miR-150 Blocks MLL-AF9–Associated Leukemia through Oncogene Repression

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Abstract
The microRNA miR-150, a critical regulator of hematopoiesis, is downregulated in mixed-lineage leukemia (MLL). In this study, miR-150 acts as a potent leukemic tumor suppressor by blocking the oncogenic properties of leukemic cells. By using MLL-AF9–transformed cells, we demonstrate that ectopic expression of miR-150 inhibits blast colony formation, cell growth, and increases apoptosis in vitro. More importantly, ectopic expression of miR-150 in MLL-AF9–transformed cells completely blocked the development of myeloid leukemia in transplanted mice. Furthermore, gene expression profiling revealed that miR-150 altered the expression levels of more than 30 “stem cell signature” genes and many others that are involved in critical cancer pathways. In conclusion, we demonstrate that miR-150 is a potent leukemic tumor suppressor that regulates multiple oncogenes.

Implications: These data establish new, key players for the development of therapeutic strategies to treat MLL-AF9–related leukemia. Mol Cancer Res; 11(8); 912–22. ©2013 AACR.

Introduction
The mixed lineage leukemia (MLL) gene, located in 11q23 region, encodes an evolutionarily conserved histone H3K4 methyltransferase that is crucial for hematopoiesis. MLL is frequently involved in chromosomal translocations observed in both acute lymphoid leukemia (ALL) and acute myelogenous leukemia (AML; ref. 1). More than 60 different MLL fusion partners have been identified (2, 3). MLL fusion proteins confer an unusual ability to transform hematopoietic cells into leukemic stem cells (LSC; refs. 3–5). The identification and characterization of key players and downstream networks by which MLL converts hematopoietic cells into LSCs are of major interest. To develop successful treatment or even elimination of MLL-associated leukemia, extensive studies on the regulatory mechanism underlying MLL fusion protein-induced transformation and progression have been carried out. In particular, Myb has been identified as an important downstream target for MLL-fusion proteins and is essential for LSC transformation (6, 7). Moreover, Zuber and colleagues showed that MLL-AF9 fusion protein contributes to leukemia maintenance by enforcing a Myb-dependent aberrant self-renewal program (7). However, important questions about the intracellular mechanisms governing MLL-induced LSC transformation and progression remain unanswered.

The discovery of miRNAs provides unique opportunities to identify a new set of players that could modulate LSC behavior. miRNAs are a class of highly conserved small endogenous RNAs (~22 nucleotides) that bind to complementary sequences in target mRNAs to trigger either a blockage in translation and/or mRNA degradation (8). Experimental and computational studies show that each miRNA targets multiple mRNAs, and that almost 50% of mammalian mRNAs are targeted by one or more miRNAs (8). Several publications have reported that deregulation of miRNA expression is associated with hematologic malignancies (9–12).

We previously described miR-150 as a key miRNA in B-cell development (13). Interestingly, miR-150 has been shown, in different cell types, to directly target Myb, a key player in MLL-related leukemia (6, 7, 14–16). However, Myb alone is essential but not sufficient for MLL fusion proteins-mediated transformation (6). Therefore, we have investigated the possible functional role of miR-150 in a MLL leukemia context. The analysis of different miRNA expression profiles in leukemias showed that miR-150 was downregulated in hematologic malignancies and in particular in MLL-related leukemia (17–21). We hypothesize that miR-150 suppression contributes to leukemic transformation by
Materials and Methods

Leukemic cell generation and colony-forming cell assay
CD45.1 C57BL/6 mice were purchased from the National Cancer Institute (Bethesda, MD). CD45.2 C57BL/6 mice were obtained from the Jackson Laboratory. All mice were maintained at the animal facilities of the Texas A&M University (College Station, TX) or the Whitehead Institute for Biomedical Research (Cambridge, MA). All animal experiments were carried out with the approval of the Texas A&M University and Massachusetts Institute of Technology (MIT, Cambridge, MA) Committee on Animal Care.

