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All-trans retinoic acid combined with 5-Aza-2′-deoxycitidine induces C/EBPα expression and growth inhibition in *MLL-AF9*-positive leukemic cells

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ABSTRACT

The present study tested whether all-trans retinoic acid (ATRA) and 5-Aza-2′-deoxycitidine (5-Aza) affect AML cell differentiation and growth *in vitro* by acting on the CCAAT/enhancer binding protein α (C/EBPα) and c-Myc axis. After exposure to a combination of these agents, cell differentiation and growth arrest were significantly higher in human and murine *MLL-AF9*-expressing cells than in *MLL-AF4/AF5q31*-expressing cells, which were partly associated with increased expression of C/EBPα, C/EBPε, and PU.1, and decreased expression of c-Myc. These findings indicate that *MLL-AF9*-expressing cells are more sensitive to ATRA and 5-Aza, indicating that different *MLL* fusion proteins possess different epigenetic properties associated with retinoic acid pathway inactivation.

1. Introduction

Sufficient induction of CCAAT/enhancer binding protein alpha (C/EBPα), a key transcription factor involved in granulocytic differentiation [1], is thought to be critical for the successful induction of terminal myeloid differentiation in acute myeloid leukemia (AML) cells [2,3]. All-trans retinoic acid (ATRA) induces C/EBPα expression in acute promyelocytic leukemia (APL) cells [4]; however, in types of AML other than APL, the retinoic acid (RA) pathway is commonly inactivated. Thus, ATRA is generally not able to induce significant granulocytic/monocytic differentiation in non-APL AML cells [5–7]. Nevertheless, several studies have demonstrated that restoration of C/EBPα expression can occur during the process of myeloid differentiation in non-APL AML cells when ATRA is used in combination with other agents [5,7,8]. Interestingly, promoter hypermethylation is a characteristic feature of *MLL*-rearranged ALL and AML, and demethylating agents are being explored as a novel therapeutic option [9–11]. In this study, epigenetic modifying agents were tested in two types of *MLL*-rearranged AML presenting different prognosis, such as *MLL-AF9* positive and *MLL-AF4/AF5q31* positive AML [12]. The effect of the combination of ATRA and 5-Aza-2′-deoxycitidine (5-Aza) on C/EBPα expression was assessed *in vitro* in these *MLL*-rearranged AML cells.

2. Materials and methods

2.1. Cell lines, cell culture, and reagents

Two human *MLL*-rearranged AML cell lines (THP-1, which contains the *MLL-AF9* fusion gene, and KOCL-48, which contains the *MLL-AF4* fusion gene) were used for this study. THP-1 was obtained from the ATCC (Manassas, VA, USA). KOCL-48 was obtained from the University of Yamanashi. Plat-E packaging cells were kindly provided by Dr. Toshio Kitamura (The Institute of Medical Science, Tokyo University) and were used for the transduction of murine hematopoietic cells. Cells were cultured in suspension in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (10 mg/ml) at 37 °C in a humidified atmosphere of 5% CO_2. ATRA (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in DMSO and stored as a 1 mM stock solution in small aliquots at −20 °C. 5-Aza (Sigma–Aldrich) was stored as a 50 μM stock solution in distilled water at −20 °C. The final concentrations used in the experiments were 1 μM ATRA and 50 nM 5-Aza.

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2.2. Retroviral constructs

An attempt was made to generate immortalized murine cells expressing *MLL-AF9* or *MLL-AF4*; however, since *MLL-AF4* cannot transform murine hematopoietic progenitor cells using retroviral transduction systems, *AF5q31* was used as an alternative to AF4. *AF5q31* is a member of the AF4 family of genes and fuses with *MLL* to cause an acute leukemia similar to that induced by *MLL-AF4* [13,14]. Retroviral constructs encoding *MLL-AF9* and *MLL-AF5q31* were generated by fusion of the 91 carboxy-terminal amino acids of human AF9 (accession number BC036089) or the 842 carboxy-terminal amino acids of human *AF5q31* (accession number BC100287) with the 1400 amino-terminal amino acids of human MLL in the retroviral vector MSCV-5′ *MLL*-Neo [13,15,16]. The MSCV *MLL*-Neo construct was kindly provided by Dr. Michael J. Thirman (University of Chicago).

2.3. Transduction of Lin^- murine hematopoietic progenitors to establish murine leukemic cell lines expressing MLL fusion proteins

Production of retroviral supernatants in Plat-E cells was performed as described previously [17]. Lineage-negative (Lin^-) bone marrow cells were obtained from C57BL6 mice 5 days after 5-fluorouracil treatment, and transduced progenitor cells were cultured in methylcellulose, as described previously [15]. The cells that survived after three passages were cultured in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (10 mg/ml), 0.05 M β-mercaptoethanol (β-ME), and recombinant murine IL-3 (10 ng/ml), IL-6 (10 ng/ml), and SCF (100 ng/ml) (each from PeproTech, Rocky Hill, NJ, USA) at 37 °C in a humidified atmosphere of 5% CO_2. These murine leukemic cells were subjected to the experiments described below. The expression of each *MLL* fusion gene was confirmed by RT-PCR analysis (Supplementary Fig. 1).

2.4. Cell proliferation

Cells were seeded at 1×10^5 cells/ml, exposed to 1 μM ATRA and 50 nM 5-Aza (or an equivalent volume of the vehicle controls) for 2 days, and were then cultured for 3 or 5 additional days. Every 24 or 48 h of culture, an aliquot of the cells was lysed under hypotonic conditions and nuclei were counted with a Coulter counter (Beckman Coulter, Brea, CA, USA). The concentration of ATRA and 5-Aza causing 50% growth inhibition (IC_{50}) was determined.

