Epigenetic silencing of Bcl-2, CEBPA and p14^{ARF} by the AML1-ETO oncoprotein contributing to growth arrest and differentiation block in the U937 cell line

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Abstract. The AML1-ETO fusion transcription factor generated by the t(8;21) translocation is considered to deregulate the expression of genes that are crucial for normal differentiation and proliferation of hematopoietic progenitors, resulting in acute myelogenous leukemia by recruiting co-repressor complexes to DNA. To investigate the role of AML1-ETO in leukemogenesis, we transfected the cloned AML1-ETO cDNA and expressed the AML1-ETO protein in U937 myelomonocytic leukemia cells. By focusing on the anti-apoptotic gene Bcl-2, the key regulator gene of granulocytic differentiation CCAAT/enhancer-binding protein α (CEBPA) and the tumor suppressor gene p14^{ARF}, we found that both AML1-ETO-expressing cell lines and t(8;21) leukemia samples displayed low levels of these three genes. Chromatin immunoprecipitation assays demonstrated that Bcl-2, CEBPA and p14^{ARF} were direct transcriptional targets of AML1-ETO. The universal binding of AML1-ETO to genomic DNA resulted in recruitment of methyl-CpG binding protein 2 (MeCP2), reduction of histone H3 or H4 acetylation and increased trimethylation of histone H3 lysine 9 as well as lysine 27 indicating that AML1-ETO induced heterochromatic silencing of Bcl-2, CEBPA and p14^{ARF}. These results suggested that the aberrant transcription factor AML1-ETO epigenetically silenced the function of the Bcl-2, CEBPA and p14^{ARF} genes by inducing repressed chromatin configurations at their promoters through histone modifications.

Introduction

The t(8;21) is the second most common chromosomal abnormality in AML, accounting for 10-15% of cases with discernible translocations, and characteristically induces a leukemia with the French-American-British (FAB) M2 phenotype. As previously documented (1-3), AML1-ETO exerts the dominant negative effect on AML1-dependent transcriptional activation, mostly through interaction of its ETO moiety with nuclear co-repressors N-CoR and Sin3A that recruit the histone deacetylases (HDACs), resulting in transcriptional repression by deacetylating histones and creating repressive chromatic structures. A simple model of AML1-ETO function in leukemogenesis reflects its dominant negative effects on AML1 target genes, to a large extent via the aberrant recruitment of epigenetic modifiers such as HDACs and DNA methyltransferases (DNMTs).

A direct transcriptional regulation by AML1-ETO through the AML1 DNA-binding activity has been demonstrated for a few genes, notably the anti-apoptotic gene Bcl-2 (4), the hematopoietic lineage regulator gene CCAAT/enhancer-binding protein α (CEBPA) (5) and the cell cycle regulator p14^{ARF} (6).

The Bcl-2 gene is a highly conserved member of the Bcl-2 family and constitutes an important regulator of apoptosis. Bcl-2 can prevent or delay apoptosis in several cell types (7). Klampfer *et al* (4) identified a consensus DNA binding sequence for AML1 (TGT/cGGT) in the 5' regulatory region of the Bcl-2 gene and demonstrated that both AML1 and AML1-ETO proteins can bind to this site. Regulation of the Bcl-2 promoter by AML1-ETO, but not by the normal AML1 proteins, indicates a unique biological activity of the fusion protein (4).

The C/EBP genes are believed to be critically involved in hematopoietic differentiation and leukemogenesis (8). Approximately 10-15% of AML samples have inactivating mutations of CEBPA, and the forced expression of C/EBPα in AML cells can induce terminal differentiation, emphasizing the important contribution of C/EBPα to AML leukemogenesis (5,9). AML1-ETO may contribute to leukemogenesis by specifically inhibiting AML1 and CEBPA-dependent activation of myeloid promoters and blocking differentiation (10).

The p53 tumor suppressor pathway is arguably the most important checkpoint control pathway in human cancer. A third component of the p53 pathway is the p14^{ARF} tumor suppressor, which regulates the p53-dependent oncogene checkpoint (11).

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Loss of p14^{ARF} impairs p53-mediated growth arrest and/or apoptosis in response to activated oncogenes. In addition, cells lacking either p53 or p14^{ARF} fail to undergo replicative crisis and are immortal (12). The p53 promoter does not contain any perfect AML1 DNA-binding sites (TGT/cGGT), but the human p14^{ARF} promoter contains eight such sites (6).

In this study, using AML1-ETO-expressing cell line U937-A/E as an *in vitro* model, we performed chromatin immunoprecipitation (ChIP) assays to investigate how the binding of AML1-ETO affected the chromatin structure of its target genes (Bcl-2, CEBPA and p14^{ARF}) and thus caused deregulated gene expression associated with growth arrest and differentiation block. Our study identified Bcl-2, CEBPA and p14^{ARF} as additional pathogenic targets for a leukemia fusion protein and provided evidence that linked the epigenetic silencing of Bcl-2, CEBPA and p14^{ARF} loci to the growth arrest and differentiation block of myeloid precursors. Thus, suppression of these gene expressions correlated with significant alterations in the chromatin structure at the promoters may play a key role in the proliferation and differentiation underlying leukemogenesis.

