Front. Med. 2017, 11(1): 87–96
DOI 10.1007/s11684-016-0469-4

RESEARCH ARTICLE

Regulatory mechanism and functional analysis of *S100A9* in acute promyelocytic leukemia cells

Yonglan Zhu[1], Fang Zhang[1], Shanzhen Zhang[2], Wanglong Deng[1], Huiyong Fan[1], Haiwei Wang[1,2], Ji Zhang (✉)[1,2]

[1]*State Key Laboratory of Medical Genomics, Ruijin Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China;* [2]*Medical Institute of Health Sciences, Chinese Academy of Sciences, Shanghai 200025, China*

Abstract **S100A9, a calcium-binding protein, participates in the inflammatory process and development of various tumors, thus attracting much attention in the field of cancer biology. This study aimed to investigate the regulatory mechanism of *S100A9* and its function involvement in APL. We used real-time quantitative PCR to determine whether PML/RARα affects the expression of *S100A9* in NB4 and PR9 cells upon ATRA treatment. ChIP-based PCR and dual-luciferase reporter assay system were used to detect how PML/RARα and PU.1 regulate *S100A9* promoter activity. CCK-8 assay and flow cytometry were employed to observe the viability and apoptosis of NB4 cells when *S100A9* was overexpressed. Results showed that *S100A9* was an ATRA-responsive gene, and PML/RARα was necessary for the ATRA-induced expression of *S100A9* in APL cells. In addition, PU.1 could bind to the promoter of *S100A9*, especially when treated with ATRA in NB4 cells, and promote its activity. More importantly, overexpression of *S100A9* induced the apoptosis of NB4 cells and inhibited cell growth. Collectively, our data indicated that PML/RARα and PU.1 were necessary for the ATRA-induced expression of *S100A9* in APL cells. Furthermore, *S100A9* promoted apoptosis in APL cells and affected cell growth.**

Keywords *S100A9*; PU.1; PML/RARα; ATRA; APL

Introduction

Acute promyelocytic leukemia (APL) is pathologically characterized by a typical translocation between chromosomes 15 and 17. This translocation results in the formation of a promyelocytic leukemia-retinoic acid receptor (PML/RARα) fusion protein, which plays an important role in the leukemogenesis of APL. The protein functions in blocking cell differentiation at the promyelocytic stage [1]. The fusion protein harbors the protein–protein interaction domain of PML and the DNA binding domain of RARα, which is a strong transcriptional repressor. In the presence of pharmacological concentrations of all-trans retinoic acid (ATRA), the dissociation of corepressor molecules from PML/RARα promotes APL blasts to terminally differentiate by restoring the expression of genes that are essential for myeloid differentiation [2]. Our previous study identified a critical mechanism of differentiation blockage in APL, by which PML/RARα disrupts the function of the hematopoietic transcription factor PU.1 and subsequently blocks the regulation of a number of genes that are essential for myeloid differentiation [3]. PU.1 is an important transcription factor for myeloid differentiation. It regulates numerous myeloid genes, including granulocyte colony-stimulating factor receptor, CD18, CD11b, and IRF1 [4,5]. PU.1 disruption has been shown to block the differentiation or maturation of myelomonocytes, which results in leukemia genesis in animal models, whereas overexpressed PU.1 improves AML blast differentiation [3]. In APL cells, ATRA restores PU.1 expression. Therefore, increased PU.1 targets molecules at the gene expression level, including PSMBs, HCK, and HOTAIRM1 [4].

The S100 protein family is a class of low-molecular-weight acidic, calcium-binding proteins, which play important roles in the biological activities of the body by interacting with calcium ions and participating in cell differentiation, cell migration, gene transcription, and cell cycle operation [6]. The S100 protein is closely related to the development of various tumors. *S100A9* is an

Received April 14, 2016; accepted July 5, 2016
Correspondence: jizhang@sibs.ac.cn

important member of the S100 calcium-binding family of proteins, which increases in level during partial inflammation and autoimmunity, especially in myeloid cells [7]. This protein is expressed during myeloid cell differentiation. S100A9 increases in abundance in granulocytes and monocytes by forming a heterocomplex with S100A8. However, the protein has a distinct function and regulatory mechanism [8]. Interestingly, S100A9 can exhibit pro- and anti-inflammatory roles depending on the tissue and cell type [9]. Increased expression of S100A9 has been observed in a number of tumors, such as colon, ovarian, breast, and thyroid cancers [10]. However, S100A9 is downregulated in several common human cancers, including head and neck squamous cell carcinoma, esophageal squamous cell carcinoma, cervical cancer, nasopharyngeal cancer, and oropharyngeal cancer [11]. Previous studies showed that S100A9 can inhibit cell growth and induce cell apoptosis through the classical mitochondrial pathway with its partner, S100A8, in various cell types [12]. Studies have also found that S100A9 is differentially expressed in adult acute myeloid leukemia and non-hematologic malignancies and increases in AML remission. These conditions indicated that an intimate connection exists between S100A9 and the pathogenesis and prognosis of leukemia [13].

In the present study, we investigated the mechanism and function involved in the differential expression of *S100A9* in APL. We discovered that PU.1 regulated the basal expression of *S100A9,* and PML/RARα repressed the expression of *S100A9* in APL. However, PML/RARα was necessary for ATRA-induced expression of S100A9 in NB4 cells. Furthermore, ATRA significantly increased S100A9 expression. In addition, the increased expression of S100A9 contributed to the apoptosis and growth inhibition of NB4 cells. Our data highlighted the regulatory mechanism of *S100A9* and important function of *S100A9* in APL.

Materials and methods

Cell culture and reagents

NB4, U937-PR9, U937, and HL-60 cells were grown in RPMI 1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco). Human embryonic kidney 293T cells were maintained in DMEM (Gibco) supplemented with 10% FBS. The cells were cultured in a humidified incubator with 5% CO_2 at 37 °C. $ZnSO_4$ (Sigma, St Louis, MO, USA) was dissolved in sterile water as a stock solution at 100 mmol/L. ATRA (Sigma) was dissolved in ethanol as a stock solution at 1 mmol/L and used in the dark during experiments. Both $ZnSO_4$ and ATRA were diluted by 1000-fold when used.

Plasmid constructions

The human *S100A9* promoter (chr1:153329870-153330800) harboring two transcription factor motifs was amplified by genomic PCR starting from U937 and inserted into the pGL3-basic vector (Promega, Madison, WI, USA) upstream of the firefly luciferase coding region at the *Xho*I and *Hin*dIII sites. The renilla luciferase plasmid pRL-SV40 was used as control for transfection efficiency.

