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Epigenetic Silencing of the Myelopoiesis Regulator microRNA-223 by the AML1/ETO Oncoprotein

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SUMMARY

Hematopoietic transcription factors are involved in chromosomal translocations, which generate fusion proteins contributing to leukemia pathogenesis. Analysis of patient's primary leukemia blasts revealed that those carrying the t(8;21) generating AML1/ETO, the most common acute myeloid leukemia-associated fusion protein, display low levels of a microRNA-223 (miR-223), a regulator of myelopoiesis. Here, we show that *miR-223* is a direct transcriptional target of AML1/ETO. By recruiting chromatin remodeling enzymes at an AML1-binding site on the *pre-miR-223* gene, AML1/ETO induces heterochromatic silencing of *miR-223*. Ectopic miR-223 expression, RNAi against AML1/ETO, or demethylating treatment enhances miR-223 levels and restores cell differentiation. Here, we identify an additional action for a leukemia fusion protein linking the epigenetic silencing of a microRNA locus to the differentiation block of leukemia.

INTRODUCTION

Hematopoietic stem cell self-renewal and differentiation along different lineages is defined by a dynamic interplay between lineage-specific transcriptional and posttranscriptional regulators, including microRNAs (miRNAs), whose key role in hematopoiesis is recently emerging (Tenen, 2003; Chen and Lodish, 2005). Epigenetic mechanisms such as DNA methylation and posttranslational modifications of nucleosomal histone proteins contribute to the correct modulation of gene expression and to the maintenance of tissue- and cell-type-specific functions (Jaenisch and Bird, 2003; Grewal and Moazed, 2003; Klose and Bird, 2006; Mikkelsen et al., 2007). Deregulation of epigenetic mechanisms cooperates with genetic alterations to the establishment and progression of cancer (Jones and Baylin, 2007; Tenen, 2003).

MiRNAs are a new class of evolutionary conserved small RNAs affecting gene expression at the posttranscriptional level by blocking translation or degrading target messenger RNAs (mRNAs) (Bartell, 2004; Ambros, 2004; Chen et al., 2004). Their expression is highly regulated according to the cell's developmental lineage and shows restricted expression profiles in adult tissues, including in the

SIGNIFICANCE

AML1/ETO is the fusion product of the t(8;21), the most frequent chromosomal translocation in acute myeloid leukemia. We show that the expression of AML1/ETO triggers heterochromatic silencing of genomic regions generating a microRNA, the miR-223, whose activity is linked to the differentiation fate of myeloid precursors. Overall, our study identifies miR-223 as an additional pathogenic target for a leukemia fusion protein and provides evidence that links the epigenetic silencing of a microRNA locus to the differentiation block of myeloid precursors. Suppression of a *miRNA* gene expands the oncogenic activity of the fusion protein, since miRNA represses the expression of multiple target proteins. Thus, repression of *miRNA* expression may represent a key event in the differentiation block underlying leukemogenesis.

hematopoietic cell system (Chen et al., 2004; Cheng et al., 2005; Zhao et al., 2005; Bartell, 2004; Chen et al., 2006; Fazi et al., 2005). MiRNAs have been found to participate in regulatory circuits that control development of skeletal and cardiac muscle and lineage-differentiation fate of hematopoietic cells (Fazi et al., 2005; Zhao et al., 2005; Chen et al., 2006). However, little information is currently available on factors that modulate *miRNA* transcription and expression at the basal or tissue-specific level (Kim and Nam, 2006).

Developmental programs of normal hematopoiesis are altered in acute myeloid leukemias (AMLs), which represents the clonal expansion of hematopoietic precursors blocked at different stages of erythroid, granulocytic, monocytic, or megakaryocytic differentiation (Tenen, 2003). Hematopoietic transcription factors and miRNAs have been found mutated or consistently altered by chromosomal translocations associated to leukemias; their role in the pathogenesis of these malignancies has been proposed (Chen, 2005; Hammond, 2006; Calin and Croce, 2006; Saito et al., 2006; Tenen, 2003).

Our previous findings showed a crucial role for the transcriptional activation of miR-223 expression in human myelopoiesis (Fazi et al., 2005). Here, we investigated whether deregulated miR-223 expression could be associated with the differentiation block underlying the pathogenesis of distinct leukemia subtypes.

RESULTS AND DISCUSSION

MiR-223 Expression Is Downregulated in AML1/ETO-Positive Primary Blasts and Cell Lines

The expression levels of miR-223 were quantified in human hematopoietic cells isolated from healthy donors and from diagnostic samples of 31 leukemia patients whose morphological and genetic features are shown in Table 1. Figure 1A shows that miR-223 is expressed at high levels in total nucleated cells from peripheral blood (PB) and bone marrow (BM) from healthy donors, respectively, consisting of mature granulocytes (~50%–70%) and committed/mature myeloid precursors. MiR-223 was detected at the lowest levels of expression in immature CD34+-hematopoietic stem/progenitor cells isolated from either normal PB, BM, or cord blood (CB) in leukemias related to the erythroblastic (M6) or lymphoid lineages and in the 6 AMLs presenting the most immature myeloid phenotype (M0 and M1, by FAB classification) (Bennett et al., 1985). Intermediate levels of miR-223 were measurable in primary samples expressing the more mature AML phenotypes (acute-promyelocytic (M3), -myelomonocytic (M4), and -monoblastic (M5) leukemias), and in chronic myeloid leukemia in blast crisis (CML-BC) (Figure 1A). Overall, these results confirmed the induction of miR-223 during myeloid differentiation (Fazi et al., 2005) and related its expression levels to the stage of maturation block underlying myeloid leukemia subtypes. However, among the myeloblastic AML-M2 subtype, miR-223 is expressed at high levels in 3/8 de novo cases and at low levels in 4/4 cases harboring the

