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Aberrant expression of SALL4 in acute B cell lymphoblastic leukemia: Mechanism, function, and implication for a potential novel therapeutic target

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Abstract

Treatment for high-risk pediatric and adult acute B cell lymphoblastic leukemia (B-ALL) remains challenging. Exploring novel pathways in B-ALL could lead to new therapy. Our previous study has shown that stem cell factor SALL4 is aberrantly expressed in B-ALL, but its functional roles and the mechanism that accounts for its upregulation in B-ALL remain unexplored. To address this question, we first surveyed the existing B-ALL cell lines and primary patient samples for SALL4 expression. We then selected the B-ALL cell lines with the highest SALL4 expression for functional studies. RNA interference was used to downregulate SALL4 expression in these cell lines. When compared with control cells, SALL4 knockdown cells exhibited decreased cell proliferation, increased apoptosis in vitro, and decreased engraftment in a xenotransplant model in vivo. Gene expression analysis showed that in SALL4 knockdown B-ALL cells, multiple caspase members involved in cell apoptosis pathway were upregulated. Next, we explored the mechanisms of aberrant SALL4 expression in B-ALL. We found that hypomethylation of the SALL4 CpG islands was correlated with its high expression. Furthermore, treatment of low SALL4-expressing B-ALL cell lines with DNA methylation inhibitor led to demethylation of the SALL4 CpG and increased SALL4 expression. In summary, to our knowledge, we are the first to show that the aberrant expression of SALL4 in B-ALL is associated with hypomethylation, and that SALL4 plays a key role in B-ALL cell survival and could be a potential novel target in B-ALL treatment.

It was estimated that 6,070 patients received a diagnosis of and 1,430 patients died of acute lymphocytic or lymphoid leukemia (ALL) in 2013. Although most patients with ALL are children younger than 10 years, this disease can occur in people of any age, and

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

approximately one third of the patients are adults. Acute leukemia occurs in 7 of 1,000,000 children younger than 15 years per year in the United States, and it is the most common childhood malignancy. The vast majority of cases are B cell lineage acute lymphoblastic leukemia (B-ALL; 75%–80%), with the remaining being acute myeloid leukemia (AML).

B-ALL is a clonal progressive malignant disease derived from B cell progenitors. The pathogenesis of B-ALL reported so far includes aberrant expression of protooncogenes, chromosomal translocations that create fusion genes encoding active kinases and altered transcription factors, and hyperdiploidy [1]. BCR-ABL, TEL-AML1, MLL rearrangements, and E2A-PBX1 are a few examples of fusion oncogenes in B-ALL. PAX5 is a transcription factor with an important role in B cell development and B-ALL. Heterozygous mutations of PAX5 contribute to leukemogenesis, and its fusion with other genes, such as ETV6, FOXP1, ZNF521, and PML, can generate oncogenic fusion proteins in B-ALL [2]. Currently, the most common treatment approaches for B-ALL consist of chemotherapy, radiation, and immunotherapy or monoclonal antibody therapy. Despite a better prognosis than for adult patients, approximately 20% of pediatric B-ALL patients remain drug resistant and can progress with leukemic relapses. A search for new pathways responsible for B-ALL pathogenesis might lead to the discovery of novel therapies.

SALL4, a member of the zinc-finger transcription factor SALL gene family, is the human homologue of the drosophila homeotic gene, *spalt*. (*sal*) [3,4]. In the past few years, several research groups [5–8] have demonstrated that SALL4 plays an essential role in the maintenance of embryonic stem cell (ESC) pluripotent and self-renewal properties by interacting with two other key regulators in ESCs—Nanog and Oct4. The loss of SALL4 expression in ESCs results in the downregulation of ESC markers, such as Oct4, and spontaneous ESC differentiation.

After birth, SALL4 expression is downregulated and absent in most adult tissues. However, SALL4 is expressed in various cancers, including a subset of solid tumors such as breast cancer [9], ovarian cancer [10], gastric cancer [11], Wilms tumor [12], and germ cell tumors [10,13–17], as well as leukemias, including almost all cases of human AML [18] and approximately 75% of B-ALL cases [19].

