is required for the pathogenesis of *MLL*-rearranged AML (16–18). Thus, our data suggest that MLL fusion proteins may directly up-regulate expression of miR-9 and not through downstream mediators (e.g., HOXA9 and MEIS1).

MLL fusion proteins can bind to the promoter regions of their critical target genes and promote their expression through recruiting DOT1L-mediated methylation of histone H3 lysine 79 (10, 13, 19–21). To determine whether MLL fusion proteins also directly bind to the miR-9 promoter regions, we performed a ChIP assay in the MONOMAC-6 cell line that carries *t*(9;11)/*MLL-AF9* (Fig. 1*E*). miR-9 has three loci, miR-9-1, miR-9-2, and miR-9-3, which all produce an identical mature miRNA product (22). We observed that MLL fusion proteins were significantly ($P < 0.05$) enriched on the promoter regions of miR-9-1, -2, and -3 loci, associated with enrichment of H3K79me2, a mark for active transcription (20). To determine whether ovrexpression of miR-9 is dependent on the presence of MLL fusions, we used *MLL-ENL*-ERtm, a mouse myeloid cell line that expresses a stable derivative of *MLL-ENL* in the presence of 4-hydroxytamoxifen (4-OHT), as a model (16, 23). We found that depletion of *MLL-ENL* following withdrawal of 4-OHT led to a significant decrease ($P < 0.05$) in miR-9 expression (Fig. 1*F*). Together, our data indicate that miR-9 is a direct target of MLL fusion proteins.

miR-9 Promotes Cell Survival and Blocks Apoptosis in Human *MLL*-Rearranged AML Cells. To determine the role of miR-9 in AML, we performed both loss- and gain-of-function studies in human *MLL*-rearranged AML cells. Transfection of miR-9 inhibitors (i.e., antagomiR oligos) into MONOMAC-6 cells led to a significant decrease in cell viability and an increase in apoptosis (Fig. 2*A*), as well as a significant decrease in cell growth/proliferation (Fig. 2*B*). Similar phenomena were observed in THP-1, another AML cell line containing *t*(9;11) (Fig. S2). Conversely, we found that forced expression of miR-9 caused a significant increase in cell viability and a decrease in apoptosis (Fig. 2*C*). In addition, in a model of monocytic differentiation of THP-1 cells induced by phorbol-12-myristate-13-acetate (PMA) (24), we found that miR-9 expression was significantly down-regulated during the differentiation (Fig. S3), implying that miR-9 may also play a role in cell differentiation.

miR-9 Plays a Critical Oncogenic Role in MLL Fusion–Mediated Cell Transformation In Vitro. Through in vitro colony-forming/replating assays, we show that, although forced expression of miR-9 alone is not sufficient to transform normal hematopoietic progenitor cells, miR-9 can significantly enhance *MLL-AF9*–mediated cell transformation (Fig. 3 *A* and *B*). Conversely, cotransduction of *MLL-AF9* and miR-9 sponge [competitive inhibitors of miR-9 (22)] result in a significant decrease of colony numbers (Fig. 3*C*) and a dramatic reduction of blast cell proportion in colony cells (Fig. 3*D*) compared with cotransduction of *MLL-AF9* and sponge control. Notably, we also found a synergistic effect in cell transformation between *HOXA9* and miR-9 (Fig. S4) but not between *HOXA9* and miR-196b [an miRNA that regulate both oncogenic and tumor-suppressor targets (10)]. Thus, miR-9 plays an important oncogenic role in cell transformation mediated by MLL fusions, probably through a synergetic effect with other oncogenic downstream targets (e.g., *HOXA9*) of MLL fusions.

