

# A minicircuitry comprised of microRNA-9 and SIRT1 contributes to leukemogenesis in t(8;21) acute myeloid leukemia

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**Abstract.** – **OBJECTIVE:** The AML1-ETO fusion protein (AE) resulting from the t(8;21) translocation is highly related to the pathogenesis and development of leukemia. microRNA-9 (miR-9) acts as a tumor suppressor gene in AE-positive acute myeloid leukemia (AML). Silent mating type information regulation 2 homolog-1 (SIRT1) is overexpressed in most cancer cells by increasing proliferation as a tumorigenic gene. The present study was performed to investigate the underlying interaction between miR-9 and SIRT1 in AE-positive AML.

**PATIENTS AND METHODS:** Expression of miR-9 and SIRT1 in AE-positive AML patients, healthy donors and AML cell lines were detected by qPCR. Relevance between miR-9 and SIRT1 was assessed by plasmid transfection, Western blot and correlation analysis. Luciferase assay was used to confirm the target gene of miR-9. Knockdown of SIRT1 in different cell lines was achieved by shRNA transfection and CCK-8 assay was used to investigate the effects on cell proliferation.

**RESULTS:** The miR-9 expression was lower in AE-positive cell lines compared to that in other AE-negative AML cell lines, while expression of SIRT1 was higher in AE-positive cell lines. Expression of miR-9 was also downregulated in adult primary t(8;21) AML patients compared to healthy donors. The over-expression of miR-9 decreased luciferase activity of wild-type SIRT1, which was recovered after transfection with mutant SIRT1. The miR-9 directly targets SIRT1 by binding to its 3'-untranslated region and reducing its protein levels. Importantly, miR-9 and SIRT1 mRNA levels were inversely correlated in AE-positive AML cell lines and t(8;21) AML primary leukemia cells. Knockdown of SIRT1 lev-

els using shSIRT1 inhibited cell proliferation in AE-positive AML cell lines.

**CONCLUSIONS:** SIRT1 is the target gene of miR-9 and the signaling pathway connecting miR-9 and SIRT1 is a therapeutic target for t(8;21) AML.

**Key Words:**

MicroRNA, SIRT1, Leukemogenesis, t(8;21), Acute myeloid leukemia.

## Introduction

Presently, cytogenetic aberrations in acute myeloid leukemia (AML) are studied in depth and are considered as the most important prognostic factors for AML patients<sup>1</sup>. The t(8;21)(q22;q22) translocation is one of the most common chromosomal abnormalities in AML, resulting in fusion of AML1 (also known as RUNX1) with ETO (also known as RUNX1T1)<sup>2</sup>. The AML1-ETO fusion protein (AE) is believed to be a key factor in AML pathogenesis but is not sufficient to induce leukemic transformation alone<sup>3,4</sup>. The concept is also supported by the clinical observation that AE transcript is also detectable in healthy individuals. Some additional genetic or epigenetic abnormalities probably favor the development of t(8;21) AML.

The deregulated microRNA (miRNA) expression may contribute to leukemogenesis by perturbing post-transcriptional gene regulation by interference with mRNAs<sup>5</sup>. Some miRNAs, including miR-181 and miR-212 have been shown

to be related to prognosis of adult AML patients<sup>6,7</sup>. Further functional studies revealed their important role in regulating hematopoietic differentiation and leukemic transformation<sup>8</sup>. Also, our previous studies<sup>9</sup> showed that heterochromatic silencing of miR-193a contributes to the AE-triggered leukemogenesis. miR-9 was also suggested to be related to prognosis of AML patients<sup>10</sup> and miR-9 expression is downregulated in t(8;21) AML cell lines<sup>11</sup>. Our previous work<sup>12</sup> demonstrated that miR-9 acted as a tumor suppressor in t(8;21) AML by inhibiting cell proliferation, inducing apoptosis, and promoting cell differentiation. However, the detailed mechanism remains to be studied.

