Original Article
MiR-9 functions as a tumor suppressor in acute myeloid leukemia by targeting CX chemokine receptor 4

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Abstract: MicroRNAs (miRNAs) play key roles in the pathogenesis of many cancers, including acute myeloid leukemia (AML). Although miRNA-9 (miR-9) is involved in the leukemogenesis of AML, the underlying mechanisms remain to be elucidated. In this study, we found that miR-9 and C-X-C chemokine receptor 4 (CXCR4) were differentially expressed in myeloid leukemia, particularly in AML. The inverse correlation between miR-9 and CXCR4 was identified in AML samples and cell lines. The AML patients simultaneously with high levels of CXCR4 and low expression of miR-9 possessed poor prognosis. In vitro, miR-9 inhibited the proliferation, apoptosis resistance, migration, and invasion of AML cells. Dual luciferase assays verified CXCR4 as a direct target of miR-9. The suppressive effects of miR-9 on AML cells were counteracted or mimicked by CXCR4 overexpression or depletion, respectively. Overall, this study reveals that miR-9 retards the aggressive behaviors of AML cells by repressing CXCR4. Thus, miR-9/CXCR4 axis may represent a potential therapeutic target for AML.

Keywords: microRNA-9, C-X-C chemokine receptor 4, acute myeloid leukemia, growth, apoptosis

Introduction

Acute myeloid leukemia (AML) is a cytogenetically and/or molecularly heterogeneous disorder characterized by uncontrolled proliferation of clonal myeloid precursors and consequently an accumulation of myeloid cells in the bone marrow (BM) and an arrest in their maturation [1, 2]. Despite great improvements in the understanding of the leukemogenic mechanisms of AML and rapid progresses in the use of more intensive therapeutic approaches, the prognosis of major AML patients remains poor [3]. The five-year survival rate of AML is only 31% in patients younger than 65 years of age, and a staggering 4% in elderly patients [4]. Therefore, it is urgent to identify more effective therapeutic target and develop precision therapy of AML by uncovering the underlying mechanisms of AML pathogenesis.

MicroRNAs (miRNAs) are a class of small non-coding RNAs with approximately 18-22 nucleotides in length that specifically bind to the 3'-untranslated region (UTR) of their target mRNAs and subsequently decrease protein levels

by destabilization of transcripts or inhibition of translation [5, 6]. Dysregulation of miRNAs has been observed in many human solid tumors and hematological malignancies and contributes to malignant transformation by intervening in critical processes, including differentiation, proliferation, and apoptosis [7, 8]. Many miRNAs have been identified as tumor supporters or tumor suppressors in AML [9, 10] and different miRNA expression profiles exist in various cytogenetic groups of AML [11-13]. In humans, three independent miR-9 genes (*miR-9-1*, *miR-9-2*, and *miR-9-3* located on chromosomes 1, 5, and 15, respectively) encode a highly conserved miRNA-9-5p (miR-9) and its passenger strand, miRNA-9-3p (miR-9*) [14]. MiR-9 can function either as an oncogene or a tumor suppressor depending on the types of cancer [15-17]. Also, miR-9 has cell context-dependent effects in AML. Maki et al. [18] stated that aberrant expression of miR-9 indicates poor prognosis in AML. MiR-9 affords an oncogenic role in mixed lineage leukemia (MLL)-rearranged AML [19], whereas miR-9 is regarded as a tumor suppressor in pediatric AML with t(8;21) [20]. Emmrich et al. [20] demonstrated that miR-9

abolishes proliferation, induces apoptosis, and promotes differentiation of t(8;21) AML cells in vitro and in vivo. In literature, miR-9 suppresses leukemogenesis of t(8;21) AML by targeting RUNX1, RUNX1T1, and RUNX1-RUNX1T1 [21] and SIRT1 [22]. However, the biological roles and the regulatory mechanisms of miR-9 in AML are still largely unclear.

C-X-C chemokine receptor 4 (CXCR4), a G protein coupled receptor, selectively interacts with CXC motif ligand 12 (CXCL12), also known as stromal cell-derived factor-1 (SDF-1) [23]. The SDF-1/CXCR4 axis is involved in tumor progression, angiogenesis, metastasis, and survival [24]. CXCR4 is one of the most common chemokine receptors and is overexpressed in many types of cancers, including leukemia [25, 26]. Several reports demonstrate that high level of CXCR4 is a poor prognostic factor in adult and pediatric AML [27-29]. Recent basic and clinical studies have revealed the safety and potential clinical utility of targeting the CXCL12/CXCR4 axis in AML with different classes of drugs, including small molecules, peptides, and monoclonal antibodies [30]. Although previous studies have illuminated the importance of miR-9/CXCR4 axis in the carcinogenesis of several cancers [31-33], the association between miR-9 and CXCR4 in AML and the underlying mechanism have yet to be elucidated.

In this study, we addressed the biological roles and the potential mechanisms of miR-9 in AML. MiR-9 was significantly downregulated whereas CXCR4 was upregulated in AML samples and cell lines and an inverse correlation was observed between miR-9 expression and CXCR4 level. Dual-luciferase reporter assays showed that miR-9 directly targets CXCR4. MiR-9 repressed the proliferation, migration, and invasion and induced G1 phase arrest and apoptosis of AML cells, which were compromised by CXCR4 overexpression or mimicked by CXCR4 knockdown. Together, these findings revealed that miR-9 functions as a tumor suppressor in AML by targeting CXCR4.

Materials and methods

Patients and BM samples

BM samples were collected from 10 healthy volunteers and 54 myeloid leukemia (ML) pa-

tients, including 36 AML patients with t(8;21) and 18 chronic myeloid leukemia (CML) cases, after informed consent was signed in accordance with the guidelines of The First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technology (Henan, China). The patients were diagnosed from February 2008 to May 2012 according to the current World Health Organization criteria and follow-up by telephone until death or April 2017. All the patients received non-chemotherapy prior to this study. The harvested samples were immediately frozen in liquid nitrogen and stored at -80°C. This study was approved by the ethics committees of The First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technology.

Cell culture

Human myeloid leukemia cell lines, including NB4, HL-60, Kasumi-1, SKNO-1, and KG-1a were purchased from American Type Culture Collection (Manassas, VA, USA). All the cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 mg/mL streptomycin, and 100 U/mL penicillin (both from Sigma-Aldrich, St. Louis, MO, USA) in a humidified air at 37°C with 5% CO_2.

CD34$^+$ mononuclear cells were isolated from human BM samples by using CD34-immunomagnetic microbeads (Miltenyi, Bergisch Gladbach, Germany). Short term culture of CD34$^+$ cells was performed in StemSpan™ Serum-Free Expansion Medium (Stemcell Technologies, Vancouver, Canada) supplemented with 100 ng/mL (SCF, TPO, FLT3-L and IL-6; all from PeproTech, Rocky Hill, CT, USA).

