Epigenetic Modification of CCAAT/Enhancer Binding Protein α Expression in Acute Myeloid Leukemia

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Abstract

Functional loss of CCAAT/enhancer binding protein α (C/EBPα), a master regulatory transcription factor in the hematopoietic system, can result in a differentiation block in granulopoiesis and thus contribute to leukemic transformation. Here, we show the effect of epigenetic aberrations in regulating C/EBPα expression in acute myeloid leukemia (AML). Comprehensive DNA methylation analyses of the CpG island of C/EBPα identified a densely methylated upstream promoter region in 51% of AML patients. aberrant DNA methylation was strongly associated with two generally prognostically favorable cytogenetic subgroups: inv(16) and t(15;17). Surprisingly, while epigenetic treatment increased C/EBPα mRNA levels in vitro, C/EBPα protein levels decreased. Using a computational microRNA (miRNA) prediction approach and functional studies, we show that C/EBPα mRNA is a target for miRNA-124a. This miRNA is frequently silenced by epigenetic mechanisms in leukemia cell lines, becomes upregulated after epigenetic treatment, and targets the C/EBPα 3′ untranslated region. In this way, C/EBPα protein expression is reduced in a posttranscriptional manner. Our results indicate that epigenetic alterations of C/EBPα are a frequent event in AML and that epigenetic treatment can result in downregulation of a key hematopoietic transcription factor. [Cancer Res 2008;68(9):3142–3151]

Introduction

Acute myeloid leukemia (AML) has been extensively studied at the cytogenetic, molecular, and transcriptional level. This knowledge has contributed to the subclassification of AML and translated into significant improvement of therapies (1–3). Leukemic transformation to AML is a multistep process requiring the alterations of genes involved in proliferation/survival and hematopoietic differentiation (4). One such gene, CCAAT/enhancer binding protein α (C/EBPα), is a key transcription factor involved in the regulation of cell proliferation and differentiation in a variety of cell types, particularly in the hematopoietic system (5, 6). Whereas under physiologic conditions C/EBPα is a master regulator for myeloid differentiation and granulocytic maturation, its absence results in a block of granulopoiesis, as shown in several studies (6, 7). C/EBPα has gained interest in the AML field, because it has been shown that down-regulation of C/EBPα protein through mutations, posttranslational modifications, and protein-protein interactions with fusion proteins AML1/ETO or CBF-B-SMMHC plays a key role in leukemic transformation (6, 8–11).

Besides genetic aberrations, epigenetic modifications, such as DNA methylation and histone-tail modifications, have been shown to initiate or augment malignant transformation (12–14). Global promoter studies, as well as gene-specific approaches, have revealed that aberrant promoter methylation is a common event in AML (13, 15). Because of the pharmacologic reversibility of epigenetic changes by drugs, such as the DNA-demethylating agent 5-aza-2′-deoxycytidine (DAC) or the histone deacetylase (HDAC) inhibitor valproic acid, epigenetic therapy seems promisingly among novel leukemia treatment strategies (16–19). Whereas it is commonly seen that epigenetic treatment leads primarily to up-regulation of genes, several groups have recently shown that DNA demethylation and HDAC inhibition can also result in downregulation of gene expression (20, 21). The molecular mechanisms underlying these findings are largely unknown, but potential mechanisms include alterations in gene expression profiles as a result of drug treatment triggering additional changes in gene expression that are independent of promoter demethylation (20).

A third epigenetic mechanism has recently gained attention: gene expression regulation through microRNAs (miRNA). These short noncoding RNAs have been shown to down-regulate gene expression by targeting the 3′ untranslated region (UTR) of their target genes. Depending on whether the specific miRNA is entirely or partially complementary to its 3′ UTR binding site, downregulation is accomplished by either mRNA degradation or translational repression, respectively (22). Besides their role in cell proliferation, differentiation, and apoptosis, recent studies have provided evidence that miRNAs are also involved in leukemogenesis (23, 24).

Until recently, little has been known about the regulation of miRNAs, but seminal studies have now shown that hematopoietic transcription factors C/EBPα and PU.1 are capable of steering miRNA-223 (miR-223) expression, which is a crucial factor in granulocytic differentiation (25, 26). Moreover, it is becoming evident

Note:
(Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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that miRNAs are not only effectors of the epigenetic machinery, but they themselves can be regulated by DNA methylation (27–29).