Some histone deacetylase inhibitors (HDACis) have been used in clinical trials as anti-proliferative agents against leukemic cells<sup>13</sup>. HDACis also cause a robust anti-leukemic response in AML mouse model driven by AML1/ETO9a expression<sup>14</sup>. Silent mating type information regulation 2 homolog-1 (SIRT1, Sirtuin 1) is a class III HDAC that plays important roles in cell proliferation, differentiation, and gene expression. SIRT1 is a tumor-suppressor factor in normal cells but is overexpressed in most cancer cells and acts as a tumorigenic gene by increasing proliferation<sup>15,16</sup>. We have demonstrated that SIRT1 is highly expressed in t(8;21) AML cell lines and its transcription is regulated by AE<sup>17</sup>.

However, whether there is a relationship between SIRT1 and miR-9, as well as their effects on leukemogenesis in t(8; 21), AML is still unclear. In this study, we found that miR-9 directly targeted SIRT1 and affected SIRT1 expression, which positively influenced the growth of AE-positive AML cells.

## Patients and Methods

### Cell Lines and Cell Culture

The human leukemia cell lines Kasumi-1, HL-60, KG-1, NB4, K562, MV4-11, HEL, THP1, U937, and U937-A/E were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS, Gibco, Grand Island NY, USA), 50 mg/mL streptomycin, and 50 IU penicillin. The U937-A/E cell line was a gift from Dr. Clara Nervi (Sapienza University, Rome, Italy) and was treated with 100  $\mu$ M ZnSO<sub>4</sub> for 16 h to induce AE expression. The human AE-positive AML cell line SKNO-1 was cultured in RPMI-1640 medium supplemented

with 10% FCS and 10  $\mu$ g/L granulocyte-macrophage colony-stimulating factor (PeproTech, London, UK). We used pRRLcPPT.hPGK to infect SKNO-1 and silence AE in SKNO-1 cells. The lentiviral vector described previously encoded the siAGF1 oligonucleotides (siAE-RNAs, sense, 5'-CCUCGAAAUCCGUACUGAGAAG-3', antisense, 5'-UCUCA-GUACGAUUUCGAGGUU-3') against the AE mRNA fusion site<sup>9</sup>. Human embryonic kidney 293T cells were maintained in DMEM (Invitrogen) supplemented with 10% FCS.

### Patients

Bone marrow samples for quantitative reverse-transcription polymerase chain reaction were obtained from AE-positive AML patients and healthy donors. The project was approved by the Human Investigation Ethics Committee of the Chinese PLA General Hospital, and informed consent was obtained from all patients before the study.

### RNA extraction and Quantitative Reverse-transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated with TRIzol reagent (Invitrogen). cDNA was synthesized using the Omniscript RT kit 200 (Cat. No. 205113, Qiagen, Hilden, Germany). The relative quantity of miR-9 was measured on 200 ng of total RNA by qRT-PCR using the Hairpin-it miRNA qRT-PCR Quantitation kit (Genepharma, Shanghai, China) in the ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA), and it was determined by the comparative CT method using snRNA U6 levels for normalization according to the manufacturer's instructions. Expression of SIRT1 was quantified by SYBR Green qRT-PCR analysis (TaKaRa, Otsu, Shiga, Japan) on a Mx3000p light cycler (Stratagene). GAPDH served as the control. The primer sequences are shown in Table I.

