

# MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly *DNMT3A* and *3B* and indirectly *DNMT1*

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**Aberrant DNA hypermethylation contributes to myeloid leukemogenesis by silencing structurally normal genes involved in hematopoiesis. MicroRNAs (miRNAs) are noncoding RNAs that regulate gene expression by targeting protein-coding mRNAs. Recently, miRNAs have been shown to play a role as both targets and effectors in gene hypermethylation and silencing in malignant cells. In the current study, we showed that enforced expression of *miR-29b* in acute myeloid leukemia**

**cells resulted in marked reduction of the expression of DNA methyltransferases *DNMT1*, *DNMT3A*, and *DNMT3B* at both RNA and protein levels. This in turn led to decrease in global DNA methylation and reexpression of *p15<sup>INK4b</sup>* and *ESR1* via promoter DNA hypomethylation. Although down-regulation of *DNMT3A* and *DNMT3B* was the result of a direct interaction of *miR-29b* with the 3' untranslated regions of these genes, no predicted *miR-29b* interaction sites were found in the *DNMT1***

**3' untranslated regions. Further experiments revealed that *miR-29b* down-regulates *DNMT1* indirectly by targeting *Sp1*, a transactivator of the *DNMT1* gene. Altogether, these data provide novel functional links between miRNAs and aberrant DNA hypermethylation in acute myeloid leukemia and suggest a potentially therapeutic use of synthetic *miR-29b* oligonucleotides as effective hypomethylating compounds. (Blood. 2009; 113:6411-6418)**

## Introduction

DNA methylation consists of an enzymatic addition of a methyl group at the carbon 5 position of cytosine in the context of the sequence 5'-cytosine-guanosine (CpG) and is mediated by DNA methyltransferases (DNMTs).<sup>1</sup> The promoter regions of approximately 50% of human genes contain regions of DNA with a cytosine and guanine content greater than expected (so-called CpG islands) that, once hypermethylated, mediate gene transcriptional silencing.<sup>2</sup> Distinct roles in genomic methylation have been reported for DNMT isoforms. Whereas *DNMT1* preferentially replicates already existing methylation patterns, *DNMT3A* and *3B* are responsible for establishing de novo methylation.<sup>2</sup> Silencing of structurally normal tumor suppressor genes by aberrant DNA hypermethylation has been reported in hematologic malignancies, including subsets of acute myeloid leukemia (AML).<sup>3,4</sup> Although the mechanisms leading to aberrant DNA hypermethylation remain to be fully elucidated, increased levels of *DNMT1* and *DNMT3A* and *3B* have been observed in malignant myeloid blasts compared with normal bone marrow (BM) mononuclear cells (MNCs), suggesting that *DNMT* overexpression contributes to gene promoter hypermethylation and in turn to leukemogenesis.<sup>4</sup>

Growing evidence supports a role for microRNAs (miRNAs) as both targets and effectors in aberrant mechanisms of DNA hypermethylation.<sup>5,6</sup> miRNAs are noncoding RNAs of 19 to 25 nucleotides in length that regulate gene expression by inducing translational inhibition or cleavage of their target mRNAs through base pairing at partially or fully complementary sites.<sup>7</sup> Several groups have shown that miRNAs are altered in human malignancies and can function as tumor suppressor genes or oncogenes through expression regulation of their target genes.<sup>7</sup> Similar to tumor suppressor genes, miRNAs with tumor suppressor activity are often located in deleted genomic areas or are silenced by mutations or promoter hypermethylation in malignant cells.<sup>5,8-10</sup> Saito et al recently demonstrated that *miR-127* is silenced by promoter DNA hypermethylation and down-regulated in human bladder cancer and reexpressed by treatment with hypomethylating agents; this coincided with down-regulation of the oncogene *BCL6*, which is a bona fide target of *miR-127*.<sup>5</sup>

Our group recently reported that miRNAs are also involved in the control of DNA methylation by targeting the DNA methylation machinery.<sup>6</sup> We showed that, in lung cancer, the *miR-29* family directly targets *DNMT3A* and *3B*,<sup>6</sup> thereby leading to down-

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regulation of these genes, reduction of global DNA methylation (GDM), and reexpression of the DNA hypermethylated and silenced tumor suppressor genes *FHIT* and *WWOX*. Nevertheless, whether down-regulation of *DNMT3A* and *3B* by enforced expression of *miR-29* suffices in inducing efficient DNA hypomethylation in malignant cells or rather additional mechanisms are necessary to attain such an effect remains to be fully elucidated.

By testing the hypomethylating role of *miR-29b* in myeloid leukemogenesis, in the current study we demonstrated that expression of *miR-29b* promotes DNA hypomethylation not only through direct targeting of *DNMT3A* and *3B* but also by decreasing the *DNMT1* expression indirectly via down-regulation of *Sp1*, a known transactivating factor of the *DNMT1* gene. These results therefore support a previously unreported role of miRNAs in aberrant DNA methylation in AML and provide a pharmacologic rationale for the use of synthetic *miR-29b* as an efficient approach for therapeutic DNA hypomethylation of leukemic blasts.

## Methods

### Patients and cell lines

These AML cell lines, Kasumi-1, MV4-11, and K562, were obtained from ATCC (Manassas, VA). We chose cell lines with either low (MV4-11) or absent *miR-29b* (K562 and Kasumi-1). MNCs from BM samples with more than 70% blasts from 3 untreated AML patients were obtained from the Ohio State University (OSU) Leukemia Tissue Bank. Morphologic (French-American-British [FAB] classification) and cytogenetics/molecular characteristics of these primary AML samples were as follows: patient 1: FAB M2, normal karyotype, FLT3 internal tandem duplications negative; patient 2: FAB M2, complex karyotype (> 5 aberrations, including -7); patient 3: FAB M2, normal karyotype. Patients signed an informed consent in accordance with the Declaration of Helsinki to store and use their tissue for discovery studies according to OSU institutional guidelines under protocols approved by the OSU Institutional Review Board.

