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*Original Article*

# miR-125b regulates cell progression in chronic myeloid leukemia via targeting BAK1

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**Abstract:** Chronic myeloid leukemia (CML) is a type of malignant tumor characterized by the accumulation of a large number of immature white blood cells in the blood and bone marrow. BAK1 was predicted to be the target gene of microRNA-451 (miR-125b). The present study was designed to illustrate the mechanism of miR-125b in regulating CML via targeting BAK1. In this study, we found that the expression of miR-125b increased strongly, whereas the expression of BAK1 decreased significantly in CML patients and CML cell lines compared with healthy controls. Moreover, the luciferase report assay confirmed the interaction between miR-125b and BAK1 mRNA. After transfection of the miR-125b mimic or miR-125b inhibitor into CML cells, we found that the inhibition of miR-125b decreased the proliferation rates and promoted apoptosis with cell cycle arrest at the G0/G1 phase in both K562 and NB-4 cells, increased the expression of BAK1 and Caspase-3, and decreased the expression of Bcl-2 and c-myc; the miR-125b mimic yielded the opposite results. In addition, siBAK1 offset the suppression effect of the miR-125b inhibitor in K562 cells, indicating that miR-125b promotes these cellular processes by inhibiting the expression of BAK1. Further in vivo experiments supported these findings because miR-125b suppression reduced CML growth in mice. Taken together, our study suggests that the down-regulation of miR-125b affects the expression of BAK1, promotes cell apoptosis and inhibits cell proliferation, leading to up-regulated expression of pro-apoptosis factors, down–regulated expression of anti-apoptosis factors in the mitochondrial apoptotic pathway, and decreased tumor size and weight of CML in vivo. These results provide a potential therapeutic strategy for CML.

**Keywords:** MiR-125b, mitochondrial apoptosis signaling pathway, chronic myeloid leukemia

## Introduction

Chronic myeloid leukemia (CML) has long been a hot topic. The disease course of CML is triphasic, beginning with a chronic phase, progressing to an accelerated phase and eventually ending in a terminal phase called the blast crisis (BC). CML is a myeloproliferative disease characterized by the Philadelphia chromosome (Ph), which is the result of a reciprocal translocation between chromosomes 9 and 22 (t[9;22][q34;q11]) [1]. Imatinib mesylate (STI-571 or Gleevec) was the first BCR-ABL tyrosine kinase inhibitor (TKI) used for the treatment of Ph+ CML [2] and is the current standard therapy. However, the failure of these kinase inhibitors to kill leukemia stem cells (LSCs) is related to disease relapse and makes them effective in controlling but not curing the disease [3].

MicroRNAs (miRNAs) are a group of non-coding regulatory RNAs, which regulate various cellular processes, such as proliferation, apoptosis, cell metabolism and angiogenesis [4, 5]. Specific miRNA expression signatures can be used to effectively classify human tumors [6]. This miRNA signatures related to specific cytogenetic changes and clinical outcomes have been reported for many diseases, including adult CLL [7], AML [8], and Hodgkin's lymphoma [9]. MicroRNA-125b is transcribed from two loci located on chromosomes 11q23 (hsa-miR-125b-1) and 21q21 (hsa-miR-125b-2). MicroRNA-125b-1 is involved in several chromosomal translocations, such as t(2;11)(p21;q23) and t(11;14)(q24;q32), which lead to myelodysplasia and acute myeloid leukemia (AML) or B-cell acute lymphoid leukemia (B-ALL), respectively [10, 11]. In both cases, miR-125b overexpres-

sion is the only consistent abnormality found in these patients, suggesting that it is a primary oncogenic event. Moreover, miR-125b-2 has been reported to co-regulate vincristine resistance in childhood acute lymphoblastic leukemia [12].

Blockage of the mitochondrial apoptosis pathway has a close relationship with CML [13]. Cell apoptosis is regulated by several protein families, including the upstream Bcl-2 family (e.g., antiapoptotic Bcl-2 and proapoptotic Bax) and the downstream Caspase family (e.g., Caspase-3) [14, 15]. Previous studies have reported that miR-125b targets pro-apoptotic Bcl-2 antagonist killer 1 (BAK1), leading to ovarian cancer cell resistance to cisplatin [16], and confers the resistance of breast cancer cells to paclitaxel through the suppression of BAK1 expression [17]. Thus, miR-125b may affect the expression of BAK1 pathway proteins and promote the proliferation and metastasis of CML cells.