miR-9 Enhances MLL Fusion–Mediated Leukemogenesis In Vivo. To investigate in vivo pathological function of miR-9, we retrovirally cotransduced BM progenitor cells (CD45.2) with MSCVneo+MSCVpig (control), MSCVneo+MSCVpig-miR-9 (miR-9), MSCVneo-*MLL-AF9*+MSCVpig (*MLL-AF9*), and MSCVneo-*MLL-AF9*+MSCVpig-miR-9 (*MLL-AF9*+miR-9) and then cultured them on methylcellulose medium for 7 d to form colonies to select transduction double-positive cells. Colony cells were then transplanted into lethally irradiated CD45.1 mice. As expected, although forced expression of miR-9 alone could not induce leukemia in transplanted mice, recipient mice transplanted with both miR-9 and *MLL-AF9* developed AML (Fig. S5*A*) with a significantly shorter latency compared with the mice transplanted with *MLL-AF9* alone (median overall survival: 64 vs. 79 d; $P = 0.001$; Fig. 4*A*). Furthermore, all mice in the *MLL-AF9*+miR-9 group exhibited a remarkable increase in the proportion of c-Kit$^+$ blast cells in the BM, spleen, and peripheral blood compared with *MLL-AF9* mice (Fig. 4*B*; Fig. S5*B*; Table S2). Morphologically, *MLL-AF9*+miR-9 leukemic BM cells exhibited a higher degree of immature/blast counts than *MLL-AF9* leukemic BM cells (Fig. 4*C*). Histological analysis also showed a greater degree of leukemic infiltration of spleen and liver in *MLL-AF9*+miR-9 mice compared with *MLL-AF9* mice (Fig. 4*D*). Collectively, *MLL-AF9*+miR-9 mice exhibited a much more aggressive leukemic phenotype than *MLL-AF9* mice, highlighting the important oncogenic role of miR-9 in the pathogenesis of *MLL*-rearranged leukemia.

Identification of Potential Target Genes of miR-9 in *MLL*-Rearranged AML. We analyzed four major miRNA target prediction programs including TragetScan, PITA, miRanda, and miRBase Targets and identified a total of 4,941 genes as putative targets of miR-9 in both human and mouse genomes as predicted by at least one of the four programs. To identify potential target genes of miR-9 in AML, we also performed mRNA expression profiling of 79 of the 100 human samples used in the miRNA expression profiling, 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 cell samples) by use of an Agilent custom-design microarray platform (14). Through correlation of expression of miR-9 with that of its putative target genes across the 79 samples, we found that 170 putative targets exhibited a significantly inverse correlation of expression ($r < -0.2$, $P < 0.05$, Pearson correlation) with miR-9 (Table S3). Of the 170 genes, 80 also exhibited a significant inverse correlation of expression ($r < -0.4$, $P < 0.05$, Pearson correlation) with miR-9 across the 18 samples of *MLL*-rearranged AML ($n = 9$) and normal controls ($n = 9$). In addition, we also performed Affymetrix exon arrays of 13 human *MLL*-rearranged AML samples and 9 normal controls (including 3 each of CD34$^+$, CD33$^+$, and MNC samples) (10). We found that of these 80 potential target genes, 31 exhibited a significant down-regulation in *MLL*-rearranged AML samples compared with the normal controls (Table S3). Moreover, in analysis of Affymetrix gene arrays of nine mouse *MLL-AF9* leukemic samples and six control samples (10, 14, 21), we found that 17 of the 31 candidate targets were also significantly down-regulated in mouse *MLL-AF9* leukemic samples relative to the normal controls (Table S3). Thus, these 17 genes (including *CPEB4*, *CYFIP2*, *ENDOD1*, *HBP1*, *JAK1*, *KLF6*, *LHFPL2*, *MAP3K8*, *RAB8B*, *RHAG*, *RHOH*, *RYBP*, *SERPINB9*, *TAL1*, *TFRC*, *TRAK2*, and *VAMP5*) are highly likely targets of miR-9 in *MLL*-rearranged AML (see Fig. 5 for their expression profiles).

