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# Methylation-associated silencing of *BASP1* contributes to leukemogenesis in t(8;21) acute myeloid leukemia

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## Abstract

The AML1-ETO fusion protein (A/E), which results from the t(8;21) translocation, is considered to be a leukemia-initiating event. Identifying the mechanisms underlying the oncogenic activity of A/E remains a major challenge. In this study, we identified a specific down-regulation of brain acid-soluble protein 1 (*BASP1*) in t(8;21) acute myeloid leukemia (AML). A/E recognized AML1-binding sites and recruited DNA methyltransferase 3a (DNMT3a) to the *BASP1* promoter sequence, which triggered DNA methylation-mediated silencing of *BASP1*. Ectopic expression of *BASP1* inhibited proliferation and the colony-forming ability of A/E-positive AML cell lines and led to apoptosis and cell cycle arrest. The DNMT inhibitor decitabine up-regulated the expression of *BASP1* in A/E-positive AML cell lines. In conclusion, our data suggest that *BASP1* silencing via promoter methylation may be involved in A/E-mediated leukemogenesis and that *BASP1* targeting may be an actionable therapeutic strategy in t(8;21) AML.

## Introduction

Brain acid-soluble protein 1 (*BASP1*) (CAP-23, NAP-22) was initially identified as an abundant membrane-bound protein in the brain and was thought to play a role in axon regeneration and neuronal plasticity<sup>1–3</sup>. *BASP1* has also been found in other cell types and at other subcellular locations, and its functions in transcriptional regulation, apoptosis, and differentiation have also been recently reported<sup>4–6</sup>.

In recent years, studies have shown that abnormal expression and methylation status of the *BASP1* gene are closely related to the occurrence and prognosis of certain tumors and that *BASP1* may also play a role as a tumor suppressor gene. For instance, in hepatocellular carcinoma (HCC), *BASP1* expression has been shown to be

down-regulated via promoter methylation; the methylation status of *BASP1* could be used in the early diagnosis of HCC<sup>7</sup>. *BASP1* gene methylation and its expression level are also associated with melanoma progression<sup>8</sup>.

The t(8;21)(q22;q22) chromosome translocation is one of the most common cytogenetic abnormalities in acute myeloid leukemia (AML), accounting for approximately 7–12% of newly diagnosed AML cases<sup>9–11</sup>. The new fusion protein AML1-ETO (A/E) is considered to be the leukemia-initiating event and also gives this subtype of AML unique molecular characteristics. The A/E fusion protein has been shown to bind specific DNA sequences and recruit DNA methyltransferases (DNMTs) to target genes, resulting in A/E-associated DNA methylation<sup>12, 13</sup>. Recent studies have shown that t(8;21) AML has a unique methylation pattern and that this abnormal methylation and silencing of tumor suppressor genes may be an important factor leading to the occurrence and development of this disease<sup>14</sup>. Here, we studied the expression of *BASP1* in various AML cell lines and identified a specific

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down-regulation and promoter methylation of the *BASP1* gene in t(8;21) AML. Our results suggest that methylation of the *BASP1* gene may be regulated by the A/E fusion protein and play a role in A/E-driven leukemogenesis.

In this study, we showed that the A/E fusion protein directly bound to the promoter of the *BASP1* gene. The A/E fusion protein recruited DNMT3a to the promoter region of *BASP1*, leading to methylation of the CpG island. As a result, the *BASP1* gene was silenced. Upregulation of the *BASP1* gene by overexpression or treatment with the DNMT inhibitor decitabine (DAC) was able to induce cell death in t(8;21) AML cell lines. Our findings suggest that in t(8;21) AML, *BASP1* plays a tumor suppressive role in vitro and that targeting *BASP1* may be an actionable treatment option in treating t(8;21) AML.

## Materials and methods

### Cell lines and clinical samples

Kasumi-1, HL-60, NB4, U937, THP-1, KG-1, MV-4-11, and HEL cells were cultured in RPMI 1640 (HyClone, GE Healthcare, Logan, UT, USA) medium containing 10% fetal calf serum (HyClone, GE Healthcare, Logan, UT, USA). SKNO-1 cells were cultured in RPMI 1640 (Gibco, Fisher Scientific, Waltham, MA, USA) containing 10% fetal calf serum (Gibco, Fisher Scientific, Waltham, MA, USA) and 10 ng/mL granulocyte-macrophage colony-stimulating factor. The 293T cells were cultured in Dulbecco's modified Eagle's medium (HyClone, GE Healthcare, Logan, UT, USA) containing 10% fetal calf serum (HyClone, GE Healthcare, Logan, UT, USA). SKNO-1-siA/E (A/E silenced by the lentiviral vector pRRL.cPPT.hPGK in SKNO-1 cells) and U937-A/E (produced by U937 electroporated with a hemagglutinin (HA)-tagged A/E complementary DNA (cDNA) and subcloned into a vector carrying the Zn<sup>2+</sup>-inducible mouse MT-1 promoter) cells were cultured in RPMI 1640 (HyClone, GE Healthcare, Logan, UT, USA) containing 10% fetal calf serum (HyClone, GE Healthcare, Logan, UT, USA) as described in a previous study<sup>13</sup>. U937-A/E cells were treated with 100 μM ZnSO<sub>4</sub><sup>2+</sup> (Invitrogen, Carlsbad, CA, USA) for 16 h to induce A/E expression. Bone marrow samples from patients and healthy donors were obtained with informed consent in accordance with the principles of the Declaration of Helsinki. This study was approved by the Human Subject Ethics Committee of the Chinese PLA General Hospital.