Table 1. Morphological and Genetic Features of Primary Leukemia Samples

Patient No.	Morphology by FAB	Karyotype
1	AML/M0	46,XY,der(12)t(12;?)(q23;?)/46,XY
2	AML/M0	Complex aberrations[a]
3	MDS-AML/M1	46,XY,del(7)(q31)
4	AML/M1	47,XX,+11
5	MDS-AML/M1	not available
6	MDS-AML/M1	46,XY,t(3;7;10)(q27;p?;p?),del(7)q31
7	AML/M2	not available
8	AML/M2	47,XX,add(12)(q23),+mar
9	AML/M2	46,XX
10	AML/M2	not available
11	AML/M2	46,XX,t(8;21)(q22;q22)/47,idem,+15
12	AML/M2	46,XY,t(8;21)(q22;q22)
13	AML/M2	46,XX,t(8;21)(q22;q22)
14	AML/M2	46,XY,t(8;21)(q22;q22)
15	AML/M3	46,XY,t(15;17)(q22;q21)
16	AML/M3	46,XY,t(15;17)(q22;q21)
17	AML/M3	46,XY,t(11;17)(q23;q21)
18	AML/M3	46,XY,t(15;17)(q22;q21)
19	AML/M4eo	46,XY
20	AML/M4	46,XY,t(4;16)(q25;q22)
21	AML/M4	not available
22	AML/M4	not available
23	AML/M4-M5	not available
24	AML/M5	not available
25	AML/M5a	45,XY,del(7),−16
26	AML/M6	46,XY
27	AML/M6	not available
28	CML blast crisis	45,XX,−7,t(9;22)(q34;q11)
29	leukemic mantle cell lymphoma	46,XY
30	ALL	46,XY,del(12)(p13;pter)/46,XY
31	ALL	46,XX

Leukemias were classified according to FAB classification (Bennett et al., 1985). MDS, myelodisplastic phase preceding AML; ALL, acute lymphocytic leukemia. Cases with no detectable aberrations by conventional karyotyping were also negative for the fusion genes PML/RARα, CBFβ/MYH11, DEK/CAN, BCR/ABL, and MLL rearrangements.

[a] 45,X,inv(Y)(p11q11),−3,del(5)(q15q35),−7,der(12),−14,del(15)(q24q26),der(17),−18,−20,+4 mar.

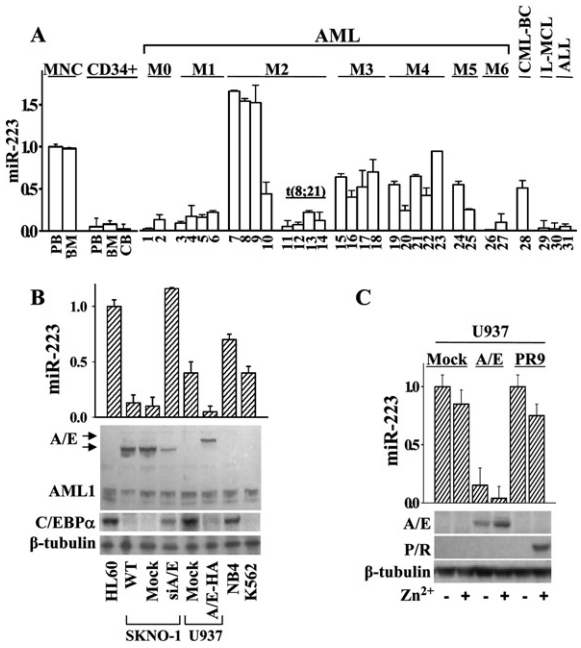

Figure 1. MiR-223 Levels in Human Hematopoietic Cells and in Leukemias

(A) Relative qRT-PCR quantization of miR-223 level in mononucleated cells (MNC) and CD34+ hematopoietic progenitors isolated from healthy donors PB, BM, CB, and from 31 leukemia patients classified by FAB (Bennett et al., 1985), which relies on blasts morphologic and cytochemical characteristics (Table 1). L-MCL and ALL are PB blasts from a leukemic mantle cell lymphoma and two acute lymphocytic leukemia patients, respectively.

(B and C) Upper panels: Relative quantization of miR-223 expression levels in the indicated leukemia cell lines. SKNO-1 wild-type cells (WT), SKNO-1 cells infected with a lentiviral empty vector (Mock), and SKNO-1 cells infected with a lentiviral vector expressing siRNAs against the fusion region of the AML1/ETO mRNA (siA/E). U937 cells stably transfected with an empty vector (Mock), HA-tagged AML1/ETO (A/E-HA), or the PML/RARα (PR9) cDNAs. The results represent the average of three independent evaluations ± SD. Lower panels: Immunoblot analysis for the detection of AML1 and AML1/ETO (A/E) with an anti-AML1 antibody. The increased molecular weight of the AML1/ETO product in A/E-HA cells in respect to SKNO-1 cells is due to the HA-tagged domain of the vector. The PML/RARα and C/EBPα protein were detected with the anti-RARα and anti-C/EBPα antibodies, respectively. The level of β-tubulin visualized the equal amount of protein loading. ZnSO4 treatment (100 μM) was used to increase the expression of AML1/ETO and PML/RARα products in U937 cells.

chromosomal translocation t(8;21) generating the AML1/ETO fusion product (Figure 1A).

In analogy to primary AML blasts, miR-223 was expressed at the highest levels in the AML-M2 HL60 cell line (Dalton et al., 1988), while low levels of miR-223 were measurable in the t(8;21)-AML-M2 cell line SKNO-1 constitutively expressing the AML1/ETO oncoprotein (Fazi et al., 2007) (Figure 1B). The NB4, U937, or K562 cell lines derived from patients respectively presenting AML-M3, AML-M5, or a CML-BC expressed miR-223 levels that were lower than HL60 but significantly higher than in SKNO-1 cells (Figure 1B).

We next infected SKNO-1 cells with a siRNA lentiviral construct knocking down the AML1/ETO product (SKNO-1 siA/E cells). This strongly increased the endogenous levels of miR-223 in respect to those measurable in SKNO-1-wild-type (WT) or -Mock cells (Figure 1B). Moreover, we ectopically expressed an HA tagged AML1/ETO cDNA in U937 cells (U937-A/E-HA) in a stable or zinc-inducible manner. In these cells, the expression levels of miR-223 were strongly reduced relative to U937-Mock cells (Figures 1B and 1C). No effect on miR-223 expression was exerted by the expression in U937 cells of PML/RARα, the AML-M3-associated fusion oncoprotein (Figure 1C). These results suggested that the AML1/ETO fusion product specifically triggers the transcriptional silencing of miR-223.