### Cell culture and transfection

Kasumi-1, MV4-11, and K562 cell lines were grown in RPMI medium supplemented with 10% fetal bovine serum or 15% fetal bovine serum (Kasumi-1) at 37°C. MNCs from BM AML samples were prepared by Ficoll-Hypaque (Sigma-Aldrich, St Louis, MO) gradient centrifugation and cultured with serum-free expansion medium (StemCell Technologies, Vancouver, BC) supplemented with granulocyte-macrophage colony-stimulating factor (50 ng/mL), interleukin-3 (20 ng/mL), interleukin-6 (20 ng/mL), and stem cell factor (100 g/mL) at 37°C. Synthetic pre-*miR-29b* oligonucleotides or scrambled oligonucleotides used as negative controls (Ambion, Austin, TX) at a final concentration of 50 nM were introduced into primary blasts or cell lines by nucleoporation (Amaxa Biosystems, Gaithersburg, MD) according to the manufacturer's instructions. Transfected cell lines were harvested at 24, 48, 72, or 96 hours. Primary blasts were harvested 24 hours after transfection. Cells were placed in cell lysis buffer for protein assay or in Trizol (Invitrogen, Carlsbad, CA) for RNA studies. MV4-11 cells were treated with 2.5 μM decitabine (Sigma-Aldrich) or phosphate-buffered saline (Sigma-Aldrich) for 72 hours.

### *miR-29b* lentivirus infection

The *miR-29b* lentivirus is a feline immunodeficiency virus-based construct purchased from Systems Biosciences (Mountain View, CA). This construct consists of the stem loop structure of *miR-29b-1* and 200 base pairs of upstream and downstream flanking genomic sequence cloned into the pMIF-cGFP-Zeo-miR plasmid (catalog no. MIFCZ301A-1). Packaging of the *miR-29b* constructs in pseudoviral particles was performed using the pPACKH1 packaging plasmid mix (catalog no. LV500A-1) according to the

manufacturer's instructions (Systems Biosciences). Lentivirus rapid titer polymerase chain reaction (PCR) kit (catalog no. LV950A-1) was used to measure the copy numbers and determine the multiplicity of infection to perform experiments. Kasumi-1 cells (10<sup>6</sup>) were infected with the *miR-29b* lentivirus with an efficiency of approximately 40% as determined by fluorescence microscopy. Empty lentivirus was used as a control for the experiments.

### Cell differentiation assay

Kasumi-1 cells were infected with empty vector or *miR-29b* lentivirus, flow cytometry sorted after 48 hours (using GFP expression, sorting efficiency 90%), followed by assessment of CD11b expression by flow cytometry at 3 and 6 days after infection. Approximately 5 × 10<sup>5</sup> kasumi-1 cells were washed twice and incubated for 1 hour at 4° in the dark with anti-CD11b (BD Biosciences PharMingen, San Diego, CA). Appropriate isotype control was used as well.

### Quantitative real-time-PCR

The single-tube TaqMan miRNA assay (Applied Biosystems, Foster City, CA) was used to detect and quantify mature miRNAs as described.<sup>11</sup> To detect *Sp1*, *DNMT1*, *DNMT3A* and *3B*, *p15<sup>INK4b</sup>*, and *ESR1* mRNA levels, we used the TaqMan gene expression assay. Raw values were normalized using *let-7i* (for *miR-29b*) and *18S* (for *DNMTs*, *Sp1* *p15<sup>INK4b</sup>*, and *ESR1* genes) as internal controls. *Let-7i* was selected because of its lack of expression variability among more than 200 AML patients tested in a previous study by our group.<sup>12</sup> Real-time PCR reactions were performed in triplicate, including no-template controls. Relative expression was calculated using the comparative C<sub>t</sub> method.<sup>13</sup>

### Northern blotting

Northern blotting was performed as previously described.<sup>8</sup> Briefly, total RNA was extracted using Trizol reagent (Invitrogen). RNA samples (20 μg) were run on 12% polyacrylamide denaturing (urea) precast gels (Bio-Rad, Hercules, CA) and then transferred onto Hybond-*n* membrane (GE Healthcare, Little Chalfont, United Kingdom). The hybridization was performed with α-32P *miR-29b* labeled probes overnight at 42°. The *miR-29b* probe sequence was the complementary one to the mature *miR-29b*. As a loading control, we used 5S rRNA stained with ethidium bromide and U6 hybridization after stripping the filter as previously described.<sup>8</sup>

### Western blotting

Western blot was performed as previously described.<sup>14</sup> Antibodies anti-*Sp1*, -*DNMT1*, -*DNMT3A*, and -*DNMT3B* were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Equivalent gel loading was confirmed by probing with antibodies against β-actin (Santa Cruz Biotechnology).

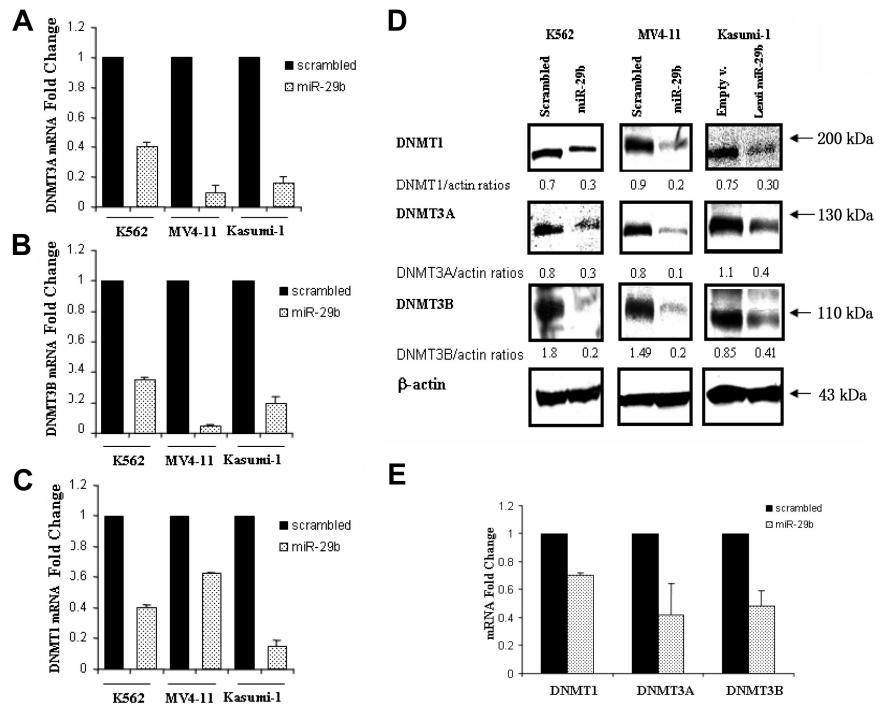
### *Sp1* silencing

A pool of 2 *Sp1* small interfering RNAs (siRNAs) were obtained commercially (Santa Cruz Biotechnology). Approximately 100 nM of *Sp1* siRNA or control oligonucleotide siRNA was transfected in K562 cells by nucleoporation as previously described in cell culture and transfection methods.