2.5. Morphological changes in treated cells

Cells were plated in 6-well dishes at a density of 1×10^5 cells/ml and cultured with either ATRA, 5-Aza, or a combination of both. After 5 days of culture, cytospin preparations were stained with May-Grünwald–Giemsa and observed under a light microscope.

2.6. Nitroblue tetrazolium (NBT) reduction

Cells (1×10^5/ml) were incubated in 6-well plates with ATRA (1 μM) and/or 5-Aza (50 nM) for 2 days. After incubation, each cell suspension was washed in phosphate-buffered saline (PBS) and a nitroblue tetrazolium (NBT) reduction test was carried out using the NBT Reduction Kit (Sigma–Aldrich) according to the manufacturer's instructions. The percentage of cells which stained blue was determined by light microscopy for at least 300 cells per sample.

2.7. Flow cytometric analysis and Annexin V assay

Cells were harvested, washed twice with 1X PBS, and incubated for 30 min with PE-conjugated anti-human CD11b (BD Biosciences, Sparks, MD, USA), or PE-conjugated anti-mouse Mac-1 (BD Pharmingen, San Diego, CA, USA) and analyzed on a FACS Calibur (BD Biosciences). Apoptotic cell death was assessed by Annexin V–FITC/propidium iodide (PI) staining using the Annexin V–FITC Apoptosis Detection Kit (R&D Systems) according to the manufacturer's instructions. Data were analyzed with Cell Quest software (BD Biosciences).

2.8. Real-time RT-PCR

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Venio, Netherlands) according to the manufacturer's instructions. The SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) was used to synthesize cDNA according to the manufacturer's instructions. Real-time RT-PCR was conducted using the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR Green I (Takara Bio, Tokyo, Japan). Relative target mRNA expression was determined using the comparative threshold (ΔC_T) method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control. The primer pairs used in this study are listed in Supplementary Table 1. A standard curve analysis with stepwise sample dilution demonstrated that all primer pairs had similar amplification efficiency.

2.9. Immunoblot analysis

Cells were lysed with Laemmli sample buffer. Samples were boiled for 5 min in sample buffer containing bromophenol blue and 1X β-ME, and equal amounts of protein were separated by SDS–PAGE. Primary antibodies were used as follows: C/EBPα (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), c-Myc (1:200, Santa Cruz Biotechnology), and β-actin (1:1000, Sigma–Aldrich).

2.10. Methylation-specific polymerase chain reaction (MSP)

Genomic DNA was extracted from cells with the QIAamp DNA Mini Kit (Qiagen), according to the manufacturer's protocol. Sodium bisulfite treatment was conducted using an EZDNA methylation kit (Zymo Research, Irvine, CA, USA) following the manufacturer's protocol. Methylation-specific PCR was conducted using the primer sets listed in Supplementary Table 1.

2.11. Statistical analysis

Statistical analysis was performed using the unpaired Student's *t*-test or paired *t*-test. A *P*-value of <0.05 was considered statistically significant.

3. Results

3.1. Effect of ATRA/5-Aza on myeloid differentiation of human AML cell lines harboring different MLL fusion genes

ATRA/5-Aza was tested in human AML cell lines with *MLL* rearrangements (THP-1 and KOCL-48). ATRA/5-Aza was compared to ATRA alone and to 5-Aza alone. As observed in APL cells, ATRA induced morphological changes in *MLL*-rearranged AML cells. Morphological changes were more significant in THP-1 cells than in KOCL-48 cells, and corresponded with the induction of CD11b on the cell surface of THP-1 cells (Fig. 1A and B). Furthermore, the combination of ATRA and 5-Aza induced slightly greater expression of CD11b compared to THP-1 cells treated with ATRA alone (Fig. 1B). A NBT reduction test also revealed that significantly more THP-1 cells could reduce NBT when 5-Aza was combined with