AML1/ETO Oncoprotein Localizes at an AML1-Binding Site on the *pre-miR-223* Gene Affecting Its Transcriptional Regulation

AML1/ETO is the fusion product of the t(8;21) translocation, the most common karyotypic abnormality of AML, which is detected in about 15% of total cases and in up to 40% of the FAB AML-M2 subtype. Expression of AML1/ETO in hematopoietic stem/precursor cells dramatically expands myeloid progenitors in vitro causing preleukemic myeloproliferative disorder in vivo. AML1/ETO maintains the ability of AML1 to bind the consensus sequence TGT/cGGT on target gene promoters and acts as a dominant-negative repressor of AML1 target genes, including *c-fms*, *GM-CSF*, *p14^{ARF}*, and the *retinoic acid receptor β* among others (Linggi et al., 2002; Frank et al., 1995; Zhang et al., 1994; Fazi et al., 2007; Nimer and Moore, 2004; Hess and Hug, 2004).

A bioinformatic search showed the presence of a putative AML1 binding site at the 5′ end of the predicted "core-promoter" sequence on the *pre-miR-223* upstream region (Zhou et al., 2007). By chromatin immunoprecipitation (ChIP) assay we investigated the in vivo localization of the AML1/ETO protein constitutively present in SKNO-1 cells or ectopically expressed in U937 cells at the AML1-binding site on the *miR-223* gene. We found that DNA sequences containing the AML1-binding site are immunoprecipitated by an α-AML1 antibody (Figures 2A and 2B, oligo1). Since these myeloid cell lines express the wild-type AML1, the AML1/ETO occupancy of this chromatin region was indicated by the detection of ETO immunocomplexes in SKNO-1 and U937-A/E-HA cells, but not in U937-Mock cells (Figures 1B and 2B). ChIP analysis performed with an α-HA antibody to distinguish the AML1/ETO-HA fusion protein, expressed by U937-A/E-HA cells from the endogenous AML1 or ETO products, confirmed the presence of AML1/ETO at this AML1 target site. The specificity of these interactions was indicated by their absence if distal sequences on *miR-223* gene lacking the AML1-binding sites were amplified in the same samples (Figures 2A and 2B oligo2). These findings suggested that the AML1 site on the *pre-miR-223* "core-promoter" sequence is a molecular target for the AML1/ETO oncoprotein in vivo.

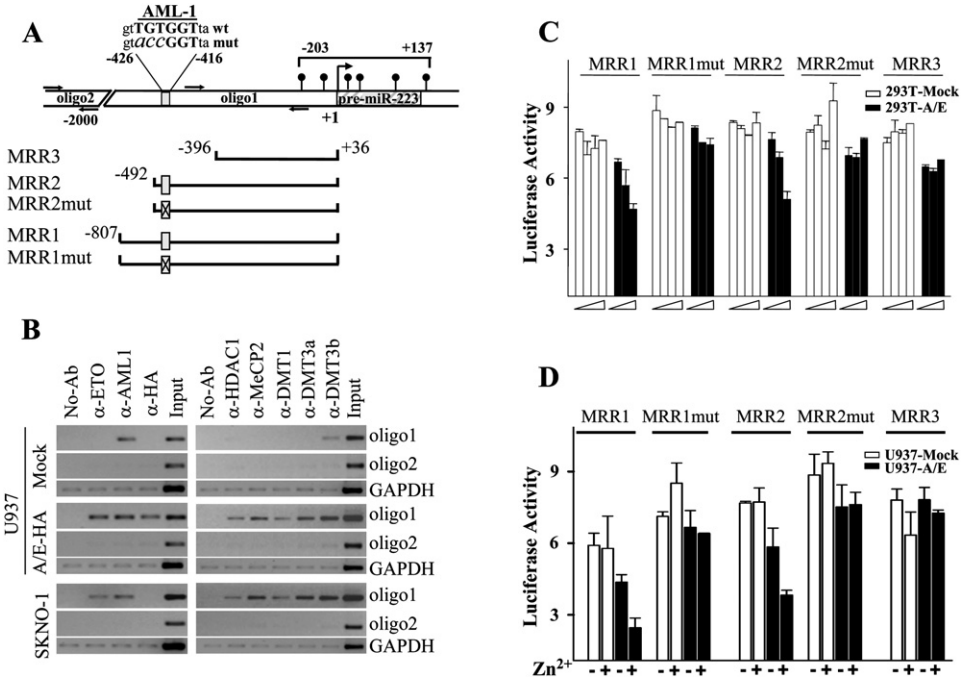

Figure 2. The AML1/ETO Oncoprotein Acts on the AML1 DNA-Binding Site on the *pre-miR-223* Upstream Sequence

(A) Schematic representation of the AML1 site (nt −426 to −416) and of the distribution of the CpG dinucleotides (black circles) (nt −203 to +137) along the *miR-223* gene. Numbers are the nucleotides relative to the 5′ end of the *pre-miR-223* (+1). Arrows indicate the location of the primers used in ChiP assay.

(B) Chromatin was immunoprecipitated using the indicated antibodies or in absence of antibody (no-Ab). PCR was performed by using oligo1 primers designed to amplify DNA sequences surrounding the AML1-binding site and the CpGs on *pre-miR-223* gene. Oligo2 primers (A) were designed for the amplification of a distal region on *miR-223* gene lacking the AML1 site to evaluate the specificity of protein binding. Input shows the amplification from sonicated chromatin. Amplification of GAPDH DNA was a control for nonspecific precipitated sequences.

(C) Human 293T cells were transiently cotransfected for 48 hr with luciferase reporter vectors containing the sequence of the *miR-223* regulatory regions (MRRs showed in [A]), and increasing amounts (10, 50, and 100 ng) of pcDNA3 vectors containing (293T-A/E) or not (293T-Mock) HA-tagged-AML1/ETO cDNAs.

(D) MMR luciferase reporter vectors were transiently transfected for 48 hr into U937 cells expressing a HA-tagged-AML1/ETO in a stable or zinc-inducible manner (U937-A/E) or an empty vector (U937-Mock) cells. ZnSO4 treatment (100 μM) was used to increase the expression of AML1/ETO. The data are expressed as activity relative to that of the empty pGL2-LUC vector alone. A cotransfected Renilla Luciferase vector pRL-SV40 was used as an internal control for normalization of the luciferase activity in each sample. The results shown are the average of three independent evaluations ± SD.