In this study, we identified the function of miR-125b in the context of CML. We found that the expression of miR-125b is significantly increased in CML cells and CML patients, leading to low expression of the target gene BAK1, which regulates the mitochondrial apoptosis pathway. Taken together, our results suggest that miR-125b may function in CML metastasis by targeting BAK1.

## Methods

### *Patients*

Thirty-three patients (16 males and 17 females) with CML and thirty healthy volunteers (14 males and 16 females) were included in this study. Patients with CML were recruited from the Department of Hematology, Xiangyang Central Hospital. Healthy volunteers were recruited from students at Hubei University of Arts and Science. The study was approved by the Ethics Committee of our institution. Informed consent was signed by the participants.

### *Cell culture*

Normal hematopoietic stem cells (HSCs) were obtained from American Type Culture Collection (ATCC, Manassas, VA) and grown in modified Eagle's medium (MEM; Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) and 1% antibiotic-antimycotic (Invitrogen, Carlsbad, CA). The CML cell lines (K562, KG-1, NB-4, HuT-78, THP-1) (ATCC, Manassas, VA) were grown in RPMI 1640, supplemented with 10% FBS and 1% antibiotic-antimycotic. All cells were incubated at 37°C in a humidified 21% $O_2$, 5% $CO_2$ atmosphere.

### *Quantitative RT-PCR (qRT-PCR)*

Total cellular RNA was isolated using the miRNeasy Mini Kit (Qiagen, Inc., Valencia, CA) and quantified using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Ten nanograms of total RNA were reverse-transcribed using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The obtained cDNA was amplified using a TaqMan miR-125b MicroRNA assay (Applied Biosystems). For real-time PCR, the above primers and TaqMan probe [6-FAM]TTGCGACTAC ACACACACACA [BHQ1a-6FAM] were mixed with TaqManH Universal PCR Master Mix (Applied Biosystems, Foster City, CA). The reaction mixtures were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min in a Stratagene qRT-PCR instrument. miR-125b expression was normalized to U6 RNA. Gene mRNA expression was normalized to β-actin. Relative gene expression was quantified using the 2-ΔΔCt method.

### *Western blot analyses*

Cell lysates (30 mg of total protein) and prestained molecular weight markers were separated by SDS-PAGE followed by transfer onto nitrocellulose membranes. The membranes were blocked in TBST (Tris-buffered saline with 0.5% Triton X-100) containing 5% nonfat milk and probed with primary antibodies against BAK1, Bcl-2, Caspase-3, c-myc and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with the secondary antibody, membranes were extensively washed. A fluorescent Western blotting detection system was used, and the band density of each gene was normalized to the corresponding density of β-actin.

### *Dual-luciferase reporter assay*

The target gene was predicted by TargetScan (http://www.targetscan.org/). A 252 bp fragment of BAK1 mRNA containing the target

sequence (CUCAGGG) of miR-125b was amplified by RT-PCR (BAK1, sense 5'-GCTCCCAACCCATTCACTAC-3' and antisense 5'-TCCCTACTCCTTTTCCCTGA-3') [17]. The fragment was designated as BAK1 3'-UTR and inserted into the pMIR-REPORT™ luciferase reporter vector (Not I and Xho I restriction enzyme sites; Ambion). Another expression vector was also constructed by inserting a mutated BAK1 3'-UTR in which the target sequence of miR-125b was mutated to CUCCGGG using the QuikChangeH Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, USA). The recombinant reporter vectors with normal and mutated BAK1 3'-UTR were co-transfected with miR-125b into K562 cells separately using TransMessenger™ Transfection Reagent (Qiagen, Germany). The luciferase assay was performed according to the manufacturer's instructions. The relative luciferase activities were normalized to that of the control cells.