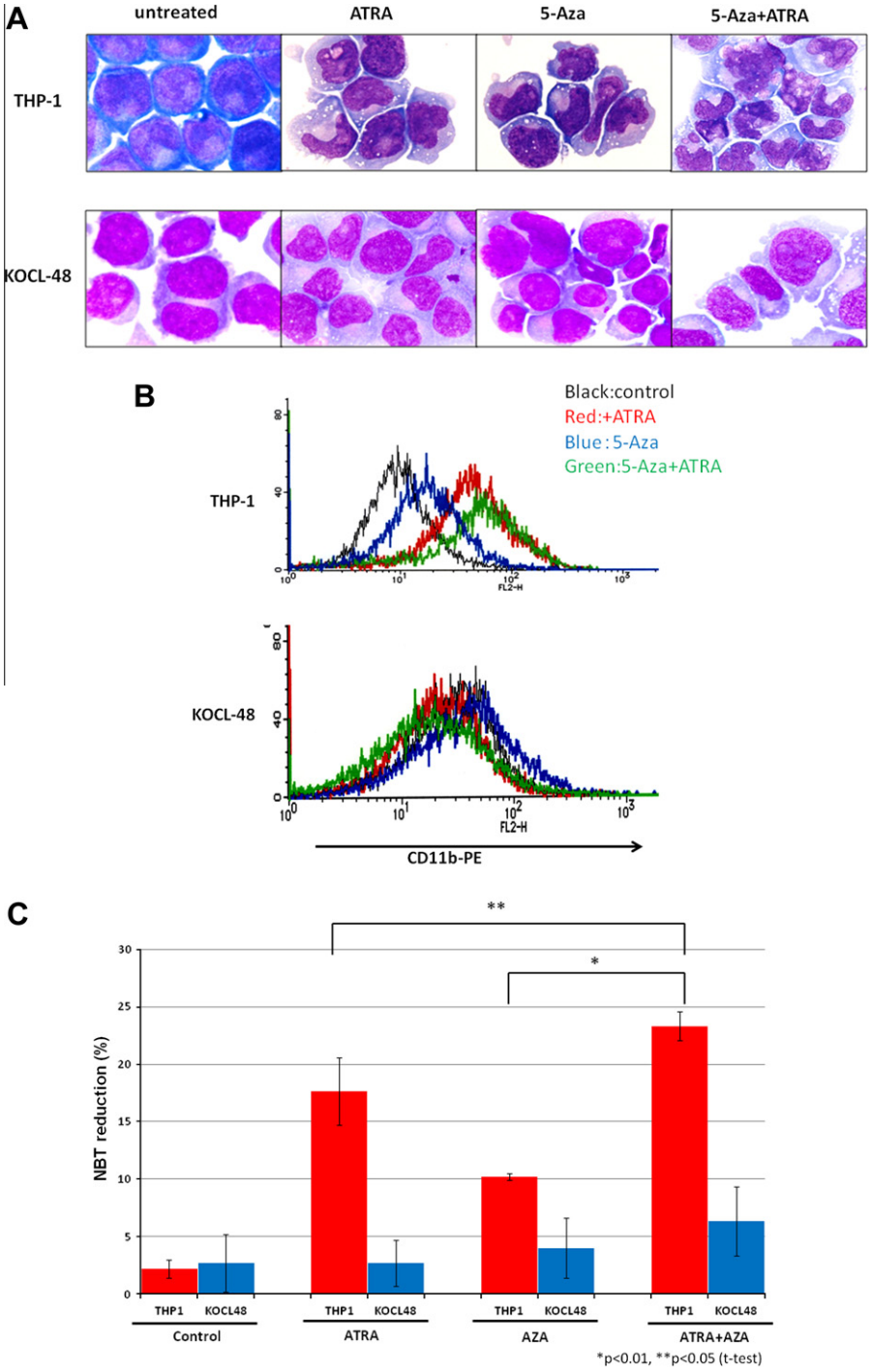


Fig. 1. The effect of ATRA/5-Aza on human *MLL*-rearranged cell lines. (A) Photomicrograph of THP-1 and KOCL-48 cell lines untreated or treated with ATRA (1 μM), 5-Aza (50 nM) or ATRA and 5-Aza (same concentrations). Cytospin preparations were stained with May-Grünwald Giemsa. The figure shows representative results from three independent experiments. (B) Effect of ATRA/5-Aza on CD11b expression in THP-1 and KOCL-48 cells by flow cytometry after 72 h of exposure. Untreated cells (black line), ATRA (red line), 5-Aza (blue line), and ATRA/5-Aza (green line). ATRA induced CD11b expression in THP-1 cells. In addition, the combination of ATRA and 5-Aza slightly intensified CD11b expression as compared to treatment with ATRA alone. In contrast, ATRA (alone or in combination with 5-Aza) had no impact on CD11b expression in KOCL-48 cells. The figure shows representative results from two independent experiments. (C) Effect of ATRA/5-Aza on NBT reduction. THP-1 and KOCL-48 cells were cultured with ATRA (1 μM) and/or 5-Aza (50 nM) for two days, and differentiation was determined by NBT reduction. Results represent the means ± SD of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; NS, no significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ATRA, but this effect was not observed in KOCL-48 cells (Fig. 1C). These findings indicated that ATRA induced myeloid differentiation significantly in THP-1 cells, but not in KOCL-48 cells. In addition, 5-Aza had additive effect on myeloid differentiation in combination with ATRA in THP-1 cells.

Next, to determine whether 5-Aza and ATRA had additive effect on the expression of transcriptional factors related to myeloid differentiation, the gene expression level of *C/EBPα* in the two cell lines was compared. The expression of *C/EBPα* mRNA was significantly increased by ATRA and 5-Aza in THP-1 cells (untreated vs.