We therefore addressed the transcriptional regulatory functions of AML1/ETO product on miR-223 expression. Luciferase reporter constructs containing different portions of the *pre-miR-223* regulatory region (MRR) surrounding the AML-1 site (schematically represented in Figure 2A) were cotransfected with increasing amounts of expression plasmids encoding for the AML1/ETO product (A/E) or for the empty vector (Mock) into 293T cells. The same MRR reporter vectors were also transfected into U937-Mock and U937-A/E cells, which have a stable expression of the AML1/ETO protein that can be further induced by zinc. In both 293T and U937 cells, the expression of AML1/ETO caused a dose-dependent decrease in the activity of the MRR1 and MRR2 constructs, both presenting the putative AML1 site on *pre-miR-223*, but not of the MRR3 lacking this binding site.

Notably, the luciferase activity of the MRR1 mut and the MRR2 mut vectors, both mutated in the AML1-binding site was not modified by AML1/ETO presence (Figures 2C and 2D), thus showing the contribution of this site to the AML1/ETO- dependent silencing of *miR-223*.

AML1/ETO Triggers the Heterochromatic Silencing of *miR-223* Genomic Regions

The oncogenic properties of AML1/ETO are linked to its ability to form oligomeric complexes with increased affinity for histone deacetylase (HDAC) and DNA methyltransferases (DNMTs) rendering AML1/ETO a potent transcriptional repressor (Liu et al., 2005, 2006; Fazi et al., 2007). DNMTs methylate the cytosine within CpG dinucleotides (CpGs), frequently gathered in clusters (“CpG islands”) (Jaenisch and Bird, 2003; Jones and Baylin, 2007). Methylated CpGs recruit DNA-methyl CpG-binding proteins (MeCPs and MBDs). Often, DNMTs, MeCPs, and MBDs are associated with other chromatin remodeling activities including HDACs (Jaenisch and Bird, 2003; Jones and Baylin, 2007; Klose and Bird, 2006). However, it has been shown that genomic underrepresented and randomly

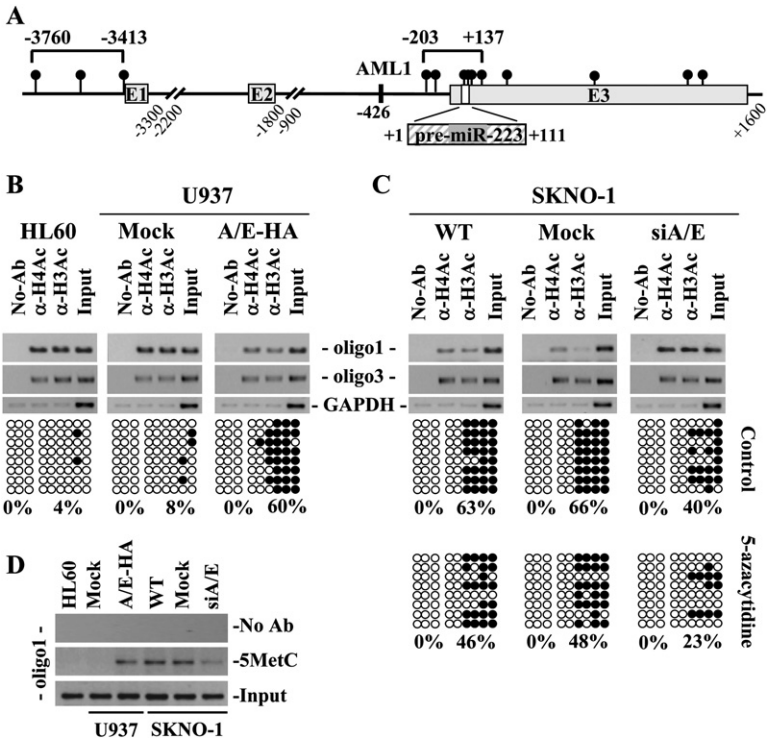

Figure 3. Epigenetic Status of *miR-223* Gene in AML1/ETO-Positive Cells

(A) Schematic representation of the genomic structure of human *miR-223* gene as reported by the UCSC Genome Browser website (http://genome.ucsc.edu/). The location of the exon sequences (E), the AML1-binding site, and the CpG dinucleotides (indicted as black circles along nt −203 to +137 and nt −3760 to −3413) on the miR-223 gene are numbered relative to the 5′ end of the *pre-miR-223* (nt +1 to +111).

(B and C) Upper panels: ChIp assays performed in the indicated cell lines using antibodies specific for the acetyl-H4 and acetyl-H3 forms or (D) for the 5-methylcytosine (5MetC). PCR amplifications were performed using oligo1, designed to amplify DNA sequences surrounding the AML1-binding site and the nearby CpGs (described in Figures 2A and 2B), and oligo 3 designed to amplify the 5′ end upstream region of *miR-223* gene (nt −3514 to −3300). Lower panels: Genomic bisulfite sequencing assay was performed to detect the methylation status of the three CpG dinucleotides dispersed along nt −3760 to −3413 (plotted as circles on the left side of each methylation subpanel) and the six CpG dinucleotides clusterized along nt −203 to +137 of *miR-223* gene sequence (circles on the right side of each methylation subpanel). Black circles and empty circles represent methylated and unmethylated CpG dinucleotides, respectively. Cells were also treated or not with 1 μM 5-azacytidine for 40 hr. For each sample, the percentages of global methylation level of these regions on the *miR-223* gene are indicated.

distributed CpG dinucleotides can act as hotspots for aberrant methylation and gene silencing (Santoro et al., 2002).