### Luciferase assays

The 3' untranslated regions (UTRs) of the *Sp1*, *DNMT1*, and *DNMT3A* and *3B* gene were amplified by PCR from genomic DNA and inserted into the pGL3 control vector (Promega, Madison, WI), using the XBA1 site immediately downstream from the stop codon of luciferase. The primer sets used were: *Sp1* FW 5'-CCTTCAGGGATTTCCTCACTG-3' and *Sp1* RV 5'-GTCCAAAAGGCATCAGGGTA-3', *DNMT1* FW: 5'-GGAGGAG-GAAGCTGCTAAGG-3' and *DNMT1* RV: 5'-TTGGTTTATAGGAGAGAT-TTATTTG-3', the *DNMT3A* and *3B* primers were previously reported.<sup>6</sup> We also generated several inserts with deletions of 4 bp from the site of perfect complementarity of the *DNMT3A*, *DNMT3B*, and *Sp1* gene using the

**Figure 1. miR-29b targets DNMT1, DNMT3A, and 3B in AML cell lines and primary AML blasts.** (A) *DNMT3A*, (B) *DNMT3B*, and (C) *DNMT1* mRNA expression after nucleoporation (K562, MV4-11) or infection (Kasumi-1) of pre-*miR-29b* or their respective controls (scrambled oligonucleotides for K562 and MV4-11 cells and empty vector lentivirus for Kasumi-1 cells). Histograms show fold changes (reduction) in mRNA expression with respect to the controls after normalization with *18s* in 3 independent experiments. Bars represent SD. (D) DNMT1, DNMT3A, and 3B protein expression after pre-*miR-29b* or control nucleoporation or infection in K562, MV4-11, and Kasumi-1 cell lines. Equivalent gel loading was confirmed by probing with antibodies against  $\beta$ -actin. (E) *DNMT1* and *DNMT3A* and *3B* mRNA expression in primary AML patient samples (n = 3) after nucleoporation of pre-*miR-29b* or a scrambled oligonucleotide. Histograms show fold changes (reduction) in mRNA expression with respect to the control after normalization with *18s*. Bars represent SD.



QIAGEN XL-site directed Mutagenesis Kit (QIAGEN, Valencia, CA). K562 cells ( $5 \times 10^6$ ) were cotransfected using nucleoporation (Amaxa Biosystems) according to the manufacturer's protocol (solution V, program T-016) using  $5 \mu\text{g}$  of the firefly luciferase report vector and  $0.5 \mu\text{g}$  of the control vector containing Renilla luciferase, pRL-TK (Promega). For each nucleoporation,  $50 \text{ nM}$  of the pre-*miR-29b* or a scrambled oligonucleotide was used. Firefly and Renilla luciferase activities were measured consecutively using the dual luciferase assays (Promega) 24 hours after transfection.

### EMSAs

Complementary oligonucleotides derived from the human *DNMT1* promoter regions that contain putative *Sp1*-binding site were synthesized, annealed, and labeled with  $^{32}\text{P}$ -dCTP and Klenow. Nuclear extracts were isolated from K562 cells after transfection with pre-*miR-29b* or scrambled oligonucleotides using Nuclear and Cytoplasmic Extraction Reagent (Pierce Chemical, Rockford, IL). Electrophoretic mobility shift assays (EMSAs) with nuclear extracts and  $^{32}\text{P}$ -labeled *DNMT1* promoter oligonucleotides were performed as previously described.<sup>14</sup>

### GDM analysis

Genomic DNA was isolated from AML cells using DNeasy tissue kit (QIAGEN), according to the manufacturer's instructions. A liquid chromatography/tandem mass spectrometry (LC-MS/MS) method was used for determination of GDM as previously reported by our group.<sup>15</sup>

### DNA methylation analysis of gene promoter regions

Quantitative DNA promoter methylation of *ESR1* and *p15* as well as the 5' upstream flanking sequence from the pre-*miR-29b-1* were performed using the EpiTYPER methylation analysis (Sequenom, San Diego, CA). DNA of scrambled or *miR-29b* nucleoporation MV4-11 cells was extracted 72 hours after transfection. A total of  $1 \mu\text{g}$  of DNA was bisulfite treated, *in vitro* transcribed, and cleaved by RNase A, before being subjected to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis for DNA methylation pattern determination as previously described.<sup>16</sup> The following primers were used to amplify the regulatory regions of *p15* and *ESR1*: *p15* forward, 5'-AGGTTTGTAGGTTTATAGGTTTTT-3'; *p15* reverse, 5'-TCTACTCTCTAACAATCATAATAATAA-3'; *ESR1* forward, 5'-GGGTAGAATTAGAGTAGTTTTGTGTT-3'; *ESR1* reverse, 5'-

AAAATCTTTACTCCAAAAATAAC-3'. A second method (pyrosequencing) was used to validate the *ESR1* results as previously described.<sup>17</sup> In brief, DNA obtained at 72 hours from MV4-11 cells transfected with *miR-29b* or scrambled oligonucleotides were bisulfite treated and PCR amplified (supplemental data, available on the Blood website; see the Supplemental Materials link at the top of the online article). A biotin-labeled primer was used to purify the final PCR product using Sepharose beads. The PCR product was bound to Streptavidin Sepharose High Performance (GE Healthcare), and the Sepharose beads containing the immobilized PCR product were purified, washed, and denatured. Then,  $0.3 \text{ amol/L}$  of pyrosequencing primer was annealed to the purified single-stranded PCR product, and pyrosequencing was done using the PSQ HS96 Pyrosequencing System. These experiments were performed by an independent laboratory (Epigen, Cambridge, MA).<sup>17</sup> The degree of methylation was expressed for each DNA locus as percentage of methylated cytosines over the sum of methylated and unmethylated cytosines.

