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Advances in Brief

Histone Deacetylase-targeted Treatment Restores Retinoic Acid Signaling and Differentiation in Acute Myeloid Leukemia[1]

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Abstract

Histone deacetylase (HDAC)-dependent transcriptional repression of the retinoic acid (RA)-signaling pathway underlies the differentiation block of acute promyelocytic leukemia. RA treatment relieves transcriptional repression and triggers differentiation of acute promyelocytic leukemia blasts, leading to disease remission. We report that transcriptional repression of RA signaling is a common mechanism in acute myeloid leukemias (AMLs). HDAC inhibitors restored RA-dependent transcriptional activation and triggered terminal differentiation of primary blasts from 23 AML patients. Accordingly, we show that AML1/ETO, the commonest AML-associated fusion protein, is an HDAC-dependent repressor of RA signaling. These findings relate alteration of the RA pathway to myeloid leukemogenesis and underscore the potential of transcriptional/differentiation therapy in AML.

Introduction

The RA-signaling[4] pathway is involved in the regulation of hemopoietic myeloid differentiation and is altered in APL, resulting in blockage of the differentiation of leukemic blasts (1–5). The APL-specific RARα fusion proteins PML/RARα and PLZF/RARα bind to and constitutively repress promoters of RA target genes via aberrant recruitment of multi-subunit complexes containing HDAC activities. HDACs induce deacetylation of nucleosomal core histone tails, leading to a chromatin conformation that correlates with gene silencing (6, 7). Consistent with a model in which HDACs are crucial molecular targets of APLs, treatment with HDAC inhibitors and/or RA reverts the blockage of differentiation of APL blasts *in vitro* and induces disease remission *in vivo* by inhibiting or releasing, respectively, the HDAC-RARα fusion repressor complex (8–11)

APL accounts for ~5–10% of AMLs (12). All other AML subtypes express RARs but are not sensitive to RA action on differentiation (13, 14). It is not known whether such unresponsiveness is a consequence of the leukemic state or whether it reflects an active repression of the RA-signaling pathway. Aberrant histone acetylation also results from chromosomal rearrangements associated to other AML subsets. In t(8;21) AML, for example, a stable association of AML1/ETO fusion protein with the nuclear HDAC complex is crucial for its ability to repress transcription of AML1 target genes and to block differentiation of hematopoietic precursors (5, 15, 16). Considering that HDACs are part of a general mechanism of gene silencing and that myeloid leukemogenesis is consistently associated with translocations of genes encoding transcription factors, we investigated whether the poor RA sensitivity of nonpromyelocytic AMLs could reflect an active, HDAC-dependent repression of RA transcription signaling pathway.

Materials and Methods

Cell Cultures. Bone marrow and/or peripheral blood were obtained from 23 informed, newly diagnosed AML patients. Cases were classified as AML-M2 or AML-M4 according to the French-American-British classification and showed an initial percentage of circulating blasts >80% (17). Cytogenetic analysis and RT-PCR to rule out the presence of the APL-associated fusion genes were performed according to standard methods as described (18–20).

The U937-AML1/ETO (U937 AE) cells were obtained by electroporation into U937 cells of an HA-tagged AML1/ETO cDNA generated and subcloned into the Zn^{2+}-inducible mouse MT-1 promoter as described (15, 21). Different neomycin-selected clones were screened for AML1/ETO expression before and after Zn^{2+} treatment using an anti-HA antibody (BabCO). The U937 MT-MHA-AE clone 16 was selected because of its almost undetectable expression of AML1/ETO prior to Zn^{2+} treatment and high and persistent (up to 96 h) expression after Zn^{2+} treatment.

AML blasts and cell lines Kasumi-1 (AML-M2), U937 (AML-M5), and U937-AE were maintained in RPMI 1640 supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% FCS at 37°C in a humidified atmosphere containing 5% CO_2. AML blasts were treated with the HDAC inhibitors TSA (WAKO Chemicals) or sodium phenylbutyrate (Sigma) for 1 h before the addition of RA (Sigma) and during the RA treatment.

Cell Proliferation and Differentiation. Cell proliferation and differentiation were evaluated and quantified by direct cell counting (trypan blue dye exclusion method) using a hemocytometer chamber, light microscopy morphological examination of Wright-Giemsa-stained cytospins, NBT dye reduction assay (at least 500 morphologically intact cells per experimental condition were counted and corrected for viability, measured by trypan blue exclusion method), cell cycle analysis of cells stained with propidium iodide, and direct immunofluorescence staining of CD11b cell surface myeloid-specific antigen (Coulter Epics XL flow cytometer; Beckman Coulter) as described previously (21, 22).

Analysis of Acetylated H3 and H4 Histones. AML blasts were fixed in 100% methanol at room temperature for 5 min, followed by acetone for 2 min at −20°C, and incubated with anti-acetylated histone H3 antibody (Upstate Biotechnology) according to the manufacturer's protocol. Immunofluorescence was detected using a Zeiss Axioplan fluoromicroscope (Zeiss s.p.a.). Immunoblot analysis of total cell homogenates (60 μg) was performed using an anti-acetylated H4 antibody (Upstate Biotechnology) as described (22). Immunoreactivity was determined using the ECL method (Amersham).

Transient Cotransfection of AML Blasts and Transactivation Assays. AML blasts were transiently transfected with the $\beta RARE_3$-tk-Luc, the TRE2-tk-Luc, or the RARβpr-Luc reporter (23, 24) by electroporation using a

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[4] The abbreviations used are: RA, retinoic acid; APL, acute promyelocytic leukemia; RARα, retinoic acid receptor α; HDAC, histone deacetylase; AML, acute myeloid leukemia; RT-PCR, reverse transcription-PCR; NBT, nitroblue tetrazolium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TGase, transglutaminase.