Materials and methods

Clinical samples. Leukemic cells of nine non-t(8;21) AML patients and nine t(8;21) AML patients who were diagnosed as the M2 subtype according to the FAB classification, were prepared from bone marrow cells or peripheral blood mononuclear cells, following approval by the Hospital Ethics Committee with signed consent provided by the patients.

Cell culture. Human myeloid U937 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37˚C in a humidified incubator with an atmosphere of 5% CO_2. U937-Mock and U937-A/E cells were maintained in RPMI-1640 medium supplemented with 10% FBS and 0.5 mg of G418/ml.

ChIP assay. ChIP assays were performed by using a ChIP Express kit (Millipore Biotechnology), according to the manufacturer's instructions.

Statistical analysis. All values in the figures are expressed as the means ± SD. To determine statistical significance, the values were compared using two-group t-tests, and P-values <0.05 were considered to indicate statistically significant differences.

Results

Expression of AML1-ETO reduces proliferation, induces apoptosis and blocks myeloid differentiation. To investigate the potential direct role of AML1-ETO in the growth, survival, and differentiation of myeloid leukemic cells, we made AML1-ETO-expressing U937 cell lines. As indicated in Fig. 1A and B, relatively high levels of AML1-ETO expression were clearly observed in U937-A/E1 and 2 at both mRNA and protein levels. The effects of AML1-ETO expression on cell growth were evaluated by comparing the growth curves of U937-A/E, U937-Mock and U937-WT cells. Analyses of the proliferative ability indicated that cell growth in AML1-ETO-transfected cells was significantly decreased in comparison to empty vector-transfected cells and non-transfected cells (P<0.01) (Fig. 1C). There was no significant difference between the proliferation rates of U937-Mock and U937-WT cells, indicating that the effect was due solely to the expression of AML1-ETO.

We performed experiments to determine how the expression of AML1-ETO affected the apoptosis. The results demonstrated that in U937-A/E cells but not in U937-WT and U937-Mock cells, apoptotic cells were statistically significantly increased, although to a lower degree, as evidenced by the Annexin V assay (Fig. 1D). In order to further confirm the effects of AML1-ETO expression on enhancing apoptosis, changes in caspase-3 protein were analyzed in U937-A/E cells. As shown in Fig. 1E, AML1-ETO expression also significantly enhanced activation of caspase-3, an indicator of cell apoptosis, as indicated by the appearance of active fragment 17 kDa of cleaved caspase-3 on the blot.

Then, we examined whether the expression of AML1-ETO fusion protein had an influence on the differentiation capacity of U937 cells. As markers for myeloid differentiation, the expression of CD11b and CD14 was monitored via FACS analysis. CD11b + cell % was 4.1-7.0% in U937-A/E1-4 cells, which was significantly lower than that in U937-Mock cells (11.4%) and U937-WT cells (11.0%) (P<0.01) (Fig. 1F). Moreover, the expression of CD14 antigen was decreased by 1.5-2-fold as compared with the control cells (P<0.01) (Fig. 1F). These data correspond to the lower differentiation morphological changes of AML1-ETO-transfected cells such as expanded cell size and increased nuclei/cytoplasm ratio with larger nuclei observed in the morphological examination of Wright-Giemsa-stained cytospins (Fig. 1G).

Therefore, it appears that AML1-ETO expression induces growth arrest in leukemic U937 transformants, as demonstrated by the reduced growth rate. Furthermore, the expression of AML1-ETO significantly inhibited the differentiation of U937-A/E cells. These cells lost their original lymphoblast-like morphology without displaying granulocytic morphology and exhibited a block of differentiation at an early stage, as previously reported (16).

Bcl-2, CEBPA and p14^{ARF} expression are downregulated by the AML1-ETO fusion protein. A quantitative reverse transcription PCR (qRT-PCR) assay was used to assess the mRNA expression of Bcl-2, CEBPA, p14^{ARF} and GAPDH. This assay was tested on U937 AML1-ETO-expressing cells and U937 non-expressing cells. We observed that AML1-ETO-expressing cells contained markedly reduced levels of Bcl-2, CEBPA and p14^{ARF} as compared with control-transfected cells or wild-type cells (P<0.001) (Fig. 2A-C).

The same assay was applied to assess Bcl-2, CEBPA and p14^{ARF} mRNA levels in primary leukemia cells of AML patients with or without t(8;21). Two cell lines derived from t(8;21) leukemia patient cells showed higher expression of Bcl-2 (4,13). However, studies using 29 (14) and 17 (15) primary t(8;21) leukemia patient samples indicated that Bcl-2 expression was generally downregulated compared to that for non-leukemic or non-t(8;21) AML samples. We also confirmed a reduced Bcl-2 mRNA level in patients with t(8;21)-containing AML (P<0.001) (Fig. 2D), consistent with a previous report (16).