### miRNAs, shRNAs, and Transfection

The miR-9 mimic and nontargeting control (NC) were synthesized by RiboBio (Guangzhou, China). Short hairpin RNAs (shRNAs) against SIRT1 and nontargeting control were also obtained from RiboBio. miRNA and shRNA cell transfection was performed using the Hiperfect Transfection Reagent (Qiagen, Hilden, Germany), according to the manufac-

**Table I.** Primer sequences used in this study.

Names	Sequence from 5' to 3'	Product size
<b>qRT-PCR</b>		
miR-9-F	CGACGCCTCTTTGGTTATCTAG	75 nt
miR-9-R	TATGGTTGTTACGACTCCTTCAC	
miR-9-Probe	TATGAGTCTGTATGGTTGGATAGGGAT	70 nt
U6-F	ATTGGAACGATACAGAGAAGATT	
U6-R	GTTTAAGCACTTCGCAAGG	
U6- Probe	TGCGCAAGGATGACACGCA	222 nt
SIRT1-F	TATGCTCGCCTTGCTGTAGA	
SIRT1-R	AACCTGTTCCAGCGTGTCTA	238 nt
GAPDH-F	GAGTCAACGGATTTGGTCGT	
GAPDH-R	TTGATTTTGGAGGGATCTCG	
<b>Full-length miR-9 for transfection</b>		
miR-9-F	CGGGGTACCTGTCCCTTCCCTCCTACTCC	300 nt
miR-9-R	CCGGAATTCATCCTCTGGTGCTGGTCAGT	
<b>3'UTR Assay</b>		
SIRT1-3'UTR-F	AACTGCAGATTGTTCCACCAGCATTAGGA	1426 nt
SIRT1-3'UTR-R	CGGAATTCTGGCAGTAATGGTCCTAGCT	
SIRT1-3'UTR-M-F	AAAATACCATACATAGCTCTAGATTAGC	
SIRT1-3'UTR-M-R	GCTAATCTAGAGCTATGTATGGTATTTT	

turer's instructions. Cells were seeded in a 10-cm dish for RNA and protein extraction, in a 24-well plate for the luciferase reporter assays, and in a 96-well plate for the Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan).

### **3'-Untranslated Region (3'-UTR) Luciferase Assay**

The wild-type human SIRT1 3'-UTR fragment containing the predicted miR-9 target sites was amplified by PCR from human genomic DNA. 3'-UTR with mutations introduced into the putative miR-9 binding site was generated by overlap extension PCR. The primer sequences are shown in Table I. All DNA fragments were inserted between the PstI and EcoRI sites of the pGL3-LUC reporter vector (Promega, Madison, WI, USA). The authenticity of the inserts was confirmed by sequencing. The constructs were co-transfected into HEK293T cells ( $5 \times 10^4$ ) along with the mimic, nontargeting control, or the miR-9 mimic using the SuperFect reagent (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The pRL-TK Renilla luciferase reporter vector (Promega, Madison, WI, USA) served as a control. Cells were collected 48 h after transfection, and luciferase activity was measured using a dual-luciferase reporter assay system (Promega, Madison, WI, USA).

### **Western Blot Analysis**

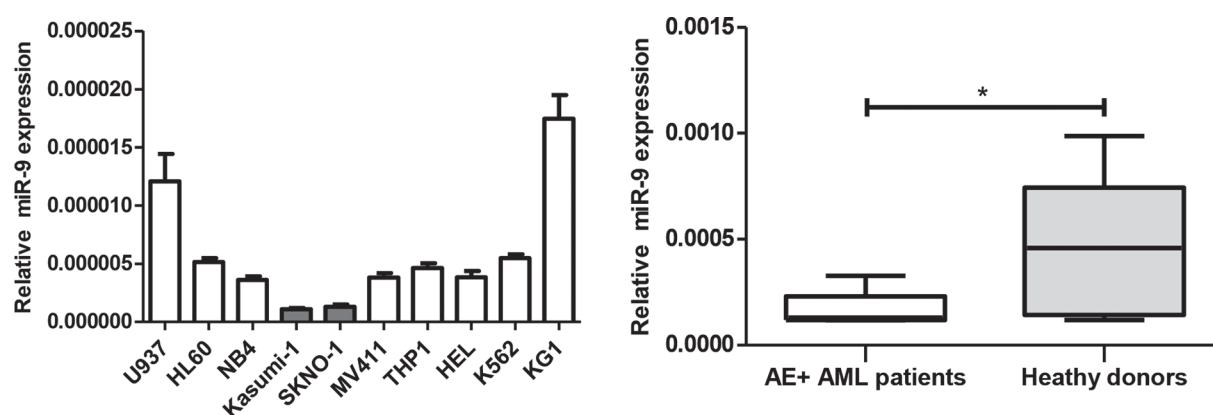
The total protein was extracted from cells using radio-immunoprecipitation assay buffer (Sigma-Aldrich, St. Louis, MO, USA), and protein expression was analyzed by Western blot. The following antibodies were used: SIRT1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).  $\beta$ -actin (C4), anti-mouse IgG horseradish peroxidase (HRP), and anti-rabbit IgG HRP (Santa Cruz Biotechnology, Dallas, TX, USA).