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Gene-Pulser II apparatus (Bio-Rad). pcDNA3 expression vector (mock) or pcDNA3s containing ETO, HA-AML1, or HA-AML1/ETO cDNAs (15, 25) were cotransfected with the above-described reporters in U937 cells. The plasmid encoding β-galactosidase (pSV-βgal) was cotransfected and used as internal control and for normalization of reactions. Six h after electroporation, cells were treated for 16 h, lysed, and then assayed using the Luciferase Assay Kit (Promega) on a luminometer (Berthold).

RNA Preparation and RT-PCR Analysis. Total RNA was extracted from Ficoll-Hypaque-isolated AML patient blasts as described (18). One microgram of total RNA was heated at 65°C for 10 min and used as a template for first-strand cDNA (cDNA) synthesis using SuperScript II RNase H Reverse Transcriptase and random hexamers as primers (Life Technologies). cDNAs were amplified for a total of 30 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s. Each PCR reaction contained 2 μl of the cDNA, 0.2 mM deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, and 2.5 units of PLATINUM Taq DNA Polymerase (Life Technologies). The RARα and AML1/ETO primers used in the reactions have been described previously (18, 26). The following oligonucleotides were used for *RAR*β and *GAPDH* transcripts: sense, 5′-AAGCT TGTCG ACGCC ACCAT GTTTG ACTGT ATGGA TG-3′, and antisense, 5′-AGCCC TTACA TCCCT CACAG-3′ for RARβ; sense, 5′-CGGGA AGCTT GTGAT CAATGG-3′, and antisense, 5′-GGCAG TGATG GCATG GACTG-3′, for GAPDH (used as an internal control). PCR products were electrophoresed on a 1.2% agarose gel. Blots were hybridized with end-labeled 5′ primers using [^{32}P]ATP and T4 kinase (Life Technologies). Radioactivities were detected using a Fuji BAS1800 scanner (Raytest) and analyzed by Advanced Image Data Analyzer (Raytest).

TGase Activity Assay. The TGase activity assay was performed on total homogenate as described previously (22) by measuring the incorporation of [^{3}H]putrescine (12.6 Ci/mmol; Amersham Corp.) into casein in duplicate or triplicate cultures.

Results and Discussion

Primary blasts from the peripheral blood and/or bone marrow of 23 newly diagnosed (non-APL) AML patients were included in this study. The morphologic and genetic features of AML blasts are shown in Table 1. None of the blasts expressed the APL-associated fusion proteins, as determined by RT-PCR using specific primers for the PML, PLZF, and RARα sequences (Refs. 18, 20; data not shown).

AML blasts were treated for 5 days in culture with RA and TSA as single agents or in combination, and then evaluated by morphologic criteria, NBT dye reduction assay, and the frequency of apoptotic cells (as determined by fluorescence-activated cell sorting of propidium iodide-stained nuclei). Treatment with RA or TSA as the sole agent induced moderate or no morphological differentiation (see Fig. 1*a* for one representative case). Remarkably, the combined RA + TSA treatment induced the appearance of cells with metamyelocyte- or neutrophil-like morphology and increased the NBT positivity up to 60–80% (Fig. 1 and Table 1). The frequency of apoptotic cells after 5 days of treatment with RA + TSA did not exceed 10–20% as evaluated by fluorescence-activated cell sorting of propidium iodide-stained cells (eight cases tested; data not shown). Therefore, it appears that RA + TSA induced terminal myeloid differentiation in blasts from all of the 23 AML cases tested, independent of the presence of a specific genetic lesion. The kinetics and extent of morphologic and functional differentiation of AML blasts after treatment with RA + TSA were comparable with those of five APL cases treated in parallel with RA as a single agent (not shown).

To investigate whether the effect of TSA correlated with modifications of histone acetylation in AML blasts, we evaluated the status of acetylation of histone H3 or H4 by immunofluorescence and Western blotting analysis, respectively, using specific antibodies. In untreated AML blasts, acetylated forms of histone H3 were almost undetectable, whereas they became detectable after treatment with RA or TSA used as single agents. Combination treatment of RA + TSA further increased acetylation of histone H3, as shown by the marked

Table 1 *Morphologic and genetic features of primary blasts from AML patients*

Patient no.	FAB[a]	Karyotype	Differentiation[b] (5-day cultures) TSA	RA	RA + TSA
1	M4	NN[c]	+	++	+++
2	M4Eo	Inv(16)(p13q22)	++	+	++++
3	M2	Complex aberrations[d]	++	+	++++
4	M4	del(20)(q13)	+	++	++++
5	M2	NN	++	+	+++
6	M2	NN	++	+	++++
7	M4	NN	++	+	+++
8	M4	NN	+	+	+++
9	M2	No mitoses	+	++	++++
10	M2	NN	+	++	+++
11	M4	t(10;11)(q23;p14)	++	+	++++
12	M2	Not available	+	+	++++
13	M2	del(4)(q21;26)	+	+	++++
14	M2	t(8;21)(q22;q22)[e]	++	++	++++
15	M2	No mitoses	+	+	+++
16	M2	NN	++	+	++++
17	M2	NN	+	+	+++
18	M4	NN	++	+	++++
19	M2	t(8;21)(q22;q22)	+	++	++++
20	M2	NN	+	+	+++
21	M2	NN	+	++	+++
22	M4	NN	++	++	+++
23	M2	No mitoses	+	+	+++

[a] FAB, French-American-British classification.

[b] Differentiation (as evaluated by morphologic criteria): +, 10–20%; ++, 20–40%; +++, 50–80%; ++++, 80–100% more mature metamyelocytes and granulocytes than control cultures.