Establishment of Kasumi-1-luciferase (luc) cell line

Kasumi-1-luc cell line was established as previously described [34]. Lentivirus pLV-luc was purchased from Inovogen Biotechnology (Delhi, India) and infected with Kasumi-1 cells. Cells were selected with puromycin (200 μg/mL; Sigma) to generate clones with stable luciferase expression. After 16 days of screening, a single clone was obtained and named Kasumi-1-luc cell line.

Cell transfection and infection

MiR-9 mimics and its negative-control RNA (miR-NC) were synthesized by Qiagen (Hilden, Germany). Plasmid pcDNA3.1-CXCR4 was obtained from Biovector (Beijing, China). Transfection was conducted through electroporation using Nucleofector instrument following the manufacturer's instructions. Kasumi-1 and SKNO-1 cells were transfected with 100 nM miR-9 mimics or miR-NC or co-transfected with miR-9 mimics and 2 µg of CXCR4 plasmid in six-well plates for in vitro experiments. Lentivirus psi-CHECK-2, psi-CHECK-2-miR-9, pLKO.1, and pLKO.1-shCXCR4 were purchased from Gene-Pharma (Shanghai, China) and infected with Kasumi-1-luc cells. All constructs were confirmed by DNA sequencing. After 14 days of screening with puromycin (100 µg/mL; Sigma), stable clones were generated and harvested for AML xenograft assays.

Luciferase reporter assay

For the dual-luciferase report assay, the wild-type (WT) 3'-UTR of CXCR4 and a variant containing mutations in the putative miR-9 binding sites were inserted downstream of the firefly luciferase gene in the pGL3 vector (Promega, Madison, WI, USA). The primers used to amplify the WT and mutate (MUT) 3'-UTRs are listed as follows. WT 3'-UTR, forward: 5'-ATACTCGAGAG-TCAACATGCCTGCCCCAAAC A-3', reverse: 5'-CA-CGCGGCCGCCTAGACAGACAAGGAAAGTTTAA-TGG-3'; MUT 3'-UTR, forward: 5'-ATTACGACAT-GTATCAATGCATAGGGAAGGAA-3', reverse: 5'-AAGGGTCGTGGCTCCCATGCTCCACGTGAAA-3'. All constructs were confirmed by DNA sequencing. The cells were co-transfected with reporter constructs, an internal control vector (pGL3), and a synthetic miR-9 mimic. After 48 h of transfection, luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

RNA extraction and quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted from samples and cells with TRIzol reagent (Invitrogen) as per the manufacturer's instructions. Complementary DNA (cDNA) was synthesized with miScript Reverse Transcription Kit (Qiagen) and then amplified using SYBR Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) to quantify miR-9 expres-

sion. U6 was used as internal control. cDNA was generated using the Reverse Transcription Kit (Promega) to quantify the mRNA levels of CXCR4. qPCR assay was carried out using a standard IQTM SYBR Green Supermix kit (Bio-Rad, Berkeley, USA), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as endogenous control. All qPCR assays were performed on an Applied Biosystems 7500 system (Applied Biosystems, Warrington, UK). The used primer pairs were miR-9, forward: 5'-CGGG GTT-GGTTGTTATCTTTGG-3', reverse: 5'-GCTTTATG-AAGACTCCACA CCAC-3'; U6 reverse: 5'-AAC-GCTTCACGAATTTGCGT-3'; CXCR4, forward: 5'-GTAGAGC GAGTGTTGCCATG-3', reverse: 5'-TT-GAAATGGACGTTTTCATCC-3'; and GAPDH, forward: 5'-ACACCCACTCCTCCACCTTT-3', reverse: 5'-TTACTCCTTG GAGGCCATGT-3'. Gene levels were measured in triplicate, quantified using $2^{-\Delta\Delta CT}$ method, and normalized to that of control.

Cell stimulation

Cell was stimulated as previously described [35]. After being serum starved for 4 h at 37°C, the cells were treated with 100 ng/mL of CXCL12 and incubated at 37°C for various time periods. At the end of the stimulation, the cells were harvested for analysis.

Cell proliferation assay

Cell proliferation was determined by Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). Kasumi-1 and SKNO-1 cells were seeded into 96-well plates at a density of 1×10^3 cells per well ($n = 5$ for each time point) in a final volume of 100 µL. The cells were cultured for 1, 2, 3, or 4 days after transfection with miR-NC, miR-9 mimics, or miR-9 mimics plus CXCR4 plasmid. In accordance with the manufacturer's instructions, CCK-8 solution (10 µL) was added to each well; absorbance at 450 nm was recorded after incubation for 2 to 4 h to calculate the number of viable cells.

Cell cycle analysis

Kasumi-1 and SKNO-1 cells were seeded into 12-well plates. After 48 h of transfection with RNA oligonucleotides, the cells were fixed with ice-cold 70% ethanol and treated with 1 mg/mL RNase overnight at 4°C. Intracellular DNA was labeled with 10 µL of propidium iodide (PI,

50 µg/mL; Sigma) at 37°C for 30 min and then analyzed using a BD FACSCalibur flow cytometer (BD Technologies, Carlsbad, CA, USA). Cell proportions in the G1, S, and G2/M phases were calculated using ModFit software (Verity Software House Inc., Topsham, ME, USA).

Apoptosis determination

Cell apoptosis was detected by using Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit I (BD Bioscience, San Diego, CA, USA) following the manufacturer's protocol. Kasumi-1 and SKNO-1 cells were treated with 10 µg/mL of Vincristine (VCR) before transfection. After 48 h of transfection with RNA oligonucleotides, cells were harvested, centrifuged, and resuspended in 100 µL of FITC-binding buffer. Approximately 5 µL of ready-to-use Annexin V-FITC (BD Bioscience) and 5 µL of PI were added to the mixture and incubated for 30 min in the dark. Annexin V-FITC and PI fluorescence was assessed by BD FACSCalibur flow cytometer (BD Technologies) and analyzed by CellQuest software (BD Bioscience).

Migration and invasion assays

A transwell insert with 8-µm diameter (Costar, Dallas, TX, USA) was employed for migration assay. A total of 5×10^3 Kasumi-1 or SKNO-1 cells were suspended in the serum-free medium and seeded into the upper chamber of the transwell, and RPMI 1640 containing 100 ng/mL of CXCL12 was added to the lower chamber. For invasion assays, 1×10^4 cells were suspended in medium without serum and added to the upper chamber of 8-µm pore size Transwells, which were precoated with 200 µL of Matrigel (BD Bioscience) at a concentration of 200 µg/mL. The medium containing 100 ng/mL of CXCL12 was added to the lower chamber as chemoattractant. After 24 h of incubation at 37°C, the filters were fixed in methanol and stained with crystal violet (Sigma). Cells that did not pass through the pores were carefully removed, and those that migrated or invaded the pores were retained. Five random fields per chamber were counted under an inverted fluorescent microscope (Carl Zeiss, Berlin, Germany).