### **Cell Proliferation**

The number of viable cells was assessed using a CCK-8 (Cell Counting Kit-8) assay (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. After 6, 24, 48, or 72 h of transfection, cells were incubated in RPMI-1640 medium containing CCK-8 for another 3 h at 37°C. The optical density (OD) value of each well was read at 450 nm using an automated microplate reader (Sunrise, Tecan, Switzerland).

### **Statistical Analysis**

SPSS 17.0 for Windows software (SPSS Inc., Chicago, IL, USA) was used to process the data. Results are expressed as means  $\pm$  standard deviation from at least three separate experiments. Student's *t*-test was performed to detect differences in the experimental results, and a *p*-value  $\leq 0.05$  was considered significant.



**Figure 1.** miR-9 levels in cell lines and primary samples. **(A)** Relative qRT-PCR quantification of miR-9 levels in the indicated leukemia cell lines. **(B)** Relative qRT-PCR quantification of miR-9 levels in mononucleated cells (MNC) isolated from 12 t(8;21) AML patients and 10 healthy donors. t(8;21) AML patients had lower miR-9 expression levels ( $p < 0.05$ ). GAPDH was used as a control.

## Results

### *miR-9 is Downregulated in AE-positive Leukemia cell Lines and Patients*

miR-9 plays an important role in AML, and high miR-9 expression is a poor predictor of survival in some adult patients with AML, such as those with MLL-rearranged AML<sup>18</sup>. However, miR-9 acts as a tumor suppressor gene in t(8;21) AML<sup>7</sup>, indicating that miR-9 plays different roles in different subgroups of AML. To address this question, we detected miR-9 expression levels by qRT-PCR in 10 AML cell lines. As shown in Figure 1a, miR-9 expression was much lower in the Kasumi-1 and SKNO-1 cell lines, in which AE was positive. To further confirm that miR-9 expression in adult patients with AML coincided with cell lines results, we studied a cohort of patients with AE-positive AML and healthy donors. As the result showed, miR-9 expression was downregulated in patients with AE-positive AML compared with that in healthy donors (Figure 1b).

### *SIRT1 is a miR-9 Target Gene Regulated directly by miR-9*

Our previous work<sup>12</sup> demonstrated that AE triggers heterochromatic silencing of miR-9 and that miR-9 functions as a tumor suppressor gene in AE-positive cell lines *in vitro*. We used three computational algorithms, miRanda, TargetScan, and miRBase, to identify miR-9 target genes and determine the mechanism of its tumor suppressive function in t(8;21) AML. The data revealed evolutionarily conserved se-

quences in SIRT1 mRNA (Figure 2a), which we had found was upregulated in t(8; 21) AML cell lines and patients<sup>19</sup>.

The 1.426 bp human SIRT1 3'-UTR was subcloned and cotransfected into 293T cells with either a miR-9 expression plasmid or the pcDNA3.0 empty vector to validate the computational data. Cells cotransfected with miR-9 showed significantly reduced luciferase activity in the wild-type SIRT1 3'-UTR compared with those cotransfected with the pcDNA3.0 empty vector; however, luciferase activity was recovered by the mutant SIRT1 3'-UTR (Figure 2b).