In a recent study, DNA methylation of the C/EBPα core promoter was found in a small subset of AML patients and biologically linked to T-cell lineage infidelity (30). Furthermore, we have shown in two studies in lung cancer and head and neck squamous cell carcinoma that C/EBPα expression is down-regulated by epigenetic mechanisms, including DNA methylation of the C/EBPα upstream promoter region (31, 32). Encouraged by these results, we sought to comprehensively investigate the role of epigenetic regulation of C/EBPα in AML.

Materials and Methods

Patient samples and cell lines. One hundred forty-six bone marrow samples from AML patients were obtained from the Cancer and Leukemia Group B tissue bank and the University of Freiburg tissue bank. The patients provided written, institutional review board–approved, informed consent. Bone marrow samples from seven healthy donors were collected after obtaining informed consent under a protocol approved by Ohio State University Institutional Review Board. Cell lines U937, THP1, HL60, K562, and Kasumi1 were obtained from the American Type Culture Collection, and NB4 from the German Collection of Cell Cultures. U937, THP1, and NB4 were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). Kasumi1 was cultured in RPMI 1640 supplemented with 20% FBS; HL60 was cultured in DMEM supplemented with 10% FBS; K562 was cultured in Iscove’s modified Dulbecco’s medium supplemented with 10% FBS. All media were supplemented with 1% streptomycin/penicillin. CD34+ cells from a healthy donor were cultured in CellGro Medium (CellGenix) supplemented with SCF (100 ng/mL), FLT3 (100 ng/mL), IL-3 (50 ng/mL), and IL-6 (20 ng/mL). Granulocyte colony-stimulating factor (G-CSF) was given at 50 ng/mL on day 0 and day 6.

DAC and trichostatin A treatment. Suspension cells were seeded at a concentration of 5 × 10^4/mL and treated for 72 and 96 h with 200 nmol/L DAC (Sigma-Aldrich), for 24 h with 300 nmol/L trichostatin A (TSA; Sigma-Aldrich), or for 72 h with 200 nmol/L DAC followed by 24 h with 300 nmol/L TSA. Medium and drugs were replaced daily.

DNA and RNA isolation. Total RNA of patient samples and cell lines was isolated using Trizol (Invitrogen) following manufacturer’s recommendations. Genomic DNA of patient samples and cell lines was isolated from the Trizol phase remaining after removal of the aqueous fraction containing total RNA, following manufacturer’s recommendations.

Bisulfite treatment, bisulfite sequencing, and combined bisulfite restriction analysis assay. One microgram of genomic DNA was used for bisulfite treatment as previously described (33). The primers and PCR conditions for bisulfite sequencing and combined bisulfite restriction analysis assay (COBRA) are summarized in Supplementary Table S1. For sequencing, PCR products were purified using a Qiagen gel extraction kit (Qiagen) and ligated into pCR2.1-TOPO according to manufacturer’s instructions. The plasmid DNA was isolated using the Miniprep kit (Qiagen). Clones were sequenced using ABI BigDye Terminator Chemistry (Applied Biosystems). For COBRA, PCR products were purified and incubated with BstUI at 60°C for 3 h or with HpyCH4IV at 37°C for 3 h. The digested DNA was then separated on an 8% polyacrylamide gel and stained with ethidium bromide.

BioCOBRA. BioCOBRA was performed as recently described (34). Briefly, 20 to 40 g of the digest of the digestion products from a regular COBRA was loaded onto a DNA 500 LabChip and assayed using the Bioanalyzer 2100. The chromatograms were visually examined; raw data were exported as CSV-files using the 2100 expert software and subsequently plotted to obtain the fluorescence values for each of the expected fragments. The methylation percentage value for each sample was calculated using the following formula: fluorescence of methylated products / (fluorescence of methylated products + fluorescence of unmethylated product). Samples were considered methylated when the average methylation of the two BstUI cutting sites was >10%. The primers used for the BioCOBRA are listed in Supplementary Table S1.

MassARRAY. Quantitative DNA methylation analysis using the MassARRAY technique was performed by Sequenom, Inc., as previously described (35). Briefly, 1 μg of genomic DNA was treated with sodium bisulfite, PCR amplified, in vitro transcribed, and then cleaved by RNase A. The samples were then quantitatively tested for their DNA methylation status using matrix-assisted laser desorption ionization-time of flight mass spectrometry. Samples were considered methylated when the average methylation within an amplicon was >10%.