Transient transfection and luciferase assays

293T cells were transfected with Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. Transfected cells were assayed for luciferase activity using dual-luciferase reporter assay system reagents (Promega, Madison, WI, USA). The detailed information for the procedure was described previously [3]. Each transfection was performed three times.

Chromatin immunoprecipitation (ChIP) assay

ChIP was undertaken according to the manufacturer's recommendation for the ChIP-IT High Sensitivity Kit (Active Motif). We used 4.5×10^6 NB4 cells for each ChIP reaction. Chromatin was sheared to a size of 200–1000 bp. In brief, NB4 cells were incubated with the following antibodies: anti-PU.1 (T-21x), anti-PML (H-238x), and anti-RARα (C-20x) from Santa Cruz, and normal rabbit IgG (ab46540). The total input and immunoprecipitated DNA was analyzed by PCR using the following primers: *S100A9*-1 (forward: 5′-TAGTGGAACCTCGGATTGGGT-3′; reverse: 5′-TGATTGGTCAGAGTGTGGCAA-3′), *S100A9-2* (forward: 5′-TAGTGGAACCTCGGATTGGGT-3′; reverse: 5′-TGATTGGTCAGAGTGTGGCAA-3′), *PLCB2*, a positive primer, (forward: 5′-GAGGGATGGCTGCTCTGGTT-3′; reverse: 5′-GCTGGGCTAAGAAGGGCGATA-3′), and *BLNK*, a negative primer, (forward: 5′-GGCCCTGACTGATGGAAATAC-3′; reverse: 5′-CAGCAGGTGACCATCCCTTTAG-3′). Each experiment was performed in triplicate, and the fold enrichment of the chip PCR product was calculated as previously described [3].

RNA extraction and quantitative real-time PCR

RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and 1 μg of the total RNA was reverse transcribed into cDNA by using SuperScript II Reverse Transcriptase kit (Invitrogen) according to the manufacturer's protocol. Quantitative real-time PCR was performed in the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the SYBR Green PCR master mix (Toyobo, Osaka, Japan). GAPDH was used as a reference gene for internal control.

The relative gene expression where calculated as follows: $10^4 \times 2^{-\Delta Ct}$, where $\Delta Ct = Ct\ (TARGET) - Ct\ (GAPDH)$. Primer sequences of *S100A9*, *PU.1,* and *GAPDH* were as follows: *S100A9* (forward: 5′-GGTCATAGAACACATCA TGGAGG-3′; reverse: 5′-GGCCTGGCTTATGGTGGTG-3′); *PU.1* (forward: 5′-GTGCCCTATGACACGGA TCTA-3′; reverse: 5′-AGTCCCAGTAATGGTCGCTAT-3′); and *GAPDH* (forward: 5′-GAAGGTGAAGGTCGGAGTC-3′; reverse: 5′-GAAGATGGTGATGGGATTTC-3′).

RNA interference experiments

Two specific sequences of siRNA against PU.1 were purchased from GenePharma. Their sequences were 5′-GCUUCGCCGAGAACAACUUTT-3′ and 5′-GAGGUC AAGAAGGUGAAGATT-3′ named as siPU.1-1 and siPU.1-2, respectively. The sequence of the negative control was 5′-UUCUCCGAACGUUGUCACGUTT-3′. NB4 cells (2×10^6) were suspended in 100 µl of Amaxa Nucleofector Solution V and then mixed with 3 µg of siRNA transfected by electroporation using the Amaxa Nucleofector II device and program X-001 (Lonza). After transfection for 24 h, the cells were added to ATRA for another 24 h.

Retroviral constructs and transfection

Full-length human *S100A9* cDNA was amplified by RT-PCR using the U937 cells and cloned into an MSCV2.2-derived vector, MigR1, at the *Not*I and *Bam*HI sites. The entire cDNA sequence that was inserted into the plasmids was verified through sequencing. MigR1-S100A9 was transfected into 293T packaging cells by using Lipofectamine 3000. The cell culture supernatants containing the virus were then collected at 48 h after transfection. NB4 cells were infected with the viral supernatant and 8 µg/ml of polybrene (Sigma) and then centrifuged at 800 $\times$ *g* for 90 min at 32 °C. After 6 h, the medium was changed.

Cell apoptosis and proliferation analysis

Annexin V-APC and PI were used (BD Biosciences) to detect apoptotic cells in GFP-positive cells via flow cytometry following the manufacturer's recommendation. We used CCK-8 assay (Dojindo Laboratories, Japan) to detect cell viability. Cells were seeded into 96-well plates at a density of 2×10^3 cells/well. After incubation for 1, 2, 3, 4, and 5 days, 10 µl of CCK-8 solution was added to each well. The optical density was measured at 450 nm, and cell proliferation was determined.

Western blot

Total protein extraction and Western blot analysis were performed as previously described [11]. Rabbit anti-S100A9 monoclonal antibody, mouse anti-β actin monoclonal antibody, and mouse anti-Bcl-2 monoclonal antibody were purchased from Abcam, Cambridge (UK). Rabbit cleaved caspase-3 was purchased from Cell Signaling Technology Inc. (Beverly, MA, USA).

Gene expression analysis

GSE10358 and GSE14468 were obtained from the Gene Expression Omnibus.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism software (GraphPad). For two-group comparisons, significance was determined using two-tailed *t*-tests. *P*-value < 0.05 was considered statistically significant.

Results

S100A9 is an ATRA-responsive gene, and PML/RARα is necessary for ATRA-induced expression of *S100A9* in NB4 cells

S100A9 is abundant in granulocytes and monocytes, and it is upregulated during the differentiation of hematopoietic cells. These properties potentially indicated that *S100A9* played an important role in myeloid differentiation. First, we analyzed the expression level of *S100A9* using the published expression profiles of 43 APL patients and 236 other AML specimens from GSE10358 [14]. As indicated in Fig. 1A, *S100A9* expression was significantly lower in APL samples than in other AML patients. All data from patients were seen in supplemental data Table S1. Real-time RT-PCR was conducted to compare *S100A9* expression among several hematopoietic cells. Fig. 1B shows that *S100A9* expression was significantly lower in NB4 cells (PML/RARα-positive) than in U937 and HL-60 cells (PML/RARα-negative). This observation indicated that *S100A9* expression was inversely correlated with PML/RARα expression. Given that APL is characterized by a unique clinical response to a differentiation inducing agent, ATRA, *S100A9* expression was examined in APL cells after ATRA treatment. As shown in Fig. 1C, RNA and protein expression levels of *S100A9* gradually increased after the NB4 cells were treated with ATRA in a time series. The same conclusion could be made on Fig. 1D showing PR9 cells treated with $ZnSO_4$ and ATRA. PR9 is a PML/RARα-inducible model [15] constructed from U937 cells treated with 100 µmol/L $ZnSO_4$ for 4 h. PR9 can eventually induce many features that are similar to those of APL. However, *S100A9* RNA expression did not change when the cells were treated with ATRA without the induction of PML/RARα, as shown in Fig. 1D. The above