### Statistical analysis

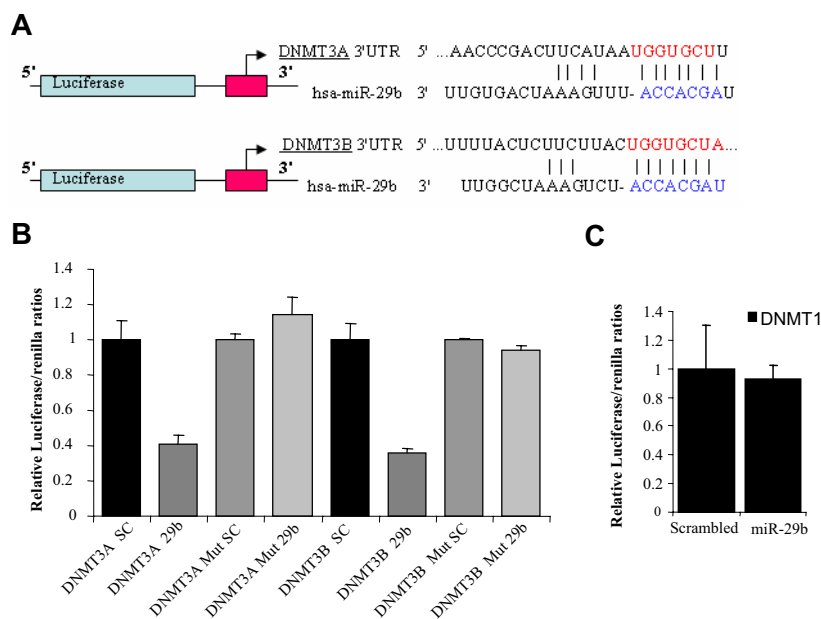
Paired Wilcoxon signed-rank test was used to compare methylation frequencies between the samples. All analyses were performed using SPSS statistics 17.0 software (Chicago, IL). *P* values are 2-sided.

## Results

### *miR-29b* down-regulates *DNMT1*, and *DNMT3A* and *3B* in AML

To test the hypothesis that *miR-29b* down-regulates DNA methyltransferases in AML, we transfected pre-*miR-29b* or a scrambled oligonucleotide into K562 and MV4-11 AML cell lines and measured levels of *DNMT1* and *DNMT3A* and *3B* mRNAs and proteins by quantitative real-time PCR and Western blotting, respectively. A third AML cell line, Kasumi-1, was infected with *miR-29b* lentivirus or empty vector because of low efficiency with the nucleoporation method in these cells. Basal *miR-29b* levels in these AML cell lines and *miR-29b* expression values after transfection/infection are reported in Figure S1.





**Figure 2. DNMT3A and 3B are targets of miR-29b.**

(A) Schema of the 2 firefly luciferase reporter constructs for *DNMT3A* and *3B*, indicating the interaction sites between *miR-29b* and the 3' UTRs of the *DNMT3s*. (B) Dual luciferase assay of K562 cells cotransfected with firefly luciferase constructs containing the *DNMT3A* or *3B* wild-type or mutated 3' UTRs and pre-*miR-29b* or scrambled oligonucleotides as indicated. The firefly luciferase activity was normalized to Renilla luciferase activity. The data are shown as relative luciferase activity of pre-*miR-29b* transfected cells with respect to the control (scrambled oligonucleotide) of a total of 9 experiments from 3 independent transfections. Bars represent SD. (C) Dual luciferase assay of K562 cells cotransfected with firefly luciferase constructs containing the *DNMT1* 3' UTR and pre-*miR-29b* or scrambled oligonucleotides. The data are shown as relative luciferase activity of pre-*miR-29b*-transfected cells with respect to the control (scrambled oligonucleotide) of 9 experiments obtained from 3 independent transfections. Bars represent SD.

Transfection of the pre-*miR-29b*, but not the scrambled controls, resulted in a marked reduction of the endogenous mRNA levels of *DNMT3A* and *3B* for all 3 cell lines (Figure 1A,B). The observed fold change reduction at 24 hours was 2.5, 11.1, and 6.1 for *DNMT3A* (Figure 1A) and 2.8, 17.7, and 4.9 for *DNMT3B* (Figure 1B) in pre-*miR-29b*-treated K562, MV4-11, and Kasumi-1 cells, respectively, compared with their respective controls. We also noted a reduction in the levels of *DNMT1* in pre-*miR-29b*-treated K562, MV4-11, and Kasumi-1 cells (Figure 1C). This was a surprising finding as *DNMT1* is not predicted to be a target for *miR-29b*. Consistent with these results, a significant down-regulation of endogenous DNMT1 and DNMT3A and 3B protein levels was also observed in all 3 AML cell lines 48 hours after pre-*miR-29b* transfection (Figure 1D).

Pre-*miR-29b*-mediated down-regulation was further validated in primary AML blasts. After 24 hours, there was a 1.4-, 2.3-, and 2.1-fold reduction in *DNMT1* and *DNMT3A* and *3B* mRNA levels in primary AML blasts transfected with pre-*miR-29b* compared with controls transfected with scrambled oligonucleotides (Figures 1E, S2).

#### **DNMT3A and 3B are bona fide targets of miR-29b**

Available miRNA target prediction programs<sup>18,19</sup> indicate that *DNMT3A* and *3B* are putative targets of *miR-29b* (Figure 2A). We confirmed this finding in AML cells by performing luciferase reporter assays. *DNMT3A* and *3B* complementary sites were cloned downstream of the firefly luciferase gene and cotransfected with pre-*miR-29b* or scrambled oligonucleotide. Luciferase activity was measured after 24 hours of transfection. Cells cotransfected with either *DNMT3A* or *3B* reporter and pre-*miR-29b* exhibited approximately 60% reduction of the luciferase activity with respect to those cotransfected with the scrambled oligonucleotide (Figure 2B). We further deleted 4 nucleotides (GGUG) from each *miR-29b*-predicted site and cotransfected the mutated constructs along with pre-*miR-29b* or a scrambled oligonucleotide in K562 cells. As shown in Figure 2B, the deletion of *miR-29b* interaction sites abrogated the *miR-29b*/*DNMT3A* and *miR-29b*/*DNMT3B* interaction evidenced by no change in the normalized luciferase activity.

