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AML1-ETO interacts with Sp1 and antagonizes Sp1 transactivity through RUNT domain

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Abstract AML1-ETO fusion protein is observed in approximately 12% of acute myeloid leukemia. In the present research, we found that AML1-ETO is able to inhibit Sp1 transactivity. We also found that this inhibition of Sp1 transactivity by AML1-ETO is achieved by interaction between Sp1 and RUNT domain of AML1. AML1b is able to abrogate the inhibition of AML1-ETO. Since Sp1 is involved in hematopoietic cell differentiation, we proposed that AML1-ETO promotes leukemogenesis by blocking cell differentiation through inhibition of Sp1 transactivity.

Structured summary:
MINT-6549474: *AML1-ETO* (genbank_protein_gi: AAB34820) *physically interacts* (MI:0218) with *Sp1* (uniprotkb:P08047) by *anti bait coimmunoprecipitation* (MI:0006)
MINT-6549439: *Sp1* (uniprotkb:P08047) *physically interacts* (MI:0218) with *AML1-ETO* (uniprotkb:AAB34820) by *anti tag coimmunoprecipitation* (MI:0007)
MINT-6549458: *Sp1* (uniprotkb:P08047) *physically interacts* (MI:0218) with *AML1a* (uniprotkb:Q01196-2) by *anti tag coimmunoprecipitation* (MI:0007)

Keywords: AML1-ETO; Sp1; Cell differentiation; Acute myeloid leukemia; Transcription regulation

1. Introduction

AML1 (RUNX1) is a member of the Runt family of transcription factors. The human AML1 gene generates three alternatively spliced variants, AML1a, AML1b, and AML1c. AML1b and AML1c both possess the DNA-binding region (RUNT domain) as well as the transcriptional regulatory domains. AML1b and AML1c are therefore considered to be broadly similar in function. In contrast, AML1a only retains the DNA-binding domain (RUNT domain), but lacks the transcriptional regulatory domains. AML1 gene is involved in chromosomal translocations in leukemia, such as t (8; 21) results in the formation of AML1-ETO fusion protein. This fusion protein contains most of the ETO protein and the N-terminal DNA-binding domain of AML1, the RUNT domain, which is similar to AML1a. AML1-ETO inhibits transcription of AML1-dependent target genes through ETO moiety and blocks hematopoietic cell differentiation [1]. In addition, AML1-ETO can also interact with other transcription factors involved in hematopoietic cell differentiation, such as C/EBP α and PU.1 [2,3].

Sp1 is a transcription factor involved in hematopoietic cell differentiation. In this study, AML1-ETO was found to inhibit Sp1 transactivity through interacting with Sp1. To our knowledge, this is the first report identifying interaction between Sp1 and AML1-ETO. Since Sp1 plays crucial roles in hematopoietic cell differentiation [4], dysregulation of Sp1 might be important for AML1-ETO-mediated leukemia development.

2. Materials and methods

2.1. Cell culture

CV1 and 293T cells were maintained in Dulbecco's modified Eagle's medium (GIBCO) with 10% fetal bovine serum at 37 °C in a humidified incubator with an atmosphere of 5% CO_2.

2.2. Plasmid construction

The human wild-type p21WAF1/CIP1 promoter luciferase reporter plasmid, WWP-Luc, was a kind gift from Dr. B. Vogelstein [5]. Its 2.4-kilobase pair genomic fragment containing the transcription start site was subcloned into the Hind III site of the luciferase reporter vector, pGL3-Basic (Promega), to generate pWWP [6]. pWP101 reporter plasmid was constructed as described previously by K. Nakano. It contains four Sp1 sites termed Sp1-3, Sp1-4, Sp1-5 and Sp1-6 [6]. pNOSP1 reporter plasmid was constructed as following: A fragment containing the partial p21WAF1/CIP1 promoter was amplified from pWWP using primes as follows: sense, 5′-CGGGGTACCATTGGCTTTCTGGCC ATT-3′, and antisense, 5′-CCGGAACCTCGCGTGCT-3′. The amplified fragment containing the sequence between 2320 and 211 of the p21WAF1/CIP1 promoter was digested with Kpn I endonuclease and then cloned into the Sma I and Kpn I sites of pWWP. The constructed pNOSP1 lost 4 Sp1 sites, the Sp1-1, Sp1-2, Sp1-3 and Sp1-4. pWWP, pWP101 and pNOSP1 were illustrated in Fig. 1A. The hemagglutinin-tagged Sp1 (identifier of Sp1 in the Uniprot database: P08047-1) expression vector (pCGNSp1) was the kind gift from Shenk and co-workers [7]. The pCMV5-AML1-ETO and pCMV5-AML1b (identifier of AML1b in the Uniprot database: Q01196-1) were kind gifts from Hiebert and co-workers [8]. GAL4 DBD/Sp1 was a kind gift from Smale and co-workers [9]. pCMV5-ETO (identifier of ETO in the Uniprot database: Q06455-1) and pcDNA3-flag2-AML1a (identifier of AML1a in the Uniprot database: Q01196-2) were constructed by our lab.