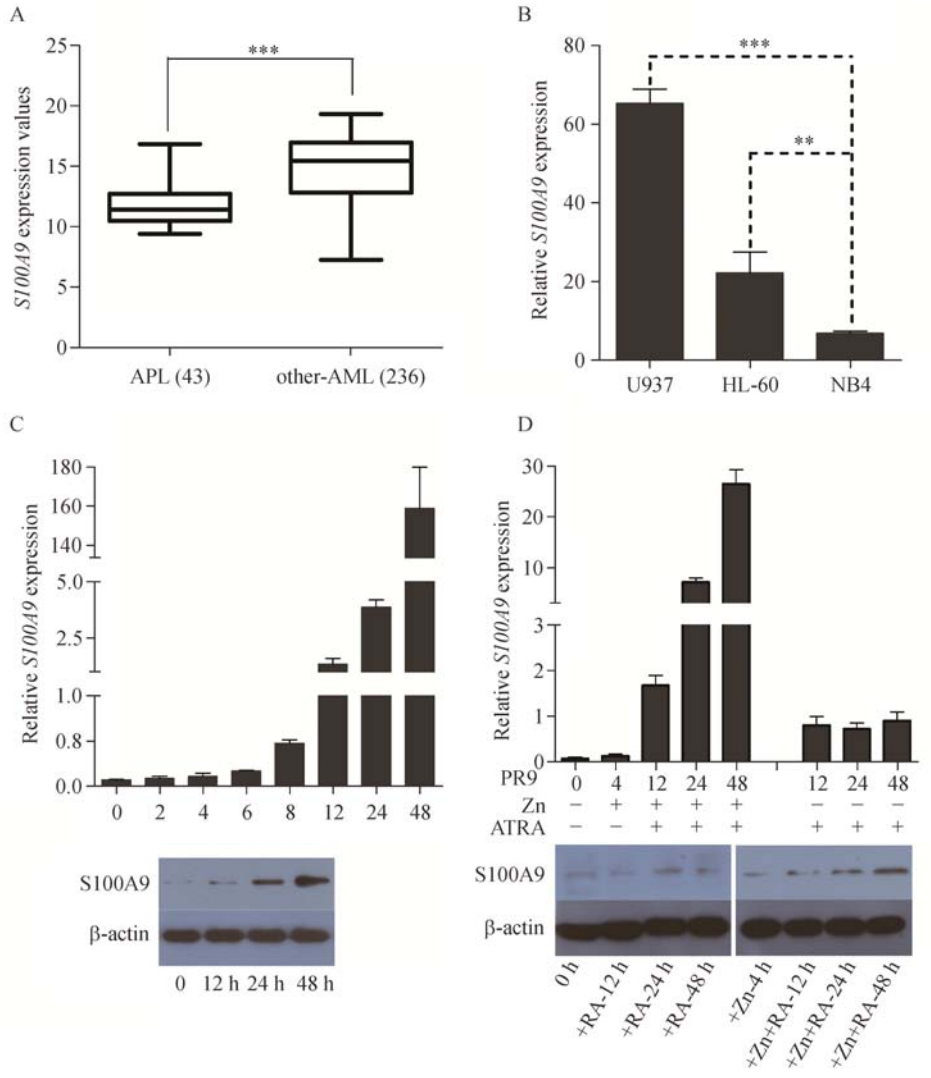

Fig. 1 ATRA-induced expression of *S100A9* is dependent on PML-RARα in APL. (A) *S100A9* expression in APL patients was significantly lower than that in other AML patients. The expression values are the absolute intensities after log transformation. Data were extracted from GSE10358. (B) The expression level of *S100A9* was significantly lower in NB4 cells than in U937 and HL-60 cells. (C) RNA and protein expression of *S100A9* gradually increased in NB4 cells when treated with ATRA at a series of time points. (D) The relative expression of *S100A9* gradually increased after PML/RARα induction of $ZnSO_4$-treated PR9 cells in the presence of ATRA at the indicated time points. However, the expression did not change in PR9 cells without $ZnSO_4$ treatment even in the presence of ATRA. The data represent the mean of three replicates ± S.D. $^{**}P < 0.01$, $^{***}P < 0.001$.

results indicated that *S100A9* was responsive to ATRA, and its expression was closely related to PML/RARα.

Considering that arsenic trioxide (As_2O_3) combined with ATRA can effectively cure APL patients, we also conducted an experiment to determine whether As_2O_3 affects *S100A9* expression. We observed that As_2O_3 had minimal effect on the expression of PU.1 on NB4 cells, but it increased *S100A9* expression. However, *S100A9* expression induced by As_2O_3 at different time points was significantly lower than that induced by ATRA (Fig. S1).

PML/RARα regulates *S100A9* activity

To determine whether PML/RARα can directly bind to the *S100A9* promoter, we conducted ChIP assays on the DNA samples of NB4 cells that were either treated or untreated with ATRA using PML and RARα-specific antibodies, respectively. Fig. 2A shows the PU.1 and PML/RARα binding sites at the *S100A9* promoter region that were determined by TRANSFAC software. Two sets of primers were designed based on the binding sites. One covered the −450 and −300 bp PU.1 and PML/RARα sites (*S100A9-1*).