ChIP analysis revealed the presence of DNMT1, DNMT3a, DNMT3b, MeCP2, and HDAC1 at the *pre-miR-223* chromatin regions occupied by AML1/ETO in U937-A/E-HA and SKNO-1 cells, whereas only a faint DNMT3 reactivity was detectable at this site in U937-Mock cells (Figure 2B). We therefore investigated if the aberrant recruitment of HDAC1, DNMTs, and MeCP2 activities by AML1/ETO modifies nucleosomal histone tails and DNA methylation status on the *pre-miR-223* upstream sequence (Figure 3A). We performed ChIP analysis using antibodies recognizing the acetylated forms of histone H3 and H4 and PCR amplification of *pre-miR-223* upstream regions adjacent the AML1 site. At this chromatin region, H3 and H4 histones are hyperacetylated in HL60 and U937-Mock cells, while decreased acetylation levels are measurable in both U937-A/E and SKNO-1 cells (Figures 3B and 3C). The reduced histone acetylation in AML1/ETO-expressing samples suggested a hindered transcription at these chromatin sites on the *pre-miR-223* gene (Klose and Bird, 2006). Moreover, ChiP assay performed with an α-5′-methylcytosine antibody to immunoprecipitate sonicated naked DNA showed that cytosines on the DNA region near the AML1 site on *pre-miR-223* gene are methylated in both SKNO-1 and A/E-HA cells, but not in Mock or HL60 cells (Figure 3D). Notably, along a 340 bp sequence (nt −203 to +137) containing *pre-miR-223*, six CpG dinucleotides are all assembled in the upstream, body, and downstream regions of the *pre-miR-223* sequence at a distance of one or two nucleosomes from the AML1 site, while only four sparse CpGs were present within about 1500 bp downstream from this region, as schematically represented in Figure 2A. Interestingly, genomic bisulfite sequencing showed a higher frequency of methylated CpG dinucleotides encompassing the endogenous *pre-miR-223* gene sequences in U937-A/E-HA (60%) and in SKNO-1 cells (63%) as compared to Mock (8%) or HL60 (4%) cells, confirming the basal hypermethylated status of these CpGs in AML1/ETO-positive cells (Figures 3B and 3C). Notably, in U937-siA/E cells in which the knockdown of AML1/ETO reactivated miR-223 expression (Figure 1B, 4A), histone H3 and H4 were hyperacetylated and the CpGs methylation level was decreased (40%) at these sites on *pre-miR-223* gene (Figure 3C).

While this paper was in preparation, Fukao et al. (2007) identified a conserved promoter region located at about 3400 bp relative to the 5′ end of the *pre-miR-223* (Figure 3A). The sequence analysis of this region revealed the presence of three dispersed CpG dinucleotides (Figure 3A). Bisulfite genomic sequencing demonstrated that these CpGs are constitutively unmethylated in human myeloid cell lines and that their methylation status is not changed either in the presence or in the absence of the AML1/ETO fusion protein (Figures 3B and 3C). Accordingly, ChIP analysis showed that the histones H3 and H4 acetylation status at this chromatin site did not change in relation to AML1/ETO expression (Figures 3B and 3C).

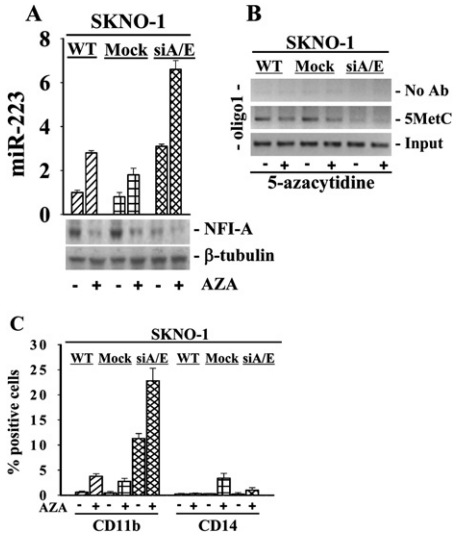

Figure 4. Enhancement of microRNA-223 Levels by siAML1/ETO and 5-azacytidine Treatment Restores Myeloid Differentiation of SKNO-1 Cells

(A) SKNO-1 cells (WT, Mock, and si-A/E), were treated or not with 1 μM 5-azacytidine (AZA) for 40 hr. MiR-223 relative expression levels were evaluated by qRT-PCR. Immunoblot analysis was performed using an anti-NFI-A antibody. The immunodetection of β-tubulin was used as loading control.

(B) ChIP assay performed with the anti-5-methylcytosine (5MetC) antibody and the oligo1 or primers.

(C) Effect of 40 hr treatment with 5-azacytidine on the percentage of cells positively stained for CD11b and CD14 surface markers as measured by FACS analysis. The results represent the average of three independent evaluations ± SD.

Of note, the DNA sequence of this upstream regulatory region (Fukao et al., 2007) also lacked a putative AML1-binding site as revealed by bioinformatic searches performed with the MatInspector Professional and the Transfac softwares. Thus, the chromatin remodeling complex aberrantly formed by AML1/ETO and the hypermethylation of the small CpG-cluster present in a close vicinity to the *miR-223* "core promoter region" containing the AML1-binding site appear to be a key mechanism for transcriptional gene silencing of *miR-223*. Whether methylation at this site could be associated to the tissue-specificity of *miR-223* gene is an interesting question arising from the recent work from Zhang et al., (2006), which requires a more in depth investigation in the human hematopoietic system.

Demethylating Treatment, RNAi against AML1/ETO, or Ectopic miR-223 Expression Enhances miR-223 Level and Restores Blasts Differentiation

We next treated the AML1/ETO-positive SKNO-1 cells with the DNMT inhibitor 5-azacytidine. This demethylating drug (1) increased by about 2- to 3-fold miR-223 expression (Figure 4A), (2) decreased both the ability of the α-5′-methylcytosine antibody to immunoprecipitate naked DNA at chromatin sites surrounding the AML1 site on *pre-miR-223* gene (Figure 4B) and the methylation status

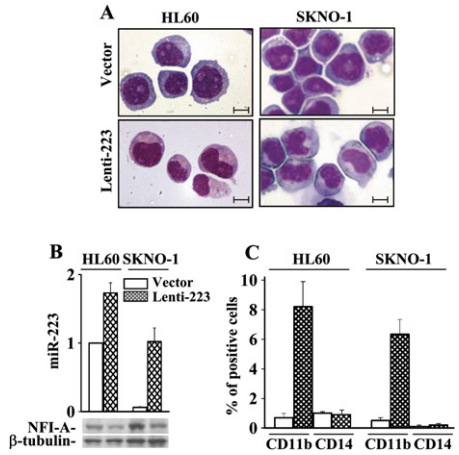

Figure 5. Ectopic miR-223 Expression Reprograms Myeloid Differentiation in SKNO-1 and HL60 Myeloid Leukemia Cells

HL60 and SKNO-1 cells were infected with a lentiviral vector expressing miR-223 (Lenti-223, hatched bars) or with the empty lentiviral vector (Vector, white bars).