We then chose *RHOH* and *RYBP*, two genes that show the greatest inverse correlation of expression with miR-9 in the set of 79 samples (Fig. 5*A*), as candidate targets for further validation. As expected, both genes are significantly down-regulated in human hematopoietic stem/progenitor cells transduced with *MLL-AF9* (Fig. 6*A*), in a manner opposite to miR-9 (Fig. 1*C*). In the presence of *MLL-AF9*, forced expression of miR-9 can cause a further significant repression of *Rhoh* and *Rybp* expression in mouse BM progenitor cells (Fig. 6*B*), and a similar pattern was observed in human MONOMAC-6 cells (Fig. 6*C*). Thus, these results supported the likelihood that miR-9 negatively regulates the expression of these potential targets.

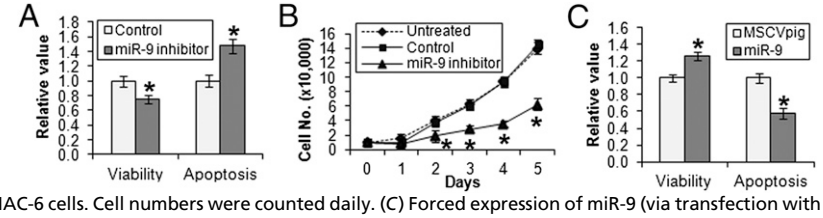

Fig. 2. Effects of miR-9 on cell viability, proliferation, and apoptosis of human MLL-rearranged AML cells in vitro. (*A*) Transfection of miR-9 inhibitor (i.e., anti-miR-9 antagomiR oligos) into MONOMAC-6 cells results in a significant decrease of cell viability and an increase in apoptosis compared with transfection of antagomiR scrambled control oligos (as control). (*B*) Inhibition of miR-9 by miR-9 inhibitor also significantly suppresses cell growth and proliferation of MONOMAC-6 compared with control and untreated MONOMAC-6 cells. Cell numbers were counted daily. (*C*) Forced expression of miR-9 (via transfection with MSCVpig-miR-9) significantly increases cell viability and decreases apoptosis of MONOMAC-6 cells compared with transfection with MSCVpig empty vector control. Cell viability and apoptosis were evaluated 48 h after transfection. *$P < 0.05$, two-tailed *t* test.