2.3. Transient transfection and luciferase assay

3.0×10^5 CV-1 cells or 2.0×10^5 293T cells per well were plated in 6-well plates. When the cells were 60–80% confluent, they were transfected by calcium phosphate precipitation technique. pUC19 plasmid was

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Abbreviations: PB, phenylbutyrate; HCK, human hematopoietic cell kinase; AML, Acute myeloid leukemia

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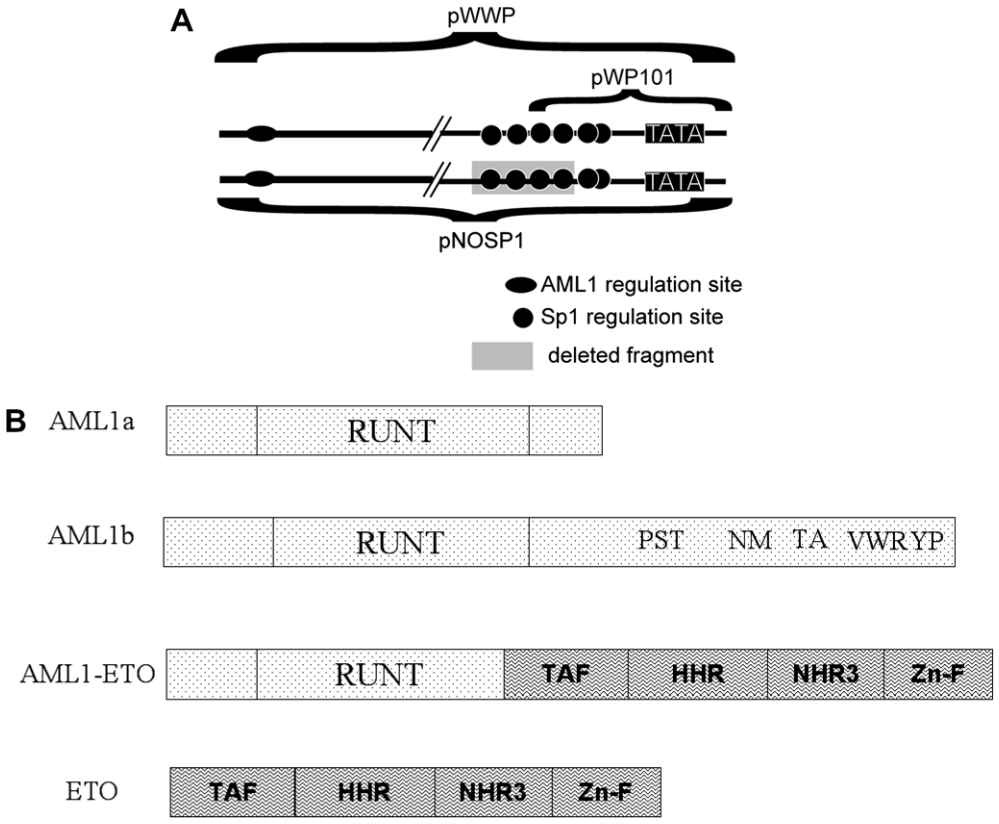