(A) Changes in morphology by light-field microscopy of Wright-Giemsa stained cells (Scale bars, 5 μm).

(B) Relative miR-223 expression levels as evaluated by qRT-PCR. Immunoblot analysis was performed using an anti-NFI-A antibody. The immunodetection of β-tubulin was used as loading control.

(C) Percentage of cells positively stained for CD11b and CD14 myeloid surface markers as measured by FACS analysis. The results represent the average of three independent evaluations ± SD.

of endogenous *pre-miR-223* CpGs (from 63% to 46% of 5-methylcytosine) (Figure 3C), (3) decreased the accumulation of the NFI-A protein, a recognized target of miR-223 action (Fazi et al., 2005), thus indicating that the demethylating action of 5-azacytidine is able to restore a functional endogenous mature miR-223 (Figure 4A), and (4) induced granulocytic maturation of the cells as indicated by the increased expression levels of the myeloid differentiation marker CD11b, but not of the monocytic marker CD14 (Figure 4C). Interestingly, in si-A/E cells miR-223 re-expression reduced the protein levels of its target NFI-A and increased the percentage of cells expressing the myeloid differentiation marker CD11b (Figures 4A and 4C). Treatment of siA/E cells with 5-azacytidine affected either methylation or phenotypic changes with respect to those measurable in untreated cells (Figures 4B, 4C, and 3C), further linking AML1/ETO expression, heterochromatic transcriptional silencing of *miR-223* to the differentiation block present in t(8;21) AML blasts.

In agreement with this evidence, stable ectopic expression of miR-223 in SKNO-1 cells obtained by infection with the lenti-223 vector reduced the accumulation of the NFI-A product and induced granulocytic maturation as measured by morphology (showing chromatin condensation with nuclear segmentation, decreased nuclear/cytoplasmic ratio, decreased cytosolic basophilia, appearance of paranuclear Golgi region, and appearance of specific granules), expression of myeloid surface differentiation marker CD11b (Figure 5) and by NBT reduction assay (data not shown).

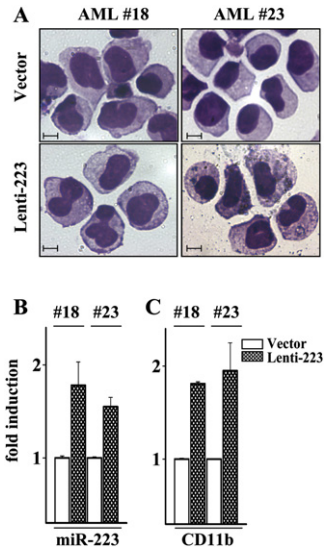

Figure 6. Ectopic miR-223 Expression Reprograms Myeloid Differentiation in Primary Blasts from Acute Myeloid Leukemia Patients

Fresh primary blasts were isolated from the peripheral blood of two newly diagnosed AML patients (18 and 23 of Table 1), showing an initial percentage of circulating blasts greater than 95%. Blasts were infected (Lenti-223, hatched bars) with the lentiviral vector expressing miR-223 or with the empty lentiviral vector (Vector, white bars). Afterwards, cells were cultured for a week and collected for morphological, immunophenotypic, and miR-223 expression level evaluation. (A) Changes in morphology by light-field microscopy of Wright-Giemsa stained cells (scale bars, 5 μm). (B) The values indicate the miR-223 expression level in primary AML blasts ectopically expressing the Lenti-223 versus that measured in the same blasts infected with the empty vector as evaluated by qRT-PCR. (C) Ratio of the CD11b myeloid differentiation antigen expression levels in Lenti-223 primary AML blasts versus that measured in empty vector infected blasts by quantitative FACS analysis The results represent the average of two independent evaluations ± SD.

The AML1/ETO knocking down by siRNAs appears to have a stronger effect in restoring SKNO-1 cell differentiation than ectoptic miR-223 expression, as indicated by the percentage of CD11b-positive cells (Figure 4C and 5C). This may suggest that the silencing of miR-223 expression by AML1/ETO only accounts for part of the differentiation block caused by AML1/ETO. Supporting this hypothesis, the expression of the CCAAT/enhancer binding protein alpha (C/EBPα) product in SKNO-1 siA/E cells is restored (Figure 1B). C/EBPα is a key transcriptional regulator of granulocytic differentiation of myeloid precursor (Radomska et al., 1998), which is indirectly silenced by AML1/ETO via protein-protein interaction blocking the positive autoregulatory regulation of *C/EBPα* own promoter (Pabst et al., 2001).

We recently described the transcriptional activation of *C/EBPα* and posttranscriptional regulation of NFI-A by miR-223 as essential for granulocytic differentiation response of acute promyelocytic leukemia blasts to the differentiating agent retinoic acid (Fazi et al., 2005). In the same study, we also reported granulocytic differentiation in miR-223-transduced acute promyelocytic leukemia patient-derived NB4 cells carrying the t(15:17) chromosomal translocation and expressing the PML/RARα fusion product (Fazi et al., 2005). Here, we show that, when engineered with the lenti-223 to overexpress the miR-223, the human myeloblastic HL60 cell line, which do not carry oncogenic fusion products, also entered a granulocytic pathway of differentiation (Figure 5).

Moreover, the infection of fresh primary blasts isolated from the peripheral blood of two consecutive newly diagnosed acute myeloid leukemia patients (18 and 23 of Table 1) with a lentiviral vector expressing miR-223, increased the expression level of miRNA-223 of about 1.6- to 1.8-fold after a week of culture. This induction resulted in primary blast granulocytic differentiation as shown by morphology and by the increased expression (about 2-fold) of the immunophenotypic myeloid differentiation marker CD11b, but not of CD14 (Figure 6 and data not shown). This indicates that the levels of miR-223 expression are critical for the development of the granulocytic differentiation program and that titrated miR-223 expression can reprogram myeloid differentiation in different leukemia subtypes independently from the presence of a specific genetic lesion.