Real-time PCR. Total RNA (1 μg) was incubated with 2 units of DNasel (Invitrogen) for 30 min at room temperature. The DNA-free RNA was reverse transcribed using 100 units of SuperScript II (Invitrogen) and 1 μg of oligo dT per reaction. Semiquantitative C/EBPα expression was measured using SYBR Green I (Bio-Rad) in an iCycler (Bio-Rad). The ABI1 proto-oncogene was used as the internal control. For primer sequences, see Supplementary Table S1. We additionally performed a PCR on DNasel incubated but non–reverse transcriptase–treated samples to ensure that no DNA contamination was present in the RNA extract, given the fact that C/EBPα is an intronless gene. For mature miR-124a, stem-loop PCR was performed using the Applied Biosystems hsa-mir-124a RT kit (part number 43735150). For the pre–miR-124a reverse transcription–PCR (RT-PCR), we used customized primers (see Supplementary Table S1). 18S was used as the internal control gene as recently described (28). Experiments were done in triplicates.

Western blot analysis. Whole-cell lysates from cell lines were prepared by incubating 2 × 10^6 cells in Laemmli buffer for 10 min at 95°C. For the two AML patient samples, frozen cell pellets of bone marrow mononuclear cells, collected before and after a 10-d Decitabine treatment, were lysed with Laemmli buffer. Proteins were separated by electrophoresis on 4% to 15% gradient polyacrylamide gels (Bio-Rad) and transferred onto a nitrocellulose membrane. C/EBPα, Cdk6, and p21 proteins were detected using rabbit polyclonal antibodies (Santa Cruz Biotechnology, Inc.) at 1:1,000 dilutions. A horseradish peroxidase–conjugated antirabbit secondary antibody (Amer sham Biosciences) was used at 1:5,000 dilutions. To control for equal loading, membranes were incubated with monoclonal mouse anti–α-tubulin (1:3,000 dilution; EMD Biosciences). Enhanced chemiluminescence (Amer sham Biosciences) was used to detect protein-antibody complexes.

Luciferase assay and luciferase target assay. A 634-bp fragment of C/EBPα 3′ UTR containing the predicted miR-124a binding site was cloned into pGL3-promoter vector (Promega) at the XbaI site, downstream of the luciferase gene according to recent descriptions (36). Using Lipofectamine 2000 (Invitrogen) K562 cells (2 × 10^5 per well) were then cotransfected with 0.8 μg of pGL3-C/EBPα 3′ UTR construct, 60 ng Renilla, and 100 nmol/L of either nontargeting RNA control oligonucleotides (Dharmacon) or miR-124a (Dharmacon). At 24 h after transfection, cells were washed thrice with PBS and lysed in passive lysis buffer (Promega). Luciferase activity was measured using the Veritas Luminometer (Turner Biosystems). Firefly luciferase activity was normalized to Renilla luciferase activity. Experiments were performed in triplicates. The in vitro methylation assay was performed as recently described (37).

Transfection assay. HL-60 (1 × 10^6 per well) cells were transfected with 200 nmol/L of either a nontargeting RNA control oligonucleotides (Dharmacon) or miR-124a (Dharmacon) using Nucleofector technology (Amaxa) according to the manufacturer’s instructions. At 48 h after transfection, whole-cell lysates were prepared, and Western blot for C/EBPα protein was performed as described above. To check whether transfection was effective, we also transfected HL-60 cells with increasing concentrations of miR-124 (40–200 nmol/L) and after 6 h washed the cell thoroughly twice with PBS, extracted RNA, and used semiquantitative RT-PCR to detect intracellular miR-124a.

Statistical analysis. Normalized DNA methylation levels were compared between each cytogenetic subgroup and the normal bone marrow (NBM) group using the two-sided Wilcoxon rank sum test, and the Bonferroni procedure was used to correct for multiple testing. Differences in mRNA expression and luciferase activity, relative to the controls, were evaluated by two-sample t tests. The two-sided level of significance was set at α = 0.05.
Results

DNA methylation in the upstream promoter of C/EBPα.