Fig. 1. (A) Diagram of pWWP, pWP101 and pNOSP1. pWWP, pWP101 and pNOSP1 were illustrated, containing different portion of p21WAF1/CIP1 promoter. Four Sp1 binding sites in the shadow were deleted in the plasmid. (B) Diagram of AMLa, AML1b, AML1-ETO and ETO. In comparison with AML1b, AML1a lacks the transcription regulation domain and only contains the RUNT domain. AML1-ETO contains most of the ETO protein and the N-terminal DNA-binding domain, the RUNT domain of AML1, which is similar to AML1a.

used to keep the total amount of DNA constant in each transfection. Phenylbutyrate (PB) was added 24 h after transfection as indicated. The final concentration of PB was 3 mmol/L. Seventy-two hours after the transfection, cell lysates were collected. The β-galactosidase and luciferase activities of the cell lysates were measured according to the instructions from the manufacturer (Promega). The protein concentration was determined by bicinchoninic acid assay. All assays were carried out at least in triplicate. Each experiment was repeated at least three times.

2.4. Coimmunoprecipitation and immunoblotting

The cells were fully homogenized by adding buffer NE (25 mM Tris pH 7.5, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 25% sucrose). The lysate was incubated on ice for 30 min to solubilize proteins and cleared by microcentrifuging for 20 min at 4 °C. For immunoprecipitation with AML1 or hemagglutinin antibodies, cell lysate was incubated with 5 μl of AML1 antibody (N-20; Santa Cruz Biotechnology) or 5 μl of hemagglutinin antibody (Y-11; Santa Cruz Biotechnology) for 2 h at 4 °C. Immunoblotting was performed with AML1, hemagglutinin or ETO (Ab-1; Calbiochem) antibody and anti-rabbit or anti-goat antibodies conjugated to horseradish peroxidase.

3. Results

3.1. AML1-ETO inhibits Sp1 transactivity

In our previous report, AML1-ETO was found to have weak inhibitory activity on a 5′ deletion construct of the p21WAF1/CIP1 promoter, pWP101 construct [10], which contains four Sp1 binding sites, but not AML1 binding sites [6,8]. This implied that AML1-ETO might regulate the p21WAF1/CIP1 promoter through Sp1 binding sites. To further define whether AML1-ETO represses Sp1-mediated transactivation of p21WAF1/CIP1 promoter, we performed reporter assays using the pWWP reporter plasmid. As shown in Fig. 2A, Sp1 enhanced the pWWP reporter activity (2.25-folds). However, when Sp1 was co-transfected with AML1-ETO, the pWWP reporter activity was repressed (0.78-fold).

By means of HDAC inhibitor to activate endogenous Sp1 [6,11], transactivity of the p21WAF1/CIP1 promoter was assessed in CV cells. In the absence of AML1-ETO, HDAC inhibitor, phenylbutyrate (PB), increased pWWP and pWP101 luciferase activity by 3.70- and 9.26-folds, respectively (Fig. 2B and C). Whereas in the presence of AML1-ETO, PB only increased pWWP and pWP101 luciferase activity by 1.16- and 2.21-folds, respectively (Fig. 2B and C). But the luciferase activity of pNOSP1 increased by PB was not affected by AML1-ETO significantly (Fig. 2D). These data suggested that AML1-ETO inhibits the transcriptional activity of Sp1. To further confirm this hypothesis, we used another reporter plasmid, pGL3-promoter (Promega), in which the firefly luciferase gene was driven by SV40 promoter containing Sp1 sites. Sp1 was reported to be able to activate SV40 promoter [12]. We found that AML1-ETO inhibits the PB-mediated activation of SV40 promoter (Fig. 2E), which is similar to what we found on p21 WAF1/CIP1 promoter.

By a different approach, cDNAs of Sp1 were fused in frame to the sequences of the DNA binding domain of the GAL4 transcription factor (GAL4 DBD/Sp1). The AML1-ETO and