Cancer is a genetic and epigenetic disease (Jones and Baylin, 2007; Zardo et al., 2002). Compelling evidence indicates a role for genetic alteration in the initiation and progression of tumors. The deregulation of epigenetic mechanisms of gene expression, such as hypermethylation of promoter and coding sequences, is also a key oncogenic mechanism for the inactivation of tumor suppressors in a wide range of tumor types. Heterochromatic gene silencing can represent an alternative oncogenic mechanism to gene mutation or deletion for the transcriptional repression of tumor suppressor genes (Zardo et al., 2002; Baylin and Ohm, 2006; Jones and Baylin, 2007). Tumor suppressor or oncogenic activities have been recently proposed for miRNAs (Chen, 2005; Hammond, 2006; Calin and Croce, 2006; Saito et al., 2006).

Here, we show that the heterochromatic silent state of genomic regions generating a miRNA, the *miR-223* gene, whose activity is linked to the differentiation fate of myeloid precursors is triggered by the expression of AML1/ETO, the oncogenic fusion product of the most frequent chromosomal translocation in AML. AML1/ETO targets the *miR-223* due to its interaction with the AML1 site at the *pre-miR-223* upstream region where it recruits HDAC and DNMT activities that deacetylate histone proteins and methylate CpGs. Newly methylated CpGs act as docking sites for the DNA-methyl CpG-binding protein MeCP2. Through changes in chromatin conformation, the AML1/ETO-associated complex resets the *miR-223* gene to a repressed ground state contributing to the differentiation block of myeloid precursors (Figure 7). Of note, that either ectopic miR-223 expression, downregulation of AML1/ETO protein levels, or the use of demethylating agents reactivate miR-223 expression and restore myeloid differentiation in t(8;21)-AML blasts. Preliminary immunophenotypic data obtained on primary blasts from

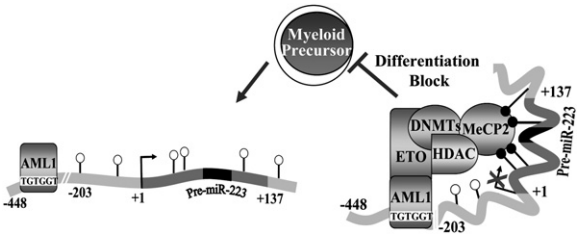

Figure 7. Schematic Model for the Heterochromatic Silencing of miR-223 Gene by AML1/ETO

In myeloid precursors, the occupancy of the AML1-binding site (TGTGGT) on the *pre-miR-223* promoter by AML1 is associated with a chromatin status permissive for transcription. The AML1/ETO oncoprotein targets this binding site, where it aberrantly recruits HDAC, DNMT and MeCP2 activities that deacetylate histone proteins and methylate CpGs. This produces a chromatin packaging nonpermissive for *miR-223* transcription, which contributes to the differentiation block of AML1/ETO+ myeloid precursors. White and black circles indicate the unmethylated and methylated CpG dinucleotides, respectively. Dark gray marks the *pre-miR-223* region, while that encoding the miR-223 mature form is marked in black. Numbers are the nucleotides relative to the 5′ end of the *pre-miR-223* (+1).

a single AML1/ETO-positive patient (row 14 of Table 1) also supported the ability of either the AML1/ETO knockdown by siRNAs or the ectopic miR-223 expression to reprogram primary leukemia cells into a granulocytic pathway of differentiation (data not shown).

This evidence establishes an important relationship between aberrant heterochromatic silencing of a tissue and developmental stage-specific miRNA transcription and differentiation block of leukemia. Suppression of a miRNA gene also carries the potential to greatly expand the fusion protein oncogenic activity since miRNA represses the expression of multiple target proteins.

The relevance of the *miR-223* silencing is also highlighted by the consequences of its ectopic expression that alone is sufficient to reprogram the myeloid differentiation program in distinct myeloid leukemia subtypes. This suggests deregulated miRNA production as a common pathway required for the differentiation block underlying myeloid leukemia pathogenesis and reveals miRNAs as additional molecular targets for therapeutic intervention in cancer.

EXPERIMENTAL PROCEDURES

Clinical Samples

The study was approved by the local internal review boards and ethic committees. Written informed consent was obtained from each subject. Normal mononuclear cells and CD34+ cells were isolated from the BM, PB, or CB of healthy donors as reported (Fazi et al., 2007). Leukemia blasts were obtained from the BM and/or PB of 31 leukemia patients. Cases were classified according to the FAB classification and showed an initial percentage of circulating blasts greater than 60% (Bennett et al., 1985). Blasts isolation and molecular analysis to detect the AML-associated fusion genes were performed as described (Fazi et al., 2007).

Cell Lines and Cell Cultures

HL60, NB4, K562, U937, U937-A/E-HA clone 9, U937-PR9, SKNO-1, and SKNO-1-siA/E-RNA cell lines were maintained in RPMI 1640 medium supplemented with 50 μg/ml streptomycin, 50 IU penicillin, and 10% FCS as described (Drexler et al., 1999; Grignani et al., 1993; Fazi et al., 2005, 2007). Primary AML blasts were cultured in IMDM medium supplemented with 50 μg/ml streptomycin, 50 IU penicillin, and 20% FCS. The lentiviral vector Lenti-223 was used for the ectopic induction of the miR223 into myeloid cell lines (SKNO-1 and HL60) and primary blasts to generate the Lenti-223 cells (Fazi et al., 2005). Cells were infected and purified by fluorescence activated cell sorting as reported (Fazi et al., 2005). Treatment with 5-azacytidine (Sigma-Aldrich, Milan, Italy) was performed at a concentration of 1 μM for 40 hr. Human embryonic kidney 293T cells were cultured in DMEM medium supplemented with 50 μg/ml streptomycin, 50 IU penicillin, and 10% FCS.

RNA Extraction and Analysis

Total RNA was extracted from cells using the TRIzol RNA isolation system (Invitrogen). The relative quantity of miR-223 was measured on 200 ng of total RNA by qRT-PCR using the *mir*-Vana Detection Kit (Ambion, Applied Biosystem, Milan) in the ABI PRISM 7000 Sequence Detection System (Applied Biosystem), and it was determined by the comparative C_T method using snRNA U6 levels for normalization as recommended by the manufacturer's instructions.