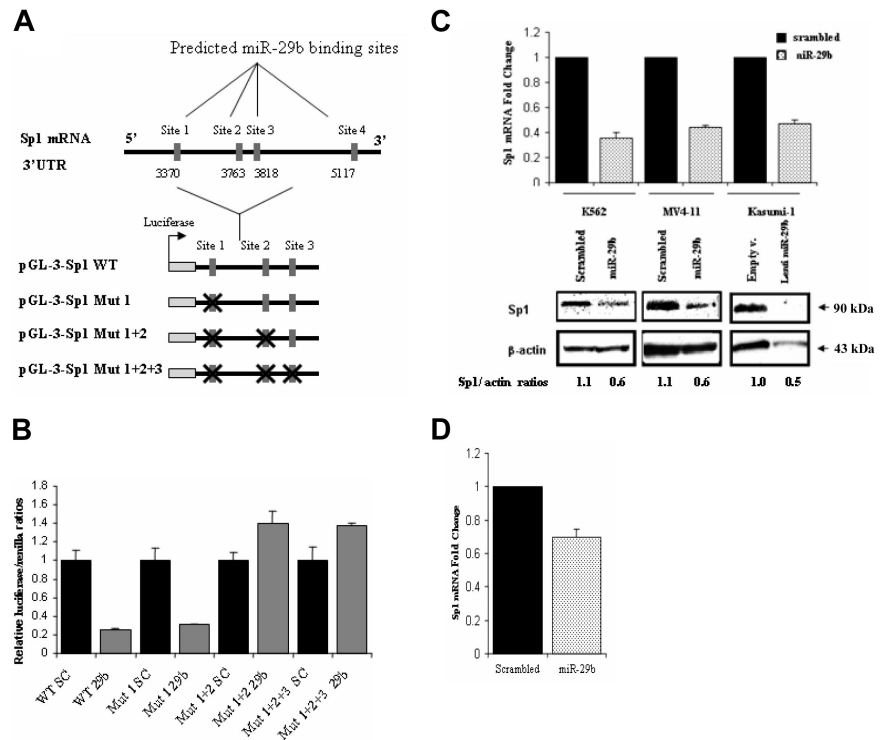
#### **miR-29b down-regulates indirectly DNMT1 by targeting its regulator Sp1**

In contrast to *DNMT3A* and *3B*, *miR-29b* is not predicted to hybridize with the *DNMT1* 3' UTR region and therefore directly target this gene.<sup>18,19</sup> To further prove that *DNMT1* is not a direct target of *miR-29b*, we cloned the *DNMT1* 3' UTR into a luciferase reporter vector and cotransfected with pre-*miR-29b* or a scrambled oligonucleotide into K562 cell lines, as done for *DNMT3A* and *3B*. Consistent with the notion that *DNMT1* is not a direct target of *miR-29b*, no difference in the luciferase activity was noted between cells treated with scrambled or pre-*miR-29b* oligonucleotides (Figure 2C). Therefore, we reasoned that *miR-29b* probably down-regulated *DNMT1* indirectly.

Recent work suggests that *Sp1*, a zinc finger transcription factor, binds directly to the promoter of *DNMT1* and positively regulates the transcription of this gene in mice and humans.<sup>14,20</sup> Here, we further confirm the *Sp1*-*DNMT1* link by showing that *Sp1*-siRNA treatment of K562 cells results in down-regulation of *Sp1* and in turn *DNMT1* mRNA and protein levels as measured by quantitative RT-PCR and Western blotting (Figure S3A,B). *DNMT1* down-regulation results in a GDM reduction of 40% with respect to the control (Figure S3C) and estrogen receptor 1 alpha (*ESR1*) gene reexpression (1.3-fold increase in *Sp1*-siRNA compared with controls; Figure S3D).

Interestingly, the 3' UTR of *Sp1* contains 4 predicted binding sites (at nucleotides 3370, 3763, 3818, and 5117) for *miR-29b* according to Pictar 2005<sup>18</sup> and 2 predicted binding sites (at nucleotides 3370 and 3818) according to TargetsCan 4.2<sup>19</sup> (Figure 3A). Therefore, we hypothesized that *miR-29b* down-regulates *Sp1* expression, which in turn inhibits *DNMT1* transactivation, thereby resulting in a decrease of *DNMT1* at both mRNA and protein levels. To validate this hypothesis, we cloned the 3' UTR region of *Sp1* that encompasses 3 of the 4 *miR-29b* binding sites (sites 3370, 3763, and 3818) predicted by Pictar into a luciferase reporter vector and cotransfected it with pre-*miR-29b* or a scrambled oligonucleotide in K562 cells. The transfection with pre-*miR-29b*, but not with the scrambled oligonucleotide, decreased the luciferase activity of the gene reporter by at least 50% in 3 independent experiments

**Figure 3. miR-29b targets *Sp1*.** (A) Genomic representation of the *Sp1* 3' UTR with the localization of the 4 predicted pre-*miR-29b* binding sites. Immediately below there is a schema of the luciferase reporter assays used in the luciferase experiments. The "X" sign over the red boxes indicates the *miR-29b* sites that were deleted. mut indicates mutated. (B) Dual luciferase assay of K562 cells cotransfected with firefly luciferase constructs containing the wild-type or mutants target site of the *Sp1* 3' UTR region with pre-*miR-29b* or a scrambled oligonucleotide. The data are shown as relative luciferase activity of pre-*miR-29b*-transfected cells with respect to the control (scrambled oligonucleotide) of 9 experiments from 3 independent transfections. Bars represent SD. (C) *Sp1* mRNA and protein expression after nucleoporation (K562, MV4-11) or infection (Kasumi-1) of pre-*miR-29b* or their respective controls, scrambled oligonucleotide, or empty vector lentivirus. Histograms show fold changes with SD. (D) *Sp1* mRNA expression in 3 primary AML samples after nucleoporation of pre-*miR-29b* or scrambled oligonucleotide. Histograms show fold change (reduction) with respect to control after normalization with *18s*. Bars represent SD.



(Figure 3B). We further deleted 4 nucleotides from each *miR-29b* predicted site and cotransfected the mutated constructs along with pre-*miR-29b* or a scrambled oligonucleotide in K562 cells. Concurrent mutations of sites 1 and 2 (ie, 3370 and 3763) or sites 1, 2, and 3 (ie, 3370, 3763, and 3818) predicted *miR-29b* binding sites abrogated the *miR-29b/Sp1* interaction evidenced by no change in the normalized luciferase activity. Mutations of only site 1 (ie, 3370) did not suffice in preventing this interaction (Figure 3B). Furthermore, ectopic expression of pre-*miR-29b* in AML cell lines and in primary AML blasts induced down-regulation of endogenous *Sp1* mRNA and protein compared with scrambled oligonucleotides (Figure 3C,D).