We have previously shown that SALL4 is critical for myeloid leukemogenesis. Transgenic SALL4 mice exhibit a preleukemic dysplastic phase that subsequently develops into AML that is transplantable [18]. SALL4 transgenic mice display an increased hematopoietic progenitor cell population and increased serial replating potential. Furthermore, loss of SALL4 in AML leads to extensive apoptosis [20,21]. The mechanism of SALL4 in myeloid leukemogenesis involves at least two critical pathways that are important for self-renewal of leukemic stem cells: Wnt/ β -catenin and Bmi-1 [18,21]. SALL4 may be one of a few genes that bridge the self-renewal properties of ESCs and myeloid leukemia. We have also reported that SALL4 is enriched in the side-population (SP) of leukemia and solid tumor cells [22]. The SP is implicated in drug resistance and cancer initiation, and it has been used to isolate cancer initiation cells [22]. Moreover, SALL4 expression is correlated with a worse prognosis in AML as well [22].

We have previously reported that SALL4 is abnormally expressed in B-ALL [19]. In this study, we explored the functional role of stem cell factor SALL4 in B-ALL and the possible mechanisms that account for its aberrant expression in this disease.

Methods

Primary patient samples and cell culture

The REH, Nalm6, 697, and Blin-1 cell lines were obtained from Dr. Leslie Silberstein's laboratory (Boston Children's Hospital, Boston, MA, USA). All cell lines were maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY; catalog no. 11875-093) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin at 37 °C in a humidified 5% CO₂ atmosphere. Twelve primary B-ALL patient samples were collected from the Dana Farber Cancer Institute under an approved institutional review board protocol (DFCI Legacy no. 01-206). The diagnosis of ALL samples was based on morphology and immunophenotype. Fresh human bone marrow cells were obtained from Lonza (Walkersville, MD USA) from healthy donors.

Data mining

Publicly available database GSE 13351 consists of gene expression profiles from primary B-ALL patients of various subtypes [23]. Gene expression profiles were generated using Affymetrix Human Genome U133 Plus 2.0 Array. The serial matrix files were downloaded from Gene Expression Omnibus. Normalized expression data were imported into Prism 3. Intergroup comparison was made using Student *t* test, with *p* < 0.05 considered statistically significant.

Real-time quantitative reverse transcriptase polymerase chain reaction

Total RNA was isolated with Trizol reagent (Life Technologies; catalog no.15596-018) according to the manufacturer's instructions, and the concentration was measured with ultraviolet spectrophotometry. Three hundred nanograms of total RNA was applied to perform quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) using iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad, Hercules, CA, USA; catalog no. 170-8893). The average threshold cycle for each gene was determined from triplicate reactions, and the expression level was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The sequences of primers for genes tested (SALL4, Caspase 3 and 8, TEL-AML1, and GAPDH) are listed in Supplementary Table E1 (online only, available at www.exphem.org).

Detection of TEL-AML1, t(15;17)

To detect *TEL-AML1*, RT-PCR was performed. Primers were designed as described previously [24], and the primer sequences are listed in Supplementary Table E1 (online only, available at www.exphem.org).

Xenotransplant

Two cell doses (1×10^4 or 1×10^6) of REH-scramble or REH-E5 cells were transplanted into NOD/scid IL-2Rg(null) mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ; The Jackson Laboratory, Bar Harbor, ME, USA) by tail vein injection. Each recipient group had three mice. Leukemic development and survival of mice were monitored daily. The recipients were sacrificed and dissected upon onset of physical symptoms of leukemia, including hind limb paralysis, weight loss, or decreased activity. Bone marrow and spleen cells were harvested, and single-cell suspensions were prepared. REH cells were detected with flow cytometry, using CD45-PE and CD19-APC antibodies (eBiosciences, San Diego, CA; catalog no. 12-9459-42, 17-0199-42).

Homing assay

For homing assay, 1×10^6 of REH-scramble or REH-E5 cells were transplanted into NOD/SCID/IL2 γ -null mice by tail vein injection. The mice were sacrificed 3 and 48 hours after transplantation. Bone marrow and spleen cells were harvested, and single-cell suspensions were prepared. The percentage of REH-scramble or REH-E5 cells was detected by flow cytometry as GFP-positive cells. The same experiments were performed with Nalm6 cells.

Bisulfite sequencing

We treated 2 μ g genomic DNA with sodium metabisulfite (Sigma-Aldrich, St. Louis, MO, USA catalog no. S9000) as described previously [25]. SALL4 exon1-intron1 region was amplified from bisulfite-treated DNA using primers listed in Supplementary Table E1 (online only, available at www.expchem.org). PCR products were subcloned into the pCR2.1 vector with a TOPO TA cloning kit (Life Technologies; catalog no. 45-0641). Ten clones from each B-ALL cell lines or primary samples were sequenced to evaluate their methylation status.