Immunoblot Assays

Immunoblot assays were performed on total cell lysates (50 μg) using the rabbit polyclonal antibodies anti-AML1/RHD (Oncogene Science), anti-RARα (Santa Cruz Biotechnology, Santa Cruz, CA), anti-NFI-A (Abcam, Cambidge, UK), or the anti-C/EBPα (Santa Cruz Biotechnology). The anti-β-tubulin mouse monoclonal IgG (Sigma-Aldrich) was used to normalize the amount of the samples analyzed. The immunoreactivity was determined by the ECL method (Amersham Biosciences).

Transactivation Assays

DNA fragments from nt −807 (MRR1) or nt −492 (MRR2) or nt −396 (MRR3) to nt +36 relative to the *pre-miR-223* were PCR amplified from human genomic DNA using the forward primers 5′-GGGCACTTTAATAGCTGCCA-3′ or 5′-GGTTGCCTAACTAGCTAATG-3′ or 5′-GAATTGAGAAGAGGGAGCAA-3′, respectively, coupled with the reverse primer 5′-TCAAATACACGGAGCGTGG-3′. All the PCR fragments were inserted in the pGL2-LUC reporter vector (Promega). The MRR1, MRR2, and MRR3 constructs were used to generate the MRR1-, MRR2-, and MRR3-mut plasmids in which the 5′-TGTGGT-3′ direct motif of the AML1 site was mutated to 5′-*ACC*GGT-3′. To generate the mutations the QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used according to manufacturer instructions. Human embryonic kidney 293T cells (2×10^5) were plated in 12-well plates and transiently cotransfected by the Lipofectamine Reagent method (Invitrogen) with 10, 50, or 100 ng of the pcDNA3 vectors containing or not the HA-tagged-AML1/ETO cDNAs (Fazi et al., 2007), 300 ng of the LUC reporter constructs described above. U937-Mock and U937-A/E cells were pretreated or not with 100 μM ZnSO4 (16 hr) and then plated (5×10^5/well) in 24-well plates. Cells were transiently transfected by the FuGENE reagent (Roche, Mannheim, Germany) with 1.5 μg of the LUC reporter constructs described above. A cotransfected pRL-SV40 Renilla Luciferase reporter vector (Promega) was used as an internal control for normalization of luciferase activity in each sample. Cells were harvested 48 hr posttransfection and assayed with Dual Luciferase Assay (Promega) according to the manufacturer's instructions.

Chromatin Immunoprecipitation Assay

The addition to cultured cells (2×10^6) of formaldehyde (1% final concentration) for 10 min at 37°C was used to crosslink the proteins to DNA. After sonication, the chromatin was immunoprecipitated overnight with 5 μl of the following antibodies recognizing AML1/RHD, ETO (Ab-1) (Oncogene Science), HA monoclonal (Babco, Richmond, CA), DNMT1 (New England BioLabs, Ipswich, MA), DNMT3a and DNMT3b (Abcam), HDAC1 (Santa Cruz Biotechnology), MeCP2,

acetyl-histone-H4, and acetyl-histone-H3 (Upstate Biotechnology, Lake Placid, NY). ChIP using the Cytosine (5-Methyl) (Abcam) antibody was performed on naked and sonicated DNA extracted from the same cell samples. A genomic *pre-miR-223* upstream gene region close to the putative AML1-binding site indicated by the MatInspector Professional (http://www.genomatix.de) and the TransFac software packages (http://www.gene-regulation.com/pub/programs.html), was amplified with the following primer sequences designed by the Primer Express software (Applied Biosystem): oligo1 (nt −400 to −186) forward 5′-GGGAGAATTGAGAAGAGGGA-3′ and oligo1 reverse 5′-GATAAG CAGGTAAAGCCCGA-3′. The other pre-miR-223 upstream regions were amplified with the following primer sequences: oligo2 (nt −2467 to −2272) forward 5′-TCTGGGATTTTTAGGCATGG-3′ and oligo2-reverse 5′-AAGAGCGTCATCAAGCCACT-3′; oligo3 (nt −3514 to −3300) forward 5′-GCATCCAGATTTCCGTTGGCTAAC-3′ and oligo3-reverse 5′-GGCAAATGGATACCATACCTGTCA-3′. PCR for GAPDH was performed using the conditions and primers already described (Fazi et al., 2005).

Cell Differentiation

Cell differentiation was evaluated by light microscopy morphological examination of Wright-Giemsa-stained cytospins; nitroblue tetrazolium (NBT) dye reduction assay (at least 500 morphologically intact cells per experimental condition were counted and corrected for viability, measured by trypan blue exclusion method); direct immunofluorescence staining of cells using an allophycocyanin (APC)-conjugated mouse anti-human CD11b antibody; and a peridinin chlorophyll protein (PerCP)-conjugated mouse anti-human CD14 antibody (Becton Dickinson, San Jose, CA) as described (Fazi et al., 2005, 2007). A minimum of 50,000 events were collected for each sample by a FACScan flow cytometer (Becton Dickinson) using CellFit software (Becton Dickinson) for data acquisition and analysis.

Bisulfite Modification and Genomic Sequencing

The methylation status of the CpG dinucleotides within two regions (nt −203 to +137 and nt −3760 to −3413), relative to the 5′ end of the *pre-miR-223* gene, was analyzed. Bisulfite sequencing assay was performed on 2.5 μg of bisulfite-treated genomic DNA from HL60, U937 (Mock, A/E-HA), and SKNO-1 (WT, Mock, and siA/E) cell lines. After bisulfite conversion performed as previously described (Zardo et al., 2002), the fragments of interest were amplified using the following specific primer pairs: oligo1 forward 5′-AGTTTTTAGT TGAGTATTGGGTG-3′; oligo1 reverse 5′-CTTATATCCAACTAACAAT CCATTC-3′; oligo2 forward 5′-AATTTGTTTTGTGATATTGAGTATTT TT-3′; oligo2 reverse 5′-TACAAAAACCAAATAAAATTAAACTTTC-3′. PCR products were gel purified and cloned into the TOPO TA Cloning/pCR2.1 TOPO kit (Invitrogen). We subjected individual bacterial colonies to PCR using vector-specific primers (sequences available upon request), and the products were sequenced for the analyses of DNA methylation.

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