[c] NN, normal karyotype. All cases with no detectable aberrations by conventional karyotyping were also negative by RT-PCR and Southern blot for the fusion genes *PML/RAR*α*, CBF*β*/MYH11, DEK/CAN, BCR/ABL,* and *MLL* rearrangements.

[d] 44,XX,der(1)ins(1;3)(p21;q?)t(1;17)(q21;p12),der(8)ins(8;3)(q12);(q?)ins(8;1)(q?;q),der(11)ins(11;12)(p14;p13),dic(12;20)(p13;p12),-17.

[e] 46,XY/46,XYt(8;21)(q22;q22)/46 idem del(9)(q13q22)/45,X,Y,t(8;21)(q22;q22)/45, idem, del(9)(q13q22).

staining of the nucleus (Fig. 2*a*). Immunoblot analysis using an anti-acetylated histone H4 antibody (Fig. 2*b*) showed that combined treatment with RA and TSA or sodium phenylbutyrate (another inhibitor of HDACs already used in clinical trials), strongly induced acetylation of histone H4. Together, these results suggest that myeloid differentiation induced by RA + TSA might be the consequence of a combined effect at the level of RA target promoters: transcriptional derepression by TSA and transcriptional activation by RA.

Therefore, we investigated whether TSA affects RA-induced activation of RA target genes in AML blasts. In a first set of experiments, we evaluated the capacity of TSA to modify the RA response of RA target promoters transiently transfected into AML blasts. For this purpose, we used two reporter constructs: (*a*) RARβpr-Luc, which contains the entire promoter region (−5 kb to +155 kb) of *RAR*β cloned 5′ to the luciferase reporter gene; and (*b*; $RARE_3$-tk-Luc, which contains three copies of the βRARE direct repeat sequence (DR5) of the *RAR*β_2 promoter, cloned 5′ to the thymidine kinase promoter and the luciferase gene. *RAR*β is a natural RA target gene. Its induction has been proposed as a general mechanism by which retinoids inhibit growth and induce differentiation in cancer cells (27). βRARE is the strongest natural RA-responsive element present in the promoter region of several RA-responsive genes (23, 27, 28). Experiments were performed on six different AML cases, using both promoters. Pharmacological doses of RA (1 μM) increased $\beta RARE_3$-tk-Luc activities and RARβpr-Luc ~2.5–3.5-fold. In the presence of 50–100 ng/ml TSA, the magnitude of the RA response of the $\beta RARE_3$-tk-Luc and RARβpr-Luc promoters increased ~10–20-fold and 6–9-fold, respectively (Fig. 3*a*).

We next evaluated the capacity of RA and TSA to activate transcription of endogenous RA target genes. Four AML cases were analyzed for the effects of TSA and RA on the expression of the RA target genes *RAR*α and *RAR*β (27). Expression of *RAR*α and *RAR*β

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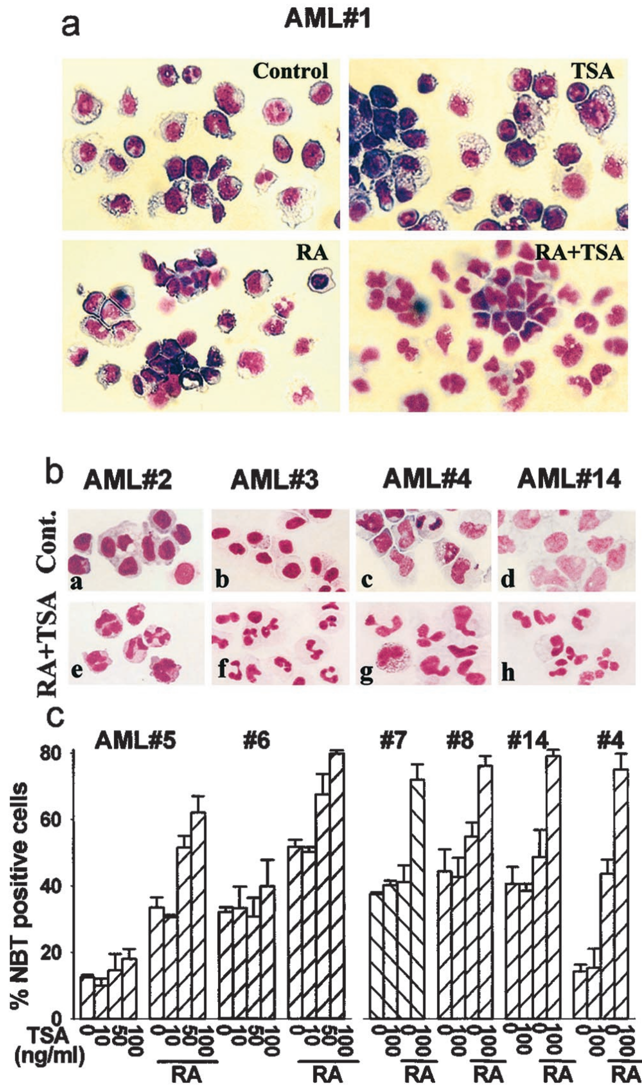

Fig. 1. Combination treatment with HDAC inhibitors and RA induces terminal differentiation of primary AML blasts. *a*, Wright-Giemsa staining of blasts isolated from a newly diagnosed AML patient (Table 1, *patient 1*) treated for 5 days with 100 ng/ml TSA alone, 1 μM RA alone, or with both agents as indicated. *b*, morphologic differentiation of blasts from four other representative AML cases (Table 1, *patients 2–4*, and *14*) induced by treatment with 1 μM RA + 100 ng/ml TSA (*RA + TSA*) for 5 days. Untreated cells are shown as controls. *c*, percentage of differentiated cells determined by NBT reduction assay in four representative AML cases (Table 1, *patients 4–8*, and *14*).