Previous studies investigated the core promoter and adjacent regions of C/EBPα in AML, concluding that epigenetic silencing is a rare event in C/EBPα regulation (38, 39). However, it has been shown that the upstream promoter, which also bears promoter activity, is the target of epigenetic silencing in lung and head and neck cancer (31, 32, 40). This prompted us to reevaluate the role of epigenetic silencing of C/EBPα in AML. To investigate the DNA methylation patterns of C/EBPα comprehensively, we conducted COBRAs and sodium bisulfite sequencing on bone marrow samples from 15 AML patients and three bone marrow samples from healthy individuals (NBM). Consistent with previous reports, we found that the core promoter [16 to −301 bp, relative to the transcription start site (TSS)], region 3 (−918 to −725 bp from TSS), and region 2 (−1,142 to −896 bp from TSS) were unmethylated (Fig. 1A–C). However, substantial DNA methylation was present in the upstream promoter region 1 (1,423 to −1,121 bp from TSS; Fig. 1B). To validate these findings, we performed quantitative DNA methylation analysis on 39 additional AML samples representing the most common cytogenetic subgroups [inv(16)(p13q22), t(8;21)(q22q22), t(15;17)(q22q12/21), t(9;11)(p22q23), and complex karyotype], three NBM samples, and five leukemia cell lines (Kasumi1, ME1, NB4, U937, HL60) using the MassARRAY technology (35). This study covered region 1 (amplicon A), parts of the core promoter (amplicon B), as well as exon 1 (amplicon C; see Fig. 1A for location of amplicons). Whereas NBM was unmethylated, 20 of 39 AML samples (51%) and four of five leukemia cell lines (Kasumi1, ME1, NB4, U937) were methylated, with 10 samples showing methylation levels of >50% (Fig. 2A). Aberrant DNA methylation in cell lines and patient samples was restricted to region 1 (amplicon A; Fig. 2A). Interestingly, AML samples that

Figure 1. DNA methylation analysis of the C/EBPα core promoter and upstream promoter region in a subset of AML samples. A, the single exon gene C/EBPα is embedded in a large CpG island; the areas of DNA methylation analysis are marked with opposing arrows and the techniques used are listed. ATG, translational start site; BS, bisulfite sequencing. B, analysis (COBRA) of 15 AML samples at the core promoter and regions 1 and 3. u, unmethylated band; m, methylated bands; +, 100% methylated control; −, negative control. C, bisulfite sequencing of six AML samples and NBM in region 2. Each row represents an individual clone. White circles, unmethylated CG dinucleotides; black circles, methylated CG dinucleotides.
showed extensive methylation were derived from patients with inv(16), t(8;21), or t(15;17), thus suggesting AML subgroup-specific epigenetic alterations.

**Aberrant DNA methylation of C/EBPα is associated with cytogenetic subgroups.** To quantitatively evaluate DNA methylation in a larger sample set and to confirm our observation of differential methylation in cytogenetic subgroups, we used the BioCOBRA assay and measured DNA methylation in region 1 in 94 AML samples, including 26 cases with normal karyotype, 14 cases with inv(16), 13 cases with t(8;21), 11 cases with t(9;11), and 15 cases with a complex karyotype, comprising three or more chromosomal aberrations. The DNA methylation levels in NBM that served as baseline were low (median, 0%; range, 0–5%). We observed significantly higher DNA methylation levels in the inv(16) and t(15;17) cytogenetic subgroups with a median of 29% and 5%, respectively (P < 0.05; Fig. 2B). No significant differences were seen in the normal karyotype (median, 2%), t(8;21) (median, 3%), t(9;11) (median, 3%), and complex karyotype patient samples (median, 4%). However, one sample from the t(8;21) subgroup showed 54% methylation and three samples from the normal karyotype subgroup showed methylation levels of 18%, 32%, and 41%. Together these data suggest that elevated DNA methylation levels of C/EBPα region 1 is associated with AML subgroups inv(16) and t(15;17); however, DNA methylation does not seem to be uniformly restricted to those two cytogenetic groups.

Additionally, we used an independent sample set of 52 AML patients [26 with normal karyotype, 11 with t(8;21), and 15 with t(15;17)] and applied MassARRAY technology (Fig. 2C). From the 51 evaluable samples, we detected DNA methylation in 6 of 25 patients with normal karyotype, 4 of 11 patients with t(8;21), and 13 of 15 patients with t(15;17), thus confirming the strong association of DNA methylation of C/EBPα region 1 with the translocation t(15;17). It has to be mentioned that, rather being associated with cytogenetic subgroups, DNA methylation of C/EBPα region 1 could also generally occur in a subset of AML patients.