Bone marrow cells from 4- to 6-week-old mice (C57BL/6) were collected and depleted with hematopoietic stem/progenitor cell enrichment kit using the iMAG system (BD Pharmingen). Lineage-negative cells (Lin−) were then cultured overnight in Iscove’s modified Dulbecco’s medium (IMDM) in the presence of Stem Cell Factor (SCF) (20 ng/mL), interleukin (IL)-3 (10 ng/mL), and IL-6 (10 ng/mL; ref. 22). Lin− cells were then spin infected with retroviral supernatant to introduce the MLL-AF9 fusion gene (MSCV-hygro-AF9) at 2,000 rpm for 1.5 hours at 32°C. Cells were cultured in methylcellulose media with SCF (20 ng/mL), IL-3 (10 ng/mL), IL-6 (10 ng/mL), Flt3-L (50 ng/mL), and 200 μg/mL hygromycin B for selection. After at least 3 rounds of replating, cells were then infected with retroviruses driving miRNA and GFP expressions (MDH-miR-150, MDH-miR-195, or empty construct MDH). For colony-forming cell assay, GFP+ MLL-AF9−sorted cells that either express GFP and miRNAs of interest (miR-150) or GFP only (MDH) were plated at 2,000 cells per well in methylcellulose media supplemented with 20 ng/mL of SCF, 10 ng/mL of IL-3, and 10 ng/mL of IL-6. Myeloid colonies were scored after 10 days in culture. The presented data correspond to the mean of at least 3 independent experiments.

Proliferation and apoptosis assay
Two days after infection, the percentage of GFP+ cells was evaluated in MLL-AF9-miR-150, MLL-AF9-miR-195, and MLL-AF9-MDH populations and GFP+ cells were mixed with noninfected MLL-AF9 cells to obtain 12% of GFP+ cells for each condition. Cells were seeded at 1 × 104 cells/mL in the IMDM medium supplemented with 10% FBS, 20 ng/mL SCF, 10 ng/mL IL-3, 50 ng/mL Flt3L, and 10 ng/mL IL-6. Percentage of GFP+ cells was evaluated by flow cytometry at 24 and 48 hours. For apoptosis analysis, cultured cells were stained using the Annexin V−PE/propidium iodide (PI) apoptosis detection kit (eBioscience) according to the manufacturer’s instructions.

Flow cytometry analysis
Antibodies were purchased from eBioscience if not specified otherwise. For lineage detection and stem cell marker detection, we used fluorescence-tagged antibodies: phycoerythrin (PE)-anti-CD11b, fluorescein isothiocyanate (FITC)-anti-Sc-a, PE-Cy5.5, FITC or Allophycocyanin (APC)-anti-c-kit, APC-anti-CD16/32 (FCRyI/III), PE-anti-CD34, APC-anti-Gr-1, PE-anti-CD19, and PE-anti-CD3e. For Lin− cell detection, we used biotin-conjugated anti-CD3e, anti-CD5, anti-B220, anti-CD11b, anti-Gr-1, and anti-Ter119 followed by streptavidin-APC or streptavidin-PE. For apoptosis analysis, we used Annexin V apoptosis detection kit from eBioscience. Cells were then analyzed using a BD BTM LSRII or an Accuri C6 Flow Cytometer System (BD Biosciences). Data were analyzed using BD FACSDiva, Flowjo software or Accuri C6 software.

Bone marrow transplantation assays
Bone marrow transplantations were conducted as previously described (13, 23). In brief, CD45.1 recipient mice were lethally irradiated (10 Gy) and retro-orbitally injected with 2 × 105 MLL-AF9-GFP+−sorted bone marrow cells (CD45.2+) and 1 × 108 bone marrow competitor cells isolated from CD45.1 C57BL/6 wild-type (WT) mice. Peripheral blood was harvested once a month for fluorescence-activated cell sorting (FACS) analysis. Mice were sacrificed when moribund. Blood, bone marrow, and spleen were harvested and processed into single cells suspensions and treated with ammonium chloride to lyse red cells (Stemcell Technologies) for flow cytometry analysis. All cells were blocked with anti-mouse CD16/CD32 (FcγRII/III) receptors; BD Pharmingen) and stained with APC-anti-CD4, PE-anti-CD19, APC-anti-Gr-1, PE-anti-CD11b, and PE-Cy7 anti-B220 (eBiosciences) for B cells, PE-anti-CD8 (BD Pharmingen), and PE-Cy7 anti-CD4 (eBiosciences) for T cells, PE-anti-CD11b (BD Pharmingen) and PE-Cy7 anti-Gr1 (eBiosciences) for myeloid cells. Peripheral blood smears were stained with May-Grünwald Giemsa. Our results are from 13 mice for the control group and 17 mice for the miR-150 group from 4 independent injections.