Western blot analysis

Proteins were extracted from samples and cells, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were blocked in TBST (Tris-buffered saline with 0.2% of Tween 20) containing 5% non-fat milk. Western blot was performed with primary antibodies targeting CXCR4, Ki67, Bax, cyclin D1, matrix metallopeptidase (MMP)-2, MMP-9 (Abcam, Cambridge, UK), Bcl-2, and β-actin (Abnova, Taiwan, China), followed by the secondary antibodies conjugated with horseradish peroxidase (Sigma). Immunoreactivity was visualized by enhanced chemiluminescence (ECL kit, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in accordance with the manufacturer's instruction.

Terminal transferase-mediated dUTP nick-end labeling (TUNEL) assay

TUNEL assay was conducted as previously described [35]. Samples were fixed with 10% formalin overnight, embedded in paraffin, non-serially sectioned (4 µm), and mounted onto poly-L-lysine-coated slides. After deparaffinization in xylene and rehydration in a graded series of ethanol solutions, the sections were rinsed with PBS and incubated with FITC-labeled terminal deoxynucleotidyl transferase nucleotide mix (Roche, Mannheim, BW, Germany) at 37°C for 60 min according to the manufacturer's protocol. The sections were washed twice in PBS and counterstained with 10 mg/mL DAPI. TUNEL-positive cells were imaged, mounted using a fluorescent microscope (Carl-Zeiss), and ultimately expressed as a percentage of the total cells determined by DAPI staining.

In vivo tumor growth assay

Animal experiments were approved by the Institutional Committee for Animal Research and performed in conformity with the national guidelines for the care and use of laboratory animals. For tumor growth assays, 6-week-old female mice with severe combined immune deficiency (SCID; Institute of Zoology, Chinese Academy of Sciences, Beijing, China) received subcutaneous injections of 1×10^6 Kasumi-1-luc cells with a stable expression of miR-9 or shCXCR4 or their controls ($n = 6$ mice/group) through the hind flank. Tumor volumes were monitored once a week and calculated as follows: tumor volume = $width^2 \times length/2$. All mice were sacrificed 5 weeks post-inoculation, and tumors were removed and weighed. The tissues were
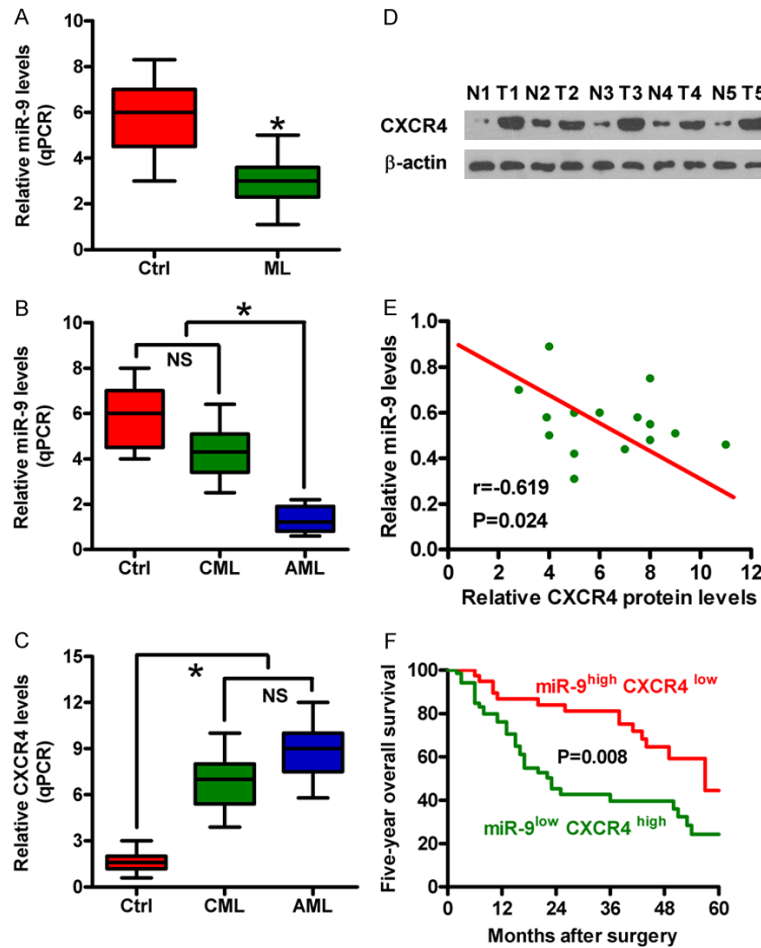
Figure 1. Expression of miR-9 and CXCR4 in AML samples. (A) qPCR assay was performed to determine the miR-9 levels in BM samples of ML patients and healthy individuals (control). U6 was used as the normal control. (B and C) Comparisons of the miR-9 expression (B) and CXCR4 mRNA level (C) in BM samples of the control, CML, and AML patients. U6 and GAPDH were used as the endogenous controls, respectively. (D) The protein level of CXCR4 in BM samples of the control and AML patients was measured by Western blot assay. β-actin were used as the normal control. (E) The inverse relation with miR-9 levels and CXCR4 protein levels in AML samples. (F) Five-year overall survival rate of AML patients with miR-9 low expression and CXCR4 high level (n = 25) and the patients with miR-9 high expression and CXCR4 low level (n = 11). Data are presented as mean ± SD of three independent experiments. *$P < 0.05$. AML: acute myeloid leukemia; CML: chronic myeloid leukemia; ML: myeloid leukemia; N: normal samples; T: AML samples; NS: Not significant.

× 10^6 Kasumi-1-luc cells infected with control lentivirus or lentivirus expressing miR-9 or shCXCR4. Tumor growth was assessed by in vivo luciferase imaging of the xenografts 14 days after the treatment. Mice were intraperitoneally injected with D-luciferin (Promega) at a dose of 150 mg/kg per mouse and anesthetized prior to image acquisition with the Xenogen IVIS imaging system. Signals in the defined regions of interest were quantified as luminescence radiance (photons/s/cm²/sr) by Living Image software (Xenogen Corporation, Berkeley, CA, USA).