### *SIRT1 is Inversely Correlated with miR-9 in AE-Positive Leukemic Cells*

We verified endogenous SIRT1 expression in t(8;21) AML cells to confirm that miR-9 regulates SIRT1 in t(8;21) AML cells. The synthetic miR-9 mimic or the mimic non-targeting control was transfected into two human t(8;21) AML cell lines (Kasumi-1 and SKNO-1). SIRT1 protein expression decreased significantly along with ectopic miR-9 expression in Kasumi-1 and SKNO-1 cells (Figure 2c). Taken together, our results demonstrate that miR-9 regulates SIRT1 expression by targeting its putative binding sites in t(8; 21) AML cells.

miR-9 expression was downregulated in AE-positive AML cell lines. To validate the correlation between SIRT1 expression and miR-9 in t(8;21) AML cells, we further detected SIRT1 mRNA and expression levels in a set of leukemic cell lines. As shown in Figure 2d, SIRT1 was expressed at a higher level in AE-positive leukemia



cell lines than that in AE-negative cell lines, which was inversely correlated with miR-9 expression. Importantly, relative SIRT1 expression levels were inversely correlated with those of miR-9 in a cohort of primary AE-positive AML samples (Figure 2e). This correlation further confirms regulation of SIRT1 by miR-9 in t(8;21) AML.

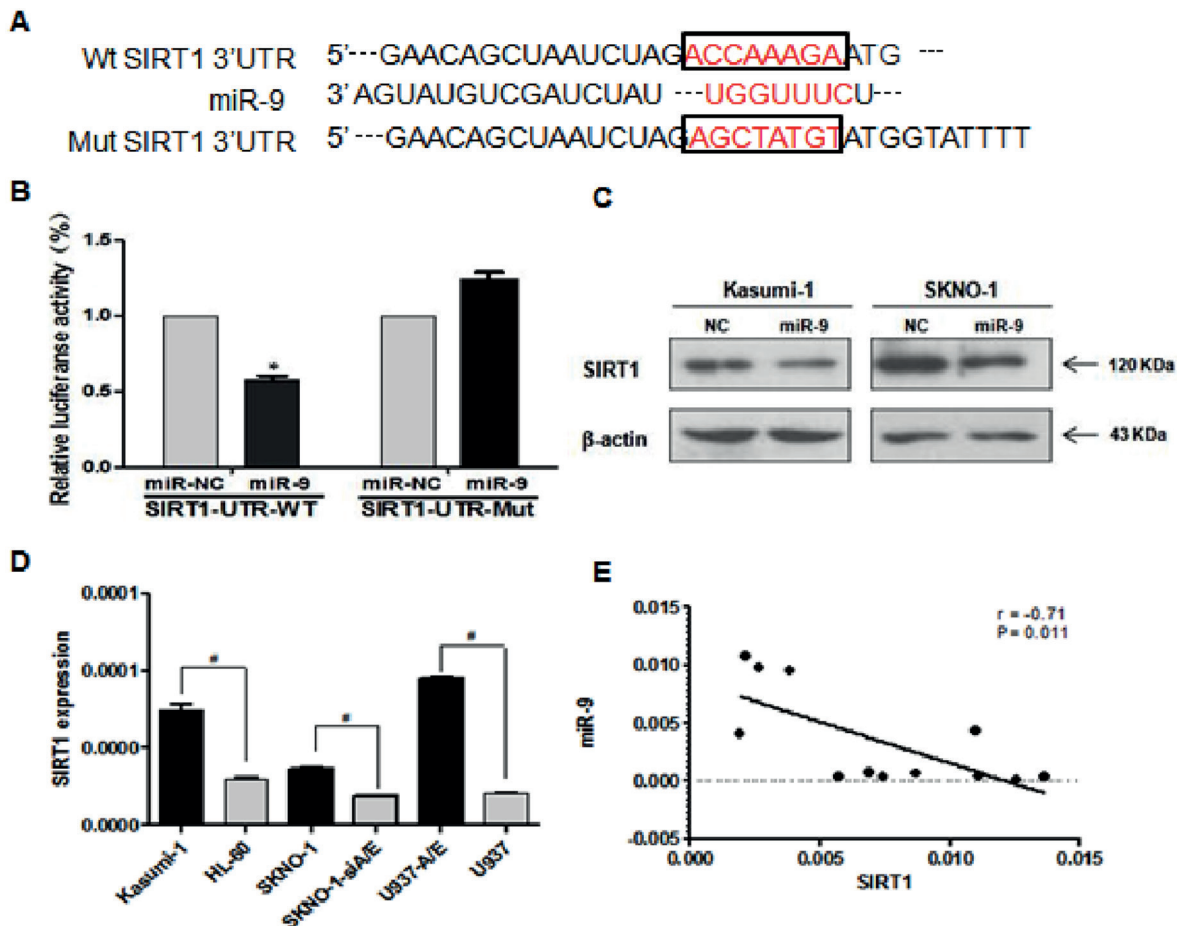
### Knockdown of SIRT1 inhibited Proliferation of AE-positive AML Cell Lines