was evaluated by semiquantitative RT-PCR analysis performed on total RNA prepared from AML blasts before or after treatment for 16 h with RA, TSA, or RA + TSA. Results revealed no modification of the levels of *RARα* and *RARβ* mRNA transcripts by TSA, slight up-regulation by RA alone, and significant up-regulation by RA + TSA treatment (Fig. 3, *b* and *c*, and data not shown). Similarly, RARα and RARβ mRNA transcripts were up-regulated by RA + TSA in two AML-M2 cell lines, HL-60 and Kasumi-1 (data not shown). We then measured cytosolic type II TGase activity in four AML cases, two of which are shown in Fig. 3*d*. *Type II TGase* is a gene transcriptionally regulated by retinoids and recently shown to be a marker of RA-induced differentiation in APL cells (8, 22). Type II TGase activity was strongly up-regulated after 3 days of treatment with RA + TSA (Fig. 3*d*). Therefore, it appears that TSA potentiates the RA induction of endogenous RA target genes *RARα*, *RARβ*, and *type II TGase*, strongly suggesting that the promoters of RA-responsive genes are repressed in AML blasts via mechanisms involving HDAC activities.

Transcriptional activation of RA target genes by RA + TSA correlates with the correction of a crucial component of the leukemic phenotype, *e.g.,* the differentiation block. Thus, transcriptional repression of RA target genes in AMLs might be part of the same mechanism(s) that lead to transformation. Because different genetic events, the majority of which are still unknown, underlie myeloid leukemogenesis, repression of RA target genes might be a common event in AMLs. To investigate this possibility, we evaluated the ability of TSA to confer sensitivity to RA-induced differentiation in cells expressing the AML-associated fusion protein AML1/ETO. It has been shown that AML1/ETO represses transcription of AML1 target genes and myeloid differentiation by an aberrant recruitment of a complex containing HDAC activity (5, 15, 16).

We therefore evaluated the biological response to RA and TSA of the Kasumi-1 cell line, derived from an AML-M2 patient with the t(8;21) translocation and expressing the AML1/ETO fusion product. In this cell line, the level of AML1/ETO expression is comparable with that detectable in blasts from t(8;21) AML patients (see cases AML 14 and AML 19 in Fig. 4*a*). Four days of *in vitro* treatment with RA or TSA as single agents induced a modest effect on the growth

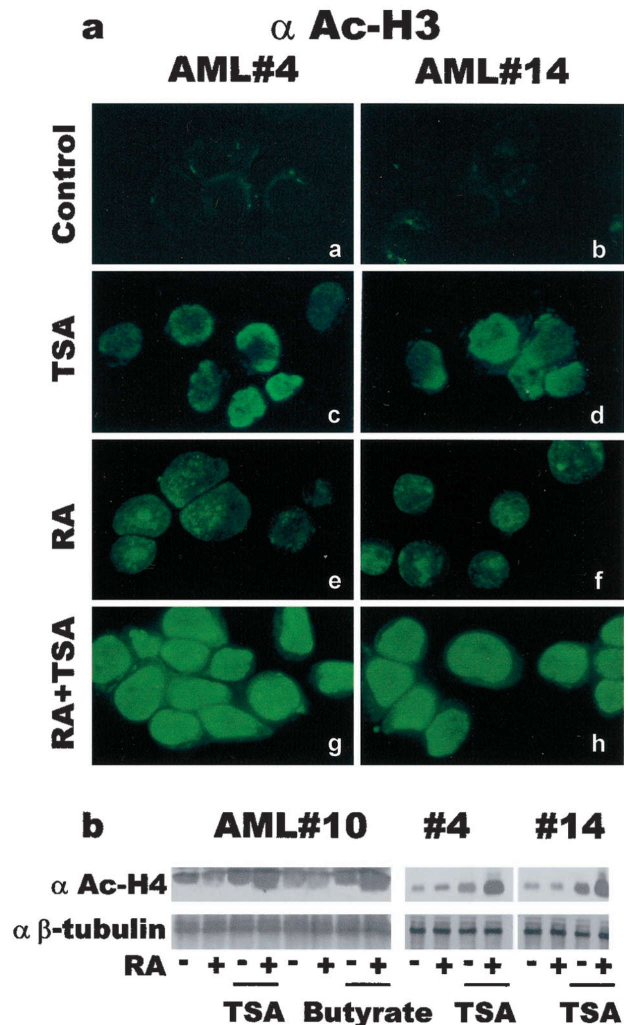

Fig. 2. Effect of HDAC inhibitors and RA on accumulation of acetylated histone H3 and H4 in AML blasts. Immunofluorescence analysis (*a*) and immunoblot analysis (*b*) of the acetylation status of histone H3 and H4 in fresh blasts from representative AML cases (Table 1, *patients 4*, *10*, and *14*) treated with the HDAC inhibitor TSA (100 ng/ml) and/or RA (1 μM) as indicated for 16 h. Immunofluorescence and immunoblot analyses of acetylated histone H3 and H4 were performed using anti-acetylated histone H3 (*α Ac-H3*) and H4 (*α Ac-H4*) antibodies, respectively. *α β-tubulin*, anti-β-tubulin antibody.