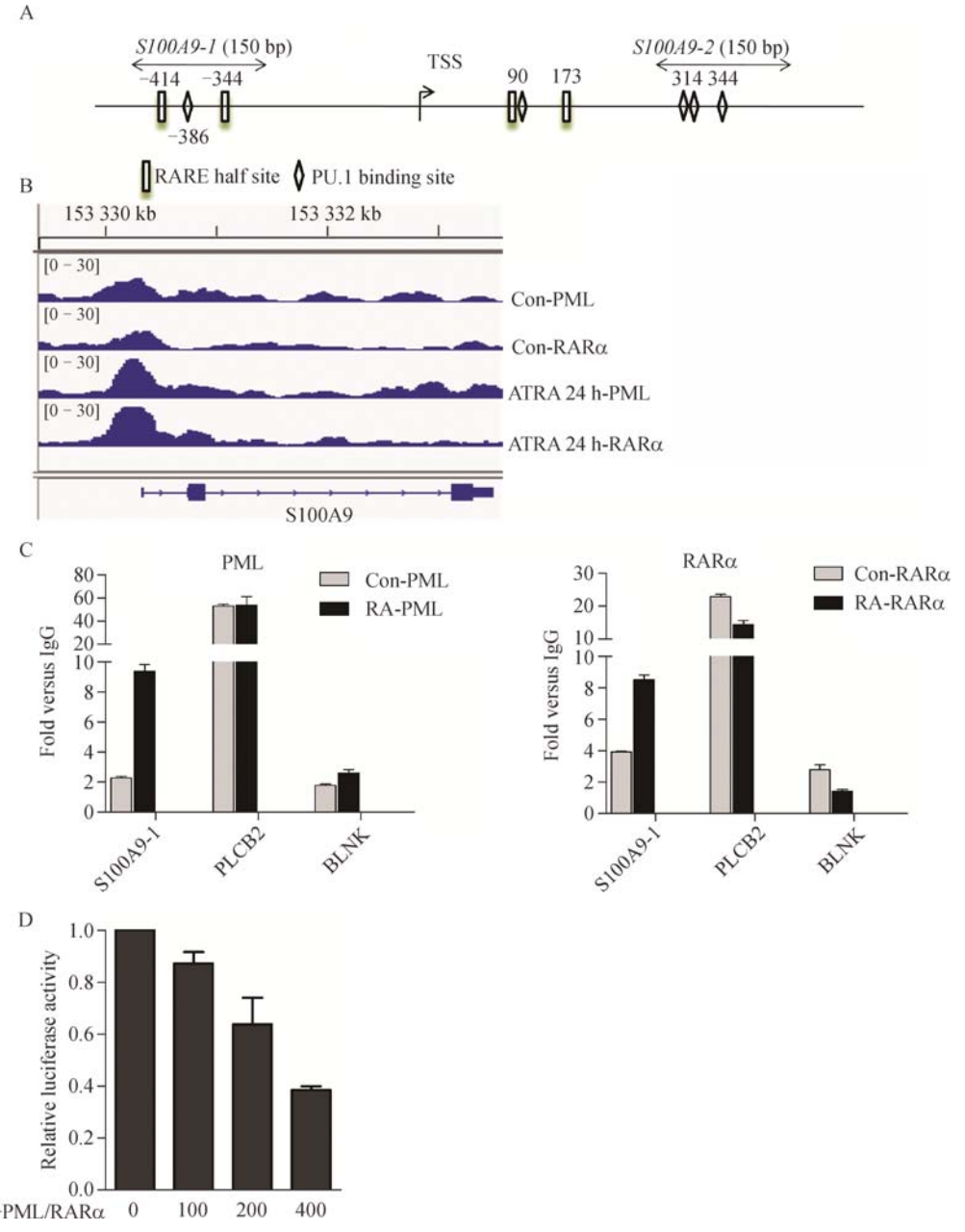

Fig. 2 PML/RARα binds to the promoter of *S100A9* in NB4 cells. (A) Schematic representation of the promoter regions of *S100A9*. PU.1 binding sites (rhombus) and RARE half-sites (rectangle) were defined using TRANSFAC with core and matrix similarity. The *S100A9-1* region spanned PU.1 and RARE sites. The *S100A9-2* region spanned PU.1 sites. (B) Schematic shows the binding of PML/RARα to the promoter regions of *S100A9*. ChIP assays of the NB4 cells were conducted using anti-PML and anti-RARα antibodies. The peaks represented the PML/RARα-enriched ChIP regions. *S100A9* was located on the Watson (+) strand of chromosome 1. (C) ChIP was conducted on NB4 cells using anti-RARα, anti-PML, or nonspecific (normal immunoglobulin G (IgG)) antibodies. ChIP-qPCR was conducted using *S100A9-1* and negative (*BLNK*) or positive (*PLCB2*) primers. Results were presented as fold enrichment of chromatin, normalized to nonspecific IgG. (D) Promoter activities of *S100A9* were repressed by PML/RARα in a dose-dependent manner. Luciferase reporter assays were conducted on 293T cells. The data represent the mean of three replicates ± S.D.

The other set covered the 300 and 450 bp PU.1 sites (*S100A9-2*). As illustrated by the genomic occupancy profiles in Fig. 2B, both PML and RARα could bind the promoter of *S100A9* with or without the presence of ATRA. However, a higher peak of enrichment on the *S100A9* promoter was observed in ATRA-treated cells than in untreated NB4 cells. To verify this finding, we conducted chromatin immunoprecipitation quantitative

real-time PCR (ChIP-qPCR) assays in NB4 cells by using the primers that contained the peak region and used anti-PML, anti-RARα, and nonspecific (IgG) antibodies. As expected, we obtained the same results (Fig. 2C), which further indicated that PML/RARα was necessary for ATRA-induced expression of *S100A9*. To further determine whether *S100A9* transcriptional expression is repressed by PML/RARα, luciferase reporter assays of 293T cells were conducted. We observed that luciferase activity of the *S100A9* promoter declined in a dose-dependent manner after transfection with PML/RARα (Fig. 2D). This behavior explains the low expression of *S100A9* in APL.

PU.1 regulates the basal expression of *S100A9* by targeting the promoter regions of *S100A9*

PU.1, which is highly expressed in granulocytic cells, regulates genes that are essential for myeloid differentiation. As shown in Fig. 2A, PU.1 binding sites were found in the *S100A9* promoter region. Simultaneously, a positive correlation was noted between *S100A9* and PU.1 mRNA levels in a sample of 415 AML patients, including M1–M6 (the expression data downloaded from GSE14468; Fig. 3A). We conducted the following experiments to verify this phenomenon. First, siRNA against the PU.1 experiment was ordered from GenePharma. The data in Fig. 3B showed the interference efficiency of siRNA. Knockdown of PU.1 reduced the ATRA-mediated activation of *S100A9* at the mRNA expression level. Subsequently, we scanned the genomic occupancy profiles (Fig. 3C, left) on the *S100A9* promoter. A high peak of enrichment was observed with ATRA treatment. Our ChIP-qPCR of NB4 cells demonstrated that PU.1 was enriched in the promoter region of *S100A9* and exhibited a slightly higher enrichment fold with ATRA treatment (Fig. 3C, right). This result