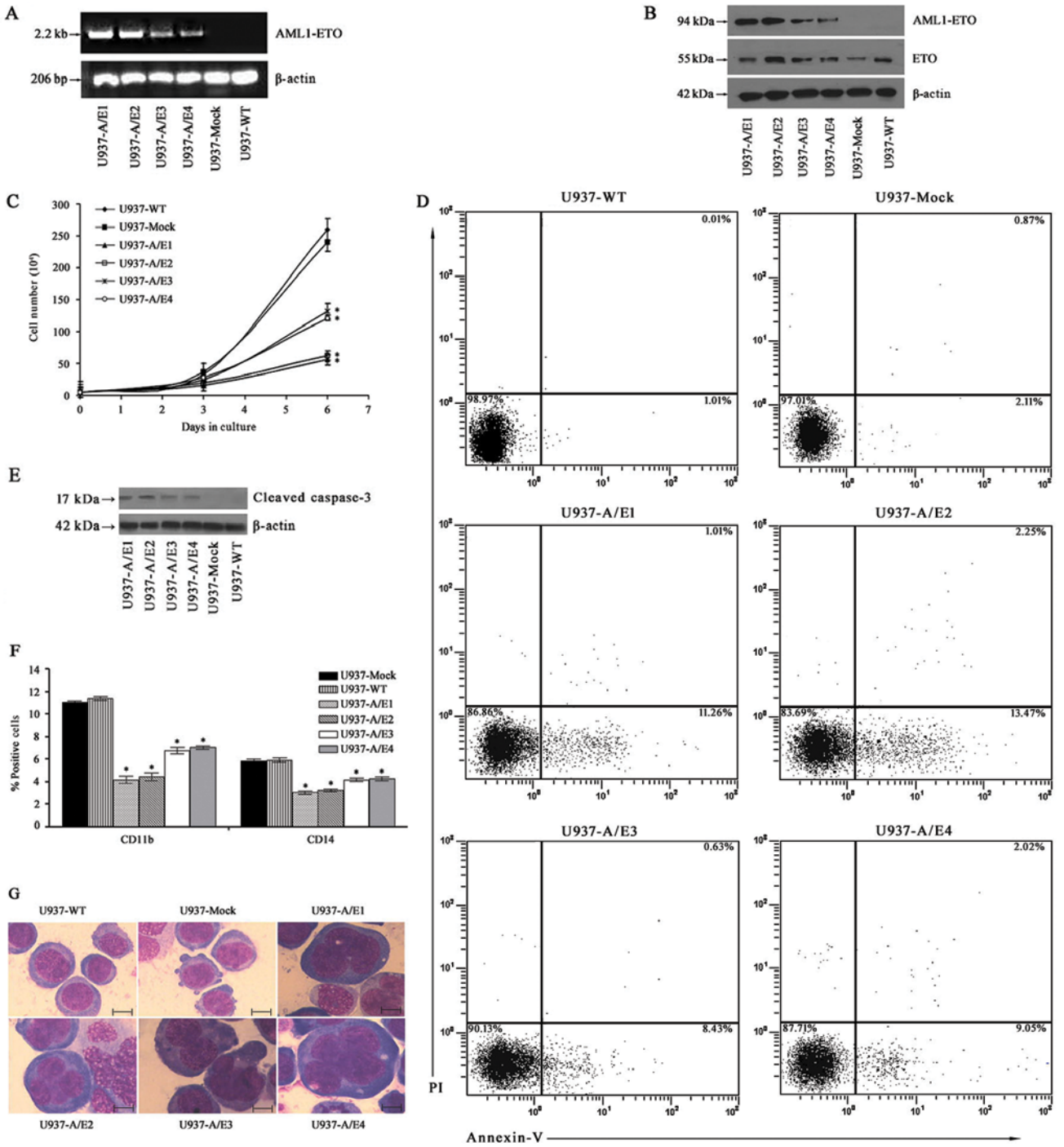

Figure 1. The expression of AML1-ETO induces growth arrest and inhibits the differentiation in leukemic U937 transformants. (A) AML1-ETO mRNA was specifically expressed in transfected U937 cells. (B) Western blot analyses showing the expression of AML1-ETO protein in U937-A/E1,2,3 and 4 individual clones. (C) Growth curves for U937-A/E1-4, U937-Mock and U937-WT cells. The viability was measured daily by trypan blue exclusion. (D) For assessment of apoptosis, Annexin V assay was performed by flow cytometry. The numbers represent the percentage of the Annexin V$^+$ apoptotic cells (mean ± SD) of triplicate samples in an independent experiment. (E) Whole-cell lysates were collected and immunoblotted for cleaved caspase-3. Equal loading was confirmed by actin immunoblot. (F) Percentage of cells positively stained for CD11b and CD14 myeloid surface markers as measured by FACS analysis. (G) Wright-Giemsa staining of cytospin preparation of U937-A/E1-4, U937-Mock and U937-WT cells (scale bars, 5 μm). The results represent the average of three independent evaluations ± SD. *P<0.01 compared respectively with U937-Mock cell lines.

This was also the case with sorted cells from patients suffering from a leukemia with or without a t(8;21), indicating that the presence of AML1-ETO led to a significant downregulation of CEBPA expression (P<0.001) (Fig. 2E).