**mRNA expression of C/EBPα in AML.** To determine if aberrant DNA methylation in the upstream promoter affected C/EBPα expression, we investigated patients with inv(16) because of their
broad range of differential methylation. Using semiquantitative RT-PCR, we observed substantial differences in mRNA levels among the patients, but no correlation with DNA methylation in region 1 was seen (Fig. 3A). The observed differences of C/EBPα mRNA levels in inv(16) patients are in accordance with previous studies (8, 11, 41).

Because we did not find a direct correlation between DNA methylation and C/EBPα expression in AML patients, we treated leukemia cell lines (HL60, U937, NB4, K562, THPI, Kasumi1) with the DNA-demethylating agent DAC and the HDAC inhibitor TSA to determine whether expression levels change after treatment. The core promoter was unmethylated in all cell lines (data not shown). In contrast, U937, NB4, K562, and Kasumi1 cells, but not HL60 and THPI cells, were highly methylated in region 1 (Fig. 3B). We applied BioCOBRA and showed that DNA methylation of C/EBPα region 1 in U937 decreased substantially after treatment with DAC, whereas TSA had—as expected—no effect on DNA methylation (Fig. 3C).

As epigenetic changes are known for their transcriptional regulatory potential, we examined C/EBPα mRNA levels in leukemia cell lines treated with 200 nmol/L DAC for 72 and 96 hours, 300 nmol/L TSA for 24 hours, or a combination 200 nmol/L DAC for 72 hours followed by 300 nmol/L TSA for 24 hours using semiquantitative RT-PCR (Fig. 3D). The unmethylated cell line HL60 and the methylated cell line NB4 showed significant down-regulation (P < 0.05) of C/EBPα mRNA levels upon DAC and/or TSA treatment. The unmethylated cell line THPI and the methylated cell lines U937, K562, and Kasumi1 showed significant up-regulation (P < 0.05) in at least one treatment time point suggesting the involvement of epigenetic factors in the regulation
of C/EBPα (Fig. 3D). The heterogeneous response of these cell lines upon epigenetic treatment reflects the complexity of transcriptional regulation of C/EBPα in AML with DNA methylation being only one part of the regulatory machinery. Moreover, as DAC is a globally acting substance, demethylation and reactivation of other factors, regulating C/EBPα, might explain the heterogeneous response in the cell lines.

To further investigate the effect of DNA methylation of C/EBPα region 1 and to show a potential biological meaning, we treated PCR-amplified region 1 with SSSI to methylate the DNA and cloned it in front of luciferase in the pGL3 vector. As control, we use nonmethylated region 1. Hereafter, we transfected the constructs in K562 cells and measured relative luciferase activity (Fig. 3E). We observed a significant decrease in relative luciferase activity in the cells with the methylated construct. While this is an artificial consequence of demethylating and HDACi treatment, we next measured C/EBPα protein expression in leukemia cell lines after treatment with DAC and TSA. Whereas the unmethylated cell lines HL60 and THPI showed high C/EBPα expression, methylated cell lines NB4 and U937 showed modest expression and Kasumi1 and K562 did not express C/EBPα protein (Fig. 4A). Surprisingly, after treatment with DAC and TSA, we observed a substantial down-regulation of C/EBPα protein in U937, HL60, and THPI cells. Because it has been well established that C/EBPα down-regulation in Kasumi1 or K562 cells is achieved through a protein-protein interaction with AML1/ETO and BCR/ABL fusion proteins, respectively, a translational up-regulation upon epigenetic treatment would have been unexpected (8). However, the down-regulation of C/EBPα protein in U937, HL60, and THPI cells could not be explained by this mechanism and therefore was intriguing (Fig. 4A).

Consistent with a previous study, which indicated that the cyclin-dependent kinase inhibitor p21 could be up-regulated in AML cell lines through demethylating treatment, we observed up-regulation of p21 protein after demethylating treatment in THPI cells, supporting that our observations were unlikely a technical issue (Fig. 4B; ref. 21).

Furthermore, we investigated whether demethylating treatment in vivo could also result in down-regulation of C/EBPα protein. We tested unselected bone marrow cells from two AML patients before and after a 10-day treatment with 20 mg/m2/d decitabine (42). In relation to the control protein tubulin, there was no change in C/EBPα protein expression, suggesting no significant effect of demethylating treatment on C/EBPα protein expression in these patients (Fig. 4D).

C/EBPα is a target of miR-124a in vitro. The surprising finding of C/EBPα protein down-regulation in leukemia cell lines after epigenetic treatment and the recent findings of epigenetic regulation of miRNAs led us to the hypothesis that a specific miRNA targeting C/EBPα might explain our findings.