To further investigate the effects of mimicry of miR-9 and SIRT1 on leukemogenesis in t(8; 21) AML, we used shSIRT1 transfection

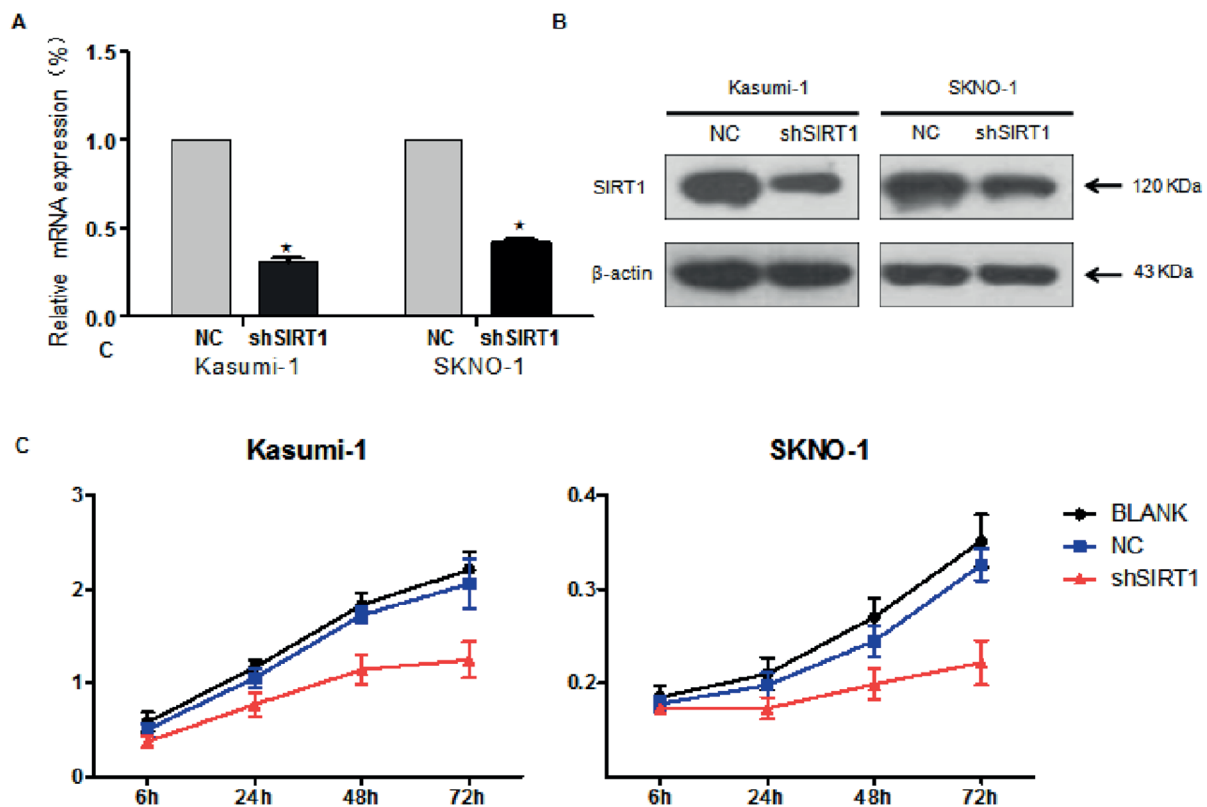
to knockdown the expression of SIRT1 in AE-positive cell lines. As shown in Figure 3a and 3b, mRNA and protein of SIRT1 were significantly knockdown after shSIRT1 transfection. The CCK-8 assay showed decreased cell proliferation in shSIRT1 transfected cells (Figure 3c).