*Transfection assay*

The transfection assay was performed as described in a previous study [16]. Briefly, the BAK1 overexpression vector was constructed by PCR amplification using BAK1 cDNA as a template and inserting the resulting BAK1 cDNA product into the pcDNA3.1 vector. Two milligrams of recombinant plasmid and 200 pmoles of miR-125b mimic, miR-125b mimic control, miR-125b inhibitor, or inhibitor control (Ambion, Austin, Texas, USA) were transfected into $3\times10^6$ K562 cells for 48 h by electroporation using a Nucleofector instrument. After transfection, the cells were allowed to recover by incubating for 4 h at 37°C. The experiment was repeated three times.

*Cell proliferation assay*

Cell viability was assessed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, cells were seeded in 96-well plates for the indicated times. Thereafter, the old medium was discarded, and fresh medium containing MTT (5 mg/ml MTT in PBS; Sangon, Shanghai, China) was added and incubated for an additional 4 h. Dimethyl sulfoxide was used to dissolve the formazan, and the absorbance at 490 nm was measured once every 24 h by a Scepter™ 2.0 Handheld Automated Cell Counter (Bio-tek, WinoCMLki, VT, USA).

*Flow cytometric analysis*

Cell cycle analysis was determined by flow cytometry (BD, UA). Briefly, K562 and NB-4 cells at $1\times10^6$ cells per well were cultured in 6-well plates and transfected with 50 nM miR-125b mimic, miR-125b inhibitor, or their respective control RNAs for 48 h. The cells were then harvested, fixed in 70% ice-cold ethanol for 24 h, and stained with propidium iodide (PI). The different cell cycle phases were analyzed using a FACS Calibur instrument.

For the apoptosis assay, cells were transfected with 100 nM miR-125b precursor (pre-miR-125b), 100 nM pre-miR-125b in combination with 100 nM miR-125b inhibitor (anti-miR-125b), or 4 g of BAK1-expressing vector. At 24 h after transfection, the cells were treated with the indicated concentrations of Taxol for 48 h and then subjected to an apoptosis assay. Apoptosis was determined by propidium iodide staining with the apoptosis detection kit (BD Pharmingen). Briefly, $1\times10^5$ treated cells were incubated with propidium iodide for 15 min at room temperature. The cells were then analyzed by flow cytometry using fluorescence-activated cell sorting analysis (BD LSR II).

*Animal experiments*

The protocols for animal experiments were approved by the Committee on the Use and Care of Animals in the animal experimental center of Hubei University of Arts and Science. To study the role of miR-125b in the tumorigenesis of glioma cell lines, two different mouse models were used. The details of the establishment of glioma xenografts are available in previous papers [7]. Briefly, CML was established by injecting $5\times10^6$ cells into the flanks of 4- to 5-week-old male BALB/c nude mice. Twelve mice were divided into two groups (n = 6). miR-125b inhibitor (50 nM in 100 μl of PBS) or control molecules (50 nM in 100 μl of PBS) were directly injected into the tumors every other day for three injections. Tumor diameters were then periodically measured with calipers. Tumor volume ($mm^3$) = maximal length (mm) × perpendicular width $(mm)^2/2$. Twenty mice were randomly divided into two groups of the same size (n = 10). miR-125b inhibitor (50 nM in 100 μl of PBS) and parental K562 cells were directly injected into the formed tumors with the help of a guide screw every other day for three injec-