Statistical analysis

Data are expressed as mean ± standard deviation (SD) from three independent experiments. The results were analyzed by Student's *t*-test and analysis of variance (ANOVA). The relationships between the expressions of miR-9 and CXCR4 were assessed by Pearson's correlation analysis. Survival curves were plotted using the Kaplan-Meier method and the survival rate was analyzed by Log-rank test. All *P* values were two-sided and obtained using SPSS 13.0 software package (SPSS Inc., IL, USA). *P* < 0.05 was considered statistically significant.

Results

sectioned and stained with TUNEL kits for apoptosis detection. TUNEL-positive cells were examined and calculated under a fluorescence microscope (Carl Zeiss).

Bioluminescence imaging and quantification

Bioluminescence imaging was implemented as previously reported [35]. Six-week-old female SCID mice were subcutaneously injected with 1

Inverse correlation between miR-9 and CXCR4 levels in AML patients

We first evaluated the miR-9 expression in BM samples of ML patients and the healthy volunteers by qPCR assays. MiR-9 expression was significantly decreased in the ML specimens compared with the controls (**Figure 1A**). Moreover, miR-9 level was much lower in the AML subtype than that in the CML subtype (**Figure**
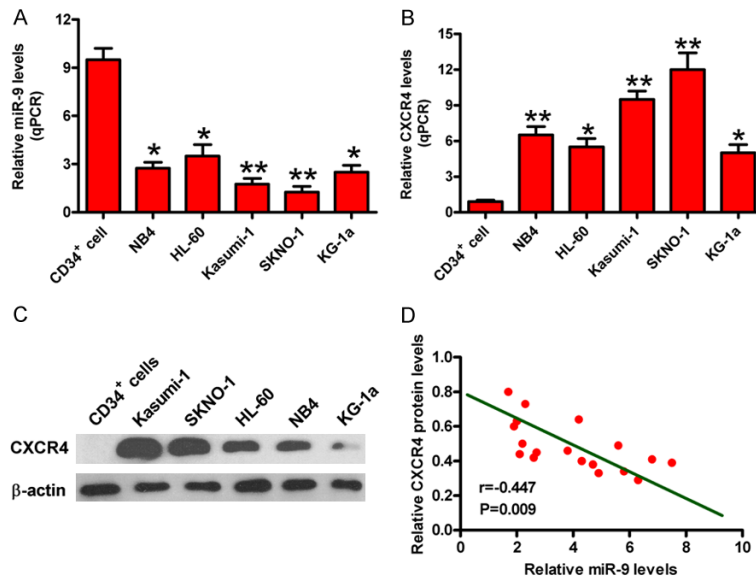
Figure 2. Levels of miR-9 and CXCR4 in AML cells. (A) qPCR assay was conducted to detect the miR-9 levels in AML cell lines (NB4, HL-60, Kasumi-1, SKNO-1, and KG-1a) and the normal CD34+ cells. U6 was used as the normal control. (B and C) The mRNA expression (B) and protein level (C) of CXCR4 in AML cells and CD34+ cells. GAPDH and β-actin were used as the normal controls, respectively. (D) The relationship between miR-9 expression and protein levels of CXCR4 in AML cells were evaluated by Pearson's correlation analysis. Data are presented as mean ± SD of three independent experiments. *P < 0.05 compared with CD34+ cells.

2A). qPCR and Western blot analyses revealed that CXCR4 expression was increased at both mRNA and protein levels in AML cell lines (**Figure 2B** and **2C**). Moreover, CXCR4 protein expression was inversely correlated with miR-9 level in AML cells (**Figure 2D**). The Kasumi-1 and SKNO-1 cells that had the lowest level of miR-9 and the highest expression of CXCR4 were selected for the subsequent experiments. These data suggest the inverse correlation between miR-9 and CXCR4 in AML cells.

MiR-9 functions as a tumor suppressor in AML cells

We explored the potential effect of miR-9 on AML cells. Kasumi-1 and SKNO-1 cells were transfected with miR-NC or miR-9 mimics. CCK-8 assay showed that the prolif-eration was inhibited in miR-9-transfected cells compared with those cells with miR-NC transfection (**Figure 3A**). Flow cytometry assay revealed that miR-9 led to cell cycle arrest at G1 phase in Kasumi-1 and SKNO-1 cells (**Figure 3B**). Moreover, miR-9 further increased the apoptosis of Kasumi-1 and SKNO-1 cells pre-treated with VCR (**Figure 3C**). Transwell assays showed that miR-9 reduced the migratory and invasive abilities of Kasumi-1 and SKNO-1 cells (**Figure 3D** and **3E**). These findings illuminate that miR-9 weakens the proliferative and aggressive behaviors of AML cells.

CXCR4 is a target of miR-9 in AML cells

To further identify the roles of miR-9 in AML cells, we sought its putative target genes by using online searching tools (TargetScan, PicStar and miRanda). CXCR4, which is highly expressed in AML cells, was found one potential binding site within its 3'-UTR for miR-9 (**Figure 4A**). We then performed dual-luciferase reporter assays to authenticate the direct interaction between miR-9 and the 3'-UTR of CXCR4. MiR-9 markedly repressed the activity of the lucifer-

1B). In contrast to miR-9, the mRNA level of CXCR4 was higher in AML patients than that in CML cases (**Figure 1C**). The protein expression of CXCR4 in AML subtype was significantly up-regulated compared with the control individuals (**Figure 1D**). In addition, miR-9 level was inversely associated with CXCR4 protein level in AML patients (**Figure 1E**). As shown in **Figure 1F**, the five-year overall survival rate of patients with high miR-9 levels and low CXCR4 expression was higher than that of patients with low miR-9 levels and high CXCR4 expression. These findings suggest that miR-9 expression is negatively associated with CXCR4 level in AML specimens and that the combination of miR-9 and CXCR4 is a prognostic indicator for AML patients.