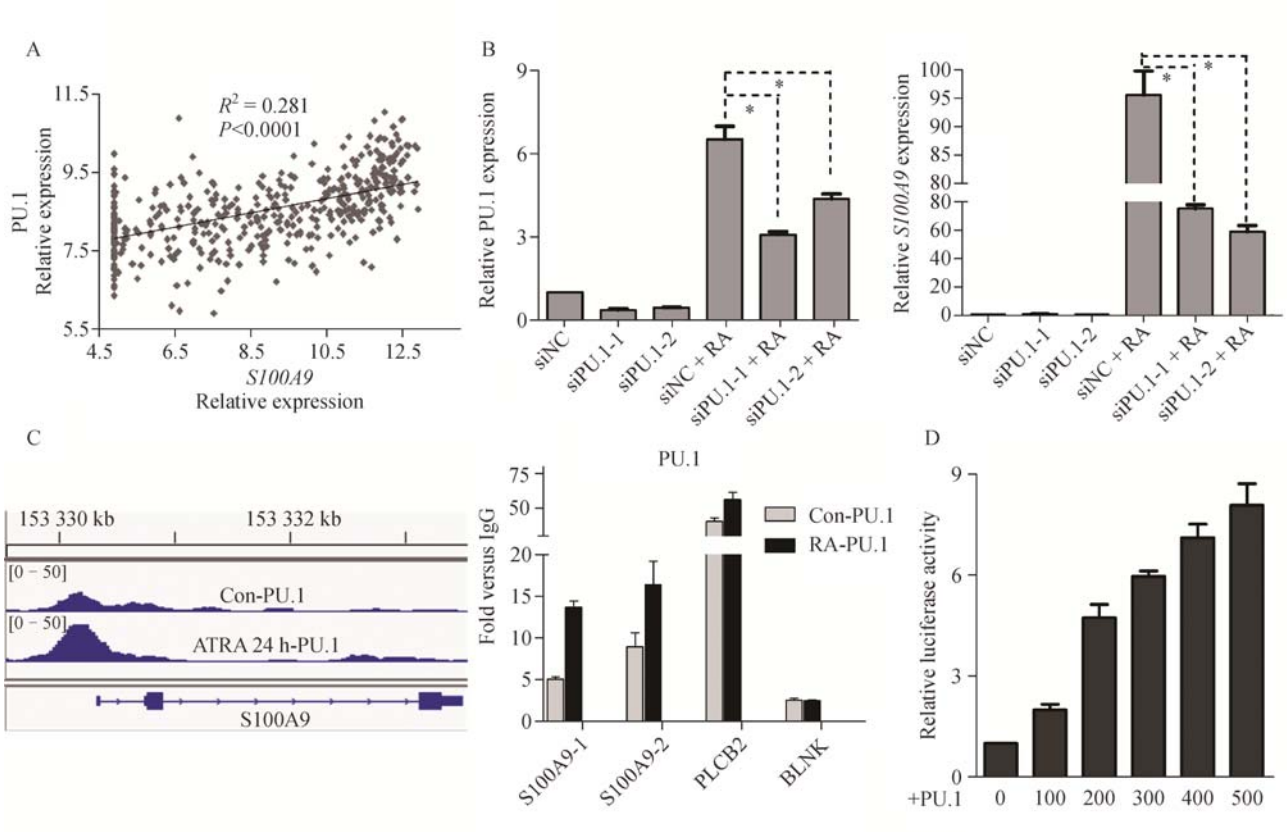

Fig. 3 PU.1 regulates the basal expression of *S100A9* by targeting the promoter regions of S100A9. (A) A positive correlation was found between *S100A9* and PU.1 mRNA levels in 415 AML (M1–M6) patients from GSE14468. (B) Knockdown of PU.1 reduced ATRA-mediated activation of *S100A9* at the mRNA expression level. The mRNA levels of PU.1 and *S100A9* were detected in NB4 cells by RT-PCR at 48 h post-transfection with siRNA (3 μg) and in the absence or presence of ATRA for 24 h. (C) PU.1 bound to the promoter of *S100A9* in APL cells. The pattern on the left is a schematic that shows PU.1 binding to the promoter regions of *S100A9*. ChIP assays of the NB4 cells were conducted using anti-PU.1 antibody. The peaks represent the PU.1-enriched ChIP regions. The image on the right shows the ChIP results of the assays for the NB4 cells that were conducted using anti-PU.1 or IgG antibodies. ChIP-qPCR was undertaken with *S100A9-1*, *S100A9-2*, *BLNK,* and *PLCB2* primers. (D) PU.1 increased the promoter activities of S100A9 in a dose-dependent manner. Luciferase reporter assays were conducted on 293T cells. The data represent the mean of three replicates ± S.D. $*P < 0.05$.

explains why ATRA could induce an increase in *S100A9* expression. To verify our hypothesis that the reductive expression of *S100A9* in APL is possibly mediated by PU.1, we conducted luciferase reporter assays on 293T cells (a non-hematopoietic cell line). As illustrated in Fig. 3D, *S100A9* promoter activity increased with increasing amounts of PU.1 expression plasmid. Collectively, the data indicated that PU.1 targeted *S100A9* and transactivated the promoters of *S100A9*.

Overexpression of *S100A9* induces apoptosis and growth suppression in NB4 cells

A previous study showed that *S100A9* induces cellular apoptosis and growth inhibition in various tumor cells [12]. To verify whether overexpression of *S100A9* is involved in the apoptosis and proliferation of our model cells, we first treated NB4 cells by retroviral transduction to induce high *S100A9* expression (Fig. 4A). We discovered that overexpressed *S100A9* inhibited NB4 cell growth (Fig. 4B). In addition, *S100A9* induced apoptosis. As shown in Fig. 4C, forced expression of *S100A9* increased the early and late total apoptosis rates from 5.64% ± 0.86% to 18.88% ± 1.32% ($P < 0.01$) in NB4 cells. In these experiments, cells with GFP were sorted for analysis to avoid a non-transfected negative background. Simultaneously, overexpressed *S100A9* increased protein expression of cleaved caspase-3 and decreased Bcl-2 protein expression in NB4 cells (Fig. 4D).

Discussion

Extensive studies have been carried out to understand the function and regulatory mechanisms of *S100A9* in cancer.