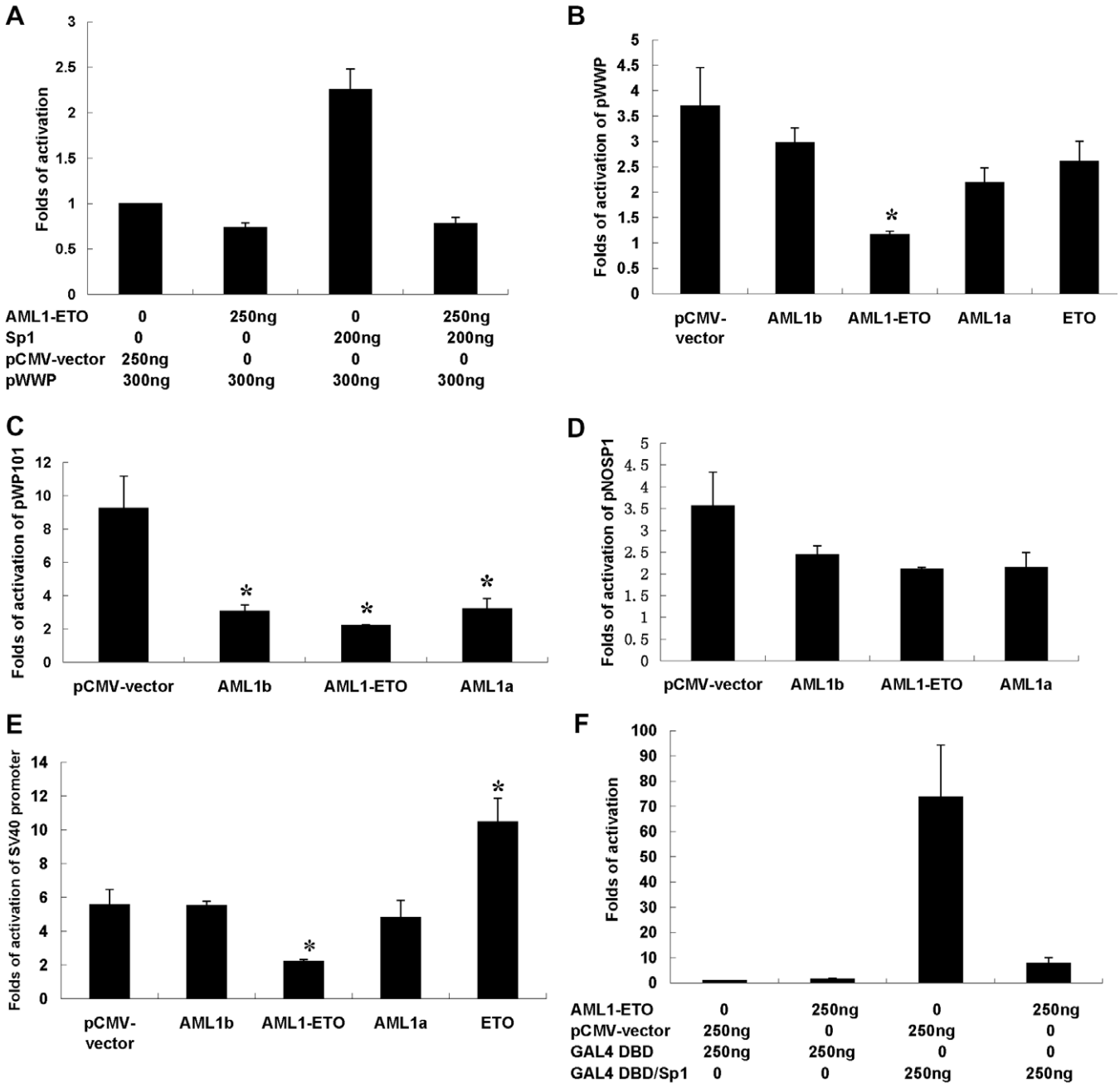

Fig. 2. AML1-ETO inhibits Sp1 transactivity in CV1 cells. (A) AML1-ETO inhibits Sp1 transactivity in CV1 cells. The difference between AML1-ETO and AML1-ETO+Sp1 is not significantly ($P > 0.05$). The difference between Sp1 and AML1-ETO +Sp1 is significantly ($P < 0.05$). (B–E) Two hundred and fifty nanograms of expression vector and 300 ng of reporter plasmid were used for transfection. PB treatments were started 24 h after transfection and generally continued for 48 h. Luciferase activities were normalized by protein concentration for the reason that we failed to find a control reporter construct that is not responsive to Sp1 [11]. The data were presented as activities relative to that of the pWWP (A and B), pWP101 (C), pNOSP1 (D) or pGL3-promoter (Promega) (E), which was not treated with PB, respectively. Each experiment was repeated three times. $^{*}P < 0.05$ (F) AML1-ETO inhibits GAL4 DBD/Sp1 transactivity. In the mammalian expression plasmid GAL4 DBD/Sp1, Sp1 is fused with DNA binding region (amino acids 1–147) of the GAL4 transcription factor. The firefly luciferase reporter plasmid is driven by GAL4 DNA-binding sites. Luciferase activities were normalized for the β-galactosidase activities in cell lysates. The data were presented as activities relative to that of the firefly luciferase reporter plasmid. The difference between empty and GAL4 DBD+ AML1-ETO is not significantly ($P > 0.05$). The difference between GAL4 DBD/Sp1 and AML1-ETO+GAL4 DBD/Sp1 is significantly ($P < 0.05$).

GAL4 DBD/Sp1 vectors were then cotransfected into CV1 cells together with a luciferase reporter plasmid driven by GAL4-binding sites. As shown in Fig. 2F, transfection of GAL4 DBD/Sp1 with the GAL4-responsive target reporter induced significantly increased luciferase activity (73.74-folds). Strikingly, co-transfection of AML1-ETO and GAL4 DBD/Sp1 together repressed the induction of luciferase activity (7.63-folds) from the GAL4-responsive target promoter, further indicating that AML1-ETO represses Sp1-dependent transcription.