Decreased miR-9 expression and increased CXCR4 level in AML cells

We next measured the levels of miR-9 and CXCR4 in AML cells. qPCR assay showed that miR-9 was underexpressed in AML cell lines (NB4, HL-60, Kasumi-1, SKNO-1, and KG-1a) compared with the normal CD34+ cells (**Figure**
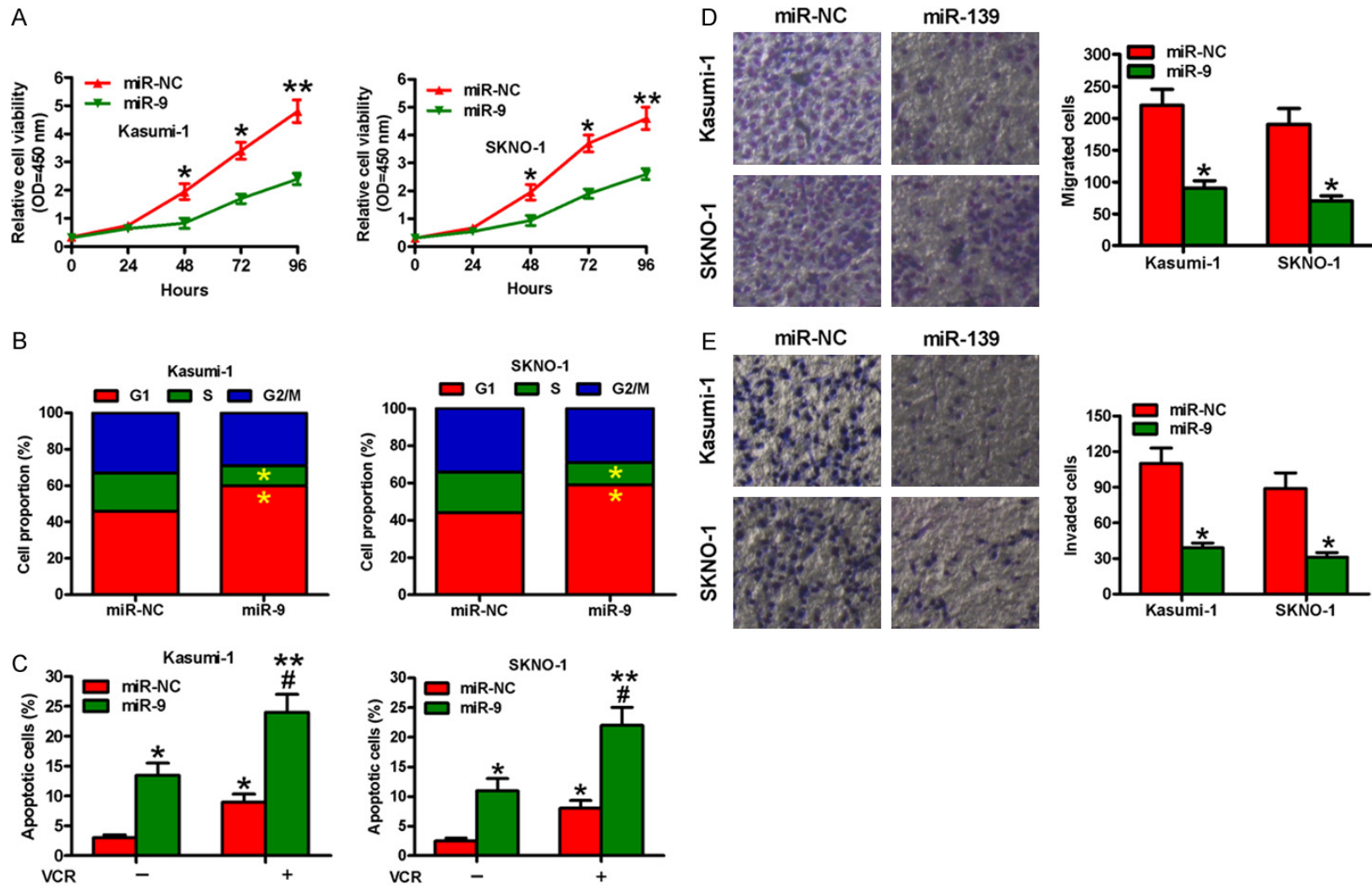
Figure 3. MiR-9 inhibited the proliferation, migration, and invasion and promoted apoptosis of AML cells. Kasumi-1 and SKNO-1 cells with or without 10 µg/mL of VCR pretreatment were transfected with 100 nM of miR-NC or miR-9 mimics. (A) CCK-8 assay was carried out to assess cell proliferation at 24, 48, 72, and 96 h after transfection. (B and C) Cell cycle distribution (B) and cell apoptosis (C) were analyzed by flow cytometry at 48 h after transfection. (D and E) Transwell assays were performed to evaluate the migration (D) and invasion (E) of cells at 48 h after transfection. Data are presented as mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$ compared with miR-NC group. VCR: Vincristine.
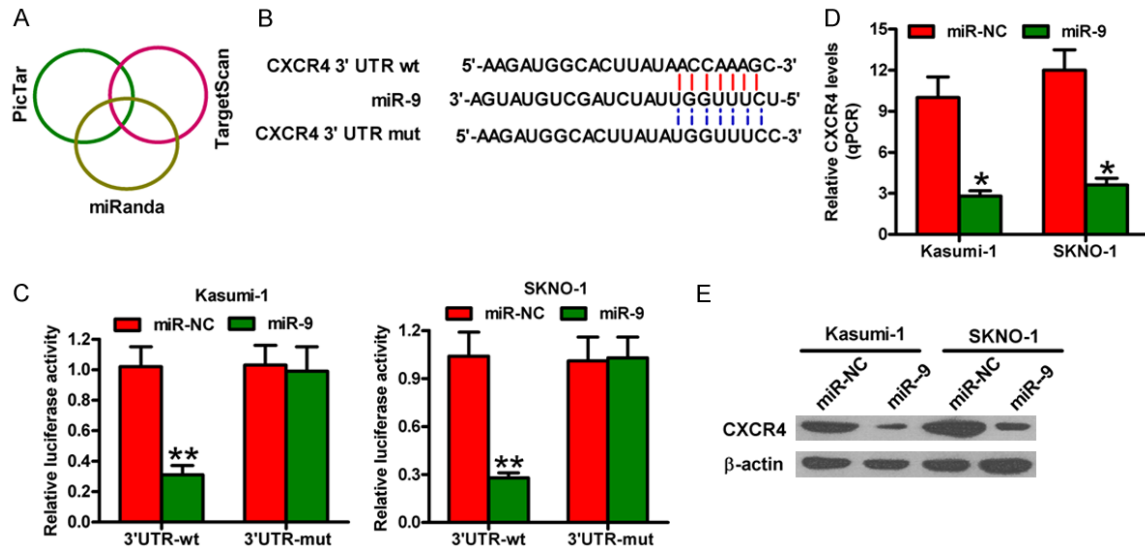
Figure 4. MiR-9 directly targeted CXCR4 in AML cells. (A) Three classical softwares were used to predict that miR-9 directly targets CXCR4. (B) Predicted binding site in 3'-UTR of CXCR4 for miR-9. (C) Dual luciferase assays were carried out after Kasumi-1 and SKNO-1 cells were co-transfected pGL3-luc-WT 3'-UTR-CXCR4 or pGL3-luc-MUT 3'-UTR-CXCR4 with miR-9 or miR-NC mimics for 48 h. mRNA (D) and protein (E) levels of CXCR4 in Kasumi-1 and SKNO-1 cells were measured by qPCR and Western blot assays. GAPDH and β-actin were used as internal controls, respectively. Data are presented as mean ± SD of three independent experiments. *$P < 0.05$ compared with miR-NC group.

ase reporter fused to the WT 3'-UTR of CXCR4 but did not suppress that of the reporter fused to the MUT version (**Figure 4B**). Next, we measured CXCR4 expression by qPCR and Western blot assays in miR-9- or miR-NC-transfected Kasumi-1 and SKNO-1 cells. As shown in **Figure 4C** and **4D**, both mRNA and protein levels of CXCR4 were decreased in miR-9-transfected Kasumi-1 and SKNO-1 cells compared with those cells transfected with miR-NC. These results show that miR-9 directly targets CXCR4 in AML cells.