5-aza-2'-deoxycytidine treatment

To inhibit DNA methylation, 1 μ mol/L of 5-aza-2'-deoxycytidine (Sigma-Aldrich, MO, USA catalog no. A3656-5MG) was added to 697 and Blin-1 every 24 hours for 3 days (days 1, 2, and 3). On day 4, cells were collected and the genomic DNA (for bisulfite sequencing) and RNA (for real-time PCR to examine the SALL4 expression) were extracted.

Statistical analysis

All statistical analysis was completed with Student *t* test, assuming normal two-tailed distribution and unequal variance.

Results

SALL4 is aberrantly expressed in human B-ALL cell lines and primary cells

Using real-time qRT-PCR or immunohistochemistry staining, we previously observed high SALL4 mRNA and protein levels in all examined human AML and some B-ALL samples compared with normal bone marrow (BM) cells [18,19]. In this study, we aimed to investigate the mechanisms and functional roles of SALL4 in human B-ALL. We first

evaluated the expression of SALL4 in primary B-ALL patients from various subtypes. By analyzing B-ALL gene expression profiles in 97 patient samples from the public database GSE133518 [23], we noticed that SALL4 expression was highest in B-ALL patients with TEL-AML1 translocation, which is the most common genetic abnormality in pediatric B-ALL (Fig. 1A). We next assessed the SALL4 mRNA level in four available B-ALL cell lines and 12 primary patient samples using qRT-PCR. We observed a 1.8–5.7-fold increase in SALL4 mRNA expression level in the four cell lines examined when compared with normal human BM control cells (Fig. 1B). Moreover, 11 of the 12 primary B-ALL samples showed constitutive expression of SALL4 (Fig. 1C), suggesting that SALL4 might play a role in B-ALL pathogenesis. Furthermore, one B-ALL cell line (REH), the highest SALL4 expressing B-ALL cell line, and one of seven SALL4-expressing patients with B-ALL were confirmed to have TEL-AML1 translocation (Supplementary Figure E1, online only, available at www.exphem.org).

Downregulation of SALL4 in B-ALL cells leads to impaired cell proliferation and increased apoptosis in vitro

We next sought to investigate the effects of SALL4 suppression in REH cells, which showed the highest SALL4 expression among four tested B-ALL cell lines. Two short hairpin RNA (shRNA) lentiviral constructs (507 and E5) that target different regions of SALL4 mRNA were made. High-titer viruses were able to infect greater than 90% of B-ALL cells (Fig. 2A). The two SALL4 shRNAs were able to downregulate its gene expression by 60% and 78% when evaluated with qRT-PCR (Fig. 2B). We next monitored the growth of SALL4 knockdown and control REH cells through a cell proliferation assay, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2Htetrazolium. SALL4 knockdown REH cells exhibited a twofold decreased growth rate compared with the control cells (Fig. 2C). The decreased cell growth could be due to increased cell death. To explore this possibility, we stained control and SALL4 knockdown REH cells with Annexin V and 7-amino-actinomycin D (7-AAD) to allow quantitation of apoptotic cells using flow cytometry assay. The percentage of apoptotic cells in SALL4 knockdown cells was twofold higher than that in the controls (Fig. 2D). Nalm6 is the second highest SALL4-expressing B-ALL cell line, and similar experiments were performed on these cells. The ability of SALL4 shRNA to downregulate its expression in Nalm6 was 50% (Fig. 2E and F). Similar results were obtained (Fig. 2G). The decreased cell growth could also be due to increased nonproliferative cells. We next examined the effect of downregulation of SALL4 on cell cycle; however, reduction of SALL4 mRNA expression in REH and Nalm6 cells did not affect cell cycle (Supplementary Figure E2, online only, available at www.exphem.org). In addition, cell division assay suggested that downregulation of SALL4 did not affect cell division either (Supplementary Figure E3, online only, available at www.exphem.org). In summary, these data demonstrated that inhibition of SALL4 in REH and Nalm6 B-ALL cells led to reduced cell proliferation because of increased apoptosis.