### Discussion

In this study, we found that miR-9 expression was lower in AE-positive cell lines than that in other AE-negative AML cell



**Figure 2.** SIRT1 was a direct target of miR-9 and inversely correlated with miR-9. (A) The potential miR-9 binding site of SIRT1-3'UTR and the mutated sequences. (B) miR-9 expressing plasmids and pCDNA3.0 control plasmids were co-transfected with a contained wild-type SIRT1 or mutant luciferase pGL-3 control vector for 48h, the results was analyzed using Luciferase assays. (C) Expression of SIRT1 protein was detected by Western blot in Kasumi-1 and SKNO-1 cells transfected with miR-9 mimics or miR-NC.  $\beta$ -actin was used as an internal control. (D) Relative qRT-PCR quantification of SIRT1 levels in three pairs of cell lines: Kasumi-1 and HL-60, SKNO-1 (wild type and si-AE), U937 (wild type and A/E). GAPDH was used as a control. ( $\#p < 0.0001$ ) (E) Relative qRT-PCR quantification of SIRT1 levels was plotted against that of miR-9 in 12 primary t(8;21) AML samples. Plotting of miR-9 vs. SIRT1 expression shows an inverse correlation between them. ( $r = -0.71$ ,  $p = 0.011$ ).



**Figure 3.** Knockdown of SIRT1 by shSIRT1 inhibited proliferation of AE positive AML cell lines. **(A)** Efficient knockdown of SIRT1 in Kasumi-1 and SKNO-1 cells was confirmed by Relative qRT-PCR quantification. GAPDH was used as a control (\* $p < 0.05$ ). **(B)** Expression of SIRT1 protein was detected by Western blot after knockdown of SIRT1 in Kasumi-1 and SKNO-1 cells.  $\beta$ -actin was used as an internal control. **(C)** Knockdown of SIRT1 induced growth inhibition in AE positive cell lines Kasumi-1 and SKNO-1 which was detected by CCK-8 at the indicated times; means  $\pm$  SD of 3 independent experiments.

lines while SIRT1 expression was higher in AE-positive cell lines. We further confirmed that miR-9 expression was also downregulated in adult primary t(8;21) AML cells compared with that in healthy controls. Besides, miR-9 and SIRT1 mRNA levels were inversely correlated in AE-positive AML cell lines and t(8;21) AML primary leukemia cells. We confirmed that miR-9 directly targets SIRT1 by binding to its 3'-untranslated region, reducing its protein levels. Therefore, SIRT1 expression induced by low miR-9 levels may be involved in the development of t(8;21) AML. The CCK-8 assay suggested that knockdown of SIRT1 levels inhibited cell proliferation in AE-positive AML cell lines.