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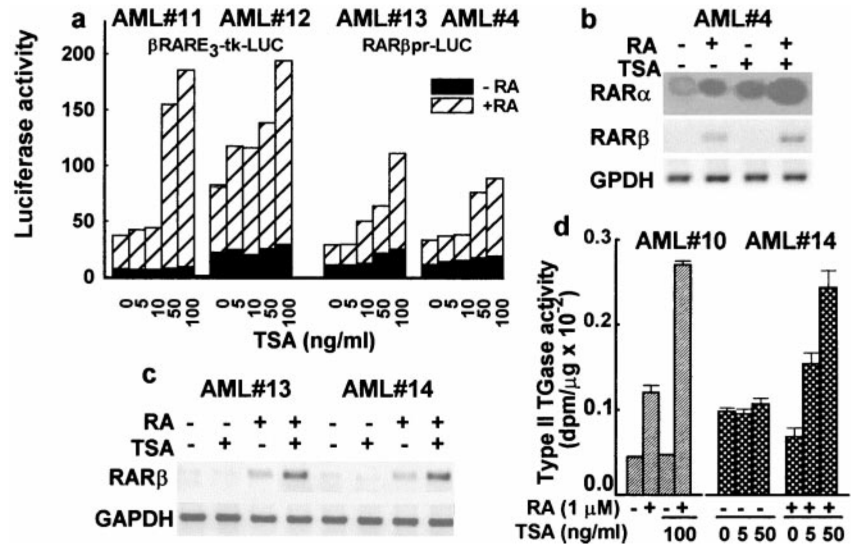

Fig. 3. Inhibition of HDAC activities affects RA signaling on RA-responsive promoters and RA target genes in AML blasts. *a*, TSA cooperates with RA to activate βRARE$_3$-tk-Luc and RARβpr-Luc reporters transiently transfected in primary blasts from AML patients. The *numbers* correspond to AML cases described in Table 1. *Filled* and *hatched columns* indicate luciferase activities in the absence or presence of RA (1 μM), respectively. In primary AML blasts, TSA (100 ng/ml) potentiates RA (1 μM) induction of RARα and RARβ mRNA expression (*b* and *c*) and type II TGase activity (*d*). *GPDH*, glyceraldehyde-3-phosphate dehydrogenase. *Bars*, SD.

and differentiation properties of Kasumi-1 cells. Combined RA + TSA treatments, instead, caused growth arrest, accumulation of cells in the G_1 phase of the cell cycle, and increased NBT positivity and expression of differentiation marker CD11b (Table 2). In addition, flow-cytometric analysis of propidium iodide-stained cells revealed only a moderate increase in the frequency of apoptosis (5–10%) after treatment with RA + TSA (not shown).

We next evaluated the RA transcriptional response in Kasumi-1 cells. Analysis of the transfected βRARE$_3$-tk-Luc promoter showed slight up-regulation (~1.2-fold) by pharmacological doses of RA (1 μM) as single agent and strong up-regulation (15-fold) by the combined treatment with TSA (50–100 ng/ml; Fig. 4*b*). In contrast, in the presence of similar concentrations of TSA, RA treatment did not significantly affect the activity of the TRE$_2$-tk-Luc reporter gene (a synthetic reporter containing the palindromic sequence TRE, which mediates both RA and thyroid hormone transactivation; Refs. 27, 28). A ligand-dependent assay showed that doses of TSA of 100 ng/ml also restored βRARE$_3$-tk-Luc response to physiological concentrations of RA (1–10 nM; Fig. 4*b*). The calculated EC_{50} values (concentrations that give 50% efficacy) for βRARE$_3$-tk-Luc transactivation potency of RA as sole agent and RA + TSA were 240 and 9 nM, respectively. In Kasumi-1 cells, TSA greatly increased the effects of RA treatment on acetylation of histones H3 and H4 and type II TGase activity (data not shown and Fig. 4*c*). These findings, together with the results obtained in blasts from two AML patients (AML patients 14 and 19) expressing the AML1/ETO fusion (Figs. 1–4 and data not shown), strongly suggest that RA signaling is repressed in AML1/ETO-expressing cells and that this repression can be relieved by inhibition of HDAC activity. Thus, AML1/ETO might act as a transcriptional repressor of the RA pathway.

To investigate this possibility more directly, we measured the effects of AML1/ETO expression on RA response using U937-AE cells, which express the AML1/ETO cDNA under a Zn^{2+}-inducible promoter, or U937 cells transiently transfected with the HA-AML1/ETO cDNA (see Fig. 4*d* for Western blotting analysis of U937-AE or