Finally, we performed EMSAs with nuclear extracts from K562 cells untransfected or transfected with pre-*miR-29b* or a scrambled oligonucleotide. We used a probe derived from the spanning *DNMT1* promoter that contains an Sp1-binding site (Figure 4). We observed that Sp1 protein was present on *DNMT1* gene promoter in untreated or scrambled oligonucleotide-treated controls whereas enforced expression of pre-*miR-29b* disrupted the association of *Sp1* with the *DNMT1* promoter. These results further support a mechanistic link between *miR-29b*-mediated down-regulation of *Sp1* and decrease in *DNMT1* expression.

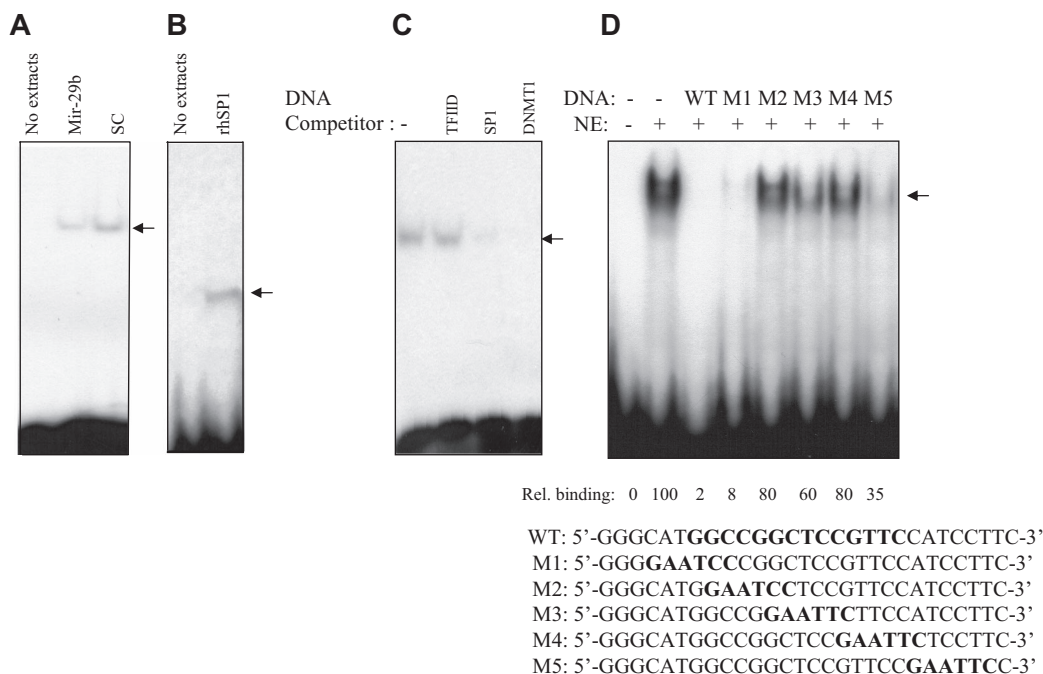
#### Overexpression of pre-*miR-29b* reduces global DNA methylation

Next we investigated whether the enforced expression of pre-*miR-29b* could also functionally result in DNA hypomethylation. GDM was measured using an LC-MS/MS method<sup>15</sup> in MV4-11 and Kasumi-1 cells lines after 72 hours of transfection or infection, respectively, with pre-*miR-29b* oligonucleotide or a lentivirus expressing *miR-29b*. Scrambled oligonucleotides and empty lentivirus vectors served as negative controls for MV4-11 and Kasumi-1, respectively. We observed a reduction of approximately 30% in GDM for the AML cells lines treated with pre-*miR-29b* oligonucleotide or *miR-29b* lentivirus compared with the negative controls

(Figure 5A,B). The reduction in GDM in MV4-11 cells by pre-*miR-29b* was comparable with that achieved with decitabine treatment at the same time point (Figure 5A).

#### Overexpression of pre-*miR-29b* restores the expression of the hypermethylated *ESR1* and *p15<sup>INK4b</sup>* in AML via promoter DNA hypomethylation

Several genes have been found methylated, silenced, and reexpressed after treatment with hypomethylating agents in AML, including *ESR1*<sup>21</sup> and cyclin-dependent kinase inhibitor 2B (*p15<sup>INK4b</sup>*).<sup>22</sup> To assess whether overexpression of *miR-29b* could also lead to reexpression of hypermethylated and silenced genes in AML, we measured the mRNA levels of *ESR1* and *p15<sup>INK4b</sup>* by quantitative RT-PCR in the MV4-11 cell line and *ESR1* for the K562 cell line after transfection with pre-*miR-29b* or a scrambled oligonucleotide. The reason we did not measure *p15<sup>INK4b</sup>* in K562 is because this gene is homozygously deleted in this cell line.<sup>23</sup> Transfection with pre-*miR-29b* induced *ESR1* expression by 86.1-fold in MV4-11 and 2.1-fold in K562 cells compared with the control after 72 hours (Figure 6A,B). Likewise, transfection with pre-*miR-29b* induced *p15<sup>INK4b</sup>* by 11.6-fold compared with the control after 72 hours (Figure 6C). Furthermore, using a quantitative approach,<sup>16</sup> we showed that, after transfection of pre-*miR-29b* oligonucleotides, reexpression of *ESR1* and *p15<sup>INK4b</sup>* coincided with reduction in the DNA methylation levels of both genes. The *ESR1* DNA methylation level was decreased in K562 cells transfected with pre-*miR-29b* compared with those transfected with scrambled oligonucleotides (mean, 58% vs 82%;  $P = .06$ , paired Wilcoxon signed-rank test; Figure 6D). Although this difference was not statistically significant, there was a trend toward significance. In the same vein, the *ESR1* DNA methylation level was significantly decreased in MV4-11 cells transfected with pre-*miR-29b* compared with those transfected with scrambled oligonucleotides (mean, 26% vs 49%;  $P = .03$ , paired Wilcoxon signed-rank test; Figures 6E, S4A). The *p15<sup>INK4b</sup>* DNA methylation levels were