The p14^{ARF} locus is rarely deleted in AML (17). Whereas p14^{ARF} mRNA levels are low in normal peripheral blood cells and bone marrow, the levels of p14^{ARF} were increased in most AML samples studied, suggesting that this checkpoint was activated (18). We tested whether AML1-ETO prevents the increase of p14^{ARF} in patients with t(8;21)-containing AML. Analysis of p14^{ARF} mRNA levels in 18 AML samples indicated that p14^{ARF} mRNA levels were lower in t(8;21)-containing AML samples. The p14^{ARF} expression values were normalized to β-actin expression. The t(8;21)-negative samples expressed

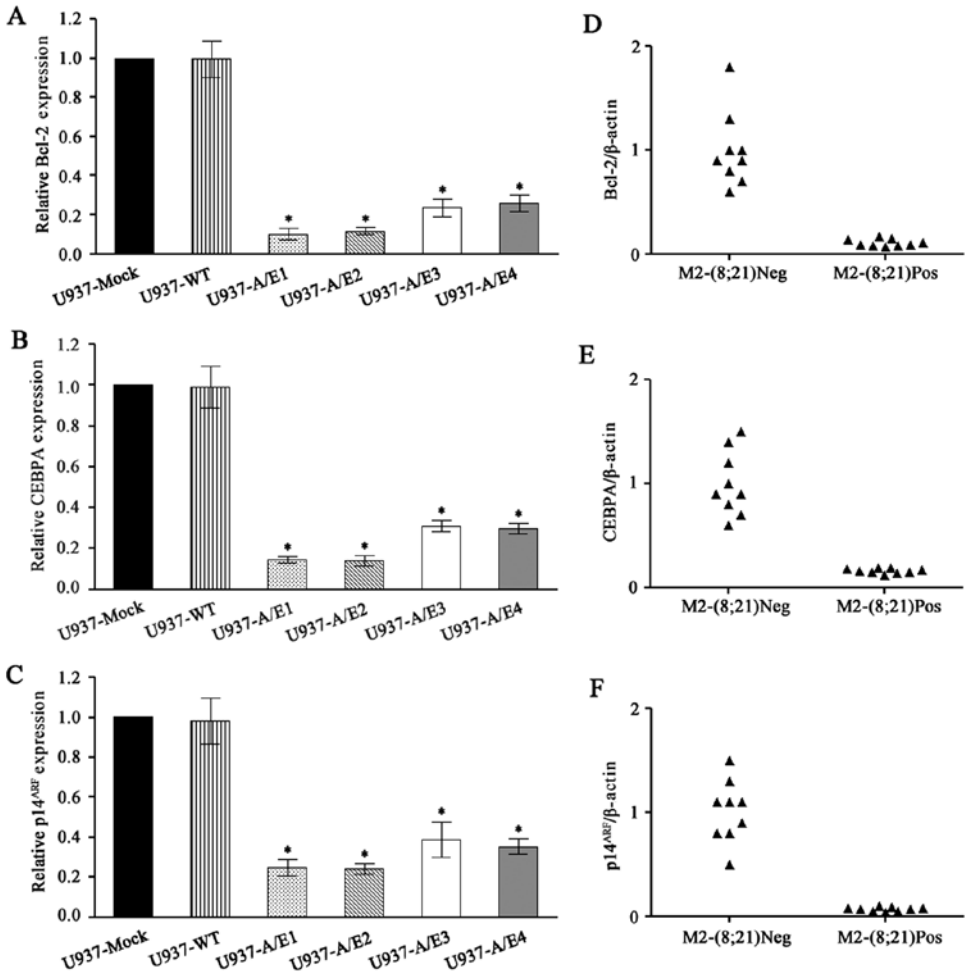

Figure 2. AML1-ETO-positive cells downregulate Bcl-2, CEBPA and p14^{ARF} mRNA. Relative qRT-PCR quantization of (A) Bcl-2, (B) CEBPA and (C) p14^{ARF} levels in AML1-ETO-expressing cells and non-expressing cells. Relative qRT-PCR quantization of (D) Bcl-2, (E) CEBPA and (F) p14^{ARF} levels in 18 AML patient samples, 9 of which contained AML1-ETO [t(8;21) positive]. β-actin expression was used for cDNA quality control. The results represent the average of three independent evaluations ± SD. *P<0.001 compared respectively with U937-Mock cell lines.

a range of p14^{ARF}, with a mean ratio of p14^{ARF}: β-actin of 1.0. By contrast, the t(8;21)-containing samples on average expressed markedly reduced levels of p14^{ARF} (P<0.001) with a mean p14^{ARF}: β-actin ratio of only 0.07 (Fig. 2F).

We therefore concluded that the expressions of Bcl-2, CEBPA and p14^{ARF} mRNA are specifically inhibited in leukemia cells that have the AML1-ETO fusion gene. These results are similar to previous findings (5,16,19,20), validating the quantitative accuracy of the RT-PCR assay.