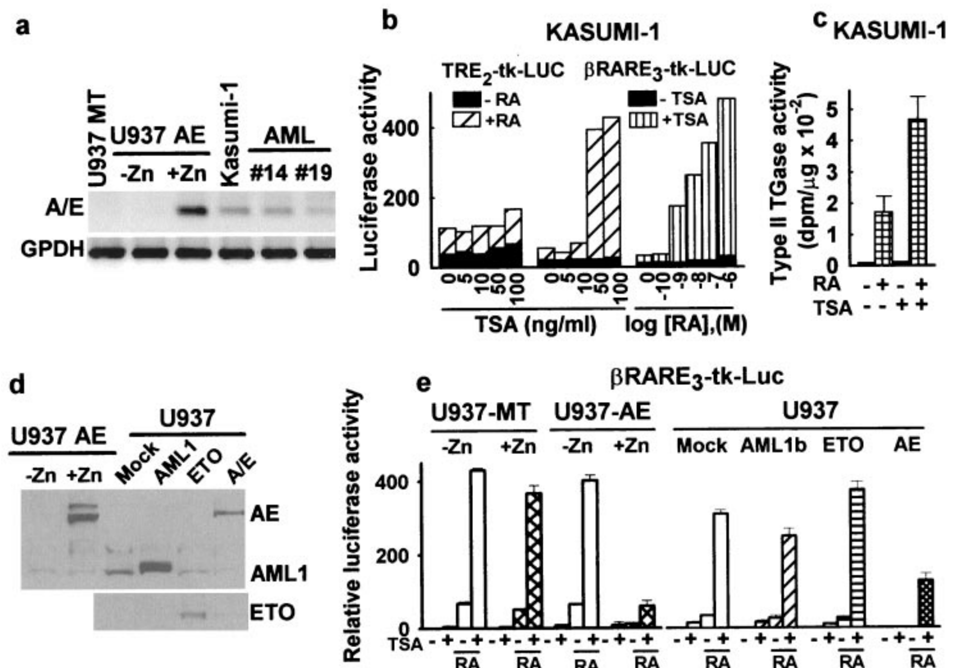

Fig. 4. Expression of AML1/ETO, the AML-associated fusion protein, induces transcriptional repression of RA signaling that is reverted by inhibiting HDAC activities. *a*, expression levels of AML1/ETO mRNA in cell lines and AML blasts (patients 14 and 19) were detected by RT-PCR using specific primers as described (26). *b*, TSA cooperates with RA to activate βRARE$_3$-tk-Luc reporter, but not the synthetic TRE$_2$-tk-Luc transiently transfected in the AML Kasumi-1 cell line, which expresses the AML1/ETO fusion product. *Filled* and *hatched columns* indicate luciferase activities in the absence or presence of RA (1 μM), respectively. A RA dose-dependent assay indicates that 100 ng/ml TSA restores the activation of βRARE$_3$-tk-Luc reporter by physiological RA concentrations (1–10 nM) in transfected Kasumi-1 cells. *c*, TSA cooperates with RA to induce type II TGase activity in Kasumi-1 cells. *d*, expression levels of AML1, ETO, and AML1/ETO in U937-AE and U937 transfected cells were quantified by immunoblot analysis using anti-HA and anti-ETO antibodies. *e*, effect of 1 μM RA, 100 ng/ml TSA and RA + TSA in induction of the reporter βRARE$_3$-tk-Luc transiently transfected into U937, U937-MT, or the U937-AE clone expressing AML1/ETO under the control of the Zn^{2+}-inducible mouse MT-1 promoter. *Open* and *hatched columns* indicate luciferase activities in the absence or presence of Zn^{2+} (100 μM), respectively.

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Table 2 *Combined effect of RA + TSA on growth and differentiation of Kasumi-1 cells*
This experiment is representative of three that gave comparable results.

Treatment (4 days)	Viable cells ($\times 10^5$)	CD11b (AU)[a]	NBT (%)	Cell cycle phase G_1	S	G_2
Control	10.9	11.8	8.1	54.1	33.9	11.9
TSA (50 ng/ml)	11.4	10.7	18.9	54.0	33.6	12.5
RA (1 μM)	9.1	15.5	22.7	57.4	26.0	16.6
RA + TSA	6.8	24.8	62.5	67.2	24.4	8.4

[a] AU, arbitrary units.

AML1/ETO-transfected U937 cells). In the absence of AML1/ETO expression, (Fig. 4*e*, *U937-MT*, *U937-AE −Zn*, and *U937 Mock*), the activity of the βRARE$_3$-tk-Luc promoter was induced ~50-fold by RA and potentiated by TSA by a factor of 10. Strikingly, the induction of AML1/ETO expression in these cells led to a marked reduction (~75%) of the RA-induced transactivation of the βRARE$_3$-tk-Luc. Notably, TSA treatment relieved transcriptional repression by AML1/ETO (Fig. 4*e*). Thus, it appears that in myeloid cells the β-RARE element, which is present in promoters of RA target genes, including myeloid genes (1), is activated by the combined RA + TSA treatment and is repressed by AML1/ETO expression.

To evaluate the specificity of the effect of AML1/ETO on the RA-signaling pathway, we next analyzed the effects of AML1, ETO, and AML1/ETO on RA-dependent transactivation. To this end, we performed cotransfection experiments in U937 cells using the expression vectors for ETO, AML1B, or AML1/ETO and the βRARE$_3$-tk-Luc promoter. Relative AML1, ETO, and AML1/ETO expression levels in U937-AE and U937 transfected cells were measured by immunoblotting analysis using an anti-HA (recognizing HA-AML1 and HA-AML1/ETO) or an anti-ETO (recognizing overexpressed ETO, but not the AML1/ETO fusion) antibody,[5] as shown in Fig. 4*d* (for a representative experiment). Results showed that (*a*) overexpression of ETO or AML1 did not repress RA- or RA + TSA-induced βRARE$_3$-tk-Luc activity (Fig. 4*e*; a slight increase in βRARE$_3$-tk-Luc activity was seen after ETO expression), and (*b*) induction of AML1/ETO expression by Zn^{2+} treatment (in the U937-AE clone) or by transient transfection (into the parental U937 cells) strongly repressed RA-induced βRARE$_3$-tk-Luc activity (Fig. 4*e*). Taken together, these results suggest that AML1/ETO (but not AML1 or ETO), when expressed in myeloid cells, acts as a specific transcriptional repressor of the RA pathway.

Indirect evidence suggest that common mechanisms underlie myeloid leukemogenesis: (*a*) The genes involved in the AML-associated translocations invariably encode transcription factors (*e.g.,* AML1, CBFβ, RARα, MLL, p300, CBP, HOX genes, and EVI1), which are physiologically involved in hematopoietic differentiation and cooperatively regulate promoters or enhancers present on myeloid specific genes (1, 5, 12). (*b*) Accumulation of undifferentiated precursors is a prominent feature of the myeloid leukemia phenotype, and ectopic expression of fusion proteins in hemopoietic precursors induces blockage of differentiation and leukemia in animal models (10, 29–32). (*c*) Aberrant recruitment of HDAC complexes is crucial to the activity of the AML-specific fusion proteins PML/RARα, PLZF/RARα, and AML1/ETO (8–10, 15, 16), suggesting that modification of the chromatin structure in the target promoters of fusion proteins represents an important mechanism of leukemogenesis. Thus, it is conceivable that different genetic alterations may result in common patterns of deregulated gene expression, leading to blockage of differentiation and favoring myeloid leukemogenesis. Here we show that in AMLs, regardless of their underlying genetic alteration, the RA-signaling pathway is constitutively repressed through an HDAC-dependent mechanism and that inhibition of HDAC activities restores the RA-differentiation response. These results indicate that repression of the RA-signaling pathway is a general pathogenetic event in AMLs and that HDACs are common targets for AMLs, highlighting the possibility of transcriptional/differentiation therapy in AMLs other than APL.