### RNA extraction and quantitative real-time polymerase chain reaction

Total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was obtained after reverse transcription. Expression of mRNA was quantified by SYBR Green real-time quantitative polymerase chain reaction (qRT-PCR) analysis (Takara,

Tokyo, Japan) on an Mx3000P Lightcycler (Stratagene, LaJolla, CA, USA). Gene expression was expressed as 2<sup>-ΔCT</sup> relative to *GAPDH*. The primers used are listed in Supplementary Table 1.

### DNA extraction, BSP, and MSP

Genomic DNA from patients, healthy donors, and cell lines was isolated with the Wizard<sup>®</sup> Genomic DNA Purification Kit (Promega, Madison, WI, USA). Genomic DNA was treated with sodium bisulfate according to the manufacturer's instructions supplied with the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany). The bisulfite sequencing PCR (BSP) and methylation-specific PCR (MSP) primer sequences were designed by Methprimer and are shown in Supplementary Table 1. For BSP, amplified PCR products were purified and cloned into pGEM-T vectors (Promega, Madison, WI, USA), and five positive clones were sequenced for the analysis of DNA methylation. MSP was conducted in a 25 μL reaction solution with GoTaq Green Master Mix (Promega, Madison, WI, USA), and PCR products were analyzed on 2% agarose gels.

### Luciferase reporter assays

DNA fragments were amplified by PCR of human genomic DNA. The primer sequences are shown in Supplementary Table 1. All DNA fragments were inserted into the pGL3-LUC reporter vector (Promega, Madison, WI, USA). Mutants were generated using overlap-extension PCR. The SuperFect reagent (Qiagen, Hilden, Germany) was used to co-transfect 293T cells at a density of 2 × 10<sup>5</sup> with the pcDNA3 vectors (0, 10, 50, or 100 ng) with or without A/E cDNA and plated in 24-well plates. The pRL-TK renilla luciferase reporter vector (Promega, Madison, WI, USA) was used as a control. According to the manufacturer's instructions, cells harvested 48 h after transfection were analyzed using the Dual-Luciferase<sup>®</sup> Reporter assay (Promega, Madison, WI, USA).

### ChIP assays

Chromatin immunoprecipitation (ChIP) assays were performed as described previously using the EZ-ChIP Assay Kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions<sup>15</sup>. Briefly, SKNO-1 and SKNO-1-siA/E cells were seeded at a density of 2 × 10<sup>6</sup> and incubated for 10 min at 37 °C to crosslink the proteins to DNA after treatment with formaldehyde (1% final concentration). After sonication, chromatin was immunoprecipitated overnight with 5 μg of antibodies against AML1 (Santa Cruz, Dallas, TX, USA), ETO (Santa Cruz, Dallas, TX, USA), DNMT1 (Abcam, Cambridge, UK), DNMT3a (Abcam, Cambridge, UK), and DNMT3b (Abcam, Cambridge, UK). A negative control experiment was performed with a normal mouse immunoglobulin G

(IgG) (Abcam, Cambridge, UK) antibody. DNA fragments obtained without antibody were used as the input controls. The genomic *BASP1* upstream region close to the active *AML1*-binding site and unrelated negative control region were amplified by SYBR Green qRT-PCR analysis (Takara, Tokyo, Japan) on an Mx3000P Lightcycler (Stratagene, LaJolla, CA, USA). Their expression was quantified as  $2^{-\Delta\text{CT}}$  relative to input. *GAPDH* served as an internal control to account for non specific precipitated sequences. The primer sequences are shown in Supplementary Table 1.

#### Western blot

Total protein extracts and western blot analysis were performed as previously described<sup>16</sup>. Rabbit polyclonal anti-BASP1 was purchased from Abcam (Cambridge, MA, USA, ab103315). Rabbit anti-human  $\beta$ -actin and horse radish peroxidase-linked anti-rabbit IgG were purchased from Cell Signaling Technology (Boston, MA, USA, 4970S). Densitometric analysis was performed using UVITEC-1D for quantification.