Downregulation of SALL 4 in B-ALL cells leads to impaired leukemic cell engraftment in vivo

After characterizing the phenotypes of SALL4 downregulation in B-ALL cells in vitro, we then examined the leukemic engraftment of SALL4 knockdown REH cells in a mouse

xenotransplantation model. REH cells (1×10^6) infected with either control or SALL4 shRNA-expressing lentiviruses were injected intravenously into immunodeficient mice (Fig. 3A). All the recipients succumbed to fatal leukemia within 4–6 weeks after transplantation. These leukemic recipient mice showed increased white blood cell counts, visibly pale BM, and splenomegaly (data not shown). Upon sacrifice of the mice, we found that the percentages of GFP⁺ REH cells were similar to donor cells in the BM and spleen of control recipients (before transplantation; black bar in Fig. 3C). However, in 507 and E5 SALL4 knockdown recipients, the proportion of GFP⁺ cells was significantly decreased compared with the donor cells (Fig. 3B and C). We also injected a lower cell dose (1×10^4 cells) of REH cells into immunodeficient mice and observed the same phenotype (Supplementary Figure E4, online only, available at www.exphem.org).

To rule out that the observed engraftment defect of SALL4 knockdown cells was a consequence of impaired homing ability, we next performed a homing assay. SALL4 knockdown or control REH or Nalm6 cells (1×10^6) were injected intravenously into immunodeficient mice. The mice were sacrificed and analyzed for the percentage of GFP⁺ cells in the BM and spleen by flow cytometry 3 hours after the injection. There was no statistically significant difference between the GFP⁺ population in the SALL4 knockdown and control groups using a 3-hour (Fig. 3D) or a 48-hour (Supplementary Figure E5, online only, available at www.exphem.org) homing assay. We conclude that downregulation of SALL4 in B-ALL cells does not affect their homing abilities, and the impaired leukemic engraftment in SALL4 knockdown cells in vivo is most likely due to impaired cell survival or increased apoptosis as observed in our in vitro experiments.

SALL4 affects B-ALL survival by affecting multiple caspase members

To explore the underlying mechanism of increased apoptosis in SALL4 knockdown REH cells, we performed gene expression profiling on apoptosis-related genes in SALL4 knockdown and control REH cells (Supplementary Table E2, online only, available at www.exphem.org). Notably, the caspases 3 and 8 that were involved in cell apoptosis pathway were upregulated in SALL4 knockdown REH cells (Supplementary Figure E6A, online only, available at www.exphem.org). On the contrary, when SALL4 was overexpressed, this pathway was downregulated (Supplementary Figure E6B, online only, available at www.exphem.org). To validate the result of the microarray plates, real-time qRT-PCR was performed. Expression of caspases 3 and 8 was upregulated in SALL4 knockdown REH and Nalm6 when compared with the control group (Fig. 4A and B). Furthermore, we performed the function assay for this pathway by evaluation of the caspase 3 and 8 activities in these cells. Caspase 3 activity was evaluated by flow cytometry, and caspase 8 activity was evaluated by Caspase-Glo 8 Assay. Both caspase 3 and 8 activities were increased in SALL4 knockdown cells when compared with the control cells (Fig. 4C and D). This result suggests that SALL4 can inhibit B-ALL leukemic cell apoptosis.

Hypomethylation of CpG sites is correlated with high SALL4 expression

After establishing the expression pattern of SALL4 in human B-ALL, we next investigated why SALL4 was aberrantly expressed in B-ALL. As methylation of SALL4 has been reported to be important for its expression regulation [26,27], we hypothesized that

hypomethylation might be one of the mechanisms underlying aberrant SALL4 expression in B-ALL. The SALL4 CpG islands were mainly located at the exon1-intron1 region. We designed six pairs of primers (e1-P1 ~P6, only e1-P5 shown) for bisulfite sequencing to cover this region and evaluated its methylation status (Fig. 5A). The region covered by primer e1-P5 was hypomethylated in REH and Nalm6 (the highest and the second highest SALL4 expressing B-ALL cell lines), but hypermethylated in low SALL4 expressing 697 and Blin-1 cells (Fig. 5B, upper panel). Furthermore, we analyzed the methylation status in seven primary B-ALL samples with high SALL4 expression, and the e1-P5 region was hypomethylated in all seven samples (Fig. 5B, lower panel). In addition, treatment of low SALL4 expressing B-ALL cell lines (697 and Blin-1) with DNA methylation inhibitor 5-Aza-2'-deoxycytidine (5-Aza-dC) led to demethylation of the SALL4 CpG island (Fig. 5C) and increased SALL4 expression (Fig. 5D and 5E).

In summary, we conclude that hypomethylation of SALL4 CpG island spanning the exon1-intron1 region covered by primers e1-P5 is associated with upregulated SALL4 expression, suggesting that hypomethylation of CpG island is one of the mechanisms accounting for aberrant SALL4 expression in B-ALL.