MiR-9 exerts its suppressive effects in AML cells by targeting CXCR4

To confirm that CXCR4 is a functional target of miR-9 in AML cells, we transfected Kasumi-1 and SKNO-1 cells with miR-NC, miR-9, or miR-9 plus a CXCR4-expressing plasmid in the presence of 100 ng/mL of CXCL12. The reduction in cell proliferation caused by miR-9 was attenuated by co-transfection with the CXCR4 plasmid (**Figure 5A**). MiR-9-induced G1 phase arrest was partly attenuated by CXCR4 overexpression (**Figure 5B**). Ectopic expression of CXCR4 partly rescued miR-9-induced apoptosis of Kasumi-1 and SKNO-1 cells. Moreover, miR-9-ehanced apoptosis of VCR-pretreated Kasumi-

1 and SKNO-1 cells was neutralized by CXCR4 overexpression (**Figure 5C**). In Transwell assays, the migration and invasion of Kasumi-1 and SKNO-1 cells were reduced after miR-9 transfection, whereas CXCR4 overexpression counteracted the above inhibition (**Figure 5D** and **5E**). To explore the molecular mechanisms by which miR-9 exerts its tumor-suppressive effects, we examined the protein expression of CXCR4 and its relative molecules, including Ki67, cyclin D1, MMP-2, MMP-9, Bax, Bcl-2, MMP-2, and MMP-9 in different groups by Western blot analysis (**Figure 5F**). MiR-9 significantly reduced the levels of CXCR4, cyclin D1, MMP-2, MMP-9, and Bcl-2 and increased Bax expression in Kasumi-1 and SKNO-1 cells, which were reversed by co-transfection with the CXCR4 plasmid (**Figure 5F**). These data suggest that miR-9 hampers the proliferation, mobility, and apoptosis evasion of AML cells by targeting CXCR4.

MiR-9 introduction or CXCR4 depletion hinders tumorigenesis of AML in vivo

A xenograft mouse model was established by subcutaneous injection of Kasumi-1-luc cells stably expressing miR-9 precursor or shCXCR4 or their vector controls. Ectopic expression of miR-9 or CXCR4 knockdown significantly reduc-
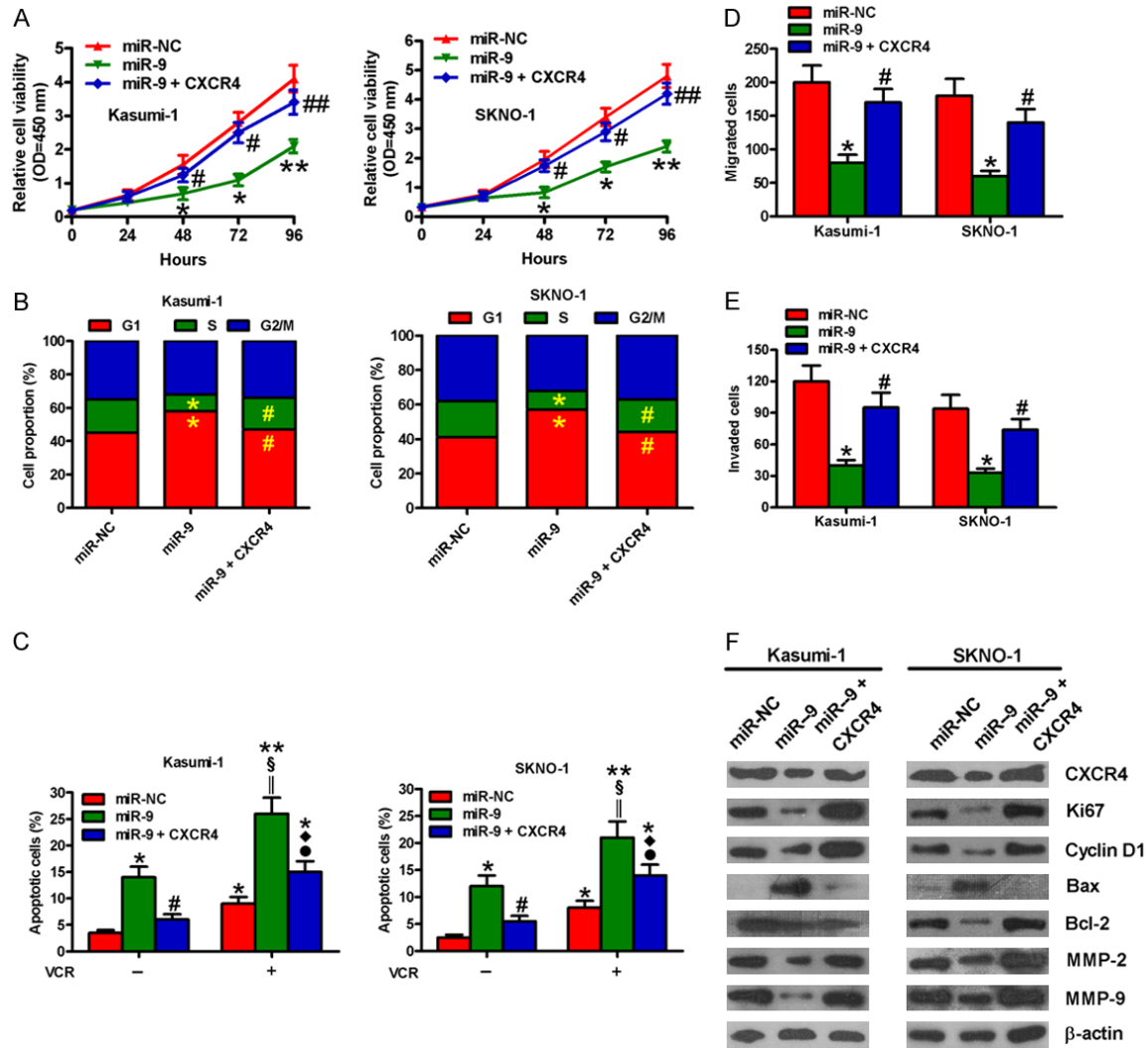
Figure 5. CXCR4 overexpression attenuated the anti-leukemic activity of miR-9 in AML cells. Kasumi-1 and SKNO-1 cells with or without 10 μg/mL of VCR pretreatment were transfected with miR-NC or miR-9 or miR-9 + CXCR4-expressing plasmid. (A) Cell proliferation was measured by CCK-8 assay at 24, 48, 72, and 96 h after transfection. (B and C) Cell cycle (B) and cell apoptosis (C) was analyzed by flow cytometrys at 48 h after transfection. (D and E) Transwell assays were performed to evaluate the migration (D) and invasion (E) of cells at 48 h after transfection. (F) Western blot results of CXCR4, Ki67, cyclin D1, Bax, Bcl-2, MMP-2, and MMP-9 expression at 48 h after transfection. β-actin was used as the normal control. Data are presented as mean ± SD of three independent experiments. $*P < 0.05$, $**P < 0.01$ compared with miR-NC + VCR (-) group, $^{#}P < 0.05$ compared with miR-9 + VCR (-) group, $^{§}P < 0.05$ compared with miR-9 + VCR (-) group, $^{\parallel}P < 0.05$ compared with miR-NC + VCR (+) group, $^{♦}P < 0.05$ compared with miR-9 + CXCR4 + VCR (-) group, $^{•}P < 0.05$ compared with miR-9 + VCR (+) group. VCR: Vincristine.