#### Cell proliferation, apoptosis, differentiation, and colony-forming unit analysis

Cell proliferation was evaluated by using the Cell-Counting Kit (CCK-8) (Dojindo, Japan), the concise method for which was that a minimum of 5000 cells per well cultured in 96-well plates were added to 10  $\mu\text{L}$  of CCK-8 solution and incubated for another 3 h at 37 °C in a 5% CO<sub>2</sub> atmosphere. Then, the absorbance was detected at a wavelength of 450 nm. The cell apoptosis assay was analyzed by a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). For every sample, cells were collected in a BD Falcon tube and stained with 5  $\mu\text{L}$  of Annexin V and 5  $\mu\text{L}$  of 7AAD (Becton Dickinson, Franklin Lakes, NJ, USA). Cell differentiation was also assessed by a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Samples stained with anti-CD11b (Becton Dickinson, Franklin Lakes, NJ, USA) were analyzed using CellQuest Pro software (Becton Dickinson, Franklin Lakes, NJ, USA). For colony-forming unit assays, cells were plated in methylcellulose H4230 (Stemcell Technologies, Vancouver, BC, Canada) at a concentration of  $1 \times 10^3$  cells per mL. The number of colonies with localized clusters of >50 cells were counted after incubation for 7 days. All experiments were performed in triplicate.

#### Statistical analysis

SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) was used to process the data. All data are expressed as the mean  $\pm$  standard deviation (SD) from at least three separate experiments. Student's *t* test was used to determine the statistical significance of the experimental

results, and a *p* value  $\leq 0.05$  was considered to be significant.