Gene expression microarray and data analysis
Total RNAs from 3 independently sorted GFP+ MLL-AF9 cells infected with MDH-miR-150 or empty control MDH were extracted using protocols described earlier. Gene expression analyses were conducted by the Genome Technology Core facility at Whitehead Institute for Biomedical Research using Agilent mouse 4 × 44 multiplexing arrays. Differential expression was determined by a moderated t test using R software with the “limma” package (corrected for false discovery rate as <0.005) and normalized using a LOWESS filter. Gene expression profiles are deposited into the Gene Expression Omnibus under access Number (GSE30771).

Luciferase reporter assay
The full 3′-untranslated region (3′-UTR) sequence of the predicted target gene or at least 250-bp flanking the predicted miR-150−binding site(s) was cloned into the psiCheck2 Vector (Promega) downstream of the Renilla
luciferase-coding region. For target site-specific mutation generation, we used a Quikchange mutagenesis kit (Stratagene) following manufacturer’s instructions. One day before transfection, HEK293 cells were seeded into 96-well white plates at 1 to 2 × 10^5 cells per well. Cells were cotransfected with 10 nmol/L of miR-150 mimic or control mimic (Dharmacon) and 10 ng of constructs by using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen). The luciferase activity was measured 48 hours posttransfection using Dual-Glo Luciferase kit (Promega) and TECAN luminescence reader. Renilla luciferase signal, which accounts for the effect of miR-150 on the 3′-UTR of the Renilla gene, was normalized with Firefly luciferase signal, which is an internal control. The presented data correspond to the mean of at least 3 independent experiments.

Quantitative RT-PCR analysis for miRNAs and gene expression

Total RNA was extracted from collected cells or tissues using Trizol extraction protocol according to the manufacturer’s instructions. miRNA expression levels were measured using TaqMan miRNA assays (Applied Biosystems) with primer pairs to each individual miRNAs. The data presented correspond to the mean of 2^−ΔΔCt from at least 3 independent experimental repeats and normalized to sno202 levels. For gene expression analysis, quantitative real-time PCR (qRT-PCR) was conducted with qScript One-Step RT-PCR kit with SYBR Green (Bio-Rad) using gene-specific primer pairs (available upon request) on Bio-Rad CFX384 according to the manufacturer’s protocol (Bio-Rad). The data presented correspond to the mean of 2^−ΔΔCt from at least 3 independent experimental repeats and normalized to β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reference genes.

Statistical analysis

For each data point derived from qRT-PCR assays represents an average of 3 technical replicates and data were averaged over independently replicated experiments (n = ≥ 3 independently collected samples) and analyzed using Student t test, presented as the mean fold-change over the mean of the control group ± SEM. Data analysis was conducted using software Graphpad Prism V5. A P value less than 0.05 was considered statistically significant. Unpaired t test with Welch correction was applied to evaluate the significance of the differences between each of the tested group. The cumulative distribution fractions (CDF) of miR-150 targets in gene expression profiles were generated and significant differences between tested groups were analyzed using nonparametric Mann–Whitney test. For overall group-effect significance, data were analyzed using two-way ANOVA analysis and Bonferroni posttest for each factor at individual times. Statistical analysis was conducted using GraphPad Prism software v5.04 (GraphPad Software). For Gene Ontology analysis, P values of term or pathway enrichment were calculated using DAVID (NIH, Bethesda, MD).

Results

miR-150 is downregulated in MLL-associated leukemia and ectopic expression of miR-150 blocks the oncogenic properties of MLL-AF9 cells in vivo

To investigate the role of miR-150 in MLL-induced leukemia, we compared miRNA expression profile datasets from previous publications (17–21). We observed that miR-150 was consistently downregulated in acute leukemia, in particular in MLL-related leukemia in which miR-150 is downregulated at least 1.6-fold compared with control samples (Supplementary Fig. S1). However, the impact of miR-150 downregulation on the oncogenic potency of MLL-expressing cells was not defined previously.

To understand the role of miR-150 in MLL-associated leukemias, we used a MLL-AF9 AML mouse model (22). First, we generated MLL-AF9–immortalized leukemic cells using a retroviral MLL-AF9 construct (22, 24, 25). After antibiotic selection by at least 3 rounds of consecutive replating, immortalized leukemic cells were analyzed by flow cytometry for c-Kit (progenitor marker) and CD11b (myeloid-specific marker) expression. Seventy-seven percent of the cells were myeloid leukemic cells exhibiting high c-Kit and CD11b levels, whereas CD19 and CD3e were undetectable (Supplementary Fig. S2A). May–Grünwald Giemsa staining also confirmed the immature morphology of these cells (Supplementary Fig. S2B).