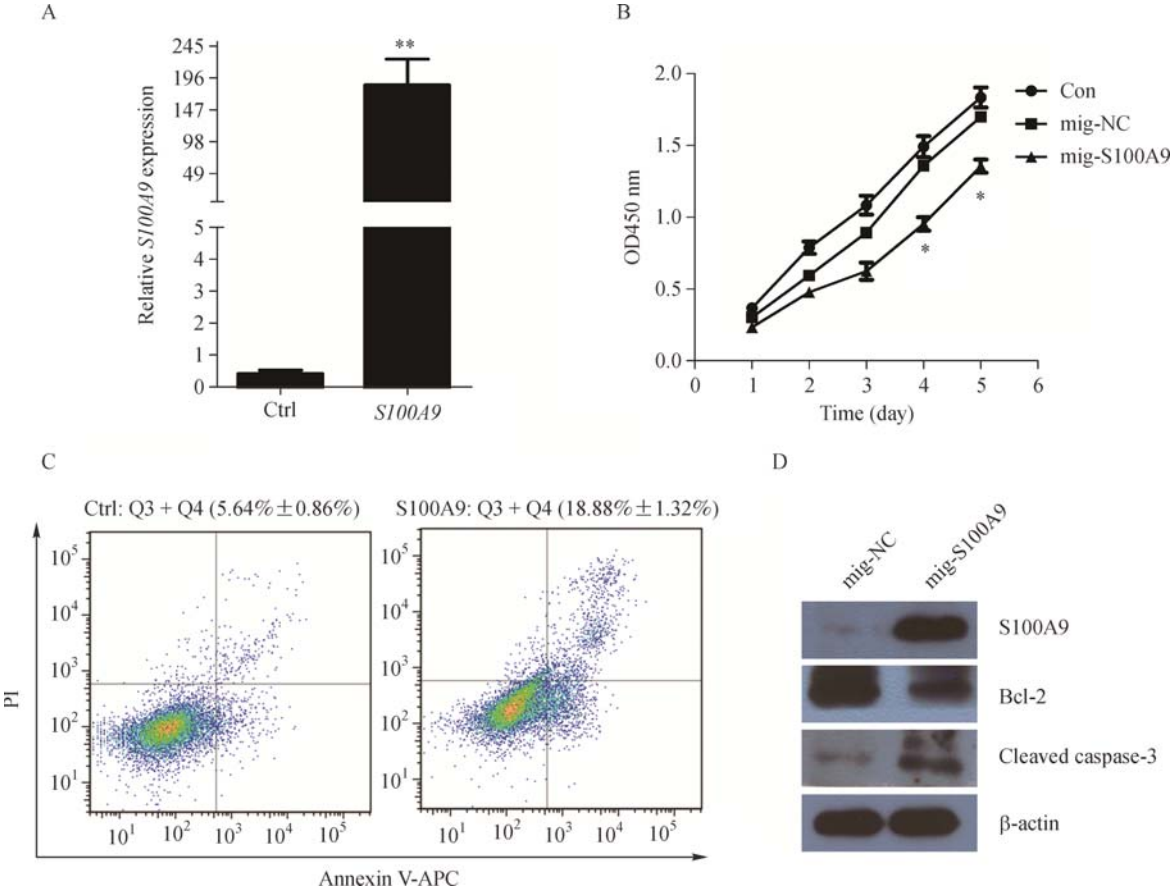

Fig. 4 Overexpression of *S100A9* induces cell apoptosis and growth inhibition in NB4 cells. (A) Relative expression of *S100A9* in NB4 cells that were harvested 72 h after MigR1 (Ctrl) or MigR1-*S100A9* infection. (B) *S100A9* overexpression inhibited the proliferation of NB4 cells. NB4 cells were infected with MigR1 (Ctrl) or MigR1-S100A9 retroviral component, and cell proliferation was detected by CCK-8 assay. Results showed that the proliferation rate of NB4 cells infected with MigR1-S100A9 was reduced significantly at days 4 and 5 when compared with the MigR1 (Ctrl) group and blank group. *$P < 0.05$. (C) Overexpression of *S100A9* induced apoptosis of NB4 cells. GFP-positive cells were analyzed by flow cytometry. The left panel depicts the apoptosis of MigR1-infected NB4 cells, and the right panel shows the result for MigR1-S100A9-infected NB4 cells. The values represent the three replicates ± S.D. (D) Western blot analysis of cleaved caspase-3 and Bcl-2 expression in total cell lysates.

In addition, many studies have revealed that *S100A9* can be used as a biomarker for acute appendicitis, Alzheimer's disease, systemic sclerosis, muscle invasive bladder cancer, and so on [16–19]. However, reports on *S100A9* in relation to leukemia are still relatively few. A previous study showed that *S100A9* is one of the most down-regulated genes in human and murine leukemia samples. *S100A9* is enriched in extracellular space in a GO term and involved in the response to stimulus and defense response [20]. In this study, we observed that *S100A9* expression was not only significantly lower in APL samples than in non-APL samples, but it was also lower in NB4 cells than in U937 and HL-60 cells (Fig. 1). However, the distinct difference between APL and other AML was the existence of PML/RARα, which indicated that PML/RARα restrained *S100A9* activity in APL.

Further experiments revealed that PML/RARα could directly bind to the *S100A9* promoter and repress *S100A9* transcriptional expression (Fig. 2). Similarly, the repression that was induced by PML/RARα could be restored by pharmacological concentrations of ATRA (Fig. 1C). Earlier reports revealed that both IPA and VD3 upregulated *S100A9* in the early stages of differentiation in HL-60 cells [21,22]. These findings implied that *S100A9* was essential for myeloid differentiation. Interestingly, in our study, ATRA alone could not upregulate the expression of *S100A9* in U937-PR9 (Fig. 1D). In addition, a higher fold enrichment of PML/RARα was observed in the *S100A9* promoter of NB4 cells treated with ATRA than in the untreated samples (Fig. 2C). Therefore, PML/RARα was necessary for the ATRA-induced expression of *S100A9*. This condition seems incomprehensible considering that PML/RARα inhibited *S100A9* expression in NB4 cells. We then speculated that PML/RARα may be a ligand that recruits more transcription factors to increase *S100A9* expression during the ATRA-induced differentiation of APL cells. A study has already reported that PML/RARα mediates the differentiation response of APL to ATRA treatment [23]. In addition, inhibiting the degradation of PML/RARα does not prevent ATRA-induced differentiation in APL [24]. Induction of PML/RARα expression can enhance the degree of cell differentiation that is induced by ATRA in PR9 cells [25]. Our data suggested that PML-RARα was involved in the regulation of ATRA-responsive genes in APL cells.

In addition, previous studies have demonstrated that *S100A9* expression can be upregulated by IL-6, IL-8, IL-1α, and P53 [26–28]. However, the vital transcription factors in AML, which are indispensable for *S100A9* basal expression, were not found. A large number of key transcriptional factors, including AML1, CBFβ, C/EBPα, Myb, and PU.1, are involved in granulocytic differentiation of hematopoietic stem cells. PU.1 controls numerous genes that are important for APL differentiation in the ATRA-induced maturation of APL blasts [4,5]. In this study, we showed that the hematopoietic transcription factor PU.1 regulates the basal expression of *S100A9* in APL cells. PU.1 is specifically expressed in hematopoietic cells. Disrupted PU.1 expression can cause terminal differentiation defect in myeloid cells [29]. In this research, we demonstrated that PU.1 targeted *S100A9* and transactivated the promoters of *S100A9* (Fig. 3). We proposed a model to better understand how PML/RARα, PU.1, and ATRA affect the regulatory transcription of *S100A9*. As illustrated in Fig. 5, PML/RARα interacted with PU.1 and inhibited *S100A9* transcription in APL cells. However, when APL cells were treated with ATRA, PML/RARα acted as a ligand and recruited PU.1 and other unidentified transcription factors to activate *S100A9* transcription. Furthermore, in accordance with previous reports stating that *S100A9* promoted apoptosis and inhibited proliferation of cancer cells [12], we discovered that overexpression of *S100A9* induced NB4 cell apoptosis and suppressed proliferation. These results suggested that *S100A9* may serve as a potential molecular therapeutic target of cancers associated with repressed expression of *S100A9*.