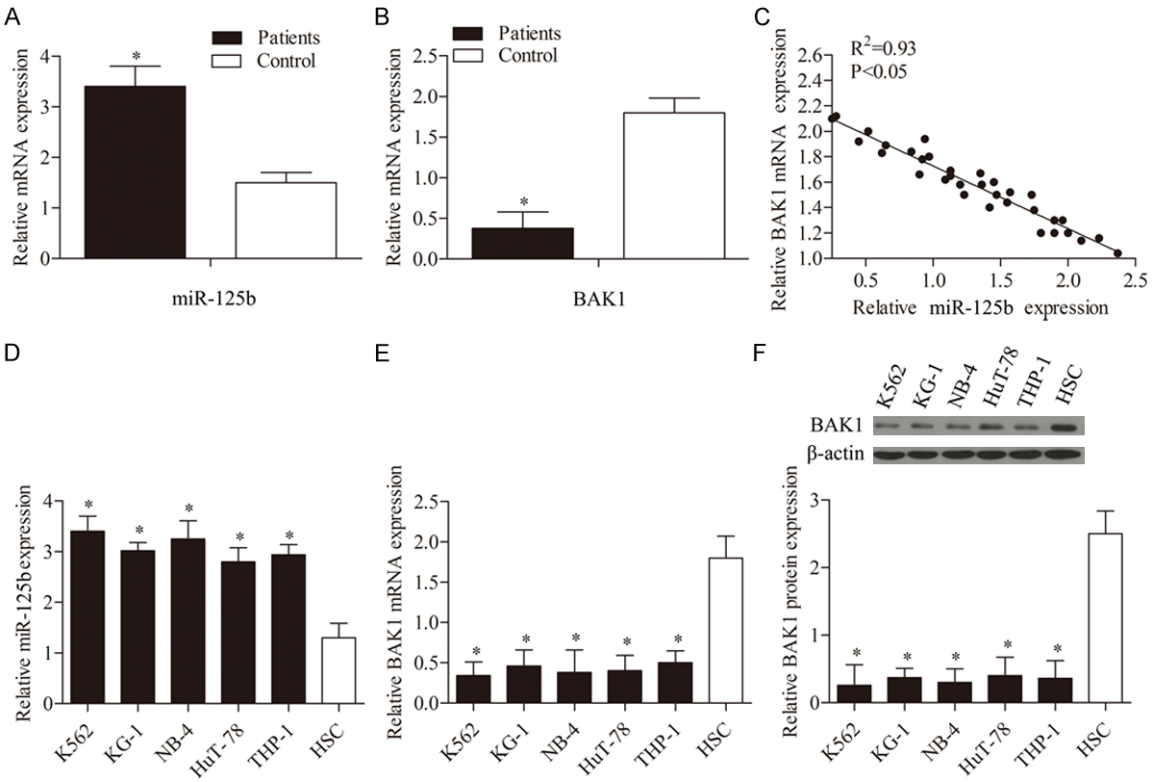



**Figure 1.** Inverse level of miR-125b and BAK1 in CML patients and normal controls. An inverse level was detected in CML cells (K562, KG-1, NB-4, HuT-78 and THP-1) and HSC cells. A. The expression levels of miR-125b in CML patients and normal patients were measured by quantitative real-time PCR (qRT-PCR). B. The mRNA expression of BAK1 in CML patients and normal controls was assessed by qRT-PCR assay. C. Correlation between the miR-125b expression level and BAK1 protein expression level in CML patients. D. The expression level of miR-125b in CML cells and HSC cells was measured by quantitative real-time PCR (qRT-PCR). E. The mRNA expression of BAK1 in CML cells and HSC cells was assessed by qRT-PCR assay. F. Western blotting assay was used to confirm the expression profile of BAK1 in the above cell lines. Relative protein expression was quantified using Image-Pro Plus 6.0 software and normalized to β-actin. Data are presented as the mean ± SD of 3 experiments. *P < 0.05 versus the control group.

tions. The death of mice was recorded to generate a survival curve using SPSS software.

*Statistical analysis*

All results were presented as the mean ± SD. The statistical significance of the studies was analyzed using Student's t test. A difference was deemed statistically significant at $P < 0.05$.

## Results

*Inverse level of miR-125b and BAK1 in CML patients and CML cells*

The expression level of miR-125b and BAK1 was detected in CML patients by qPCR. The results indicated that the expression level of miR-125b was significantly higher in CML patients than in healthy individuals ($P < 0.05$) (**Figure 1A**). The BAK1 expression level was decreased in CML patients compared with normal controls ($P < 0.5$) (**Figure 1B**), and there was a strong negative correlation between the expression level of miR-125b and the mRNA expression level of BAK1 in CML patients (**Figure 1C**). The expression level of miR-125b and BAK1 was also detected in K562, KG-1, NB-4, HuT-78, THP-1 and HSC cell lines by qPCR. The results indicated that the level of miR-125b was significantly higher in CML (K562, KG-1, NB-4, HuT-78 and THP-1) cells compared with HSC cells ($P < 0.01$) (**Figure 1D**). In addition, the mRNA and protein expression levels of BAK1 were examined by qRT-PCR and Western blot analysis, respectively (**Figure 1E** and **1F**). The results showed that both the mRNA and protein levels of BAK1 were significantly elevated in CML patients and CML cells compared with normal control cells.