AML1-ETO triggers the heterochromatic silencing of Bcl-2, CEBPA and p14^{ARF} promoter regions. 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 targeting genes, including Bcl-2, CEBPA and p14^{ARF} (5,16,19,20). ChIP using primers that encompassed the Bcl-2, CEBPA and p14^{ARF} promoter regions was performed with anti-ETO antibody to verify these AML1-ETO targets enriched within the AML1-ETO bound genomic sequences in U937-AML1-ETO cells and as a negative control in U937-empty vector. All comparisons in the cell lines were made between AML1-ETO-expressing and non-expressing cells. DNA sequences specifically precipitated by anti-ETO antibody in AML1-ETO-expressing cells (but not in AML1-ETO-negative cells) most likely represent the AML1-ETO-specific targets. We detected the Bcl-2, CEBPA and p14^{ARF} promoter sequences in anti-ETO immune complexes, but not in control immune complexes (Fig. 3A), indicating that Bcl-2, CEBPA and p14^{ARF} are direct and specific targets of the t(8;21) fusion protein.

The oncogenic properties of AML1-ETO are linked to its ability to form oligomeric complexes with increased affinity for HDAC and DNMTs rendering AML1-ETO a potent transcriptional repressor of AML1-target genes (21). ChIP analysis also revealed the presence of methyl-CpG binding protein 2 (MeCP2) at the Bcl-2, CEBPA and p14^{ARF} promoter regions occupied by AML1-ETO in U937-A/E1-4 (P<0.001) (Fig. 3B). We therefore investigated whether the aberrant recruitment of MeCP2 activities by AML1-ETO modifies nucleosomal histone tails on the Bcl-2, CEBPA and p14^{ARF} promoters. Using ChIP analysis, we focused on several modifications of histone H3 (AcH3, tri-mK27 and tri-mK9) and the acetylated forms of histone H4 in the same cell lines that were used for AML1-ETO target identification. These modifications are mutually exclusive, whereby H3-K9 trimethylation or H3-K27 trimethylation is a hallmark of inactive chromatin