To determine the impact of miR-150 on the oncogenicity of MLL-AF9–transformed cells, we adopted a transplantation assay in mice (Fig. 1A). MLL-AF9 leukemic cells (CD45.2+) were infected with the retrovirus encoding miR-150 and the GFP reporter gene (MLL-AF9-miR-150 cells) or the empty vector containing GFP only (MLL-AF9-MDH cells; ref. 13). After sorting, 2 × 10^5 GFP− MLL-AF9 cells (CD45.2−) with or without ectopic miR-150 expression were mixed with 1 × 10^6 Lin− bone marrow competitor cells (CD45.1+) and injected into lethally irradiated syngeneic mice (CD45.1−). Once a month, flow cytometry analysis was conducted to follow the evolution of disease by the presence of GFP+ leukemic cells in the peripheral blood.

Strikingly, none of the mice injected with MLL-AF9-miR-150 cells developed AML (17 mice total from 4 independent experiments), whereas all mice injected with MLL-AF9-MDH cells died of leukemia after 30 to 140 days (13 from 4 independent experiments; Fig. 1B). When MLL-AF9-miR-150 mice were sacrificed at the end of the experiment (after 1 year), there were no circulating GFP+ cells detected nor any significant changes of spleen size in MLL-AF9-miR-150 groups, whereas all mice injected with MLL-AF9-MDH cells displayed typical myeloid leukemic symptoms, such as enlarged spleen size and the presence of myeloblasts in the peripheral blood (Fig. 1C and D). Flow cytometry analysis confirmed the onset of AML in control mice with a CD11b+ phenotype in the peripheral blood, spleen, and bone marrow samples (Supplementary Fig. S3). These results show that miR-150 is a potent leukemic suppressor able to block the leukemia-initiating properties of MLL-AF9–expressing cells in vivo.
Restoration of miR-150 in MLL-AF9 cells impairs the colony initiation potency of LSCs

To understand the inhibitory mechanism of miR-150 in MLL-AF9 expressing cells, we evaluate the frequency of leukemia-initiating cells in a colony-forming assay (4, 5, 22). To this end, MLL-AF9–transformed cells were infected with a retrovirus encoding miR-150 and the GFP reporter gene (MLL-AF9-miR-150 cells) or the empty vector containing GFP only (MLL-AF9-MDH cells). qRT-PCR analysis from 3 batches of sorted GFPþ cells showed that miR-150 levels increased from 12- to 45-fold (Supplementary Fig. S4) in MLL-AF9-miR-150 cells compared with control cells, that is, less than the expression of miR-150 in mature B cells and higher than in the mature macrophages. After 10 days of culture in methylcellulose, we observed a significant decrease in the number of colonies derived from sorted MLL-AF9-miR-150 cells compared with control cells transduced with either empty vector (MLL-AF9-MDH) or miR-195, a miRNA with no significant change from gene expression profiles (refs. 17–21; Fig. 2A). We hypothesized that decreased expression of miR-150 in MLL-AF9 leukemic cells contributes to the leukemic progression by providing to leukemic cells an advantage due to enhanced proliferation and/or facilitating their escape from programmed cell death. To test the effects of miR-150 expression on cell growth, GFPþ MLL-AF9 cells expressing miR-150, miR-195, or the control vector were mixed with GFP−/C0 MLL-AF9 cells to obtained 12% of GFPþ cells in each condition. We observed a slight decrease of the percentage of GFPþ cells over time for MDH and miR-195 that could be explained by some loss of GFP expression as frequently observed for GFP constructs. In contrast, we observed a significant decrease of GFPþ cells for miR-150, reflecting the antiproliferative effect conferred by miR-150 ectopic expression (Fig. 2B).