**Figure 4. DNMT1 promoter activity was down-regulated by pre-*miR-29b* through repression of *Sp1*.** (A) EMSA of *DNMT1* promoter DNA and nuclear extracts prepared from K562 cells transfected with *miR-29b* construct (*miR-29b*) or scrambled control (sc). The arrow represents the DNMT1-protein complex. (B) EMSA of *DNMT1* promoter DNA with human recombinant *Sp1* protein (0.5 mg, rh*Sp1*, Promega, catalog no. E6391) or no protein extracts. These data further indicate that *Sp1* is binding to the *DNMT1* promoter DNA. (C) EMSA of *DNMT1* promoter DNA using K562 nuclear extracts with unlabeled DNA competitors (*Sp1*-binding elements and *DNMT1* promoter DNA, WT). As a control, we used no DNA competitor (–) and a nonspecific unlabeled probe (TFIID) that contains the TATA box sequence 5'-GCAGAGCATATAAGGTGAGGTAGG A-3'. This sequence is not related to the *DNMT1* promoter DNA. The fact that this oligo did not compete shows specificity of DNA competition by *DNMT1* DNA and by *Sp1* DNA. (D) EMSA of *DNMT1* promoter DNA using K562 nuclear extracts and unlabeled wild-type (WT) or mutants (M) excess (20 times) DNA competitors. The first 2 lanes (from the left) are without competitors. The sequences of the *DNMT1* promoter DNA and various *DNMT1* mutants with linker scanned mutations (shown in bold-type) are shown below (M1-M5).

significantly decreased in MV4-11 cells transfected with pre-*miR-29b* compared with those transfected with scrambled oligonucleotides (mean, 75% vs 91%;  $P = .001$ , Paired Wilcoxon signed-rank test; Figures 6D, S4B). Using an alternate method (pyrophosphate sequencing), we confirmed that *ESR1* DNA methylation level was significantly decreased in pre-*miR-29b* MV4-11 transfected cells compared with the scrambled oligonucleotides (mean, 76% vs 62%;  $P = .04$ , paired Wilcoxon signed-rank test; Figure S4C,D).

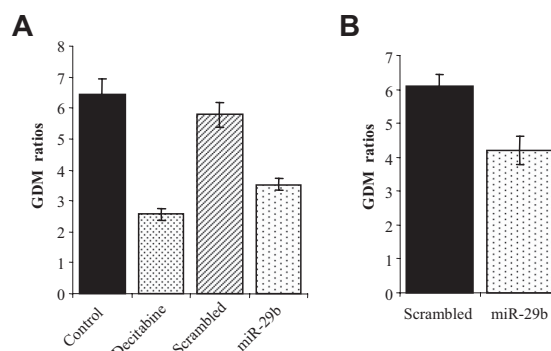
#### Overexpression of pre-*miR-29b* induces partial differentiation of AML blasts

To assess the biologic effects of overexpressing *miR-29b* in AML blasts, we infected Kasumi-1 cells with *miR-29b* lentivirus or empty vector and measured CD11b expression by FACS. As shown in Figure 7, Kasumi-1 cells infected with *miR-29b* exhibited higher levels of CD11b expression compared with the empty vector at 6 days (38% increase in CD11b expression in *miR-29b* lentivirus compared with empty vector).

## Discussion

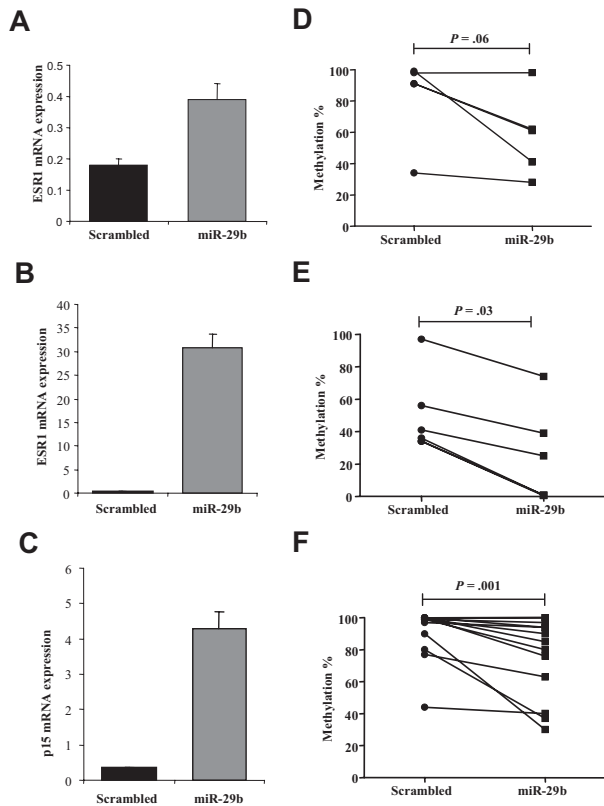
In the current study, we characterized the role of *miR-29b* in the regulation of DNA methylation in AML. Our data showed that *miR-29b* targets *DNMTs*, thereby resulting in global DNA hypomethylation and reexpression of hypermethylated, silenced genes in AML. The current results are consistent with our previous work in lung cancer with regard to the targeting activity of *miR-29b* on *DNMT3A* and *3B* and expand the epigenetic regulatory role of this miRNA to myeloid malignancies.<sup>6</sup> We also showed that *miR-29b* down-regulates *DNMT1* by targeting *Sp1*, which is a zinc finger

transcription factor that regulates a large numbers of genes involved in the cell cycle, proliferation, apoptosis, and malignant transformation.<sup>24</sup> It has been shown that *Sp1* binds to the promoter of *DNMT1* and transactivates the *DNMT1* gene in the mouse.<sup>20</sup> Our group recently reported that the proteasome inhibitor bortezomib induces DNA hypomethylation by interfering with the *NF- $\kappa$ B/Sp1*-dependent transcription of *DNMT1* in AML cell lines and patients' primary blasts.<sup>14</sup> Here we showed that *miR-29b* down-regulates *Sp1*, thereby interfering with the *Sp1*-dependent transcription of *DNMT1*.



**Figure 5. Overexpression of pre-*miR-29b* in AML cell lines reduces GDM.** MV4-11 (A) and Kasumi-1 (B) cell lines were nucleoporated (MV4-11) or infected (Kasumi-1) with pre-*miR-29b* (oligonucleotides and lentivirus) or their respective controls. DNA was obtained from both cell lines after 72 hours of nucleoporation or infection, and GDM was measured by LC-MS/MS.<sup>14</sup> Results from treatment with 2.5  $\mu$ M decitabine, a hypomethylating agent, or phosphate-buffered saline (control) are also shown for the MV4-11 cell line as positive controls. The data are shown as absolute GDM. Bars represent range of 2 independent experiments.