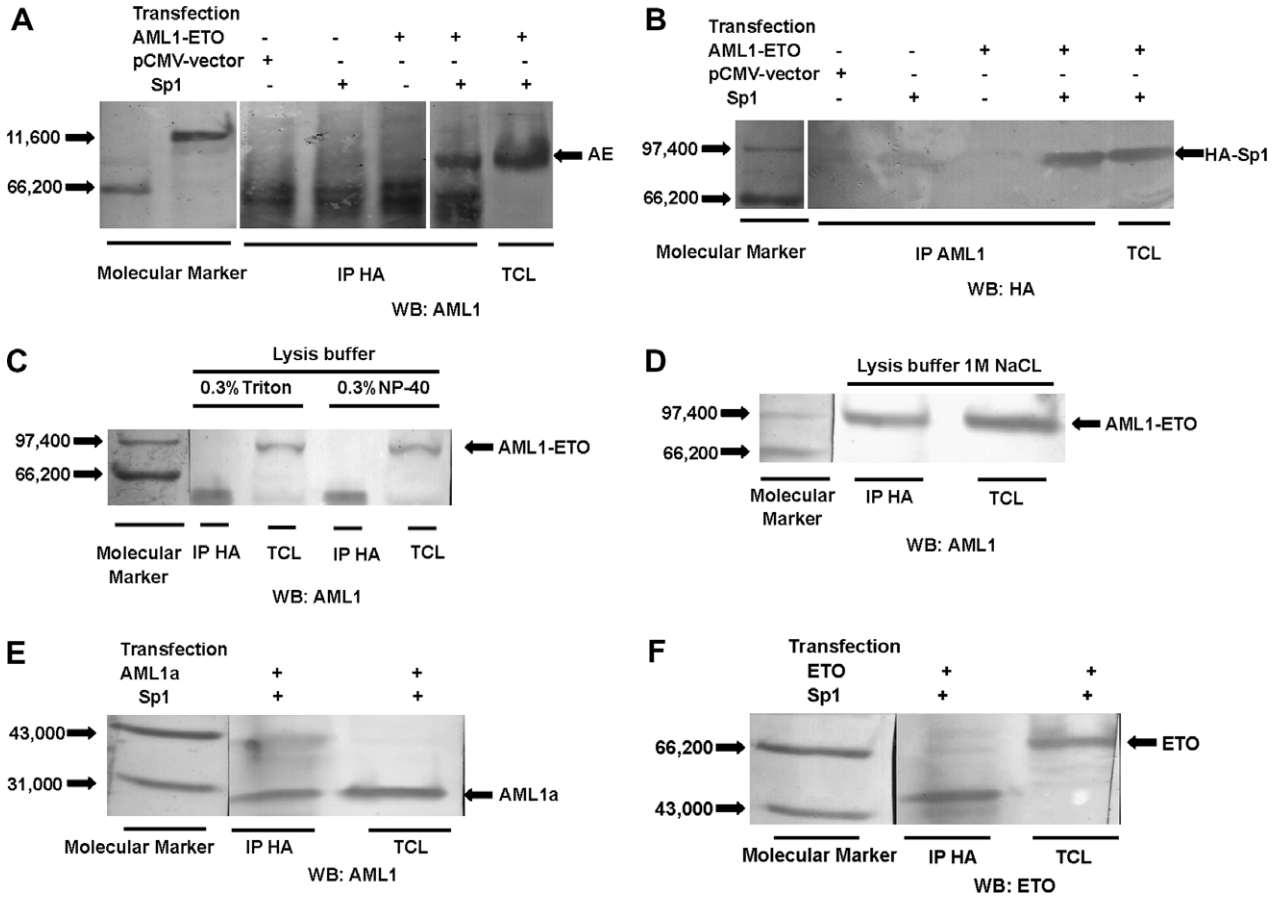

Fig. 3. AML1-ETO binds to Sp1 through RUNT domain. 293T cells were transiently transfected with mammalian expression constructs encoding hemagglutinin-tagged Sp1 and AML1-ETO, AML1a or ETO. Immunoprecipitation (IP) was performed with hemagglutinin (HA) or AML1 antibody. Eight hundred microliters cell lysates were incubated with 5 μl of AML1 antibody. Bound proteins were subjected to immunoblotting with AML1 or hemagglutinin antibody. Forty microliters total cell lysates were loaded on a SDS–polyacrylamide gel electrophoresis as a control. Association between AML1-ETO and Sp1 was shown (A). By alternative use of AML1 or hemagglutinin antibodies, the similar association between AML1-ETO and Sp1 was observed (B). The cells were fully homogenized by adding buffer NE + 0.3% Triton X-100, buffer NE + 0.3% NP-40 (C) or buffer NE+1 M NaCl (D). The complex of AML1-ETO and Sp1 is resistant to 1 M NaCl, but not to 0.3% Triton X-100 or 0.3% NP-40 (C and D). AML1a but not ETO associated with Sp1 (E and F). TCL, total cell lysates. AE, AML1-ETO.

3.2. AML1-ETO binds to Sp1 through RUNT domain

To investigate how AML1-ETO represses Sp1-dependent transcription, we analyzed whether AML1-ETO is able to bind to Sp1. 293T cells were transfected with plasmids expressing AML1-ETO and hemagglutinin epitope-tagged Sp1. In the coimmunoprecipitation experiments, we observed the association between AML1-ETO and Sp1 (Fig. 3A and B). In addition, the complex of AML1-ETO and Sp1 was resistant to 1 M NaCl, but not to 0.3% Triton X-100 or 0.3% NP-40 (Fig. 3C and D).