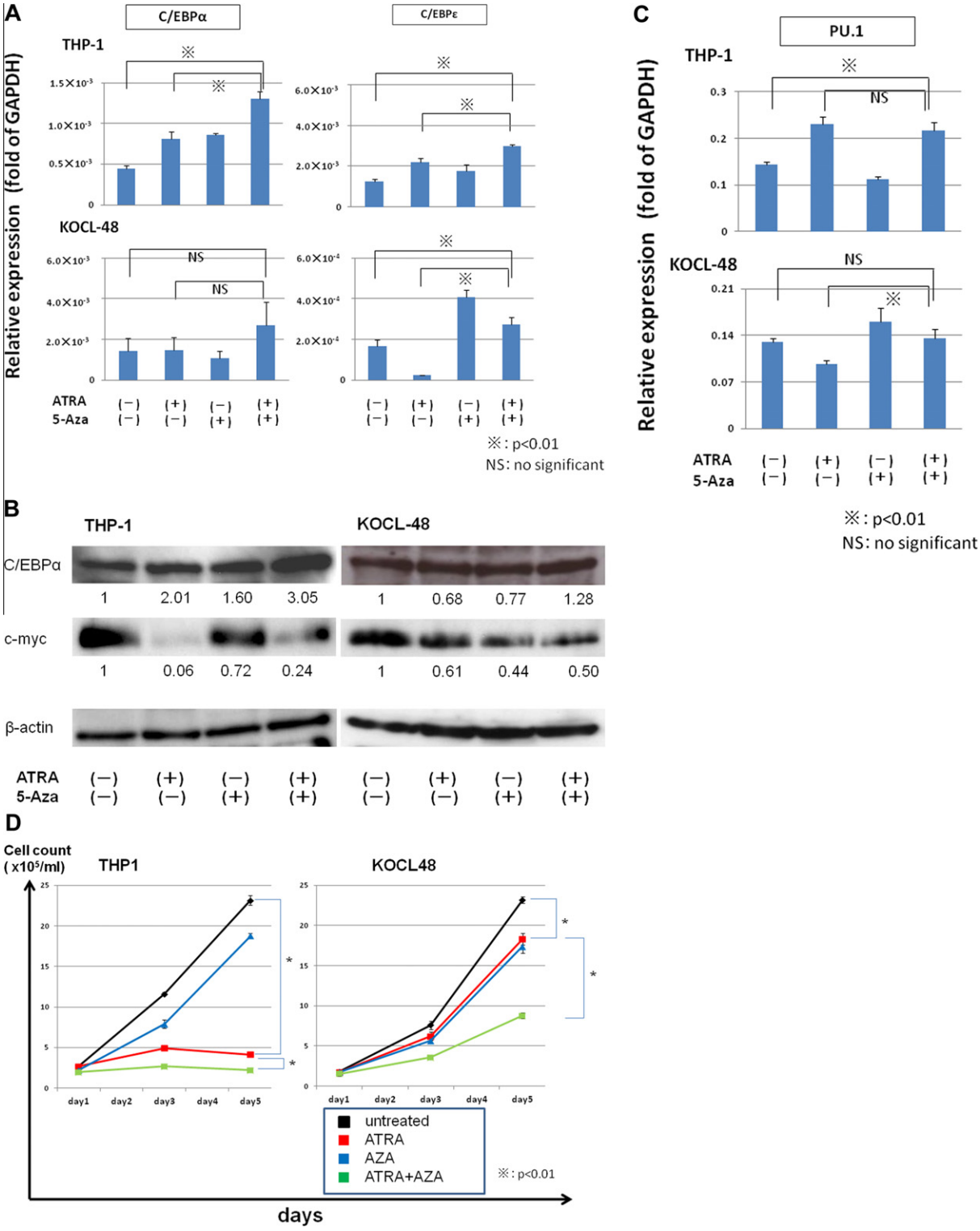


Fig. 2. C/EBPα expression, growth inhibition and apoptosis in human THP-1 and KOCL-48 cells exposed to ATRA/5-Aza. (A) Expression of *C/EBPα* and *C/EBPε*, in THP-1 and KOCL-48 cells by real-time RT-PCR, normalized to GAPDH, after 72 h of exposure to ATRA. ATRA increased the expression of *C/EBPα* and *C/EBPε* in THP-1 cells. However, ATRA did not induce *C/EBPα* and *C/EBPε*, in KOCL-48 cells. Furthermore, 5-Aza synergized with ATRA to induce the expression of *C/EBPα* and *C/EBPε* in THP-1 cells. Results represent the mean ± SD of three independent experiments. $^{*}P < 0.01$; NS, no significance. (B) Expression of C/EBPα and c-Myc in THP-1 and KOCL-48 cells exposed to ATRA and/or 5-Aza for 72 h by immunoblot analysis. The expression level of c-Myc negatively correlated with that of C/EBPα. Each lane was loaded with 20 μg of total protein. Levels of β-actin served as a control. The numbers in each column represent the expression level as determined by Image J software Ver. 2.0. The figure shows representative results from two independent experiments.(C) Expression of PU.1 in THP-1 and KOCL-48 cells by real-time RT-PCR, normalized to GAPDH, after 72 h of exposure to ATRA. ATRA increased the expression of PU.1 in THP-1 cells. However, ATRA did not induce PU.1 in KOCL-48 cells. 5-Aza did not have a synergistic effect on the induction of PU.1 in ATRA-treated THP-1 cells. Results represent the mean ± SD of three independent experiments. $^{*}P < 0.01$; 0.01; NS, no significance. (D) The growth of THP-1 and KOCL-48 cells exposed to ATRA, 5-Aza, or the combination was assessed by counting nuclei every 48 h. Growth inhibition by ATRA or ATRA/5-Aza was more prominent in THP-1 cells than in KOCL-48 cells. Results represent the mean ± SD of three independent experiments. $^{*}P < 0.01$. (E) Apoptosis in THP-1 and KOCL-48 cells exposed to ATRA and 5-Aza, alone or in combination, for 72 h, as revealed by Annexin V/PI staining. Annexin V/PI-double positive cells were counted as apoptotic. Results represent the mean ± SD of three independent experiments. $^{*}P < 0.01$.

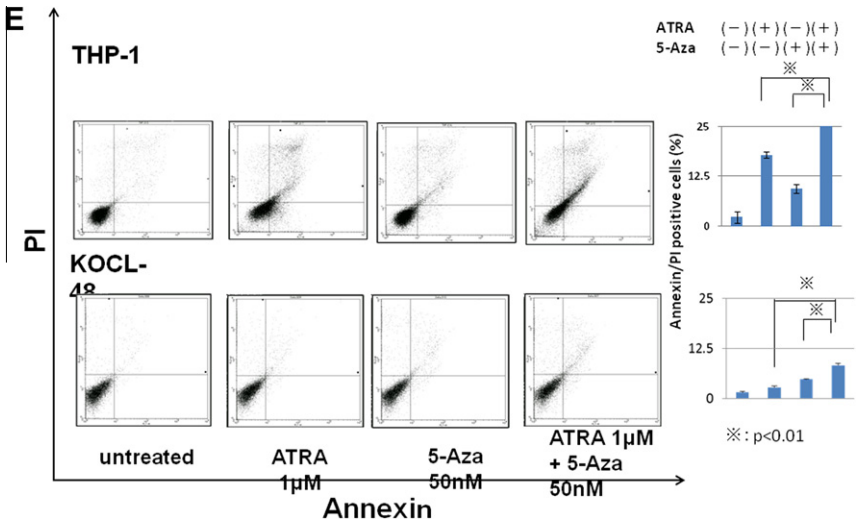


Fig. 2. (*continued*)