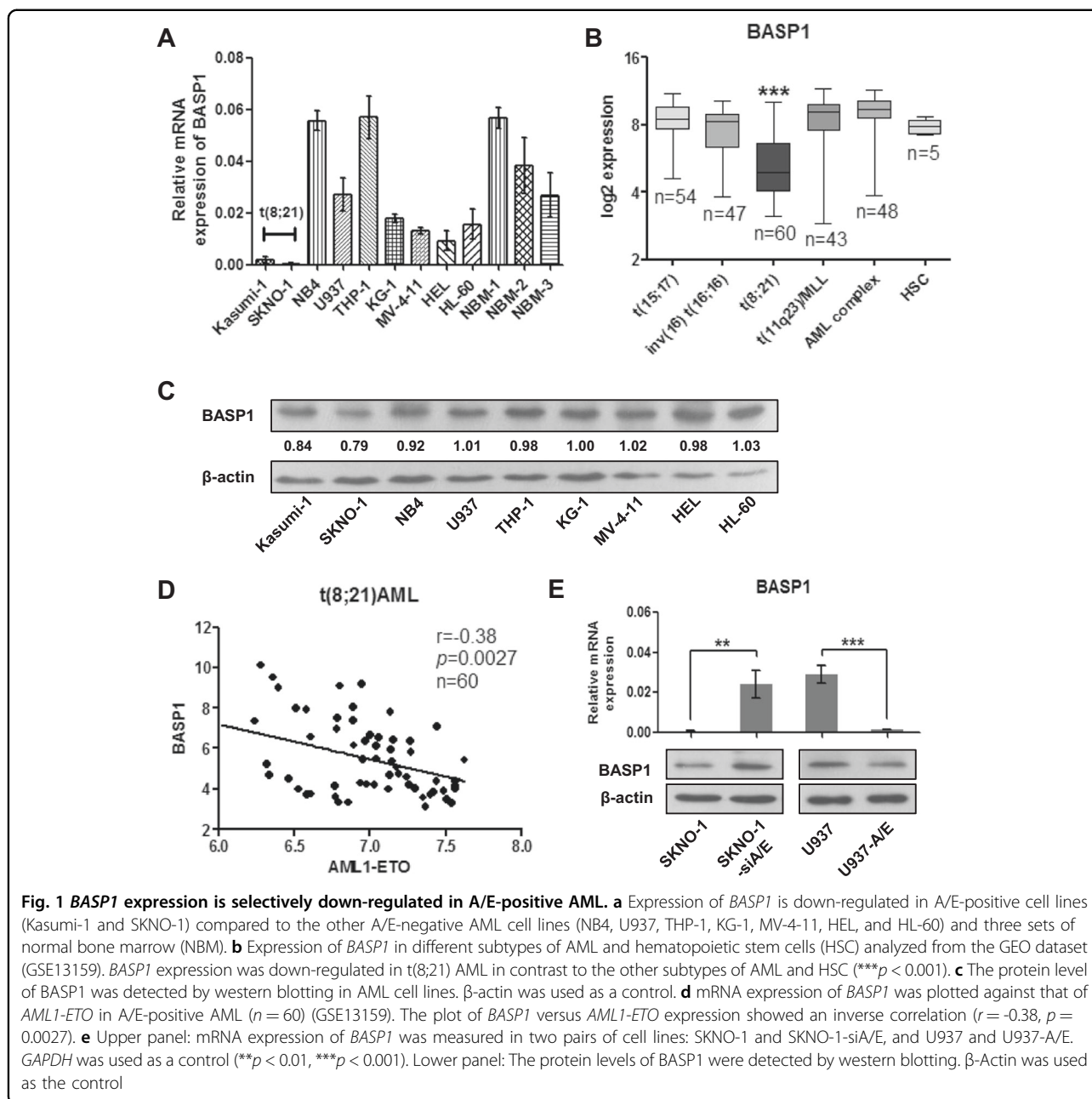
## Results

### *BASP1* is selectively silenced in A/E-positive AML

In an initial screen of *BASP1* expression in AML, we examined the mRNA expression of *BASP1* in nine AML cell lines (Kasumi-1, SKNO-1, NB4, U937, THP-1, KG-1, MV-4-11, HEL, and HL-60) and three sets of normal bone marrow (NBM) from healthy donors. Expression of *BASP1* showed an interesting pattern: it was robustly down-regulated in A/E-positive AML cell lines (Kasumi-1 and SKNO-1) in contrast to the other A/E-negative AML cell lines and NBMs (Fig. 1a). The results were further validated in a published GEO microarray dataset (GSE13159) that included 60 t(8;21) AML, 54 t(15;17) AML, 47 inv<sup>16</sup> or t(16;16) AML, 43 t(11q23)/MLL-rearrangement AML, 48 complex aberrant karyotype AML, and 5 hematopoietic stem cells (HSCs) (Fig. 1b). This trend of *BASP1* was also confirmed by western blotting of the AML cell lines (Fig. 1c). Surprisingly, the expression of *BASP1* was adversely correlated with the expression of *AML1-ETO* in t(8;21) AML (Fig. 1d). To determine the role of A/E in the regulation of *BASP1* expression, A/E was knocked out in SKNO-1 (SKNO-1-siA/E) by using a lentiviral vector, and HA-tagged A/E cDNA was electroporated into U937 cells to produce the U937-A/E-HA clone in a zinc-inducible manner, as previously described<sup>13</sup>. The efficiency of *AML1-ETO* knockdown and overexpression was evaluated by western blotting, as described in our previous study<sup>17</sup>. *BASP1* mRNA expression and protein levels were up-regulated in SKNO-1-siA/E compared to SKNO-1 and were down-regulated in U937-A/E compared to U937 (Fig. 1e), suggesting that *BASP1* expression may be selectively silenced by A/E in A/E-positive AML.

### *BASP1* silencing in A/E-positive AML by promoter methylation

To further clarify the mechanism of *BASP1* down-regulation in A/E-positive AML, we analyzed the genomic sequence of the *BASP1* promoter region (−2000 to −1 bp) by performing a bioinformatics search (<http://www.urogene.org/methprimer/>) and identified one CpG island with CpGs that were densely clustered close to the transcription start site (Fig. 2a). Bisulfite sequencing of the genome region from −179 to −400 bp of the *BASP1* promoter showed that this CpG island was hypermethylated in A/E-positive AML cell lines (Kasumi-1 and SKNO-1), but not in A/E-negative AML cell lines (HL-60 and SKNO-1-siA/E) and NBM (Fig. 2b). MSP showed that the *BASP1* promoter was methylated in almost all primary t(8;21) AML samples ( $n = 9$ ), but not in NBM ( $n = 3$ ) (Fig. 2c). To elucidate whether DNA methylation was able