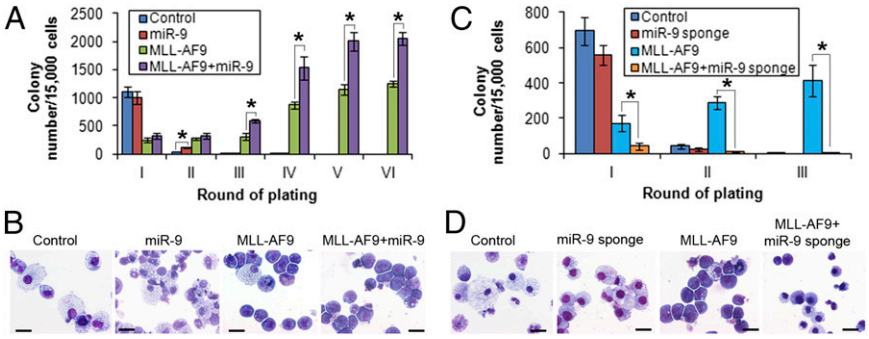

Fig. 3. In vitro colony-forming/replating assays. (*A*) Forced expression of miR-9 promoted MLL-AF9–mediated cell transformation. Normal mouse BM progenitor cells were retrovirally transduced with MSCVneo+MSCVpig (i.e., Control), MSCVneo+MSCVpig-miR-9 (i.e., miR-9), MSCVneo-*MLL-AF9*+MSCVpig (i.e., MLL-AF9), or MSCVneo-*MLL-AF9*+MSCVpig-miR-9 (i.e., MLL-AF9+miR-9) and then plated into methylcellulose medium under double selection of puromycin and G418 to form colonies. The colony cells were replated every 7 d for up to six passages. Mean± SD values of colony numbers are shown. (*B*) Cytospin morphology analysis of first passage of colony cells (see Fig. 3*A*) via Wright-giemsa staining. (*C*) Block of miR-9 function inhibited MLL-AF9–mediated cell transformation. Normal mouse BM progenitor cells were retrovirally transduced with MSCVneo+pBABE-puro-scrambled sponge (i.e., Control), MSCVneo+pBABE-puro-miR-9 sponge (i.e., miR-9 sponge), MSCVneo-MLL-AF9+pBABE-puro-scrambled sponge (i.e., MLL-AF9), or MSCVneo-MLL-AF9+pBABE-puro-miR-9 sponge (i.e., MLL-AF9+miR-9 sponge), and colony forming and replating were conducted as described above for up to three passages. Mean ± SD values of colony numbers are shown. (*D*) Cytospin morphology analysis of first passage of colony cells (see Fig. 3*C*) via Wright-giemsa staining. The length of bars in *B* and *D* represents 10 μm. $*P < 0.05$; $**P < 0.01$; two-tailed *t* test.

EVI1 Has No Apparent Effect on Expression of miR-9 in *MLL*-Rearranged AML. Senyuk et al. reported recently that ecotropic viral integration site 1 (EVI1) could repress the expression of miR-9, with antagonistic effects on myelopoiesis and EVI1-induced leukemogenesis (25). Interestingly, previously studies indicate that *Evi1* is a transcriptional target of MLL fusion proteins (26). Consistent with a previous report that *EVI1* was aberrantly overexpressed in ∼40–50% of *MLL*-rearranged AML patients (27), we found that *EVI1* was expressed at a higher level in four (44%) of our nine patients with *MLL*-rearranged AML than its average level in the nine patients (Fig. S6*A*). We classified these four samples as "EVI1-high" and the remaining five samples as "EVI1-low." As expected, *EVI1* is expressed at a significantly higher level in the EVI1-high group than in both the EVI1-low group and the normal control group, but there is no significant difference between the latter two groups (Fig. S6 *A* and *B*). Notably, there is a significantly positive correlation of expression ($r = 0.29$, $P = 0.01$; Pearson correlation) between miR-9 and *EVI1* across our 79 human samples (Fig. S6*A*). This positive correlation is largely attributed to the co-overexpression of miR-9 and *EVI1* in the subset of *MLL*-rearranged AML, and when *MLL*-rearranged AML samples were excluded from the correlation analysis, no significance ($r = -0.028$, $P = 0.81$; Pearson correlation) was observed across the remaining 70 samples. In contrast to the previous report that forced expression of EVI1 could significantly repress expression of miR-9 in normal hematopoietic progenitor cells (25), we show here that in *MLL*-rearranged AML, miR-9 expression level in the EVI1-high group is even higher than in the EVI1-low group, although the difference is not statistically significant (Fig. S6 *C* and *D*). Thus, our data indicate that MLL fusion–mediated up-regulation of miR-9 expression likely overrides EVI1-mediated suppression in *MLL*-rearranged AML cells.

Discussion

Here we show that as many as 29 miRNAs are significantly up-regulated, whereas only 1 miRNA (i.e., miR-495) is significantly down-regulated, in *MLL*-rearranged AML compared with normal controls and other AMLs. It should not be a surprise that the miRNAs specifically dysregulated in *MLL*-rearranged AML are predominantly up-regulated ones, because previous studies from us and others have shown that MLL fusion proteins function predominantly as transcriptional activators, rather than inhibitors, in regulating expression of downstream targets (10, 13, 16, 19, 28). In particular, miR-9 is the most specifically and consistently up-regulated miRNA in *MLL*-rearranged AML compared with normal controls and other AMLs. Similar to miR-17-92 and miR-196b (10, 13), we demonstrate that miR-9 is also a direct target of MLL fusion proteins.