ATRA + 5-Aza and ATRA vs. ATRA + 5-Aza, $P < 0.01$). In contrast, 5-Aza and ATRA did not induce the expression of *C/EBPα* significantly in KOCL-48 cells (Fig. 2A). Immunoblot analysis also revealed that the up-regulation of C/EBPα was more prominent in THP-1 cells than in KOCL-48 cells (Fig. 2B). In addition, c-Myc, which was negatively regulated by C/EBPα through direct binding to the promoter region [18], was downregulated in response to ATRA/5-Aza treatment in THP-1 cells (Fig. 2B). These findings demonstrated that 5-Aza and ATRA additively induced C/EBPα expression in THP-1 cells but not in KOCL-48 cells. To determine whether the expression of *C/EBPα* was affected by promoter methylation, the methylation status of the promoter region of *C/EBPα* was evaluated in ATRA/5-Aza- treated THP-1 and KOCL-48 cells by MSP analysis. Surprisingly, the methylation status of the core and distal promoter regions of *C/EBPα* did not correlate with the *C/EBPα* expression level, suggesting that 5-Aza and ATRA did not induce *C/EBPα* expression by enhancing the demethylation of the *C/EBPα* promoter (data not shown).

Because the expression of *PU.1* is regulated positively by C/EBPα [19], *PU.1* expression was evaluated by qRT-PCR in the two cell lines treated with ATRA and/or 5-Aza. Although the qRT-PCR analysis revealed that ATRA induced the expression of *PU.1* in THP-1 cells, 5-Aza and ATRA did not have an additive effect on *PU.1* expression (Fig. 2C). These findings are consistent with the results of the analysis of CD11b expression, which is positively regulated by PU.1 [20].

Collectively, these results demonstrate ATRA and 5-Aza cooperate to induce myeloid differentiation in THP-1 cells. However, this effect was not sufficient to induce terminal differentiation, as was observed in APL cells. Finally, we found that ATRA induced *C/EBPε* expression (Fig. 2A), which is directly regulated by RARα in THP-1 cells [6]. These findings suggest that the RA pathway is not completely inactivated in THP-1 cells. Conversely, ATRA did not induce *C/EBPε* expression in KOCL-48 cells, (Fig. 2A), suggesting that the RA pathway was almost completely inactivated in that cell line.

3.2. ATRA and 5-Aza cooperate to inhibit the growth of THP-1 cells

Because ATRA and 5-Aza cooperated to induce C/EBPα expression with suppression of c-Myc expression in THP-1 cells (Fig. 2B), we hypothesized that those agents might inhibit cell proliferation. Growth inhibition was higher in THP-1 cells than in KOCL-48 cells upon exposure to ATRA alone and the drug combination (Fig. 2D). Furthermore, the combination of ATRA/5-Aza induced apoptosis efficiently in both cell lines, although the additive effect was greater in THP-1 cells (Fig. 2D). To determine the IC_{50} of ATRA and 5-Aza in THP-1 and KOCL-48 cells, the two cell lines were treated with a titrating dose of ATRA and 5-Aza. THP-1 cells were more sensitive to ATRA/5-Aza-induced growth inhibition compared to KOCL-48 cells (Supplementary Fig. 2A and B). To determine whether ATRA and 5-Aza could cooperate to inhibit the growth of these two cell lines, both cell lines were treated with a titrating dose of ATRA concurrently with 0 or 50 nM 5-Aza. 5-Aza potently cooperated with ATRA and resulted in a significant decrease in the IC_{50} of ATRA in THP-1 cells (Fig. 3A and B). In contrast, 5-Aza did not induce a significant decrease in the IC_{50} of ATRA in KOCL-48 cells (Fig. 3A and C). These findings suggested that 5-Aza might exert its effect as a sensitizer for ATRA in THP-1 cells, but not in KOCL-48 cells.

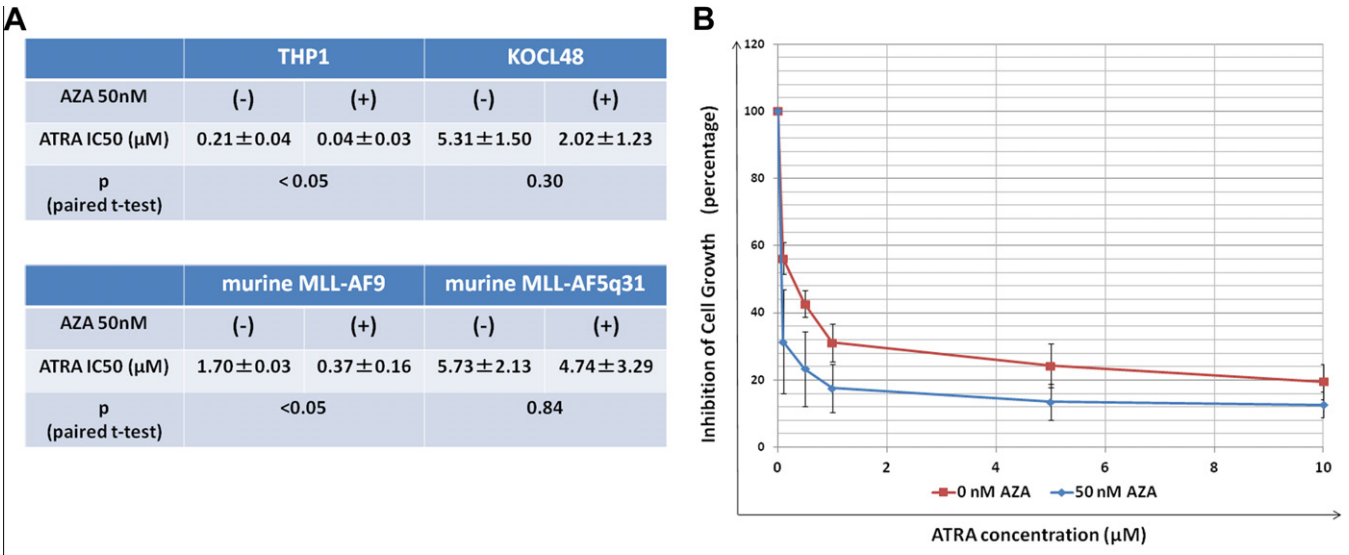
3.3. The effect of ATRA/5-Aza on murine hematopoietic progenitor cells transfected with MLL fusion genes