We used the publicly available TargetScan software to search for miRNAs with a potential regulation of C/EBPα. We used the publicly available TargetScan software to search for miRNAs with a potential regulation of C/EBPα. We used the publicly available TargetScan software to search for miRNAs with a potential regulation of C/EBPα. We used the publicly available TargetScan software to search for miRNAs with a potential regulation of C/EBPα. We used the publicly available TargetScan software to search for miRNAs with a potential regulation of C/EBPα.
was identified as candidate with the highest predicted likelihood to bind the C/EBPα 3′ UTR. Further support for C/EBPα mRNA and miR-124a interaction came from a study in HeLa cells investigating putative target sequences for miR-124a (43). The fact that miR-124a was predicted to be only partially complementary to the C/EBPα 3′ UTR binding site strengthened our hypothesis that miR-124a might block translation of C/EBPα mRNA rather than degrading it. To validate the predicted interaction between miR-124a and C/EBPα, we prepared a luciferase construct by inserting the C/EBPα 3′ UTR downstream of the luciferase reporter gene in the pGL3 promoter vector. This reporter construct was then cotransfected in K562 cells with either control nontargeting RNA oligonucleotides or miR-124a. Relative luciferase activity was significantly decreased (P < 0.05) 24 hours after miR-124a transfection compared with transfection with the control miRNA, indicating that miR-124a interferes with C/EBPα mRNA via a direct interaction with the 3′ UTR (Fig. 5A). Next, we transfected the C/EBPα-positive cell line HL60 with miR-124a or the negative control oligonucleotide. After 48 h, C/EBPα protein decreased substantially, supporting our hypothesis that miR-124a negatively regulates C/EBPα (Fig. 5B).

Figure 5. Functional analysis of miR-124a in leukemia cell lines and DNA methylation and expression analyses of precursors and mature miR-124a. A, dual luciferase assay of K562 cells cotransfected with a pGL3-promoter construct containing the C/EBPα 3′ UTR and miR-124a and a control nontargeting RNA oligonucleotide (control miR), respectively, for 24 h. Firefly luciferase activity was normalized using Renilla luciferase activity as internal transfection control. The luciferase value of the miR-124a transfection was then normalized to the average of the control miR transfection. *, P < 0.05. Error bars, SE from triplicates. B, Western blot analysis of C/EBPα protein in HL60 after transient transfection with miR-124a and a control nontargeting RNA oligonucleotide, respectively, for 48 h. 42-kDa band of C/EBPα is shown. Tubulin served as loading control. C, COBRA analysis of the miR-124a-3 gene, which is embedded within a CpG island, in leukemia cell lines and NBM. D, COBRA analysis of the mir-124a-3 gene in U937 before and after treatment with 200 nm DAC and/or 300 nm TSA. E, MassARRAY analysis of the mir-124a-1 gene in HL60 and U937 cells before and after treatment with DAC for 72 and 96 h. The location of the miR stem loop within the amplicon is marked. av., average methylation of all CpGs within the amplicon. F, expression of precursor miR-124a in HL60 and U937 cells before and after treatment with DAC in relation to the untreated cell line using semiquantitative RT-PCR. 18S was used as internal control gene. For U937, expression is shown in relation to the 24-h TSA time point (because the untreated U937 did not show any miR-124a expression). Error bars, SE from triplicates.*, significant up-regulation (P < 0.05).
**MiR-124a is epigenetically silenced in leukemia cells and reactivated after DAC treatment.** Interestingly, the miR-124a-1 and miR-124a-3 genes but not miR-124a-2, are located within CpG islands. To investigate whether DNA methylation is involved in miR-124a regulation, we first evaluated the DNA methylation status of the miR-124a-1 and miR-124a-3 CpG island in leukemia cell lines by COBRA and MassARRAY and found that miR-124a-3 was highly methylated in all of them but not in NB4 (Fig. 5C). After treatment of cell lines with 200 nm DAC and/or 300 nm TSA, substantial DNA demethylation of miR-124a-3 was seen in all cell lines (Fig. 5D shown for U937). miR-124a-1 gene was also highly methylated in HL60 and U937 cell lines and could be demethylated by DAC treatment as shown by MassARRAY (Fig. 5E). As we were not able to detect baseline expression or up-regulation of any single pri-pre miR-124a (most likely due to technical limitations), we designed primers specific for all pre-miR-124a precursors, however, not amplifying mature miR-124a. In cell lines HL60 and U937, we detected substantial up-regulation of miR-124a precursors upon DAC treatment (Fig. 5F). Moreover, expression analysis using semiquantitative RT-PCR revealed a significant up-regulation (P < 0.05) of mature miR-124a that correlated with DNA demethylation (Fig. 5G). Taken together, these data show a significant up-regulation of miR-124a precursors and mature miR-124a upon demethylating treatment. Whether only one of the three precursors or all three are getting up-regulated could not be differentiated during this study.