Discussion

B-ALL is a heterogeneous disease entity, with one third of the cases bearing normal karyotype. Adult and pediatric B-ALLs differ in prognosis. Despite its relatively good outcome, approximately 20% of pediatric B-ALL patients are still classified as high-risk and are resistant to current combined therapy. Adult patients have an even worse prognosis. New targets and pathways need to be explored for the development of better therapeutic options for this disease.

We have reported the aberrant expression of SALL4 in B-ALL [19], and others have observed that SALL4 is highly expressed in a subgroup of pediatric high-risk patients with B-ALL [28]. In this study, by data mining on BALL public expression profiles, we noticed that the expression of SALL4 was higher in patients with TEL1-AML1 translocation (Fig. 1A). REH, the highest SALL4 expressing B-ALL cell line, and one of the seven high-SALL4- expressing B-ALL patients also had this translocation (Supplementary Figure E1, online only, available at www.exphem.org). Similarly, 4 of 10 high-risk pediatric patients with B-ALL and high SALL4 expression were associated with genetic abnormality of ETV6 (TEL1) [28]. The observed upregulation of SALL4 in the TEL-AML1 subgroup is intriguing. It is possible that this fusion protein somehow affects the regulation of SALL4 expression in B-ALL. On a separate note, hypermethylation and the absence of SALL4 expression has been reported in colon cancer [27]. Therefore, we investigated whether methylation status of SALL4 is correlated with its expression in B-ALL. Indeed, we found a correlation of higher SALL4 expression with hypomethylation status of its CpG islands, suggesting that hypomethylation is one of the mechanisms accounting for aberrant SALL4 expression in B-ALL.

SALL4 has also been implicated in the pathogenesis of AML. We and others have shown that SALL4 promotes cell survival in AML [20,21], and high SALL4 expression is

associated with drug resistance and poor prognosis in AML [22]. We next explored the functional roles of SALL4 in B-ALL. When SALL4 was downregulated in SALL4-expressing B-ALL cell lines, such as REH and Nalm6, the growth of these cells was decreased and apoptosis was increased in vitro. Furthermore, we examined the leukemogenic ability of SALL4 knockdown REH cells in a xenotransplant murine model. By monitoring the engraftment of leukemic cells, we observed that SALL4 knockdown GFP⁺ REH cells had a survival disadvantage when compared to its controls in vivo. This disadvantage was not due to a homing defect. These results suggest that SALL4 plays an important role in maintaining BALL cell survival, similar to what we have observed for SALL4 in AML [29] and SALL4-expressing solid tumors, such as hepatocellular carcinoma [30].

We next explored the mechanism of SALL4 function in BALL. Based on the increased apoptosis phenotype upon downregulation of SALL4, we chose to perform gene expression array on the apoptosis pathways. We found that caspases were affected the most by downregulation or upregulation of SALL4. We further validated that SALL4 could repress apoptosis through inhibition of caspases 3 and 8 (Fig. 4A–D). We have shown previously that SALL4 affects the proliferation or survival of AML cells through multiple pathways [20], and that Bmi-1 and c-Myc can be directly or indirectly regulated by SALL4 [18,21]. It is possible that the inhibitory function of SALL4 on apoptosis through caspases 3 and 8 in B-ALL is also mediated by these pathways. The role of SALL4 in B cell development remains unclear; however, it has been suggested by others that SALL4 can regulate early B cell factor (EBF), a gene that is involved in B cell lineage differentiation [26].

To our knowledge, we are the first to report that the stem cell factor SALL4 plays an important role in maintaining B-ALL survival by affecting caspases, and hypomethylation is, at least in part, responsible for the aberrant expression of SALL4 in B-ALL. The treatment outcome of B-ALL has been improved by chemotherapy, stem cell transplantation, and antibody-based therapies. Despite these therapies, some patients still cannot achieve complete remission, and some bear the risk of relapse even if they have achieved complete remission. Based on our studies, we propose that SALL4 could become a novel therapeutic target for B-ALL, as we have proposed for AML and hepatocellular carcinoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Aberrant SALL4 expression in primary B ALL samples and cell lines. **(A)** Data mining of SALL4 expression from public database GSE13351 revealed that SALL4 expression was higher in group 1 with TEL-AML1 translocation. The cytogenetic characteristics of other groups were listed. SALL4 expression in B-ALL cell lines **(B)** and primary cells **(C)**. The expression level of SALL4 mRNAs was normalized to internal control GAPDH. Normal human bone marrow cells were used as controls ($N = 3, \pm SD$).