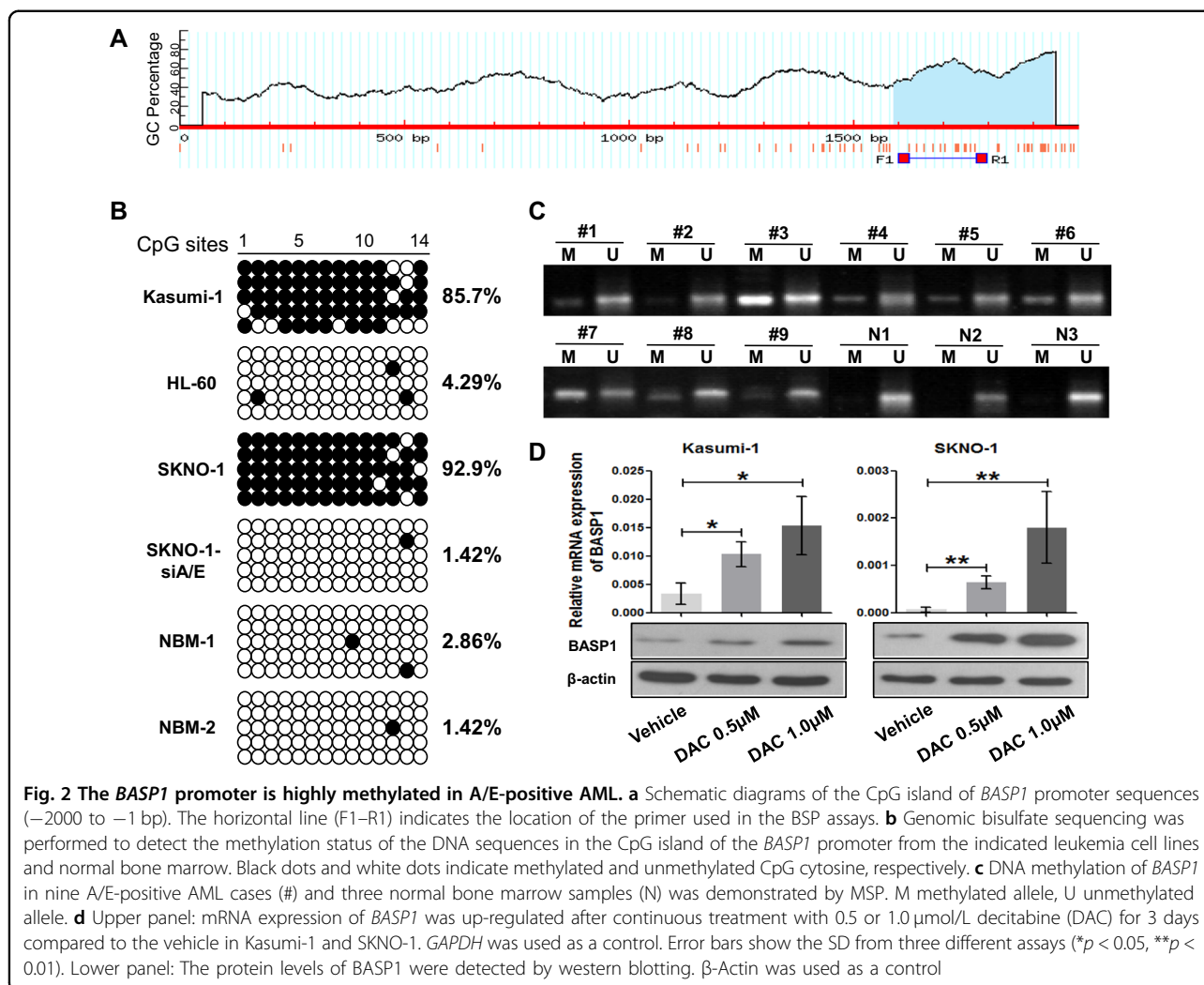


to account for the regulation of *BASP1* expression, we treated Kasumi-1 and SKNO-1 cells with a DNMT inhibitor, DAC, for 72 h. The mRNA expression and protein levels of *BASP1* were significantly increased after treatment with DAC in a dose-dependent manner (Fig. 2d). These results indicated that DNA methylation may contribute to the transcriptional silencing of *BASP1* in A/E-positive AML.

#### A/E triggers epigenetic silencing of *BASP1* through direct promoter binding

To address the question of whether the epigenetic silencing of *BASP1* in t(8;21) AML is directly mediated by

A/E, we searched the JASPAR database ([http://jaspar.genereg.net/cgi-bin/jaspar\\_db.pl?rm=browse&db=core&tax\\_group=vertebrates](http://jaspar.genereg.net/cgi-bin/jaspar_db.pl?rm=browse&db=core&tax_group=vertebrates)) and found four putative AML1-binding sites in the *BASP1* promoter region (−1500 to −1 bp). We cloned various lengths of the *BASP1* promoter sequence with different wild-type A/E-binding sites into a pGL3-basic vector and monitored luciferase activity with co-transfection of the A/E plasmid into 293T cells. As shown in Fig. 3a, the promoter activity of *BASP1* was significantly decreased after co-transfection with the A/E plasmid (10 ng) when the luciferase reporter contained sequences of a