Butyrate has been used as single agent in the treatment of an AML patient resistant to conventional chemotherapy, and was shown to trigger terminal differentiation of leukemic blasts *in vivo* (33). The usage of butyrates and their derivatives as HDAC inhibitors, however, is limited by their poor specificity, as well as by their transient and reversible activities. Indeed, high drug plasma concentrations must be sustained to obtain a biological effect (11). In contrast, TSA is a highly specific, stable, and potent HDAC inhibitor. We recently demonstrated that *"in vivo "* modulation of gene transcription by micromolar concentrations of TSA is not toxic in adult mice and does not perturb mouse embryonic or postnatal development,[6] thereby suggesting that TSA might represent a useful agent for transcriptional/differentiation therapy in AMLs.

[5] V. Gelmetti and P. G. Pelicci, unpublished results.

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References

1. Tenen, D. G., Hromas, R., Licht, J. D., and Zhang, D. E. Transcription factors, normal myeloid development, and leukemia. Blood, *90:* 489–519, 1997.
2. Lawson, N. D., and Berliner, N. Neutrophil maturation and the role of retinoic acid. Exp. Hematol., *27:* 1355–1367, 1999.
3. Labrecque, J., Allan, D., Chambon, P., Iscove, N. N., Lohnes, D., and Hoang, T. Impaired granulocytic differentiation in vitro in hematopoietic cells lacking retinoic acid receptors α1 and γ. Blood, *92:* 607–615, 1998.
4. Du, C., Redner, R. L., Cooke, M. P., and Lavau, C. Overexpression of wild-type retinoic acid receptor α (RARα) recapitulates retinoic acid-sensitive transformation of primary myeloid progenitors by acute promyelocytic leukemia RARα-fusion genes. Blood, *94:* 793–802, 1999.
5. Redner, R. L., Wang, J., and Liu, J. M. Chromatin remodelling and leukemia: new therapeutic paradigms. Blood, *94:* 417–428, 1999.
6. Xu, L., Glass, C. K., and Rosenfeld, M. G. Coactivator and corepressor complexes in nuclear receptor function. Curr. Opin. Genet. Dev., *9:* 140–147, 1999.
7. Kouzarides, T. Histone acetylases and deacetylases in cell proliferation. Curr. Opin. Genet. Dev., *9:* 40–48, 1999.
8. Grignani, F., De Matteis, S., Nervi, C., Tomassoni, L., Gelmetti, V., Cioce, M., Fanelli, M., Ruthardt, M., Ferrara, F. F., Zamir, I., Seiser, C., Grignani, F., Lazar, M. A., Minucci, S., and Pelicci, P. G. Fusion proteins of the retinoic acid receptor-α recruit histone deacetylase in promyelocytic leukaemia. Nature (Lond.), *391:* 815–818, 1998.
9. Lin, R. J., Nagy, L., Inoue, S., Shao, W., Miller, W. H. J., and Evans, R. M. Role of the histone deacetylase complex in acute promyelocytic leukaemia. Nature (Lond.), *391:* 811–814, 1998.
10. He, L. Z., Guidez, F., Tribioli, C., Peruzzi, D., Ruthardt, M., Zelent, A., and Pandolfi, P. P. Distinct interactions of PML-RARα and PLZF-RARα with corepressors determine differential responses to RA in APL. Nat. Genet., *18:* 126–135, 1998.
11. Warrell, R. P., Jr, He, L. Z., Richon, V., Calleja, E., and Pandolfi, P. P. Therapeutic targeting of transcription in acute promyelocytic leukemia by use of an inhibitor of histone deacetylase. J. Natl. Cancer Inst. (Bethesda), *90:* 1621–1625, 1998.
12. Löwenberg, B., Downing, J. R., and Burnett, A. Acute myeloid leukemia. N. Engl. J. Med., *341:* 1051–1062, 1999.
13. Fenaux, P., and Degos, L. Differentiation therapy for acute promyelocytic leukemia. N. Engl. J. Med., *337:* 1076–1077, 1997.
14. Chomienne, C., Ballerini, P., Balitrand, N., Daniel, M. T., Fenaux, P., Castaigne, S., and Degos, L. All-*trans* retinoic acid in acute promyelocytic leukemias. II. *In vitro* studies: structure-function relationship. Blood, *76:* 1710–1717, 1990.
15. Gelmetti, V., Zhang, J., Fanelli, M., Minucci, S., Pelicci, P. G., and Lazar, M. A. Aberrant recruitment of the nuclear receptor corepressor-histone deacetylase complex by the acute myeloid leukemia fusion partner ETO. Mol. Cell. Biol., *18:* 7185–7192, 1998.

[6] C. Nervi, V. Buffa, F. Fazi, U. Borello, P. G. Pelicci, and C. Gossu, submitted for publication.