Apoptosis was investigated by flow cytometry with Annexin V/PI staining on MLL-AF9 cells expressing or not miR-150. Our results showed a significant increase in the percentage of apoptotic MLL-AF9-miR-150 cells at 48 hours after infection compared with MLL-AF9-MDH or MLL-AF9-miR-195 control cells (Fig. 2C). Further cell-cycle analysis revealed that 48 hours after infection of miR-150 significantly decreased the percentage of cells in the S-phase compared with the empty vector control (MDH) or miR-195 transduction (Fig. 2D). These results showed that ectopic expression of miR-150 in MLL-AF9 leukemic cells inhibits cell survival by both repressing cell proliferation and inducing apoptosis.
Multiple oncogenes that are often upregulated in MLL leukemia are bona fide miR-150 targets

Myb has previously been described as a miR-150 target in different cell types and also participates in MLL fusion protein-mediated transformation; therefore, we investigated Myb mRNA levels in MLL-AF9–transformed cells infected or not with miR-150 (6, 7). As shown in Fig. 3A, we observed a downregulation of Myb mRNA in miR-150–overexpressing cells compared with MLL-AF9 control cells (Fig. 3A). Moreover, in cells transfected with a construct containing the Myb 3’-UTR with miR-150–binding sites (site 1 and 2) linked to a luciferase reporter gene (WT), miR-150 overexpression decrease luciferase activity, whereas in cells transfected with the same construct containing mutations in both miR-150–binding sites (mut), luciferase activity was not affected by miR-150 expression (Fig. 3B and Supplementary Fig. S5).

These results suggest that downregulation of miR-150 observed in MLL-leukemia, and the miR-150–dependent derepression of Myb, could be an important contributor to the transformation process induced in MLL-AF9 AML. Because Myb has been shown to be essential but not sufficient for MLL fusion protein mediated transformation (7), we hypothesized that miR-150 is a tumor suppressor miRNA regulating expression of other important genes that also play a role in MLL-leukemia.

To identify other miR-150 targets, we conducted gene expression profile analyses of MLL-AF9-miR-150 and MLL-AF9-MDH infected cells using 3 independent batches of GFP ÷-sorted cells. Among a total of 21,611 unique genes detected, 319 genes were upregulated more than 1.5-fold, whereas 1,551 genes were downregulated more than 1.5-fold in MLL-AF9-miR-150 cells compared with control cells. For genes that are downregulated more than 1.5-fold in MLL-AF9-miR-150 cells, bioinformatic analysis indicated an enrichment of genes involved in leukocyte differentiation or negative regulation of apoptosis (Fig. 3C). By using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, miR-150 was found to significantly suppress genes that are involved in several cancer pathways (Fig. 3D)

The list of genes with decreased mRNA levels in cells expressing miR-150 was compared with the list of predicted miR-150 target determined by 2 miRNA target prediction algorithms, TargetScan (26) and PicTar (27). Among 1,188 miR-150–predicted targets that are detected in our gene expression profile, 106 genes containing at least one conserved miR-150–binding site were downregulated. Cumulative gene expression distribution analysis confirmed the
enrichment of genes containing a predicted miR-150–binding site(s) in their 3′-UTR among the downregulated genes compared with genes without miR-150–predicted binding site (Fig. 4A).

CBL and Egr2 were selected as putative miR-150 targets as they are known to be deregulated in leukemia and contain predicted miR-150–binding sites in their 3′-UTR (28–33).

We confirmed downregulation of these genes by qRT-PCR as shown in Fig. 4B. To show if these genes are directly targeted by miR-150, we used a luciferase reporter assay with constructs containing the 3′-UTR of CBL or Egr2 with miR-150–predicted target sites inserted downstream of the Renilla luciferase open reading frame in the psicheck2 vector. The c-Myb 3′-UTR mut corresponds to the same construct with an internal mutation in the binding site for miR-150. Each construct was cotransfected in HEK293 cells with miR-150 mimics or control mimics and luciferase activity was assessed 2 days after transfection. Renilla activity was normalized to the firefly internal psiCheck control. Presented results correspond to the relative luciferase activity normalized to transfections with control mimics (n = 9; **, P < 0.001). C, a Gene Ontology study was conducted to analyze the biologic function of the genes that are downregulated more than 1.5-fold in MLL-AF9-miR-150 cells as compared with MLL-AF9-MDH cells. D, KEGG pathways analysis shows an enrichment of genes involved in cancer pathways among the genes downregulated in MLL-AF9-miR-150 cells.

To validate whether these genes are bona fide miR-150 targets and involved in miR-150–mediated tumor suppression in the MLL leukemia context, we used specific short hairpin RNA (shRNA) to knockdown these genes and we also included Myb as a positive control. As shown in Fig. 5A using qRT-PCR experiments, we obtained good knockdown efficiency for CBL, Egr2, and Myb. MLL-AF9–transformed cells were then transduced with these shRNA and apoptosis was analyzed by Annexin V/PI staining. When CBL, Egr2, and Myb were knocked down in MLL-AF9 cells, increased apoptosis was observed compared with control cells (Fig. 5B). These results indicate that ectopic expression of miR-150 induces apoptosis of MLL-AF9 leukemic cells by simultaneous inhibition of multiple oncogenes.