**Fig. 2** The *BASP1* promoter is highly methylated in A/E-positive AML. **a** Schematic diagrams of the CpG island of *BASP1* promoter sequences (−2000 to −1 bp). The horizontal line (F1–R1) indicates the location of the primer used in the BSP assays. **b** Genomic bisulfate sequencing was performed to detect the methylation status of the DNA sequences in the CpG island of the *BASP1* promoter from the indicated leukemia cell lines and normal bone marrow. Black dots and white dots indicate methylated and unmethylated CpG cytosine, respectively. **c** DNA methylation of *BASP1* in nine A/E-positive AML cases (#) and three normal bone marrow samples (N) was demonstrated by MSP. M methylated allele, U unmethylated allele. **d** Upper panel: mRNA expression of *BASP1* was up-regulated after continuous treatment with 0.5 or 1.0 μmol/L decitabine (DAC) for 3 days compared to the vehicle in Kasumi-1 and SKNO-1. *GAPDH* was used as a control. Error bars show the SD from three different assays (\* $p < 0.05$ , \*\* $p < 0.01$ ). Lower panel: The protein levels of *BASP1* were detected by western blotting. β-Actin was used as a control

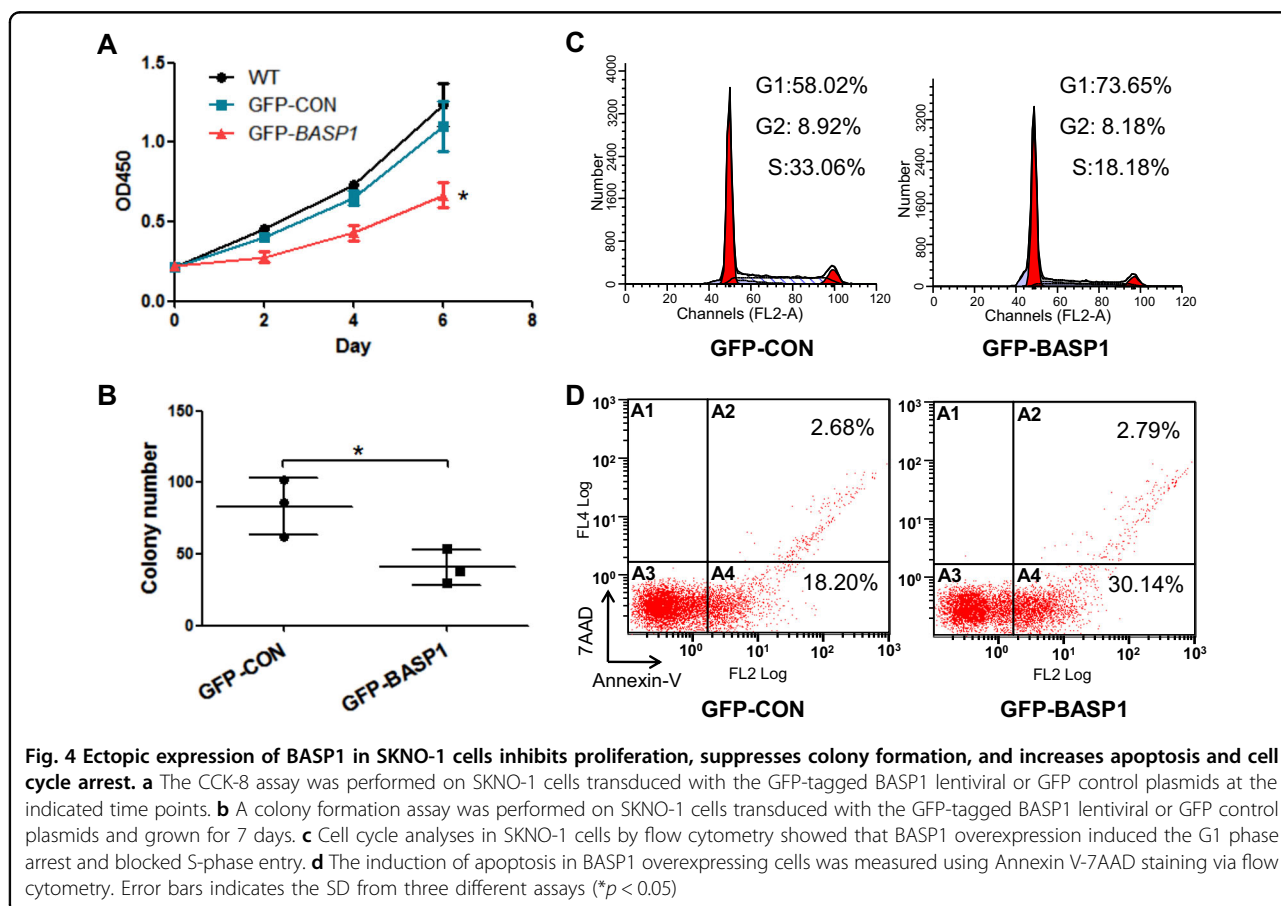
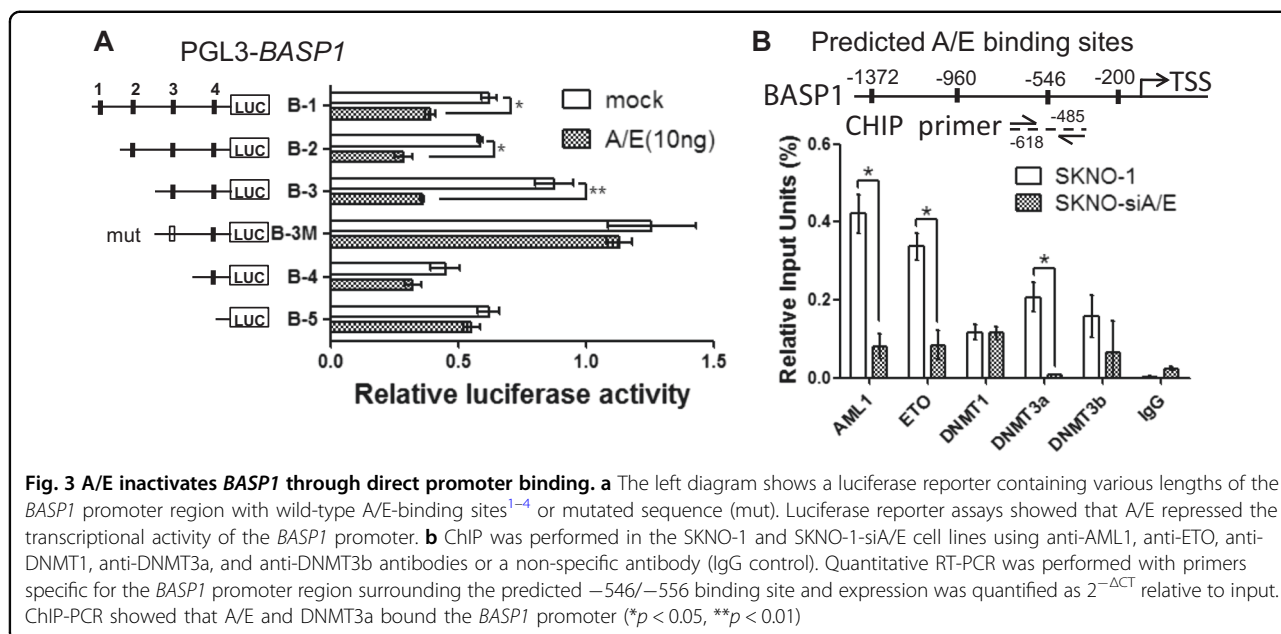
functional binding site (B-3), but not when the luciferase reporter contained no functional binding sites or a mutated sequence (B-3M). Furthermore, increasing the amount of the A/E plasmid to 50 and 100 ng did not further inhibit the luciferase activity of the *BASP1* promoter (data not shown), suggesting that the presence of a low dose of A/E was sufficient to maximize inhibition of *BASP1* promoter activity. These results suggest that AML1-binding site B-3 (nucleotides at −546 to −556) in the promoter region of *BASP1* may be related to the A/E-dependent silencing of *BASP1*.