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16. Wang, J., Hoshino, T., Redner, R. L., Kajigaya, S., and Liu, J. M. ETO, fusion partner in t(8;21) acute myeloid leukemia, represses transcription by interaction with the human N-CoR/mSin3/HDAC1 complex. Proc. Natl. Acad. Sci. USA, *95:* 10860–10865, 1998.
17. Bennett, J. M., Catovsky, D., Daniel, M. T., Flandrin, G., Galton, D. A., Gralnick, H. R., and Sultan, C. Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. Ann. Intern. Med., *103:* 620–625, 1985.
18. Mandelli, F., Diverio, D., Avvisati, G., Luciano, A., Barbui, T., Bernasconi, C., Broccia, G., Cerri, R., Falda, M., Fioritoni, G., Leoni, F., Liso, V., Petti, M. C., Rodeghiero, F., Saglio, G., Vegna, M. L., Visani, G., Jehn, U., Willemze, R., Muus, P., Pelicci, P. G., Biondi, A., and Lo Coco, F. Molecular remission in PML/RARα-positive acute promyelocytic leukemia by combined all-*trans*-retinoic acid and idarubicin (AIDA) therapy. Blood, *90:* 1014–1021, 1997.
19. Mancini, M., Nanni, M., Cedrone, M., Diverio, D., Avvisati, G., Riccioni, R., De Cuia, M. R., Fenu, S., and Alimena, G. Combined cytogenetic, FISH and molecular analysis in acute promyelocytic leukemia at diagnosis and in complete remission. Br. J. Haematol., *91:* 878–884, 1995.
20. Grimwade, D., Gorman, P., Howe, K., Langabeer, S., Oliver, F., Walker, H., Culligam, D., Waters, J., Pomfret, M., Goldstone, A., Burnett, A., Freemont, P., Sheer, D., and Solomon, E. Characterization of cryptic rearrangements and variant translocations in acute promyelocytic leukemia. Blood, *90:* 4876–4885, 1999.
21. Grignani, F., Ferrucci, P. F., Testa, U., Talamo, G., Fagioli, M., Alcalay, M., Mencarelli, A., Peschle, C., Nicoletti, I., and Pelicci, P. G. The acute promyelocytic leukaemia specific PML/RARα fusion protein inhibits differentiation and promotes survival of myeloid precursor cells. Cell, *74:* 423–429, 1993.
22. Benedetti, L., Grignani, F., Scicchitano, B. M., Jetten, A. M., Diverio, D., Lo Coco, F., Avvisati, G., Gambacorti-Passerini, C., Adamo, S., Levin, A. A., Pelicci, P. G., and Nervi, C. Retinoid-induced differentiation of acute promyelocytic leukemia involves PML-RARα-mediated increase of type II transglutaminase. Blood, *87:* 1939–1950, 1996.
23. de Thè, H., del Mar VivancoRuiz, M., Tiollais, P., Stunnenberg, H., and Dejean, A. Identification of a retinoic acid response element in the retinoic acid receptor β gene. Nature (Lond.), *343:* 177–180, 1990.
24. de Verneuil, H., and Metzger, D. The lack of transcriptional activation of the v-erbA oncogene is part due to a mutation present in the binding domain of the protein. Nucleic Acids Res, *18:* 4489–4497, 1990.
25. Minucci, S., Maccarana, M., Cioce, M., De Luca, P., Gelmetti, V., Segalla, S., Di Croce, L., Giavara, S., Matteucci, C., Gobbi, A., Bianchini, A., Colombo, E., Schiavoni, I., Badaracco, G., Hu, X., Lazar, M. A., Landsberger, N., Nervi, C., and Pelicci, P. G. Oligomerization of RAR and AML1 transcription factors as a novel mechanism of oncogenic activation. Mol. Cell, *5:* 811–820, 2000.
26. van Dongen, J. J., Macintyre, E. A., Gabert, J. A., Delabesse, E., Rossi, V., Saglio, G., Gottardi, E., Rambaldi, A., Dotti, G., Griesinger, F., Parreira, A., Gameiro, P., Diaz, M. G., Malec, M., Langerak, A. W., San Miguel, J. F., and Biondi, A. Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. Report of the BIOMED-1 Concerted Action: investigation of minimal residual disease in acute leukemia. Leukemia, *13:* 1901–1928, 1999.
27. Chambon, P. A decade of molecular biology of retinoic acid receptors. FASEB J., *10:* 940–954, 1996.
28. Mangelsdorf, D. J., and Evans, R. M. The RXR heterodimers and orphan receptors. Cell, *83:* 841–850, 1995.
29. Grisolano, J. L., Wesselschmidt, R. L., Pelicci, P. G., and Ley, T. J. Altered myeloid development and acute leukemia in transgenic mice expressing PML/RARα under control of cathepsin G regulatory sequences. Blood, *89:* 376–387, 1997.
30. He, L. I., Tribioli, C., Rivi, R., Peruzzi, D., Pelicci, P. G., Soares, V., Cattoretti, G., and Pandolfi, P. P. Acute leukemia with promyelocytic features in PML/RARα transgenic mice. Proc. Natl. Acad. Sci. USA, *94:* 5302–5307, 1997.
31. Early, E., Moore, M. A. S., Kakizuka, A., Nason-Burchenal, K., Martin, P., Evans, R. M., and Dmitrovsky, E. Transgenic expression of PML/RARα impairs myelopoiesis. Proc. Natl. Acad. Sci. USA, *93:* 7900–7904, 1996.
32. Brown, D., Scott, K., Lagasse, E., Weissman, I., Alcalay, M., Pelicci, P. G., Atwater, S., and Bishop, M. A PML/RARα transgene initiates murine acute promyelocytic leukemia. Proc. Natl. Acad. Sci. USA, *945:* 2551–2556, 1997.
33. Novogrodsky, A., Dvir, A., Ravid, A., Shkolnik, T., Stenzel, K. H., Rubin, A. L., and Zaizov, R. Effect of polar organic compounds on leukemic cells. Butyrate-induced partial remission of acute myelogenous leukemia in child. Cancer (Phila.) *51:* 9–14.

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