Because it has recently been shown that Cdk6, a gene involved in cell cycle progression and a potential oncogenic factor, was downregulated by reactivation of epigenetically silenced miR-124a in colon cancer (28), we investigated Cdk6 expression in U937, THP1, and NB4 cell lines. Cdk6 was highly expressed in U937 and NB4 cells and moderately expressed in the THP1 cell line. While we saw no down-regulation in U937 and NB4 cells, Cdk6 protein decreased substantially in THP1 cells after demethylating treatment (Fig. 4B).

Next, we transfected miR-124a in U937 cells to achieve higher intracellular miR-124a levels than upon DAC treatment. This resulted in a substantial down-regulation of Cdk6 (Fig. 4C). These finding supports our hypothesis that up-regulation of epigenetically silenced miR-124a can lead to down-regulation of two of its target genes in AML.

Finally, we investigated the DNA methylation status of the miR-124a-1 and miR-124a-3 genes in 52 AML samples [26 with normal karyotype, 11 with t(8;21) and 15 with t(15;17)] and in G-CSF induced and control CD 34+ hematopoietic progenitors. Independent of cytogenetic subgroups, we saw substantial methylation of miR-124a-1 and miR-124a-3 in the majority of samples (Fig. 6a and B). Interestingly, DNA methylation of the normal control CD34+ cells was significantly lower before and upon differentiating treatment compared with the majority of AML samples, suggesting that methylation of these miR-124a genes might be an acquired event during leukemogenesis. In summary, these data indicate that miR-124a is epigenetically regulated in vitro and methylation of miR-124a-1 and miR-124a-3 is a frequent finding in AML.

**Discussion**

The crucial role of the transcription factor C/EBPα in lineage determination during normal hematopoiesis is well established. Reduced expression or loss of function in hematopoietic malignancies has been studied extensively, and loss of C/EBPα function is thought to contribute as an early event to leukemogenesis by inhibiting myeloid differentiation (7, 44). In the present study, we investigated the epigenetic contribution to C/EBPα deregulation and show that aberrant DNA methylation in the upstream promoter of C/EBPα is a frequent event in AML. A distinct pattern of aberrant DNA methylation in region 1 was seen in 51% of AML patient samples, whereas the core promoter and all other investigated regions remained unmethylated. Most interesting was the finding of significantly higher DNA methylation levels in AML samples cytogenetically characterized by inv(16) and t(15;17). This AML subgroup–specific pattern suggests a biological relevance for the aberrant DNA methylation, and the reason for this preferential methylation is focus of ongoing research. A direct correlation of DNA methylation with mRNA levels was not detectable in patient samples with inv(16), possibly due to a constitutively active core promoter. The relationship to gene expression is difficult to evaluate because we believe that epigenetic modulation of the upstream promoter is not completely abolishing expression but rather reduces expression and thus modulates the expression level. Altered gene expression levels rather than on-off switches have been reported to possess drastic effects (e.g., PU.1 expression levels; ref. 45). Therefore, we speculate that such a mechanism is operating in those AML subgroups demonstrating C/EBPα methylation and cooperating with additional molecular alterations in this subgroup. Alternatively, one could speculate that epigenetic alterations are just the final marking of a gene locus that has become silenced or showed reduced expression. In this case, aberrant DNA methylation would be a biomarker for a yet unknown event occurring in specific subgroups.

Interestingly, C/EBPα protein levels were affected by the presence of a miRNA, miR-124a, which is also regulated by promoter methylation. This interplay of two epigenetically modulated genes offers a novel explanation for the finding of down-regulation of a key hematopoietic transcription factor after pharmacologic unmasking of methylated gene promoters.