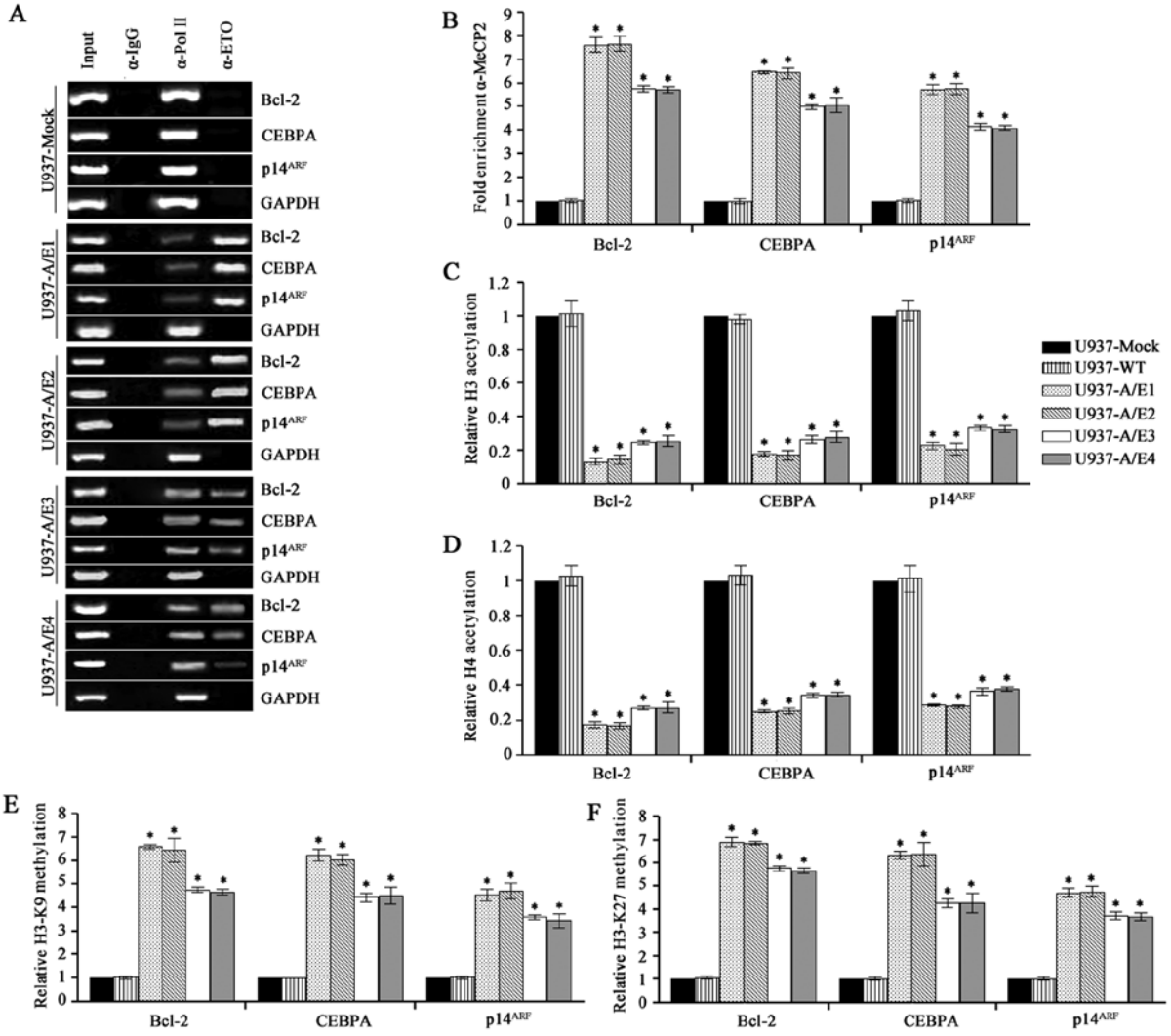

Figure 3. Verification of AML1-ETO targets (Bcl-2, CEBPA and p14^{ARF}) and identification of epigenetic status at these genes in AML1-ETO-positive cells. (A) AML1-ETO bound to the Bcl-2, CEBPA and p14^{ARF} promoters. ChIP assays were performed using specific antibodies for ETO, RNA polymerase II (Pol II), as well as non-immune IgG. Immunoprecipitated chromatin was analyzed by quantitative real-time PCR with primers specific for the regions of the Bcl-2, CEBPA and p14^{ARF} promoters. Input showed the amplification from sonicated chromatin. Amplification of GAPDH DNA was a control for nonspecific precipitated sequences. (B) The enrichment of MeCP2 at the Bcl-2, CEBPA and p14^{ARF} promoters was analyzed by qChIP. Chromatin modifications by ChIP assays were analyzed in the indicated cell lines using antibodies specific for the (C) acetyl-H3 and (D) acetyl-H4 forms or (E) for the trimethylation of H3 lysine 9 and (F) trimethylation of H3 lysine 27 forms. Data are expressed as fold-differences relative to control conditions (in which IgG is used instead of specific antibodies in the ChIP). The results represent the average of three independent evaluations ± SD. *P<0.001 compared respectively with U937-Mock cell lines.

and acetylation of H3 or H4 is found at active loci (22,23). As illustrated in Fig. 3C and D, H3 and H4 histones are hyperacetylated at the Bcl-2, CEBPA and p14^{ARF} promoter regions in U937-Mock and U937-WT cells, while decreased acetylation levels are measurable in U937-A/E1-4 cells (P<0.001). The reduced histone acetylation in AML1-ETO-expressing cells suggested a hindered transcription at these chromatin sites on the Bcl-2, CEBPA and p14^{ARF} genes. ChIP assay performed using antibodies against H3-K9 trimethylation and H3-K27 trimethylation demonstrated that AML1-ETO-expressing cells had a marked trimethylation level of H3-K9 and H3-K27 at the Bcl-2, CEBPA and p14^{ARF} promoters. By contrast, few or no promoters with trimethylation of H3-K9 and H3-K27 were observed in AML1-ETO-non-expressing cells (P<0.001) (Fig. 3E and F). The higher level of histone methylation in AML1-ETO-expressing cells paralleled with significantly lower levels of H3 and H4 acetylation. These changes are consistent with the induction of a repressive chromatin configuration by AML1-ETO in its direct target genes.

Treatment of demethylating agent or HDAC inhibitor partially reverses Bcl-2, CEBPA and p14^{ARF} suppression. We next treated the AML1-ETO-positive and -negative U937 cells with either the DNMT inhibitor 5-Aza or the HDAC inhibitor TB, respectively. Both 5-Aza and TB increased the expression of Bcl-2, CEBPA and p14^{ARF} by ~2- to 3-fold (P<0.001) (Fig. 4A-C). In addition, 5-Aza impaired the ability of anti-MeCP2 antibody to immunoprecipitate naked DNA surrounding the region of AML1 binding sites on Bcl-2, CEBPA and p14^{ARF} gene promoters (P<0.001) (Fig. 4D). On the other hand, a significant increase (P<0.001) in chromatin H3 and H4 acetylation of Bcl-2, CEBPA and p14^{ARF} was observed in cells treated with TB compared with untreated cells (Fig. 4E and F).