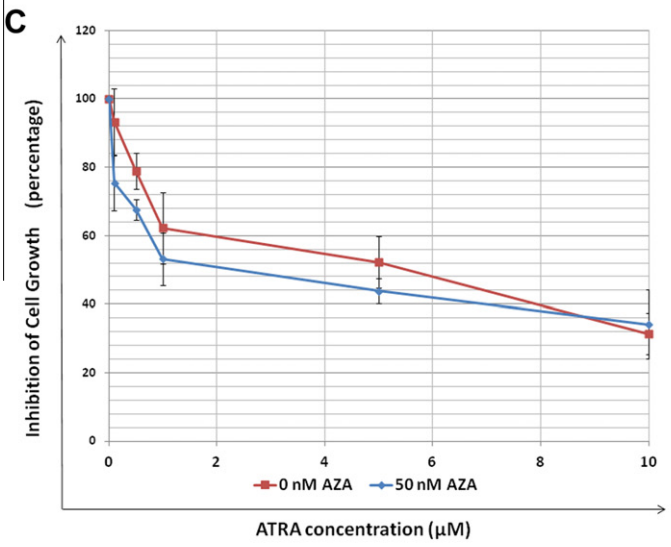
The contrasting results obtained with the two human *MLL*-rearranged AML cell lines raised the possibility that sensitivity to ATRA/5-Aza may depend on the particular *MLL* fusion partners. To test this hypothesis, immortalized murine cells transduced with two different *MLL* fusion proteins, *MLL-AF9* and *MLL-AF5q31*, were exposed to ATRA alone, 5-Aza alone, or a combination of the two drugs. FACS analysis revealed both cell lines expressed Mac-1, not expressed B220, suggesting both cell lines show myeloid phenotype (data not shown). As was observed in human AML cells, morphological changes in response to ATRA or to the combination of the two drugs were noted only in *MLL-AF9*-expressing cells (Fig. 4A). These changes were associated with the up-regulation of Mac-1 expression, suggesting that *MLL-AF9*-expressing cells, but not *MLL-AF5q31*-expressing cells, were sensitive to ATRA (Fig. 4B). However, there was no significant difference in Mac-1 expression in cells treated with ATRA alone or cells treated with ATRA/5-Aza. As was observed in human AML cells, an up-regulation of *C/ebpα* expression was noted only in *MLL-AF9*-expressing cells and was detectable even when the cells were exposed to ATRA alone (Fig. 4C), although the effect was greater when cells were exposed to the drug combination (ATRA vs. ATRA/5-Aza, $P < 0.01$). In addition, the expression level of *C/ebpα* relative to that of GAPDH was significantly higher in *MLL-AF9*-positive cells than in *MLL-AF4/AF5q31*-positive cells (Fig. 4C). Growth inhibition in response to ATRA/5-Aza was also significant in *MLL-AF9*-expressing cells

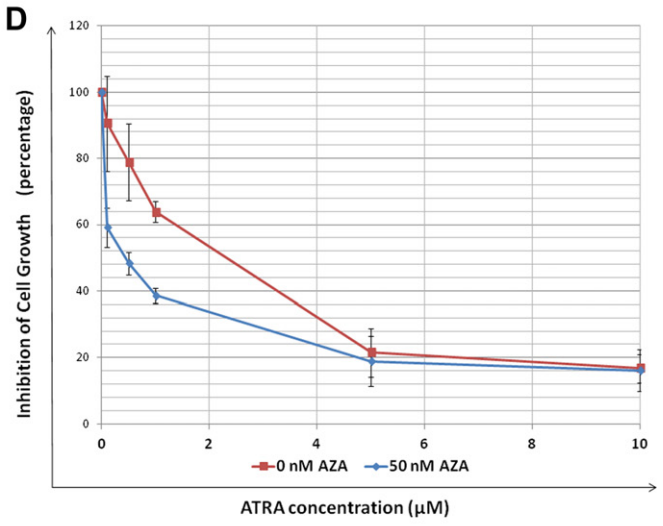
A

	THP1		KOCL48	
AZA 50nM	(-)	(+)	(-)	(+)
ATRA IC50 (μM)	0.21±0.04	0.04±0.03	5.31±1.50	2.02±1.23
p (paired t-test)	< 0.05		0.30	

	murine MLL-AF9		murine MLL-AF5q31	
AZA 50nM	(-)	(+)	(-)	(+)
ATRA IC50 (μM)	1.70±0.03	0.37±0.16	5.73±2.13	4.74±3.29
p (paired t-test)	<0.05		0.84	