The DNA methylation status of C/EBPα in AML has been studied previously (38, 39). Both studies concluded that DNA methylation of C/EBPα in AML is a rare event. However, very recently, Wouters and colleagues provided first evidence for the importance of C/EBPα methylation in a small subgroup of AML (30).

However, these studies did not examine the most upstream promoter region (region 1), in which we found aberrant DNA methylation (−1,423 to −1,121 bp from TSS). Aberrant promoter methylation has also been described in lung cancers and head and neck cancers. Again, the core promoter was not affected by epigenetic silencing in these entities (31, 32). It is noteworthy that the DNA methylation patterns within the CpG island showed tumor-type specificity with C/EBPα methylation being restricted to region 1 in head and neck cancer and AML, whereas in lung cancer also region 2 was differentially methylated. A possible explanation for this finding could be that different regulatory regions are used in different tissues, and epigenetic mechanisms interrupt the interaction of the relevant binding proteins with these regions through chromatin conformation changes. Interestingly, the sequence of the upstream methylated region (−1,423 to −1,121 bp from TSS) is highly conserved between humans, mice, and dogs according to UCSC Genome Browser (March 2006 assembly). Also, this sequence contains two SP1 and USF binding sites, which are known transcriptional activators of C/EBPα (31, 46). This mechanism has been shown for USF1/2 transcription factor binding in the C/EBPα upstream promoter in lung cancer (31).
The biological relevance of differential DNA methylation of C/EBPα region 1 in AML is likely, as there are two cytogenetic subgroups, inv(16) and t(15;17), which are preferentially targeted. It has been shown that leukemia fusion proteins, such as PML/RARα [t(15;17)], are capable of recruiting DNA methyltransferases to their target genes, thereby inducing epigenetic silencing (47, 48). Therefore, one could postulate that C/EBPα may be a target of PML/RARα and, moreover, that the inv(16) fusion protein CBFB-SMMHC might possess DNA methyltransferase recruiting capacity.

As this offers an explanation for our observation of differential C/EBPα methylation in AML, the consequence may be a selection advantage of these cells, thereby contributing to the malignant clone. In the context of recent findings that C/EBPα protein is down-regulated in AML posttranslationally by fusion proteins, such as AML1/ETO [t(8;21)] or CBFB-SMMHC [inv(16)], our data support the probable collaboration of genetic and epigenetic aberrations in leukemogenesis (8, 11).

Epigenetic silencing and activation of miRNAs after demethylating treatment have been described. These reports focused on the interaction of reactivated miRNAs with the 3' UTR of proto-oncogenes (27–29). Saito and colleagues showed that epigenetically silenced miR-127 could be up-regulated by demethylating treatment and targets the proto-oncogene BCL6 (27). Lujambio and colleagues observed that the epigenetic silencing of miR-124a in colon cancer cell lines resulted in activation of cyclin D kinase 6 (CDK6), an oncogenic factor (28). In our study we report the targeting of a candidate tumor suppressor gene (C/EBPα), as well as a proto-oncogene (CDK6), by a reactivated miRNA. Especially for AML, it is intriguing to speculate that epigenetic down-regulation of miR-124a may up-regulate Cdk6, a cell cycle regulator previously shown to be associated with centrosome and numerical chromosome aberrations in AML (49).

Finally, we want to emphasize that our findings should not be considered as negating the promising results of clinical trials with epigenetic drugs in patients with hematopoietic malignancies, especially because we do not see down-regulation of C/EBPα in DAC-treated patients (16, 17). On the contrary, our data might help explain why some patients respond very well to epigenetic therapy while others do not. Moreover, it should be highlighted that a large number of genetic pathways are likely to be affected by these systemic therapies, thereby making the effect of their deregulation difficult to predict. Although we show that miR-124a methylation is common in AML patients, functional consequences, especially in patients undergoing epigenetic treatment, require careful and

Figure 6. DNA methylation of miR-124a-1 and miR-124a-3 in AML patients and CD34+ cells. Quantitative DNA methylation analysis of miR-124a-1 (A) and miR-124a-3 (B) using MassARRAY in 52 AML samples (for miR-124a-3 only 37 samples amplified) with normal karyotype, t(8;21), or t(15;17) and CD34+ cells treated with or without G-CSF for 3 to 10 d. Each row represents a sample and each column represents a single CG site or a combination of CG sites. Color coding reflects the degree of methylation with blue being 100% and yellow being 0%; gray, amplicon did not amplify; CH3, methylation. The location of the miR stem loop within the amplicon is marked.

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