Both tumor suppressor and oncogenic roles of miR-9 have been reported in various solid tumors (22, 29–31). However, its

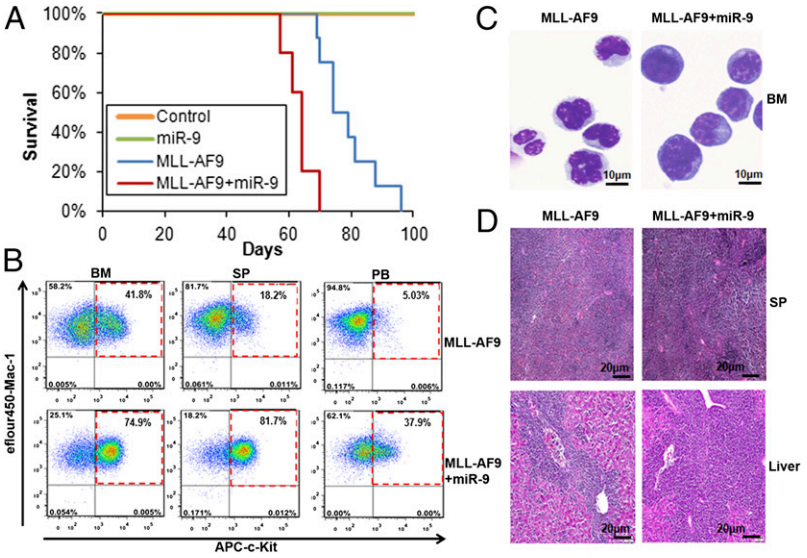

Fig. 4. miR-9 promotes *MLL-AF9*–mediated leukemogenesis in vivo. (*A*) Kaplan-Meier survival curves of lethally irradiated CD45.1 recipient mice, which were reconstituted with colony cells derived from CD45.2 mouse BM progenitor transduced with MSCVneo+MSCVpig (control, $n = 5$), MSCVneo+MSCVpig-miR-9 (miR-9; $n = 5$), MSCVneo-*MLL-AF9*+MSCVpig (*MLL-AF9*; $n = 8$), or MSCVneo-*MLL-AF9*+MSCVpig-miR-9 (*MLL-AF9*+miR-9; $n = 5$). Overexpression of miR-9 accelerates *MLL-AF9*–mediated leukemia ($P = 0.001$, log-rank test) compared with the *MLL-AF9* alone group. (*B*) Flow cytometric analysis of bone marrow (BM), spleen (SP), and peripheral blood (PB) from *MLL-AF9* and *MLL-AF*+miR-9 representative mice stained for c-Kit/Mac-1. (*C*) Wright-Giemsa–stained BM cytospin. (*D*) H&E-stained SP and liver sections showing massive leukemic infiltration.