AML1a only has RUNT domain and is similar to the portion of AML1 contained in AML1-ETO (Fig. 1B). To identify the region of AML1-ETO that is responsible for interaction with Sp1, we transfected 293T cells with plasmids expressing hemagglutinin epitope-tagged Sp1 and AML1a or ETO. We found that Sp1 is able to interact with AML1a, but not ETO (Fig. 3E and F). These indicated that AML1-ETO interacts with Sp1 through the N-terminus of AML1 including the RUNT domain, since the RUNT domain constitutes major part of AML1a. These results support our findings in transcription activity assays (Fig. 2B–D).

3.3. AM L1b is able to abrogate the inhibition of AML1-ETO competitively

We had shown that both AML1 -ETO and AML1b are able to bind to both Sp1 protein and AML1 binding sites through the RUNT domain. Then AML1-ETO and AML1b are able to compete for binding to them. To determine whether AML1b is able to abrogate the inhibition of AML1-ETO through competition, CV1 cells were co-transfected with pWWP, AML1-ETO and AML1b, then treated with HDAC inhibitor. It was found that excess dose of AML1b could relief the transcription inhibition mediated by AML1-ETO (Fig. 4). Since both of AML1-ETO and AML1b are able to bind to Sp1 and AML1 binding sties, we postulated that AML1b bind to them and prevented AML1-ETO from binding to AML1 binding sties and Sp1.