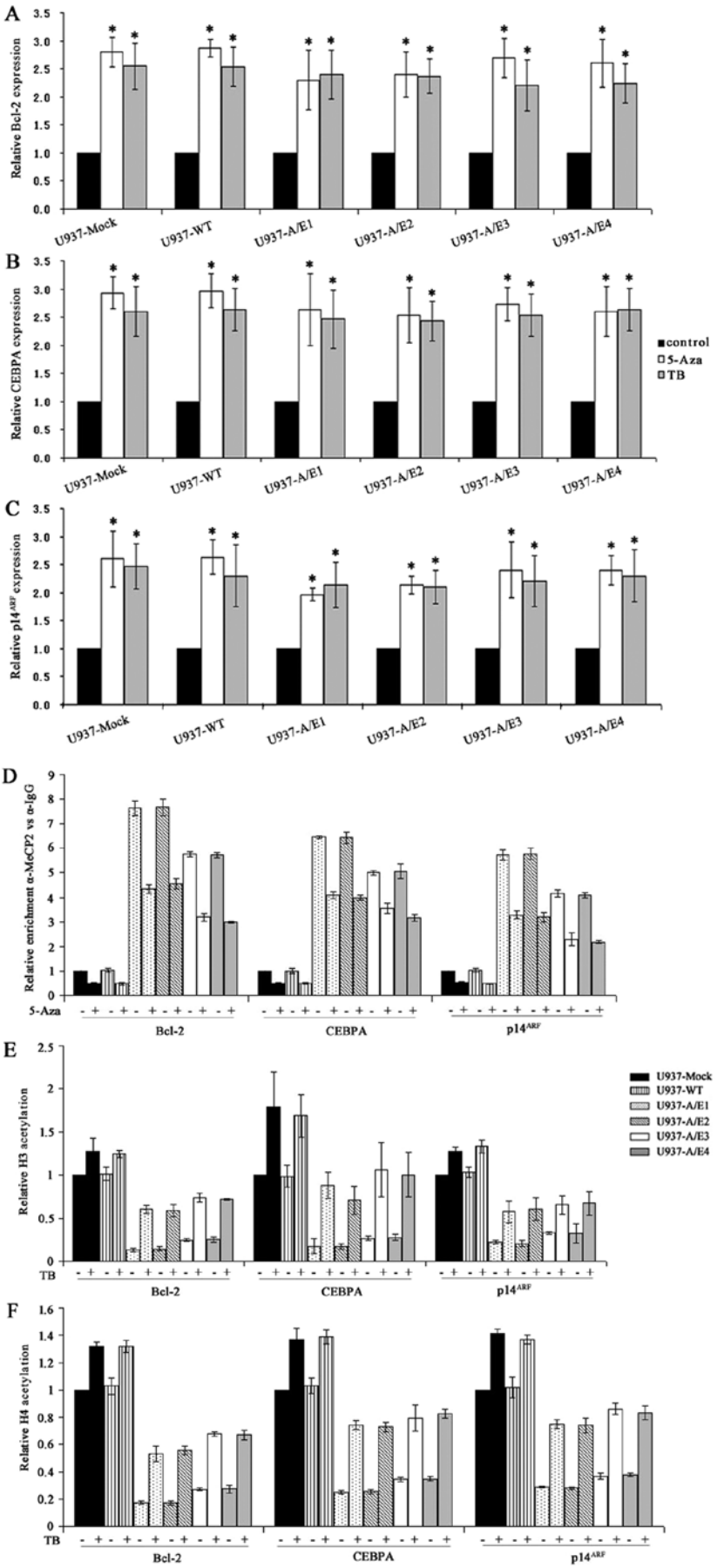

Figure 4. 5-Aza or TB treatment induces Bcl-2, CEBPA and p14^{ARF} expression. Relative levels of mRNA expression of (A) Bcl-2, (B) CEBPA and (C) p14^{ARF} in 5-Aza or TB-exposed and non-exposed U937 cells. ChIP assay performed with antibodies for (D) MeCP2, (E) acetyl-H3 and (F) acetyl-H4 following (D) 5-Aza or (E and F) TB treatment in AML1-ETO-positive cells. IgG isotype control was used. The results represent the average of three independent evaluations ± SD. *P<0.001 compared respectively with untreated U937 cells.

Consistent with the increased Bcl-2, CEBPA and $p14^{ARF}$ mRNA levels, 5-Aza treatment showed demethylation of CpGs and TB treatment resulted in enhanced accumulation of acetylated histone H3 or H4 at the Bcl-2, CEBPA and $p14^{ARF}$ promoters. These results indicated that DNA methylation and HDAC were simultaneously and independently operative in this model, and both contributed to gene regulation in U937 cells.

Discussion

Cancer is a genetic and epigenetic disease (24,25). The contribution of epigenetic mechanisms for a correct cell function is highlighted by the effects of their deregulation in cooperation with genetic alterations leading to the establishment and progression of tumors. Heterochromatic gene silencing represents an alternative oncogenic mechanism to gene mutation or deletion for the transcriptional repression of tumor suppressor genes (24).

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 (9). Hypermethylation in the upstream region of the promoter-associated CpG island of CEBPA has previously been detected in lung cancer as well as in head and neck squamous cell carcinoma (26,27). In hematopoietic tumor cell lines, CpG island hypermethylation of the proximal CEBPA promoter region was associated with transcriptional silencing, and treatment with the demethylating agent 5-aza-2'-deoxycytidine resulted in C/EBPα reexpression and promoter demethylation (28). Wouters *et al* provided first evidence for the importance of C/EBPα methylation in a small subgroup of AML (29). The epigenetic contribution to C/EBPα deregulation has been investigated and the aberrant DNA methylation in the upstream promoter of C/EBPα has been shown to be a frequent event in AML (28).