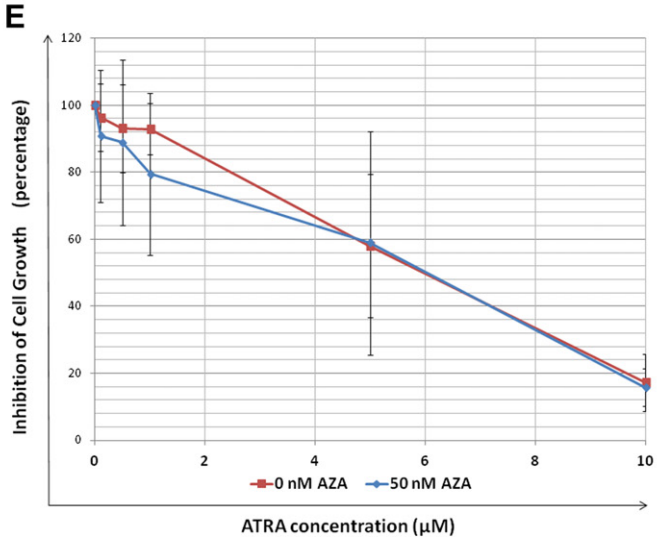


Fig. 3. Sensitivity to ATRA/5-Aza in human/murine cells expressing *MLL-AF9* or *MLL-AF4/AF5q31* fusion genes. (A) Combination treatment resulted in a significant reduction in the IC_{50} of ATRA in THP-1 cells expressing *MLL-AF9* and murine *MLL-AF9*-expressing cells. In contrast, the combination treatment did not significantly reduce the IC_{50} of ATRA in KOCL-48 expressing *MLL-AF4* or murine *MLL-AF5q31*-expressing cells. (B and C) Two human cell lines with *MLL* rearrangement (THP-1 and KOCL-48) were exposed to serial dilutions of ATRA in the absence or presence of 50 nM of 5-Aza, and cell growth was assessed. (B) THP-1, C; KOCL48. (D and E) Two murine cell lines expressing *MLL*-AF9 or *MLL-AF5q31* were exposed to serial dilutions of ATRA in the absence or presence of 50 nM of 5-Aza, and cell growth was assessed. Results represent the mean ± SD of three independent experiments. (D) Murine cells expressing *MLL-AF9*, (E) murine cells expressing *MLL-AF5q31*.