4. Discussions

Acute myeloid leukemia (AML) is characterized by the abnormal proliferation and differentiation of myeloid progenitor cells. AML1-ETO fusion protein is observed in approxi-

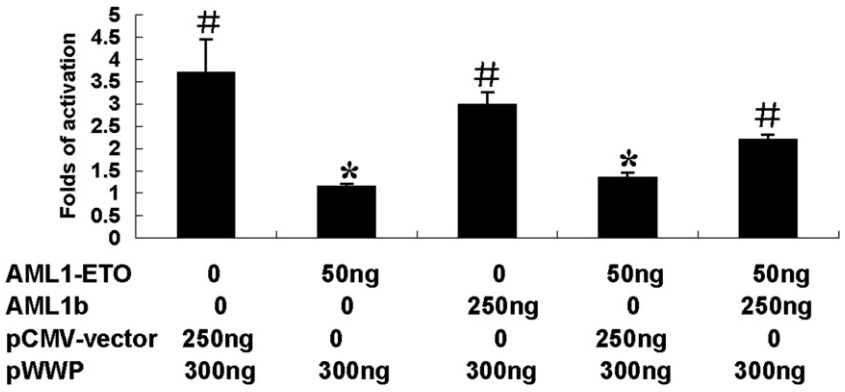

Fig. 4. AML1b is able to abrogate the inhibition of AML1-ETO competitively: different amounts of expression vector and reporter plasmid, pWWP, were used for transfection. PB treatments were started 24 h after transfection and generally continued for 48 h. Luciferase activities were normalized by protein concentration for the reason that we failed to find a control reporter construct that is not responsive to Sp1. The data were presented as activities relative to that of the pWWP, which were not treated with PB. Each experiment was repeated three times. *Compare with empty group $P < 0.05$. # Compare with AML1-ETO group. $P < 0.05$.

mately 12% of AML, suggesting that it is extremely important in leukemia development. The biological feature of AML1-ETO has shown that it has many effects on leukemogenesis [13,14]. To understand the molecular mechanism of AML1-ETO in leukemogenesis, we investigated how AML1-ETO affects p21WAF1/CIP1 and transcription factor Sp1.

In this report, we demonstrated that AML1-ETO, AML1a and AML1b all inhibit the PB induced luciferase activity of pWP101 (Fig. 2C). Hence we postulated that RUNT domain is necessary for this inhibition effect since AML1-ETO, AML1a and AML1b all have RUNT domain. ETO moiety in the AML1-ETO fusion protein is able to enhance this inhibition effect since AML1-ETO has most potent inhibition effect on Sp1 (Fig. 2B and C). Neither AML1a norAML1b represses the luciferase activity of pWWP increased by PB. But both AML1a and AML1b inhibit the luciferase activity of pWP101 increased by PB. We thought that the 2.4-kilobase pair wild-type p21WAF1/CIP1 promoter is regulated by more transcription factors than pWP101. pWP101 contains the luciferase reporter gene driven by only 101 bp upstream of the transcription start site of p21WAF1/CIP1. It maybe has no transcription factor binding sites other than Sp1. pWP101 activity is more dependent on Sp1 than pWWP. Therefore AML1a and AML1b only inhibit the luciferase activity of pWP101 increased by PB, but not the luciferase activity of pWWP.

The previous reports had found that Sp1 is a transcription factor involved in hematopoiesis. Sp1 transactivity increased in decitabine and Vitamin D3 induced differentiation of leukemia cells [4]. The human hematopoietic cell kinase (HCK) is specifically expressed in myeloid cells and to a minor extent in B-lymphoid cells. Sp1 up-regulates HCK expression at the transcriptional level during myeloid differentiation of hematopoietic cells [15]. All these data indicate that Sp1 is a transcription factor involved in hematopoietic cell differentiation. Here, we demonstrated that AML1-ETO inhibited Sp1 transactivity significantly. AML1-ETO might block hematopoietic cell differentiation through suppressing Sp1 transactivity.

In conclusion, we found that AML1-ETO, through its RUNT domain, interacts with Sp1 and inhibits Sp1 transactivity. This provides a novel mechanistic explanation for AML-ETO mediated differentiation block, which represents an important step towards transformation of t (8; 21) leukemia.