Here, we showed that the myeloid transcription factor C/EBPα was specifically downregulated in AML patients with the AML1-ETO of the FAB-M2 subtype or U937 AML1-ETO-expressing cells. U937-A/E clones exhibited lower differentiation morphological changes such as expanded cell size and increased nuclei/cytoplasm ratio with larger nuclei associated with a decreased expression of cell surface markers CD11b and CD14. This altered differentiation potential is correlated with the downregulation of C/EBPα upon expression of AML1-ETO. Therefore, the epigenetic dysregulation including MeCP2 binding, H3 and H4 hypoacetylation as well as hypertrimethylation of H3-K9 or K27 may be a common alternative or complementary mechanism of interfering with C/EBPα function.

It has previously been reported that the AML1-ETO fusion protein was able to induce anti-apoptotic Bcl-2 expression *in vitro* (4), while Burel *et al* (16) and Lu *et al* (20) as well as the present study showed an AML1-ETO-induced decrease in Bcl-2 expression. On the contrary, AML1-ETO increased the expression of Bak protein, a pro-apoptotic member of the Bcl-2 family that plays an important role in regulating mitochondrial membrane permeability during apoptosis (30). The induction of AML1-ETO in U937T-A/E cells causes a progressive cell cycle arrest in G0/G1 phase. Moreover, ectopic expression of Bcl-2 delays apoptosis without preventing AML1-ETO-induced G1/G0 arrest (16). Our results are in agreement with this and showed that AML1-ETO could markedly downregulate the expression of Bcl-2 by inducing repressive chromatin structure at its promoter. It has been suggested that the overexpression of the anti-apoptotic protein Bcl-2 in chronic lymphocytic leukemia (CLL) is caused by hypomethylation of the promoter region of the Bcl-2 gene (31). However, methylation of the 5' region of apoptosis-associated genes is a common finding in patients with bladder carcinoma (32). This finding is noteworthy as DNA hypermethylation is often associated with decreased gene expression, and in the case of Bcl-2, this would be expected to promote apoptosis rather than tumor growth. Inhibition of proliferation or apoptosis would not be favorable to the propagation of clonal cells harboring the t(8;21) translocation. If growth arrest and apoptosis are general features associated with the expression of AML1-ETO, we hypothesize that AML1-ETO-modulated apoptosis-regulating genes and/or proteins may become the targets for secondary 'hit' that contributes to the pathogenesis of AML1-ETO-associated leukemia. It may be inferred that some genetic or/and epigenetic alterations of apoptosis-related genes have appeared in these AML1-ETO-positive AML cells, which may overcome the apoptosis-enhancing effect of AML1-ETO.

Methylation at the $p14^{ARF}$ promoter to suppress gene expression has been observed in some tumor cell lines, particularly in colorectal cancer (33,34). In our study, we found that the recruitment of MeCP2 to $p14^{ARF}$ chromatin in AML1-ETO-expressing cell lines correlates with lower levels of H3 and H4 acetylation and higher levels of H3 (Lys9 and Lys27) trimethylation resulting in the silence of the $p14^{ARF}$ gene. AML1-ETO suppressed the $p14^{ARF}$ promoter and reduced endogenous levels of $p14^{ARF}$ expression in multiple cell types (6). Our results support this and provide an explanation for the observed reduced $p14^{ARF}$ expression. Thus, AML1-ETO-mediated suppression of $p14^{ARF}$ may disrupt both p53-dependent and p53-independent growth suppression pathways to extend the lifespan of myeloid progenitor cells, allowing more opportunities to acquire additional mutations, ultimately leading to leukemia.

Epigenetic alterations are increasingly recognized as important contributors to human cancer pathogenesis and DNMT and HDAC inhibitors have recently been incorporated into the treatment of AML1-ETO leukemias (35-37). Their ability to reverse the inhibition of myeloid-specific genes helps to re-establish a normal differentiation program. Our findings indicate that demethylating agents or HDAC inhibitors can relieve Bcl-2, CEBPA and $p14^{ARF}$ suppression in AML1-ETO-expressing cells through a mechanism that involves inversion of epigenetic alterations.

Despite the changes in expression pattern of Bcl-2, CEBPA and $p14^{ARF}$ in primary bone marrow cells of AML1-ETO-positive AML-M2 patients were similar to those in U937-A/E cells when compared to the AML1-ETO-negative cells, to date we have not found the significant and consistent alterations of DNA/histone modifications at these genes in primary cells of AML1-ETO-positive AML patients (data not shown). This may be due to the heterogeneity of the primary marrow cells of the patients and the limitation of techniques. However, one would expect to apply epigenetic markers for diagnosis, strati-

fication and especially as an indicator in epigenetic regulatory treatment in leukemia patients in the future.

Collectively, we provided the first evidence for modifications of the chromatin structure at the Bcl-2, CEBPA and p14^{ARF} promoters occupied by the AML1-ETO fusion protein. Our data are therefore consistent with a model by which the binding of AML1-ETO leads to alterations in the chromatin structure of its target genes. These findings underscore the importance of epigenetic alteration mediated silencing of these genes in leukemogenesis. It is noteworthy to compare the chromatin structure of different AML1-ETO target genes in order to better understand the molecular details of the deregulation of gene expression by this oncoprotein. If so, these mechanisms may be potential targets for therapeutic strategies based on the reversal of epigenetic silencing in t(8;21)-positive leukemias.

Acknowledgements

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