Figure 2.

Downregulation of SALL4 in B-ALL cell lines leads to reduced cell growth and increased apoptosis. REH and Nalm6 cells were transduced with control scramble vector (CTL), or SALL4 shRNA lentiviral vectors (507 or E5) and then cultured for 48 hours. (A) Representative flow cytometry profile (left) and percentage (right) of lentivirus infected (GFP⁺) REH cells. (B) Total RNA of REH cells was isolated, and qRT-PCR was performed to determine the level of SALL4 mRNA upon viral infection. Expression levels were normalized to GAPDH. (N = 3, error bars, SD between three independent experiments). (C) MTS assay showed that SALL4 knockdown reduced growth curve on viable REH cells. The assay was performed in triplicate. (D) REH cells (1×10^6) were transduced with control vector (CTL) or different SALL4 shRNA lentiviral vectors (507 or E5), then cultured for 48 hours. Apoptotic cells were examined with Annexin V and 7-AAD staining. Left, representative flow cytometric analysis of 7-AAD and Annexin V staining (right panel, percentage of apoptotic cells). (E) Percentage of lentivirus infected (GFP⁺) Nalm6 cells. (F) Relative SALL4 expression level of Nalm6 upon viral infection. Expression levels were normalized to GAPDH. (G) Percentage of apoptotic cells in Nalm6 transfected with E5 or Sc. Data are from three independent experiments and represented as mean $\pm$ SD. Similar results were obtained from Nalm6 cells (E–G). * $p < 0.05$.

Figure 3. Downregulation of SALL4 significantly decreases the engraftment of REH cells in a mouse xenotransplantation model. **(A)** Schematic diagram of the xenotransplantation protocol. **(B)** Representative flow cytometric profile of GFP⁺ REH cells (CD45⁺CD19⁺). **(C)** SALL4 knockdown REH cells (GFP⁺) exhibited reduced engraftment in BM and spleen of recipient immunodeficient mice (N = 3). **(D)** No difference in homing between SALL4 knockdown and the control group was observed. Three hours after transplantation, recipient mice were

sacrificed and analyzed for the percentage of GFP⁺ cells in BM and spleen by flow cytometry (N = 3). * $p < 0.05$.

Figure 4.

Caspases 3 and 8 are upregulated and activated upon SALL4 knockdown. (**A**, **B**) The expression of caspases 3 and 8 was examined by real-time qPCR in SALL4 knockdown REH (**A**), Nalm6 (**B**), and control-treated cells. (Error bars, SD of three or four independent experiments). (**C**) Caspase 3 activity increased in SALL4 knockdown Nalm6 when compared with control-treated cells. Seventy-two hours after lentiviral transduction, SALL4 knockdown and control-treated Nalm6 cells were collected for caspase 3 activity assay. *Left*, Representative flow cytometric profile and percentage of active caspase 3 cells after gated

on GFP⁺ cells. *Right*, Bar graph showing the overall percentage of the caspase 3–positive cells in two groups with control group normalized to 1. (Error bars, SD of three independent experiments). **(D)** Increased caspase 8 activity in SALL4 knockdown REH and Nalm6. Caspase-Glo 8 assay was performed after 48 hours (REH) or 72 hours (Nalm6) after lentiviral transduction.

Figure 5.

Hypomethylation of the CpG sites is correlated with SALL4 expression. (A) Diagram on SALL4 exon1/intron1 region. Six pairs of primers were designed to cover this region (one of the primers set e1-P5 is shown). The translation start site is represented by a filled, inverted triangle. Exon1 is represented by a short, gray rectangle with CpG islands in a long, gray rectangle. Each open circle represents one CpG. (B) Methylation status of the e1-P5 region in B-ALL cell lines (REH, Nalm6, 697 and Blin-1; upper panel). Each column represents a single CpG site. Methylation status of the e1-P5 region in B-ALL cell lines and seven primary samples (Nos. 1-7; lower panel). (C) Methylation status of the e1-P5 region in B-ALL cell lines (697 and Blin-1) after 5-Aza-dC treatment. The scale bar at bottom shows the percentage of methylation. Ten clones of each B-ALL cell line or primary sample were sequenced for evaluation of their methylation status.

(D) SALL4 expression of 697 after 5-Aza-dC treatment. No-treatment 697 was set as 1. (E) SALL4 expression of Blin-1 after 5-Aza-dC treatment. No-treatment Blin-1 was set as 1.