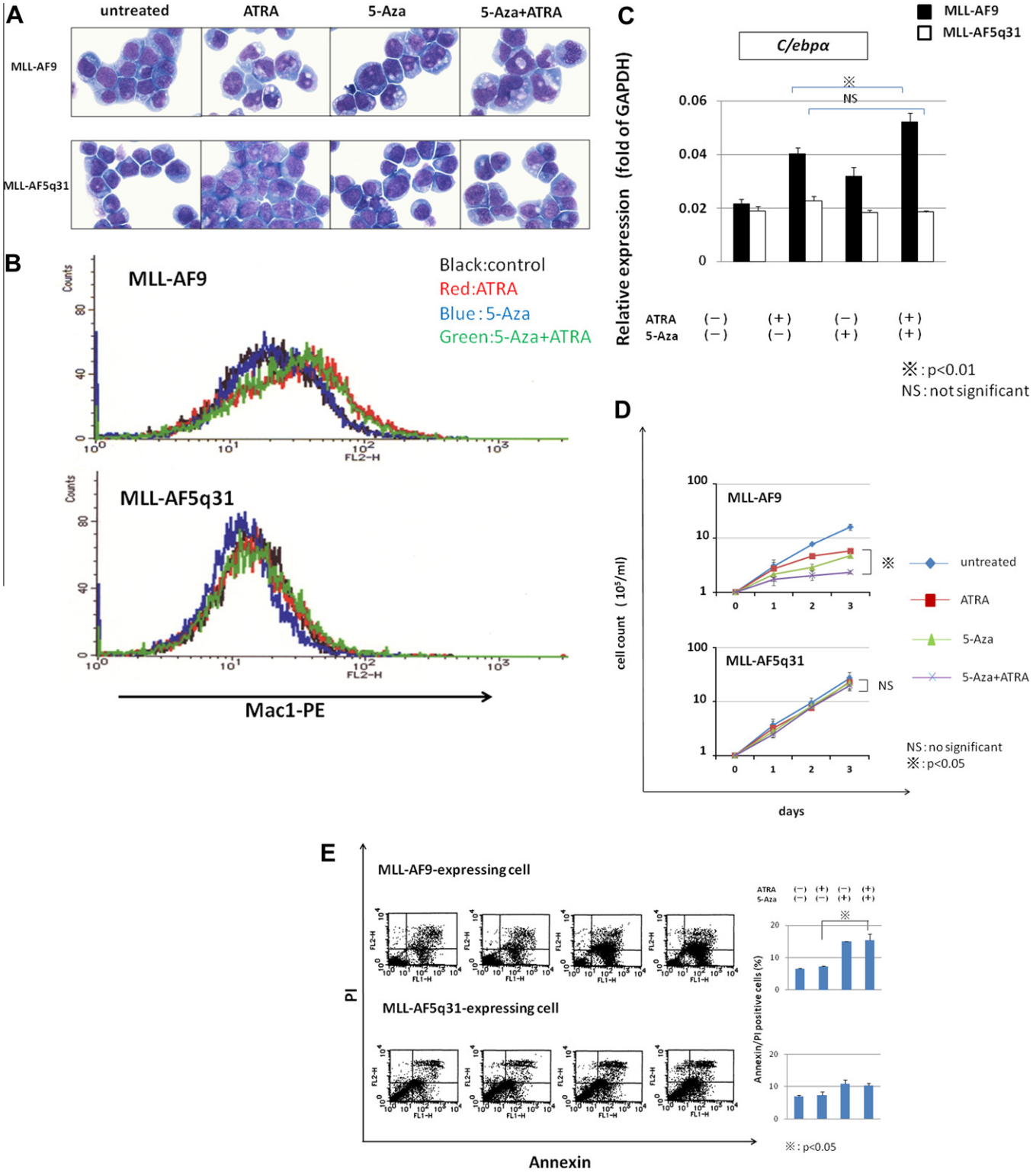


Fig. 4. The effect of ATRA/5-Aza on murine *MLL*-rearranged immortalized cells. (A) Photomicrograph of *MLL-AF9*- and *MLL-AF5q31*-expressing murine immortalized cell lines exposed to ATRA (1 μM), 5-Aza (50 nM), or ATRA and 5-Aza (same concentrations). Cytospin preparations were stained with May-Grünwald Giemsa. The figure shows representative results from three independent experiments. (B) Effect of ATRA/5-Aza on Mac-1 expression in *MLL-AF9*- and *MLL-AF5q31*-expressing murine immortalized cell lines by flow cytometry after 72 h of exposure to ATRA. Untreated cells (black line), ATRA (red line), 5-Aza (blue line), and ATRA/5-Aza (green line). ATRA induced Mac-1 expression in *MLL-AF9*-expressing cells but not in *MLL-AF5q31*-expressing cells. 5-Aza did not have a synergistic effect on ATRA-induced Mac-1 expression in *MLL-AF9*-expressing cells. The figure shows representative results from two independent experiments. (C) Expression of C/ebpα in *MLL-AF9*- and *MLL-AF5q31*-expressing murine immortalized cell lines by real-time RT-PCR after 72 h of exposure to ATRA. Expression is normalized to GAPDH. ATRA induced C/ebpα expression in *MLL-AF9*-expressing cell, but not in *MLL-AF5q31*-expressing cells. The two drugs also cooperated to induce C/ebpα expression in *MLL-AF9*-expressing cells. Results represent the mean ± SD of three independent experiments. $^{**}P < 0.05$; $^{*}P < 0.01$; NS, no significance. (D) Growth of *MLL-AF9*- and *MLL-AF5q31*-expressing murine immortalized cell lines exposed to ATRA or 5-Aza, either alone or in combination. Growth was assessed by counting nuclei every 24 h. Results represent the mean ± SD of three independent experiments. $^{*}P < 0.01$; NS, no significance. (E) Apoptosis in murine *MLL-AF9*-expressing and *MLL-AF5q31*-expressing cells exposed to ATRA or 5-Aza, either alone or in combination, by Annexin V/PI staining after 72 h of exposure to ATRA. Annexin V/PI-double positive cells were counted as apoptotic. Results represent the mean ± SD of three independent experiments. $^{*}P < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

but minimal in *MLL-AF5q31*-expressing cells (Fig. 4D). Furthermore, the combination of ATRA/5-Aza induced apoptosis more efficiently in *MLL-AF9*-expressing cells (Fig. 4E). The IC_{50} of ATRA and 5-Aza was lower in *MLL-AF9*-expressing cells than in *MLL-AF5q31*-expressing cells, as shown in sFig. 2C–D and A. In addition, 5-Aza potently cooperated with ATRA and significantly decreased the IC_{50} of ATRA in *MLL-AF9*-expressing cells (Fig. 3A and D). In contrast, 5-Aza did not induce a significant decrease in the IC_{50} of ATRA in *MLL-AF5q31*-expressing cells (Fig. 3A and E).These findings suggested that expression of the *MLL-AF4/AF5q31* fusion gene might inactivate the RA pathway more profoundly than *MLL-AF9*.

4. Discussion

The promoter methylation of genes closely associated with myeloid differentiation results in a significant blockade of cell differentiation. In APL cells, promoter hypermethylation occurs through the aberrant recruitment of DNA methyltransferase by the PML-RARα oncoprotein [21,22]. As a result, treatment of APL with ATRA in combination with DNA methyltransferase inhibitors was proposed as a possible therapeutic approach [22]. In addition to APL, the combination of demethylating agents and ATRA, which induces transcription factors associated with myeloid differentiation such as C/EBPα, might also counteract the differentiation block observed in *MLL*-rearranged AML cells. Accordingly, the activity of the combination of ATRA and 5-Aza was tested *in vitro* in *MLL*-rearranged AML cell lines.

Since the outcome of AML with the t(4;11)(q21;q23)/*MLL-AF4* fusion is poorer than that of AML with the t(9;11)(p21–22;q23)/*MLL-AF9* fusion [11], *MLL-AF9*-positive cells were compared to *MLL-AF4/AF5q31*-positive cells in experimental conditions sufficient to induce cell differentiation and apoptosis in APL cells. Interestingly, we found that *MLL-AF9*-expressing cells were relatively sensitive to ATRA, but *MLL-AF4/AF5q31*-expressing cells were not, suggesting that the RA pathway was completely inactivated in *MLL-AF4/AF5q31*-expressing cells. In addition, the experiments using murine cells transduced with *MLL* fusion genes revealed that the sensitivity to ATRA was determined by the particular *MLL* fusion partner. The combination of ATRA/5-Aza induced the up-regulation of C/EBPα (C/ebpα) and both factors cooperated to inhibit growth in human or murine cells expressing *MLL-AF9*, but not in cells expressing *MLL-AF4/AF5q31*, suggesting that 5-Aza enhanced the effect of ATRA in cells in which the RA pathway was not completely inactivated. However, the current study demonstrated that the methylation status of the promoter region of *C/EBPα* did not correlate with the expression of C/EBPα, which is consistent with a previous report [23]. Thus, the mechanism which 5-Aza enhances the activity of ATRA in AML cells expressing *MLL-AF9* should be determined. According to previous reports, inactivation of the RA pathway by oncogenic fusion proteins is associated not only with DNA methylation but also with chromatin deacetylation/demethylation [5,7,24]. Thus, further studies are required to understand the relationship between *MLL* fusion proteins, DNA methylation, and chromatin modification, the latter of which might re-activate the RA pathway in AML, including *MLL*-rearranged AML.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.131.

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