To study whether A/E binds directly to the promoter region of *BASP1* and leads to the methylation of the *BASP1* promoter region, we performed CHIP-PCR assays in the SKNO-1 and SKNO-1-siA/E cell lines using anti-AML1, anti-ETO, anti-DNMT1, anti-DNMT3a, and anti-DNMT3b antibodies or a non-specific antibody (normal IgG). Quantitative RT-PCR was performed with primers specific to the *BASP1* promoter region surrounding the predicted −546/−556 site or with the unrelated negative

control primers. The results showed that A/E and DNMT3a were present at the *BASP1* promoter region surrounding the functional AML1-binding site, but not for DNMT1 and DNMT3b (Fig. 3b and Supplementary Figure 1). This suggests that A/E and DNMT3a may form a co-repressor complex to inhibit the expression of *BASP1* by binding directly to its promoter region and then methylating the DNA.

#### **BASP1 plays a tumor suppressor role in A/E-positive cell lines in vitro**

To evaluate the effects of *BASP1* in t(8; 21) AML, we transduced SKNO-1 cells with the GFP-tagged *BASP1* or GFP control lentiviral plasmid. Transfection was confirmed by flow cytometry (Supplementary Figure 2). *BASP1* restoration significantly reduced cellular proliferation, as monitored by the CCK-8 assay to quantify the colony-forming ability and colony-forming assay in SKNO-1 cells (Fig. 4a, b). *BASP1* restoration also blocked cells in the G1 phase of the cell cycle and reduced the



number of cells in the S phase (Fig. 4c). In addition, *BASP1* restoration increased the number of apoptotic SKNO-1 cells by 1.6-fold compared to the negative

control (Fig. 4d). We also examined the biological effects of *BASP1* on the expression of CD11b by using flow cytometry and found no significant differences between

BASP1-restored cells and the control group (data not shown). These data indicate that BASP1 may play a key role in the regulation of cell proliferation by inducing apoptosis and cell cycle arrest in A/E-positive AML.