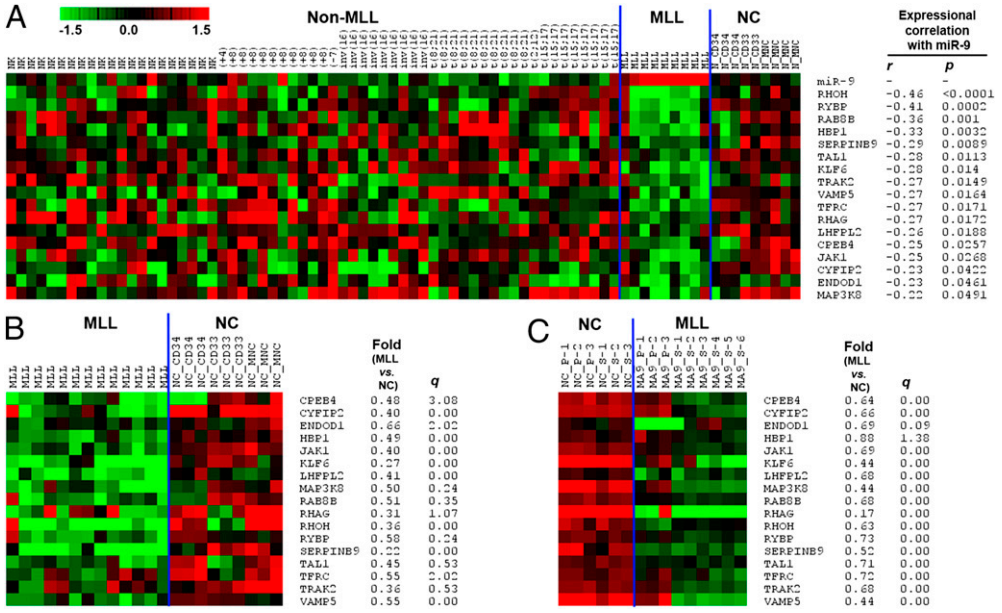

Fig. 5. Expression profiles of 17 candidate target genes of miR-9 in *MLL*-rearranged AML. Expression data were mean centered, and the relative value for each sample is represented by a color, with red representing high expression and green representing low expression (scale shown in the upper left). (*A*) Expression profiles of miR-9 and its 17 candidate target genes in the 79 human sample set (including 9 *MLL*-rearranged AML, 61 non–*MLL*-rearranged AML, and 9 normal control samples) and their expressional correlations are shown. (*B*) Expression profiles of the 17 candidate target genes in the 22 human sample set including 13 *MLL*-rearranged AML and 9 normal control samples. (*C*) Expression profiles of the 17 candidate target genes in the 15 mouse sample set including 9 *MLL*-rearranged AML and 6 normal control samples. The fold changes and corresponding *q* values (as detected by SAM) of the 17 target genes in *MLL*-rearranged AML samples compared with normal controls are shown in *B* and *C*.

role in leukemia is unclear. Here we show that, although gain- or loss-of-function of miR-9 alone does not exhibit any significant effect on cell transformation, its forced expression can significantly enhance MLL-AF9– or HOXA9-mediated transformation of normal mouse BM progenitor cells in vitro. Depletion of miR-9 expression can significantly inhibit viability/growth and promote apoptosis of human *MLL*-rearranged AML cells, and the opposite is true when expression of miR-9 is forced. Moreover, forced expression of miR-9 can significantly accelerate MLL-AF9–mediated leukemogensis in transplanted mice, leading to a more aggressive leukemic phenotype. Thus, our data clearly indicate that miR-9 plays an essential oncogenic role in the pathogenesis of *MLL*-rearranged AML. Notably, a very recent study reported a potential tumor suppressor role for miR-9 in EVI1-mediated leukemogenesis (25). EVI1 is also a direct downstream target of MLL fusion proteins (26). We show that co-overexpression of miR-9 and EVI1 occurs in around half of the *MLL*-rearranged AML cases, and the expression level of miR-9 in the EVI1-high subgroup is even relatively higher than that in the EVI1-low subgroup of *MLL*-rearranged AML, suggesting that EVI1-mediated repression is overridden by MLL fusion–mediated up-regulation of miR-9 expression in *MLL*-rearranged AML. Therefore, those data together suggest that miR-9's role in tumorigenesis is context dependent.

In addition, we identified 17 potential targets of miR-9 through analyses of a series of human and mouse leukemia sample sets (Fig. 5; Table S3). The miR-9–mediated repression of two representative potential targets (i.e., *RHOH* and *RYBP*) has been validated. RYBP (RING1 and YY1-binding protein), a polycomb complex-associated protein, can stabilize p53 by modulating MDM2 and thus has tumor suppressor activity (32). In contrast, *RHOH*, encoding a hematopoietic-specific, GTPase-deficient member of the Rho GTPase protein family, was first identified as a partner of *BCL6* in a fusion transcript resulting from *t*(3;4)(q27; p13) in a non-Hodgkin lymphoma cell line (33) and was aberrantly overexpressed in B-cell chronic lymphocytic leukemia (CLL), in which it played an essential oncogenic role (34). However, in AML, low expression of *RHOH* has been shown to be an independent unfavorable prognostic factor for both overall and disease-free survival of the patients and contributes to chemotherapy