ed the tumor growth (**Figure 6A**), tumor volume (**Figure 6B**) and tumor weight (**Figure 6C**) compared with the control groups. The number of the apoptotic cells was increased in the tumors derived from miR-9-introduced or CXCR4-silenced group compared with that in the control groups (**Figure 6D**). MiR-9 restoration or CXCR4 depletion effectively downregulated the levels of CXCR4, Ki67, cyclin D1, Bcl-2, MMP-2, and MMP-9, but upregulated Bax expression in

vivo (**Figure 6E**). These data indicate that miR-9-suppressed AML growth is mediated by CXCR4 reduction in vivo.

Discussion

In this study, we found that miR-9 is a tumor suppressor in AML by targeting CXCR4. Key findings were as follows: First, miR-9 expression was decreased in ML patients, particularly
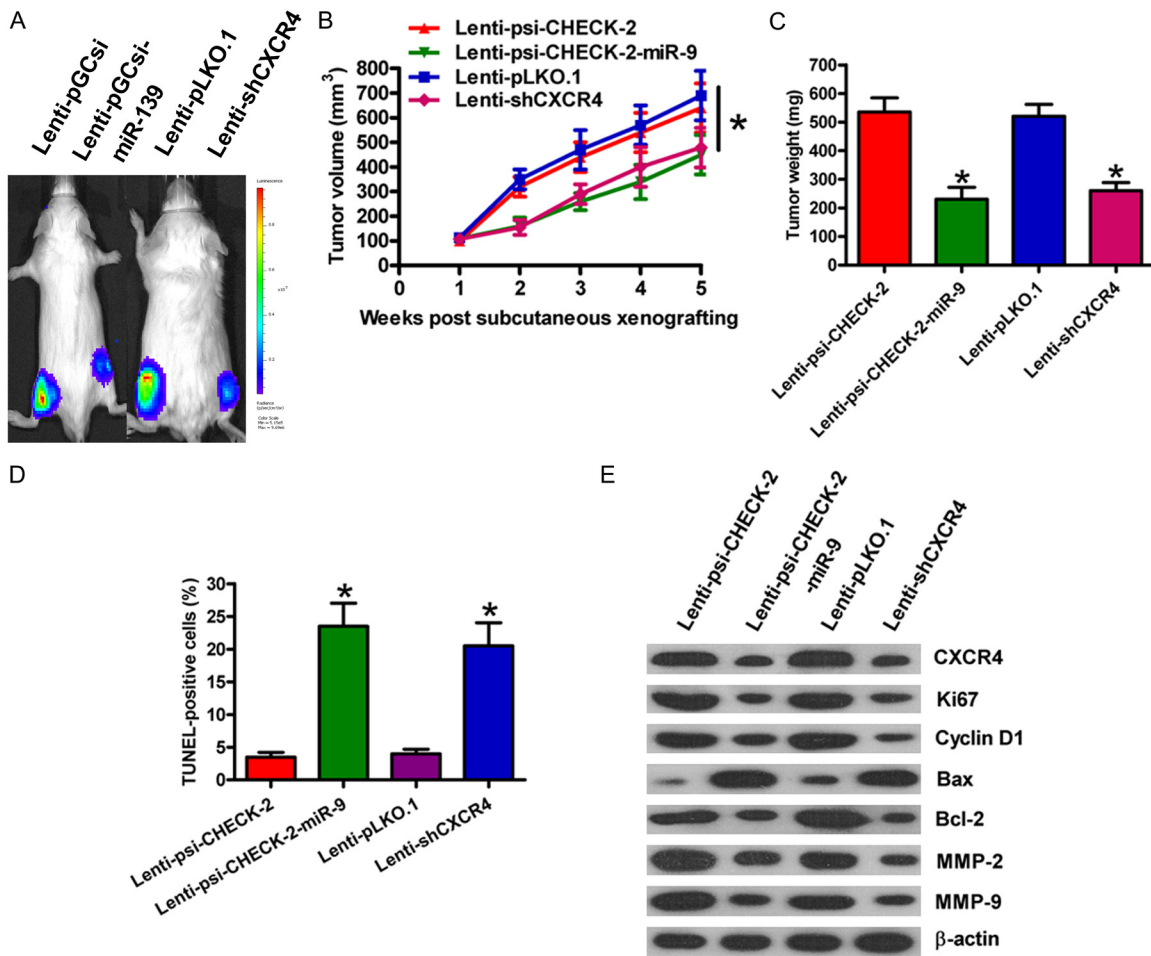
Figure 6. Overexpression of miR-9 or knockdown of CXCR4 impaired AML growth in vivo. SCID mice were subcutaneously injected with Kasumi-1-luc cells infected with control lentivirus (Lenti-psi-CHECK-2 or Lenti-pLKO.1) or a recombinant lentivirus expressing miR-9 precursor (Lenti-psi-CHECK-2-miR-9) or shCXCR4 (Lenti-shCXCR4). A. In vivo luciferase image for detection of xenograft tumor growth in mice implanted with various cells. B. Tumor volume was monitored once a week for 5 weeks. C. Tumor weight was measured after 5 weeks of implantation. D. TUNEL assay was conducted to determine the percentage of apoptotic cells. E. Western blot results of CXCR4, Ki67, cyclin D1, Bax, Bcl-2, MMP-2, and MMP-9 expression in different groups. β-actin was used as the normal control. Data are presented as mean ± SD of three independent experiments. *$P < 0.05$ vs. Lenti-psi-CHECK-2 or Lenti-pLKO.1 group; **$P < 0.01$ vs. Lenti-psi-CHECK-2 or Lenti-pLKO.1 group.

in AML. Second, an inverse correlation between miR-9 and CXCR4 expression was confirmed in AML samples and cell lines. Third, the patients with high miR-9 levels and low CXCR4 expression has higher five-year overall survival rate than the patients with low miR-9 level and high CXCR4 expression. Fourth, miR-9 inhibited the proliferation, migration, and invasion and induced cell cycle arrest and apoptosis of AML cells in vitro and hindered AML tumorigenesis in vivo. Fifth, CXCR4 was a direct target of miR-9. Lastly, the repressive effects of miR-9 on AML were rescued by CXCR4 overexpression or

mimicked by CXCR4 depletion. Overall, miR-9 reduced the malignant characteristics of AML by targeting CXCR4.