The combined treatment of ATRA and As_2O_3 has been widely used for APL patients in clinical settings. Results have achieved very high complete remission rates and long-term outcomes [30]. As_2O_3 is considered the first line of treatment for elderly patients [31]. Previous studies have indicated that the combination of the molecules is better than treatment with ATRA or As_2O_3 alone [32]. Our data demonstrated that ATRA substantially increased the expression of *S100A9*, whereas As_2O_3 slightly affected *S100A9* expression. These results indicated the importance of *S100A9* in APL treatment. Our findings further supported that combination therapy, rather than As_2O_3

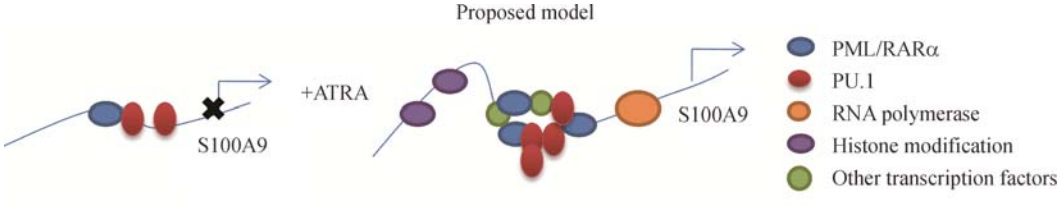

Fig. 5 Possible model for the transcription regulation of *S100A9* is proposed in APL.

alone, is a wise and desirable method for most APL patients.

In conclusion, the present study investigated the transcriptional regulatory mechanism of *S100A9* in APL cells. This study provides a better understanding of how PML/RARα blocks differentiation at the promyelocytic stage. Moreover, we found that PML/RARα and PU.1 regulated *S100A9* transcription during neutrophil differentiation, and overexpressed *S100A9* induced APL cell apoptosis and growth inhibition. Such results indicated that *S100A9* may be a crucial tumor suppressor in APL and a potential therapeutic target in other cancer types with underexpressed *S100A9*. This work adds to our understanding of the basal regulatory mechanism of the S100 family and the role of *S100A9* in the development of carcinomas.

Acknowledgements

This work was supported in part by the National Natural Science Foundation of China (No. 81370655), and the grants from Ministry of Science and Technology of China (Nos. 2013CB966802 and 2012AA02A505). We also greatly appreciated institutional supports from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, China.

Compliance with ethics guidelines

Yonglan Zhu, Fang Zhang, Shanzhen Zhang, Wanglong Deng, Huiyong Fan, Haiwei Wang, and Ji Zhang declare that there is no conflict of interest. This article does not contain any studies with human or animal subjects performed by any of the authors.

Electronic Supplementary Material Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s11684-016-0469-4 and is accessible for authorized users.

References

1. de Thé H, Chen Z. Acute promyelocytic leukaemia: novel insights into the mechanisms of cure. Nat Rev Cancer 2010; 10(11): 775–783
2. Hu J, Liu YF, Wu CF, Xu F, Shen ZX, Zhu YM, Li JM, Tang W, Zhao WL, Wu W, Sun HP, Chen QS, Chen B, Zhou GB, Zelent A, Waxman S, Wang ZY, Chen SJ, Chen Z. Long-term efficacy and safety of all-trans retinoic acid/arsenic trioxide-based therapy in newly diagnosed acute promyelocytic leukemia. Proc Natl Acad Sci USA 2009; 106(9): 3342–3347
3. Wang K, Wang P, Shi J, Zhu X, He M, Jia X, Yang X, Qiu F, Jin W, Qian M, Fang H, Mi J, Yang X, Xiao H, Minden M, Du Y, Chen Z, Zhang J. PML/RARα targets promoter regions containing PU.1 consensus and RARE half sites in acute promyelocytic leukemia. Cancer Cell 2010; 17(2): 186–197
4. Wei S, Zhao M, Wang X, Li Y, Wang K. PU.1 controls the expression of long noncoding RNA HOTAIRM1 during granulocytic differentiation. J Hematol Oncol 2016; 9(1): 44
5. Qian M, Jin W, Zhu X, Jia X, Yang X, Du Y, Wang K, Zhang J. Structurally differentiated cis-elements that interact with PU.1 are functionally distinguishable in acute promyelocytic leukemia. J Hematol Oncol 2013; 6(1): 25
6. Donato R. S100: a multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles. Int J Biochem Cell Biol 2001; 33(7): 637–668
7. Nacken W, Roth J, Sorg C, Kerkhoff C. S100A9/S100A8: myeloid representatives of the S100 protein family as prominent players in innate immunity. Microsc Res Tech 2003; 60(6): 569–580
8. Hunter MJ, Chazin WJ. High level expression and dimer characterization of the S100 EF-hand proteins, migration inhibitory factor-related proteins 8 and 14. J Biol Chem 1998; 273(20): 12427–12435
9. Srikrishna G. S100A8 and S100A9: new insights into their roles in malignancy. J Innate Immun 2012; 4(1): 31–40
10. Salama I, Malone PS, Mihaimeed F, Jones JL. A review of the S100 proteins in cancer. Eur J Surg Oncol 2008; 34(4): 357–364
11. Khammanivong A, Wang C, Sorenson BS, Ross KF, Herzberg MC. S100A8/A9 (calprotectin) negatively regulates G2/M cell cycle progression and growth of squamous cell carcinoma. PLoS ONE 2013; 8(7): e69395
12. Yui S, Nakatani Y, Mikami M. Calprotectin (S100A8/S100A9), an inflammatory protein complex from neutrophils with a broad apoptosis-inducing activity. Biol Pharm Bull 2003; 26(6): 753–760
13. Kuwayama A, Kuruto R, Horie N, Takeishi K, Nozawa R. Appearance of nuclear factors that interact with genes for myeloid calcium binding proteins (MRP-8 and MRP-14) in differentiated HL-60 cells. Blood 1993; 81(11): 3116–3121
14. Tomasson MH, Xiang Z, Walgren R, Zhao Y, Kasai Y, Miner T, Ries RE, Lubman O, Fremont DH, McLellan MD, Payton JE, Westervelt P, DiPersio JF, Link DC, Walter MJ, Graubert TA, Watson M, Baty J, Heath S, Shannon WD, Nagarajan R, Bloomfield CD, Mardis ER, Wilson RK, Ley TJ. Somatic mutations and germline sequence variants in the expressed tyrosine kinase genes of patients with *de novo* acute myeloid leukemia. Blood 2008; 111(9): 4797–4808
15. Grignani F, Ferrucci PF, Testa U, Talamo G, Fagioli M, Alcalay M, Mencarelli A, Grignani F, Peschle C, Nicoletti I, Pelicci PG. The acute promyelocytic leukemia-specific PML-RAR α fusion protein inhibits differentiation and promotes survival of myeloid precursor cells. Cell 1993; 74(3): 423–431
16. Bealer JF, Colgin M. S100A8/A9: a potential new diagnostic aid for acute appendicitis. Acad Emerg Med 2010; 17(3): 333–336
17. Horvath I, Jia X, Johansson P, Wang C, Moskalenko R, Steinau A, Forsgren L, Wågberg T, Svensson J, Zetterberg H, Morozova-Roche LA. Pro-inflammatory S100A9 protein as a robust biomarker differentiating early stages of cognitive impairment in Alzheimer's disease. ACS Chem Neurosci 2016; 7(1): 34–39
18. van Bon L, Cossu M, Loof A, Gohar F, Wittkowski H, Vonk M, Roth J, van den Berg W, van Heerde W, Broen JC, Radstake TR. Proteomic analysis of plasma identifies the Toll-like receptor agonists S100A8/A9 as a novel possible marker for systemic sclerosis phenotype. Ann Rheum Dis 2014; 73(8): 1585–1589