On the basis of previous studies, we then examined expression of genes that were previously reported to be directly involved in MLL-associated leukemias using qRT-PCR analysis. Among the 14 tested genes, several crucial leukemic regulators were downregulated in MLL-AF9-miR-150–infected cells, including Meis1, CDK2, and HoxA7 (P < 0.05) but not in MLL-AF9 control cells.
Because these genes did not contain any miR-150–predicted binding site in their 3'–UTR, they are probably indirect miR-150 targets that could be regulated by CBL, Egr2, or Myb. To test this hypothesis, we evaluated their expression levels in MLL-AF9 cells infected with shRNA against CBL, Egr2, or Myb. As shown in Fig. 6B, CDK2 and HoxA7 are downregulated in the absence of Egr2 or Myb, whereas CBL knockdown only affects the expression of Meis1.

**miR-150 transduction into MLL-AF9 cells significantly alters the expression of stem cells genes**

It is well known that MLL-AF9 can transform normal hematopoietic stem cells (HSC) or granulocyte and macrophage progenitors (GMP) into LSC by activating pathways that are important for stem cells (3, 5, 22, 34). Elegant profiling analyses suggest that LSCs and HSCs share some crucial pathways for cell survival and self-renewal (3, 5, 22, 35). Thus, it is possible that the suppressive effects of miR-150 in MLL-AF9–transformed cells are mediated through subsets of these networks. We compared our gene expression profiles with 2 datasets comparing murine MLL LSC with HSC or GMP cells (36) with the expression of selected HSC and myeloid progenitor signature genes (37). We found that ectopic expression of miR-150 primarily affects genes that are enriched in HSCs and often upregulated in LSCs instead of GMPs. Out of 114 stem cell signature genes, 30 (26.3%) are downregulated more than 1.5-fold and 1 (0.8%) was upregulated by miR-150. Moreover, out of 290 myeloid progenitor signature genes, 38 (13%) are downregulated and 7 (2.4%) are upregulated by miR-150 and several ontology terms were significantly enriched, including cell cycle, chromosome organization, and leukocyte activation (Fig. 7B). These results suggest a crucial suppressive role of miR-150 in MLL-AF9 leukemic cells and loss of its expression facilitates leukemic cell survival partially by altering cell identity and activating cancer pathways.
Discussion

Several miRNAs play important roles in hematopoiesis and their deregulation can contribute to leukemogenesis (23, 38–44). However, the mechanisms of action of only a few oncogenic miRNAs have been investigated. In this study, we identified miR-150 as a new regulator for MLL-associated leukemia, one of the most aggressive leukemias with a poor prognosis. miR-150 is a key miRNA involved in hematopoiesis via suppression of Myb and other potential target genes (6, 7, 13, 14, 45, 46). Altered expression of crucial developmental regulators is often linked with diseases and cancer transformation, so it was not surprising...
to observe that altered expression of miR-150 was detected in multiple hematopoietic disorders. Indeed, miR-150 is downregulated in MLL-related leukemias. However, the function of miR-150 in leukemias has not been established and was therefore investigated in this study using a MLL-AF9 myeloid leukemia mouse model.

Our data show that restoring miR-150 expression in MLL-AF9–transformed primary leukemic cells prevents the development of leukemia in vivo and significantly reduces leukemic colony formation in vitro. Furthermore, MLL-AF9 leukemic cells transduced with miR-150 exhibited increased apoptosis and a decreased cell growth compared with control cells. These results suggest that elevation of miR-150 levels, in MLL-AF9 cells inhibits the survival of leukemic cells.