AML is a heterogeneous malignancy that is morphologically and cytogenetically classified into different subtypes [1, 2]. Cytogenetic and/ or molecular abnormalities are the most important prognostic factors for AML [36, 37]. Accumulating evidence shows that miRNAs are involved in the leukemogenesis of AML, including miR-9 [38]. Both tumor-suppressive and oncogenic roles of miR-9 have been reported in

various tumors [15-17]. The functional roles of miR-9 in tumorigenesis are cell type-specific. For instance, upregulation of miR-9 promotes the proliferation, migration, and invasion of human breast cancer and gastric cancer cells [39, 40]. Conversely, miR-9 is downregulated and suppresses the proliferation and promotes the apoptosis in human oral squamous cell carcinoma and ovarian cancer cells [31, 33]. In literature, high level of miR-9 is a predictor of poor survival in adult AML [18]. However, miR-9 is recently found as an independent poor prognostic factor for AML regardless of the karyotype and genetic mutations [41]. Actually, miR-9 expression differs and exerts controversial functions in AML depending on cell context. Nowek et al. [42] demonstrates that miR-9 and miR-9* are significantly upregulated and their levels vary according to AML subtype, with the highest expression in MLL-related leukemia harboring 11q23 abnormalities and the lowest expression in AML cases with t(8;21) and biallelic mutations in CEBPA. Chen et al. [19] shows that miR-9 is the most specifically upregulated miRNA in MLL-rearranged AML compared with both normal control and non-MLL-rearranged AML and that depletion of miR-9 inhibits cell growth/viability and promotes apoptosis in human MLL-rearranged AML cells, and the opposite is true when miR-9 is forced expression. However, miR-9 suppresses the growth of t(8;21) AML in vitro and in vivo and induces monocytic differentiation in KASUMI-1 cells by targeting the LIN28B/let-7/HMGA2 axis [20]. Introduction of miR-9-1 also induces differentiation and represses proliferation of SKNO-1 cells [21]. MiR-9 is lowly expressed in t(8;21) AML samples and KASUMI-1 and SKNO-1 cells [22]. Consistently, this study revealed that miR-9 was markedly downregulated in t(8;21) AML cases, and the forced expression of miR-9 hindered the proliferation, apoptosis resistance, migration, and invasion of KASUMI-1 and SKNO-1 cells in vitro and reduced AML growth in vivo, supporting the tumor-suppressive roles of miR-9 in the leukemogenesis of t(8;21) AML.

CXCR4 is a chemokine receptor defined by its ability to induce directional migration of cells depending on the concentration gradient of CXCL12 [43]. CXCR4 is highly expressed on many non-malignant and malignant cells, including AML cells [25, 26]. The interaction be-

tween AML cells and BM microenvironment is postulated as an important mediator of resistance to chemotherapy and disease relapse [44]. CXCR4 and CXCL12 are critical components involved in the crosstalk between AML cells and BM stroma [45]. Binding of CXCL12 produced by the stroma to CXCR4 induces CXCR4 phosphorylation and internalization and activates pro-survival and proliferative signaling pathways, ultimately leading to survival, proliferation, and migration of AML cells [46, 47]. Several studies point out that high level of CXCR4 is positively associated with poor prognosis in AML patients [28, 48]. Small-molecule inhibitors or antibodies against CXCR4 are currently being successfully employed for AML treatment [30]. Tavor et al. [46] reports the anti-leukemic effect of CXCR4 neutralization in an AML xenograft model. CXCR4 inhibitor reduces the survival and trafficking of AML cells in vitro and in vivo [49, 50]. Recently, CXCR4 is identified as a target of several miRNAs, including miR-9, which may represent a new tool for CXCR4 downregulation and subsequently suppress the progression of cancers [31-33]. CXCR4 inhibition by miR-146a in AML cells impairs leukemic cell proliferation [51]. Consistently, in this study, we demonstrated that CXCR4 was highly expressed and inversely correlated with miR-9 in AML samples and cells. CXCR4 overexpression or depletion reversed or mimicked the anti-leukemic activities of miR-9 in vitro and in vivo, respectively. Our findings suggested that targeting miR-9/CXCR4 axis may be a potential treatment for AML.

Sustained proliferation, uncontrolled cell cycle progression, and apoptosis resistance are the hallmarks of cancers [52]. Several molecules are highly involved in the leukemogenesis of AML. Ki67 is a simple and valid measurement of intensive proliferation of AML cells [53]. Cyclin D1 is closely associated with the proliferation of AML cells, and reduction of cyclin D1 leads to cell cycle arrest at the G0/G1 phase [54]. The Bcl-2 family proteins, such as Bax and Bcl-2, are frequently related with therapy resistance and are attractive targets for the development of anti-AML agents [55]. Previous study suggests that MMP-2 and MMP-9 are implicated in the invasive phenotype of AML because AML cells express and secrete high levels of MMP-2 and MMP-9 [56]. In the current study, we found that miR-9 reduced the expressions

of Ki67, cyclin D1, Bcl-2, MMP-2, and MMP-9, but increase the level of Bax in AML cells in vitro and in vivo, which was reversed by CXCR4 overexpression or mimicked by CXCR4 knockdown. These results indicated that miR-9/CXCR4 axis is involved in the leukemogenesis of AML by regulating the expressions of proliferation- prompted Ki67 and cyclin D1, and motility-increased MMP-2 and MMP-9, and apoptosis-associated Bax and Bcl-2.

In summary, miR-9 is downregulated and inversely correlates with CXCR4 expression in AML samples and cells. In vitro and in vivo studies confirmed that miR-9 is an inhibitor of the proliferation, migration and invasion, and an inducer of apoptosis in AML cells by targeting CXCR4. These results suggest that downregulation of miR-9 and upregulation of CXCR4 together promote AML leukemogenesis and highlight the potential role of miR-9/CXCR4 axis in therapy for AML patients.

Disclosure of conflict of interest

None.

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