**Figure 6. Pre-*miR-29b* restores expression of hypermethylated *ESR1* and *p15<sup>INK4b</sup>* in the MV4-11 cell line.** (A) Quantitative RT-PCR for *ESR1* in K562 and MV4-11 cells (B), and *p15<sup>INK4b</sup>* (C) after 72 hours of nucleoporation with pre-*miR-29b* or scrambled oligonucleotides. Histograms represent mean values with SD. (D) Scatter plots of the quantitative DNA methylation data of *ESR1* promoter regions in K562 (D) and MV4-11 cells (E) after nucleoporation with pre-*miR-29b* (■) or a scrambled oligonucleotide (●) obtained using the MassArray system.<sup>15</sup> The lines connecting the dots indicate the corresponding CpG pairs. (F) Scatter plots of the quantitative DNA methylation data of *p15<sup>INK4b</sup>* in MV4-11 cells after nucleoporation with pre-*miR-29b* (■) or a scrambled oligonucleotide (●). Each dot represents a single CpG or a group of CpGs analyzed. The lines connecting the dots indicate the corresponding CpG pairs. The scatter plots were created using GraphPad Prism, version 5.00 for Windows (GraphPad Software, San Diego, CA). The *P* values were obtained comparing the CpG methylation levels between scrambled and pre-*miR-29b*-transfected cells using paired Wilcoxon signed-rank test. Error bars indicate SD.

The discovery that *miR-29b* down-regulates not only *DNMT3A* and *3B* but also *DNMT1* has important functional ramifications because it has been reported that selective genetic disruption of *DNMT3B* in colon cancer cell lines reduced GDM only by 3%. However, genetic disruption of both *DNMT1* and *DNMT3B* completely abolished DNA methyltransferase activity and reduced GDM by 95%.<sup>25</sup> Consistent with these results, we now show that

*miR-29b* can efficiently modulate DNA hypomethylation because of its targeting of both *DNMT3s* and *DNMT1*.

To our knowledge, this is also the first report showing that overexpression of *miR-29b* results functionally in global DNA hypomethylation and gene reexpression of the hypermethylated and silenced *p15<sup>INK4b</sup>* and *ESR1* in AML cell lines. Elegant studies in mice support a tumor suppressor role of *p15<sup>INK4b</sup>* in AML as *p15<sup>INK4b</sup>*-deficient mice are more susceptible to develop myeloid leukemia.<sup>26</sup> The *ESR1* gene is also frequently silenced by methylation in AML,<sup>22</sup> and we previously showed that reexpression of these genes correlates with clinical response in patients treated with the hypomethylating agent decitabine.<sup>27</sup> In the current study, reexpression of both *p15<sup>INK4b</sup>* and *ESR1*, induced by pre-*miR-29b*, coincided with significant DNA hypomethylation of the promoter regions of these genes, thereby validating the hypomethylation activity of *miR-29b*.

In this work, we also provide insights about the biologic effects of overexpressing *miR-29b* in AML. Restoring *miR-29b* expression in Kasumi-1 cells induces partial granulocytic differentiation. Similar differentiation effects have been shown in AML cell lines treated with hypomethylating agents.<sup>28</sup> It has been shown that *miR-29b* induces apoptosis in cholangiocarcinoma cells by targeting *MCL-1*.<sup>29</sup> We recently confirmed this finding in AML.<sup>30</sup> The ectopic expression of *miR-29b* in AML cells induces apoptosis by targeting *MCL-1*.<sup>30</sup> Therefore, restoring *miR-29b* expression in AML blasts induces partial differentiation and apoptosis.

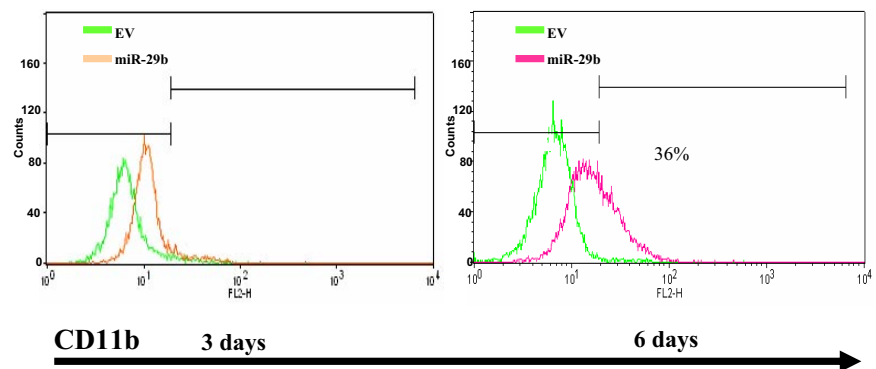
We also think that these findings have potentially relevant therapeutic implications. Targeting *DNMT1* with decitabine or azacitidine in AML cell lines and patients only partially reduces hypermethylation despite compelling inhibition of *DNMT1*,<sup>15</sup> suggesting an underlying activity of other DNMT isoforms that are not efficiently inhibited by these agents. Therefore, one could envision that combining *DNMT1* inhibitors (decitabine or azacitidine) with synthetic *miR-29b* oligonucleotides may result in a synergistic hypomethylating effect, more robust gene reexpression, and in turn better disease response in AML.

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**Figure 7. Overexpression of pre-*miR-29b* induces partial differentiation of AML blasts.** (A) Flow cytometry analysis of CD11b expression in Kasumi-1 cells infected with lentivirus-expressing *miR-29b* or empty vector. Green represents empty vector (EV); red, *miR-29b* lentivirus.



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## Authorship

Contribution: R.G., S.L., M.F., Z.L., C.E.A.H., E.C., S.S., J.P., J.Y., V.H., L.J.R., and L.-C.W. performed research; R.G., S.L., Z.L., S.V., W.B., K.C., M.A., N.M., L.J.R., D.P., C.D.B., J.C.B., C.M.C.,

and G.M. analyzed data; and R.G., S.L., and G.M. wrote the manuscript. All authors critically reviewed and edited the manuscript.

Conflict-of-interest disclosure: C.M.C. has applied for a patent on clinical use of miR-29 family in cancer. The remaining authors declare no competing financial interests.

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