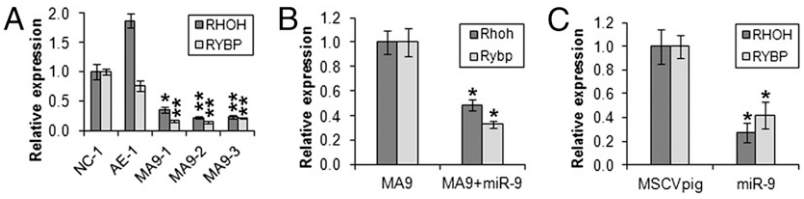

Fig. 6. *RHOH* and *RYBP* are potential direct targets of miR-9. (*A*) Relative expression of *RHOH* and *RYBP* in normal human cord blood $CD34^+$ cells transduced with MSCV-*MLL-AF9* (MA9-1, -2, and -3), MSCV-AML1-ETO (AE-1), or empty vector (NC-1) (15). (*B*) Relative expression of *Rhoh* and *Rybp* in normal mouse BM progenitor cells transducted with MSCVneo-*MLL-AF9*+MSCVpig (MA9) or MSCVneo-*MLL-AF9*+MSCVpig-miR-9 (MA9+miR-9). Cells used for the analysis are passage II colony cells from the in vitro colony-forming/replating assay shown in Fig. 3A. (*C*) Relative expression of *RHOH* and *RYBP* in human MONOMAC-6 cells 48 h after transfection with empty vector (MSCVpig) or miR-9 (MSCVpig-miR-9). Expression level of each target gene in the control group in each plot was set to 1 for comparison and statistical analysis. $*P < 0.05$; $**P < 0.01$; two-tailed *t* test.

resistance in leukemia cells (35), implying a tumor suppressor role in AML. Similarly, *TAL1* is a common target of chromosomal rearrangements in T-cell acute lymphoblastic leukemia (T-ALL) and is aberrantly expressed in up to 60% of pediatric T-ALL, in which it plays an oncogenic role (36); however, the down-regulation of *TAL1* has been frequently observed in AML (37, 38). Thus, it is likely that both *RHOH* and *TAL1* are oncogenes in lymphoblastic leukemia but serve as tumor suppressors in myeloid leukemia. Indeed, a recent study showed that Notch signaling, a main oncogenic trigger of T-ALL, plays an essential tumor suppressor role in myeloid leukemia (39). Several other potential targets such as *HBP1*, *KLF6*, and *SERPINB9* are also tumor suppressors in myeloid leukemia (40–42). Therefore, it is quite possible that miR-9 plays a critical oncogenic role in the pathogenesis of *MLL*-rearranged AML through targeting a group of tumor suppressor genes.

Thus far, we showed that MLL fusion proteins could directly up-regulate expression of miR-9, miR-17-92 (8, 11, 13), and miR-196b (10), and all of them play oncogenic roles in the pathogenesis of *MLL*-rearranged leukemia. It would be important to study how these miRNAs work together in promoting leukemogenesis. These miRNAs may regulate different sets of critical targets in distinct pathways and thus contribute to leukemogenesis through different pathways and may also regulate some common target genes or different components in the same pathways to achieve a synergistic effect in inhibiting the corresponding pathways. Clearly, our studies demonstrate that miRNAs are functionally important mediators in the pathogenesis of *MLL*-rearranged leukemia. Comprehensive understanding of their roles and identification of their essential target genes would broaden and deepen our understanding of the complex molecular mechanisms underlying MLL fusion–mediated leukemogensis, which may lead to the development of effective targeted therapy strategies to treat this presently therapy-resistant disease.

Materials and Methods

Exiqon miRCURY LNA arrays, Agilent's custom-design microarrays, and Affymetrix exon or gene arrays have been used for miRNA and mRNA expression profiling. ChIP, cell apoptosis and viability assays, colony-forming/replating assays, and bone marrow transplantation (BMT) were performed as described previously (8, 10, 13, 14, 21), with some modifications. Additional details can be found in *SI Materials and Methods*.

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