In a very recent publication, Jiang and colleagues showed that transplantation of mice with bone marrow cells overexpressing MLL-AF9 and miR-150 increases the overall survival compared with control MLL-AF9–transplanted mice (110 vs. 56 days) but all of the MLL-AF9–miR-150 mice died from AML (48). In our hands, all of the MLL-AF9-miR-150 mice survived until the end of the experiment (1 year posttransplantation). The differences observed could be explained by the methodologies used. In Jiang and colleagues’ article, normal bone marrow cells were transduced with MLL-AF9 and miR-150 and then directly injected in lethally irradiated mice (47). In our experiments, we first infected the Lin− bone marrow cells with MLL-AF9 and cultured them in methylcellulose media. After at least 3 round of replating and antibiotic selection, these cells were infected with miR-150 or MDR retroviruses and then sorted for GFP+ cells before transplantation in irradiated mice. In other word, we transplanted only double positive MLL-AF9 and miR-150 cells in the mice. In the Jiang and colleagues’ experiment, transplanted mice can also have cells infected only with MLL-AF9 or only with miR-150, which could explain why their mice died with MLL-AF9 and miR-150. So, our study allows the conclusion that miR-150 blocks the development of MLL-AF9–related leukemia.

An individual miRNA can suppress hundreds of genes directly and exert a significant impact on networks governing crucial cell processes (48). Indeed, our array analysis shows that genes bearing miR-150 target sites are more downregulated than genes without binding sites in their 3′-UTR. Gene ontology and pathway analyses show that genes downregulated in MLL-AF9 cells with miR-150 ectopic expression are enriched in genes involved in cancer pathways and cell cycle. Of note, among the genes that were downregulated and involved in the cancer pathway (KEGG pathway analysis), 24% were miR-150 predicted target genes. After screening more than 30 predicted miR-150 predicted targets using qRT-PCR and luciferase reporter assays, we confirmed that Myb is a miR-150 direct target and also identified 2 new miR-150 direct targets that are often highly expressed in MLL-associated leukemias; namely CBL and Egr2 (29–31, 35, 49). To confirm that the tumor suppressive activity of miR-150 is mediated through inhibition of these target genes, we used gene-specific shRNAs to knockdown Myb, CBL, and Egr2 and observed an induction of apoptosis in MLL-AF9–transformed cells.

Elegant studies by Armstrong and Cleary’s groups have shown that leukemic transformation can occur either at the HSC or GMP level (3, 5, 22, 34). Valuable information gained from gene expression profiles from HSC- or GMP-transformed LSCs suggests that LSCs acquire normal HSC properties as a result of stem/progenitor cell-specific gene expression and recruitment of pathways that are crucial for normal stem cell maintenance (3, 5, 22, 34). To understand the impact of miR-150 on MLL-AF9 LSCs, we conducted arrays to compare gene expression patterns of primary MLL-AF9-miR-150–infected cells with LSC, HSC, and GMP cells. Of note, 26% of genes that are enriched in HSCs or LSCs were downregulated in the presence of miR-150, whereas only 13% of myeloid progenitor signature genes were affected. Furthermore, multiple genes that are known to be important for HSCs and LSCs are also downregulated in MLL-AF9–miR-150 cells, including HOX A7, Mei1, and CDK2. These results suggest that miR-150 may repress genes that are pivotal for LSCs cancer initiation potency and survival.

In summary, we show that miR-150, a key miRNA in hematopoiesis, acts as a tumor suppressor through direct repression of multiple oncogenes in MLL-AF9–induced leukemias. miR-150 inhibits MLL-AF9 leukemic cell growth in vitro by induction of apoptosis and blocks the progression of MLL-AF9 leukemias in vivo. Current studies are focused on the mechanisms of miR-150 suppression in leukemias, thereby provide novel approaches for treating this aggressive disease.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: M. Bousquet, G. Zhuang, P. Wong, S. Safe, B. Zhou Development of methodology: M. Bousquet, G. Zhuang, P.S. Cherukuri, A.T. Shir, P. Wong, S. Safe, B. Zhou Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Bousquet, G. Zhuang, A.T. Shir, S. Wang, G. Wang, S. Safe, B. Zhou Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Bousquet, G. Zhuang, C. Meng, A.T. Shir, G. Ge, G. Wang, S. Safe, B. Zhou Writing, review, and/or revision of the manuscript: M. Bousquet, C. Meng, W. Ying, S. Safe, B. Zhou Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Zhuang, C. Meng, S. Wang, S. Safe, B. Zhou Study supervision: S. Safe, B. Zhou

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miR-150, a Tumor Suppressor in MLL-AF9 Leukemia

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References


32. Paluszczuk M. MiR-150, a Tumor Suppressor in MLL-AF9 Leukemia. Published OnlineFirst April 19, 2013; DOI: 10.1158/1541-7786.MCR-13-0002-T
Repression Associated Leukemia through Oncogene Repression

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