19. Kim WT, Kim J, Yan C, Jeong P, Choi SY, Lee OJ, Chae YB, Yun SJ, Lee SC, Kim WJ. S100A9 and EGFR gene signatures predict disease progression in muscle invasive bladder cancer patients after chemotherapy. Ann Oncol 2014; 25(5): 974–979
20. Li Z, Luo RT, Mi S, Sun M, Chen P, Bao J, Neilly MB, Jayathilaka N, Johnson DS, Wang L, Lavau C, Zhang Y, Tseng C, Zhang X, Wang J, Yu J, Yang H, Wang SM, Rowley JD, Chen J, Thirman MJ. Consistent deregulation of gene expression between human and murine MLL rearrangement leukemias. Cancer Res 2009; 69(3): 1109–1116
21. Ishii Y, Kasukabe T, Honma Y. Immediate up-regulation of the calcium-binding protein S100P and its involvement in the cytokinin-induced differentiation of human myeloid leukemia cells. Biochim Biophys Acta 2005; 1745(2): 156–165
22. Roth J, Goebeler M, van den Bos C, Sorg C. Expression of calcium-binding proteins MRP8 and MRP14 is associated with distinct monocytic differentiation pathways in HL-60 cells. Biochem Biophys Res Commun 1993; 191(2): 565–570
23. Wang JG, Barsky LW, Davicioni E, Weinberg KI, Triche TJ, Zhang XK, Wu L. Retinoic acid induces leukemia cell G1 arrest and transition into differentiation by inhibiting cyclin-dependent kinase-activating kinase binding and phosphorylation of PML/RARα. FASEB J 2006; 20(12): 2142–2144
24. Nervi C, Ferrara FF, Fanelli M, Rippo MR, Tomassini B, Ferrucci PF, Ruthardt M, Gelmetti V, Gambacorti-Passerini C, Diverio D, Grignani F, Pelicci PG, Testi R. Caspases mediate retinoic acid-induced degradation of the acute promyelocytic leukemia PML/RARα fusion protein. Blood 1998; 92(7): 2244–2251
25. Grignani F, Gelmetti V, Fanelli M, Rogaia D, De Matteis S, Ferrara FF, Bonci D, Grignani F, Nervi C, Pelicci PG. Formation of PML/RAR alpha high molecular weight nuclear complexes through the PML coiled-coil region is essential for the PML/RARα-mediated retinoic acid response. Oncogene 1999; 18(46): 6313–6321
26. Kim JH, Oh SH, Kim EJ, Park SJ, Hong SP, Cheon JH, Kim TI, Kim WH. The role of myofibroblasts in upregulation of S100A8 and S100A9 and the differentiation of myeloid cells in the colorectal cancer microenvironment. Biochem Biophys Res Commun 2012; 423(1): 60–66
27. Bando M, Zou X, Hiroshima Y, Kataoka M, Ross KF, Shinohara Y, Nagata T, Herzberg MC, Kido J. Mechanism of interleukin-1α transcriptional regulation of *S100A9* in a human epidermal keratinocyte cell line. Biochim Biophys Acta 2013; 1829(9): 954–962
28. Li C, Chen H, Ding F, Zhang Y, Luo A, Wang M, Liu Z. A novel p53 target gene, *S100A9*, induces p53-dependent cellular apoptosis and mediates the p53 apoptosis pathway. Biochem J 2009; 422(2): 363–372
29. McKercher SR, Torbett BE, Anderson KL, Henkel GW, Vestal DJ, Baribault H, Klemsz M, Feeney AJ, Wu GE, Paige CJ, Maki RA. Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. EMBO J 1996; 15(20): 5647–5658
30. Chau D, Ng K, Chan TSY, Cheng YY, Fong B, Tam S, Kwong YL, Tse E. Azacytidine sensitizes acute myeloid leukemia cells to arsenic trioxide by up-regulating the arsenic transporter aquaglyceroporin 9. J Hematol Oncol 2015; 8(1): 46
31. Zhang Y, Zhang Z, Li J, Li L, Han X, Han L, Hu L, Wang S, Zhao Y, Li X, Zhang Y, Fan S, Lv C, Li Y, Su Y, Zhao H, Zhang X, Zhou J. Long-term efficacy and safety of arsenic trioxide for first-line treatment of elderly patients with newly diagnosed acute promyelocytic leukemia. Cancer 2013; 119(1): 115–125
32. Shen ZX, Shi ZZ, Fang J, Gu BW, Li JM, Zhu YM, Shi JY, Zheng PZ, Yan H, Liu YF, Chen Y, Shen Y, Wu W, Tang W, Waxman S, De Thé H, Wang ZY, Chen SJ, Chen Z. All-trans retinoic acid/As_2O_3 combination yields a high quality remission and survival in newly diagnosed acute promyelocytic leukemia. Proc Natl Acad Sci USA 2004; 101(15): 5328–5335