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References

[1] Wang, J., Hoshino, T., Redner, R.L., Kajigaya, S. and Liu, J.M. (1998) 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.

[2] Westendorf, J.J., Yamamoto, C.M., Lenny, N., Downing, J.R., Selsted, M.E. and Hiebert, S.W. (1998) The t(8;21) fusion product, AML-1-ETO, associates with C/EBP-alpha, inhibits C/EBP-alpha-dependent transcription, and blocks granulocytic differentiation. Mol. Cell Biol. 18, 322–333.

[3] Vangala, R.K., Heiss-Neumann, M.S., Rangatia, J.S., Singh, S.M., Schoch, C., Tenen, D.G., Hiddemann, W. and Behre, G. (2003) The myeloid master regulator transcription factor PU.1 is inactivated by AML1-ETO in t(8;21) myeloid leukemia. Blood 101, 270–277.

[4] Koschmieder, S., Agrawal, S., Radomska, H.S., Huettner, C.S., Tenen, D.G., Ottmann, O.G., Berdel, W.E., Serve, H.L. and Müller-Tidow, C. (2007) Decitabine and vitamin D3 differentially affect hematopoietic transcription factors to induce monocytic differentiation. Int. J. Oncol. 30, 349–355.

[5] el-Deiry, W.S., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W. and Vogelstein, B. (1993) WAF1, a potential mediator of p53 tumor suppression. Cell 75, 817–825.

[6] Nakano, K., Mizuno, T., Sowa, Y., Orita, T., Yoshino, T., Okuyama, Y., Fujita, T., Ohtani-Fujita, N., Matsukawa, Y., Tokino, T., Yamagishi, H., Oka, T., Nomura, H. and Sakai, T. (1997) Butyrate activates the WAF1/Cip1 gene promoter through Sp1 sites in a p53-negative human colon cancer cell line. J. Biol. Chem. 272, 22199–22206.

[7] Parks, C.L. and Shenk, T. (1996) The serotonin 1a receptor gene contains a TATA-less promoter that responds to MAZ and Sp1. J. Biol. Chem. 271, 4417–4430.

[8] Lutterbach, B., Westendorf, J.J., Linggi, B., Isaac, S., Seto, E. and Hiebert, S.W. (2000) A mechanism of repression by acute myeloid leukemia-1, the target of multiple chromosomal translocations in acute leukemia. J. Biol. Chem. 275, 651–656.

[9] J Brightbill, H.D., Plevy, S.E., Modlin, R.L. and Smale, S.T. (2000) A prominent role for Sp1 during lipopolysaccharide-mediated induction of the IL-10 promoter in macrophages. J. Immunol. 164, 1940–1951.

[10] Wei, H., Liu, X.R., Liu, H., Rao, Q., Wang, M. and Wang, J.X. (2007) Effects of AML1-ETO on transcription activity of p21WAF1/CIP1 gene promoter. Zhonghua Xue Ye Xue Za Zhi 28, 545–548.
[11] Xiao, H., Hasegawa, T. and Isobe, K. (1999) Both Sp1 and Sp3 are responsible for p21waf1 promoter activity induced by histone deacetylase inhibitor in NIH3T3 cells. J. Cell Biochem. 73, 291–302.
[12] Dynan, W.S. and Tjian, R. (1983) The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. Cell 35, 79–87.
[13] Burel, S.A., Harakawa, N., Zhou, L., Pabst, T., Tenen, D.G. and Zhang, D.E. (2001) Dichotomy of AML1-ETO functions: growth arrest versus block of differentiation. Mol Cell Biol. 21, 5577–5590.
[14] Peterson, L.F., Yan, M. and Zhang, D.E. (2007) The p21Waf1 pathway is involved in blocking leukemogenesis by the t(8; 21) fusion protein AML1 -ETO. Blood 109, 4392–4398.
[15] Hauses, M., Tönjes, R.R. and Grez, M. (1998) The transcription factor Sp1 regulates the myeloid-specific expression of the human hematopoietic cell kinase (HCK) gene through binding to two adjacent GC boxes within the HCK promoter-proximal region. J. Biol. Chem. 273, 31844–31852.