## Discussion

As a transcription factor, the A/E fusion protein plays a critical role in the pathogenesis of t(8;21) AML and regulates gene expression through various mechanisms, that is, by direct DNA binding and recruitment of DNMTs, HDACs, P300, and PRMT1;<sup>12, 18–20</sup> by interacting with other transcription factors;<sup>15, 21, 22</sup> or by forming stable multiprotein complexes, including CBF $\beta$ /HEB/E2A/LYL1/AML1<sup>23</sup>. Our previous studies showed that A/E can bind to the promoters of *miR-9-1* and *SIRT1* to form a regulatory circuit, resulting in the down-regulation of miR-9 and upregulation of SIRT1 and contributing to leukemogenesis<sup>15, 17</sup>. In this study, we showed for the first time that hypermethylation of the *BASP1* gene promoter leads to specific down-regulation of *BASP1* in t(8;21) AML. Our results showed that the A/E fusion protein could bind to the AML1-binding site on the *BASP1* promoter sequence together with DNMT3a and inactivate transcription of *BASP1* by CpG island hypermethylation. *BASP1* may play a role as a tumor suppressor gene in vitro in t(8;21) AML.

*BASP1* contains a nuclear localization sequence domain that can localize to promyelocytic leukemia bodies within the nucleus. Release of *BASP1* from the nucleus to the cytoplasm was observed in apoptotic cells<sup>24</sup>. We also observed enrichment of *BASP1* in the nucleus after transfection of the *BASP1* lentiviral vector in 293T cells. Further, ectopic expression of *BASP1* promoted apoptosis of A/E-positive leukemia cells. As a transcriptional co-regulator of Wilms' tumor-1 (WT1) protein, *BASP1* is involved in the regulation of many genes related to apoptosis pathways, such as BCL-2 family, c-Myc, p21, and others<sup>4, 6</sup>. A study in thyroid cancer cells showed that *BASP1* overexpression increases the expression of bax, promote cytochrome C release, and activate caspase-3, which ultimately results in apoptosis<sup>25</sup>. Again, further work is needed to determine the exact mechanism of *BASP1*-induced apoptosis in t(8;21) AML cells. In addition, *BASP1* has been reported to be involved in cell differentiation. After ectopic expression of *BASP1* in K562 cells, more than 90% of cells were differentiated by PMA induction<sup>26</sup>. We found that *BASP1* expression was gradually increased during the differentiation of myeloid cells using publicly available expression data (Supplementary Figure 3). However, we were unable to find any difference in the CD11b expression level after overexpression of *BASP1* compared to controls in A/E-positive cells, but cell cycle arrest occurred. Therefore, we hypothesize that the expression level of *BASP1* may be related to the degree of

cell differentiation, but is probably not directly involved in the induction of cell differentiation in t(8;21) AML. Inhibition of proliferation in A/E-positive AML cells by ectopic expression of *BASP1* may occur mainly through cell cycle arrest and induction of apoptosis.

Expression of WT1 might play an important role in A/E-mediated leukemogenesis<sup>27</sup>. Many studies using murine models have demonstrated that A/E alone was not able to independently induce leukemia<sup>28, 29</sup>. However, all mice rapidly developed AML after transplantation of A/E-transduced bone marrow cells co-expressing WT1<sup>27</sup>. *BASP1* has been identified as a transcriptional cosuppressor of WT1 and exerts its transcriptional activity by binding to the promoters of target genes together with WT1. Down-regulation of *BASP1* weakens the inhibitory effect of WT1 on target genes causing the transcriptional activation domain to be released<sup>4, 30</sup>. *BASP1* has also been identified as a specific MYC target that can inhibit v-Myc-induced cell transformation<sup>31</sup>. MYC is frequently activated in AML, and WT1 can also induce upregulation of MYC, which is correlated with the induction of leukemia, notably for sustained cell proliferation<sup>32</sup>. It will be important in the future to study the effect of *BASP1* expression on signaling pathways regulated by WT1 and MYC to better understand the possible mechanisms of *BASP1* promoter hypermethylation in the leukemogenicity of t(8;21) AML.

In conclusion, our results provide new insights into the role of *BASP1* in leukemogenesis. We demonstrate that *BASP1* plays a role as a tumor suppressor gene in vitro and is silenced via promoter methylation in t(8;21) AML. We suggest that manipulation of *BASP1* expression may be a new strategy for the treatment of t(8; 21) AML.

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**Conflict of interest**

The authors declare that they have no conflict of interest.

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