The tightly controlled miR-9 expression is very important for cellular integrity for miR-9 is encoded in three locations in the human genome and highly conserved among species. In our cohort of adult AML samples, miR-9

expression was significantly lower in t(8;21) positive AML compared with healthy controls. It was consistent with the report that miR-9 expression is downregulated in pediatric patients with t(8;21) AML using microRNA screening tools<sup>11</sup>. In contrast, previous literature<sup>10</sup> suggested that high expression of miR-9 is a predictor of poor survival in adult AML. Although miR-9 expression was not an independent prognostic factor in multivariate analysis in AML, we clearly suggested that ectopic expression of miR-9 in AML cell lines and primary AE-positive patient samples conferred a tumor-suppressive phenotype<sup>12</sup>. Several miR-9 target genes have been identified in previous studies, including FoxO3 and FoxO1 in hematopoietic cells<sup>20</sup>, PRDM1, E2F1, FGFR1, and CDK6 in lymphoid malignancies<sup>21-23</sup>, BRCA1 in ovarian cancer<sup>24</sup>, HMGA2 and LIN28B in AML<sup>11</sup>, RYBP and RHOH in MLL-AML (19). Moreover, miR-9 could directly target and

downregulate SIRT1 in pancreatic  $\beta$ -cells<sup>18</sup>, which is consistent with the results that miR-9 and SIRT1 mRNA expression were inversely correlated in the t(8;21) AML cell lines in the present paper.

Sirtuins are NAD<sup>+</sup>-dependent class III HDACs. The mammalian sirtuin family consists of seven members (SIRT1-7), of which SIRT1 is the most studied and plays a pivotal role in many physiological processes, such as aging, metabolism, apoptosis, DNA repair and tumorigenesis<sup>20,25,26</sup>. SIRT1 can act as tumor suppressor or tumor promoter depending on the cellular and genetic context. The dual roles of SIRT1 in tumorigenesis may depend on its organ distribution or upstream and downstream effectors. Some studies have suggested that SIRT1 functions in the pathogenesis and development of hematological malignancies. SIRT1 is overexpressed in adult T-cell leukemia, diffuse large B-cell lymphoma, chronic lymphocytic leukemia, and chronic myeloid leukemia (CML)<sup>26-28</sup>. Inhibiting SIRT1 also suppresses BCR/ABL transformation, increases its sensitivity to tyrosine kinase inhibitors (TKIs), and even enhances eradication of CML leukemia stem cells when combined with TKIs<sup>8,29,30</sup>. Sasca et al<sup>31</sup> showed that SIRT1 protein, but not RNA, was highly expressed in AML harboring receptor tyrosine kinases (RTKs) mutations or RAS-GTPases mutations depending on aberrant FLT3 tyrosine kinase activity. These findings suggested that expression of SIRT1 may be facilitated by particular genetic abnormalities. In the present study, we showed that SIRT1 mRNA is overexpressed in t(8;21) AML cell lines. SIRT1 inhibitor decreased cell proliferation in the AE-positive cell lines, indicating that SIRT1 may act as a tumor promoter in t(8;21) AML. Inhibiting SIRT1 may restore p53 activity and render leukemic blasts sensitive to TKIs or chemotherapeutic agents. Our data also indicated that targeting SIRT1 is an attractive therapeutic strategy for t(8;21) AML.

## Conclusions

We revealed that miR-9 directly targeted and regulated SIRT1, which is an oncogene in t(8;21) AML. The minicircuitry comprised of miR-9 and SIRT1 expression affected the growth of t(8;21) AML cells and may be asso-

ciated with regulating of t(8;21) AML development. Meanwhile, inhibiting SIRT1 expression decreased the growth of t(8;21) AML cells, suggesting it may be a potential therapeutic target for t(8;21) AML.

## Conflict of interest

The authors declare no conflicts of interest.

## Acknowledgments

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