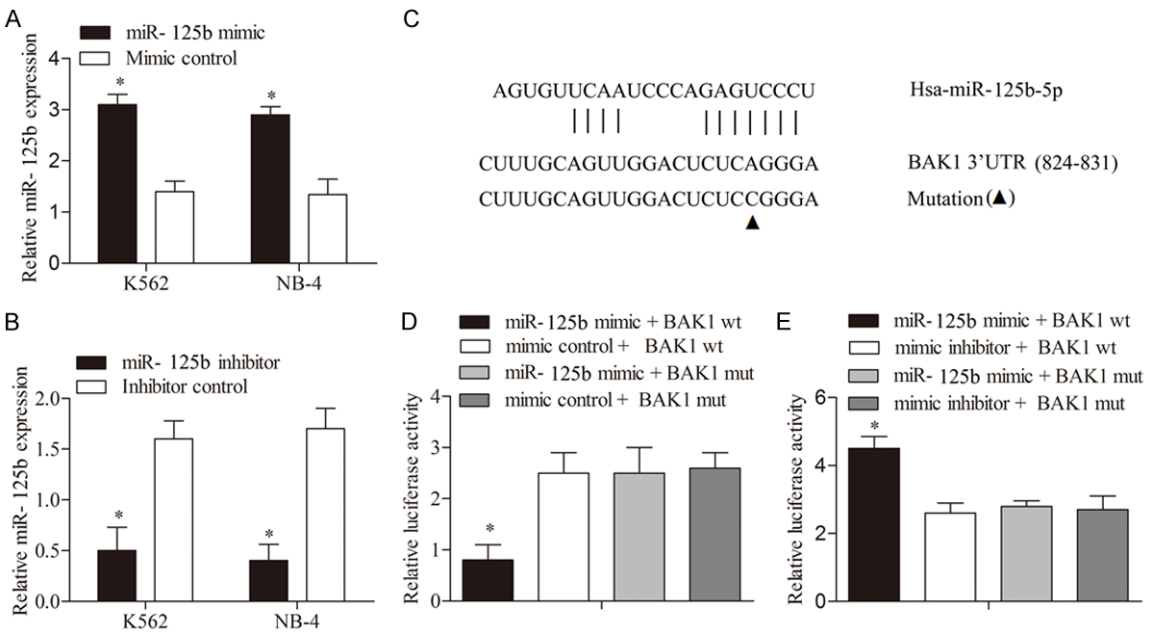



**Figure 2.** The targeting relationship between miR-125b and BAK1. The target gene was predicted by TargetScan database and identified by the luciferase activity. A. The expression level of miR-125b in K562 and NB-4 cells co-transfected with the miR-125b mimic or mimic control was measured by qPCR. B. The expression level of miR-125b in K562 and NB-4 cells co-transfected with the miR-125b inhibitor or inhibitor control was measured by qPCR. C. The wild-type and mutant BAK1 3'-UTR contained the target sequence of miR-125b. D. K562 cells were co-transfected with the miR-125b mimic or mimic control luciferase reporter vectors containing the wild-type (wt) or mutant (mut) BAK1 3'-UTR. E. A similar luciferase assay was performed in K562 cells treated with the miR-125b inhibitor or control. Luciferase activity was presented as firefly luciferase normalized to renilla luciferase. Data represent the mean ± SD of 3 experiments. *P < 0.05 versus the control group.

*BAK1 is targeted by miR-125b*

Given that the level of miR-125b was increased in CML cell lines (especially in K562 and NB-4 cells), we investigated its role in CML cell biology s. A miR-125b mimic was used to amplify the expression of miR-125b, whereas a synthetic inhibitor specific for miR-125b was employed to suppress the expression of endogenous miR-125b in CML cell lines. The efficiency of this miR-125b mimic or inhibitor was confirmed by qPCR assay (**Figure 2A** and **2B**). BAK1 targeting by miR-125b was predicted by the bioinformatic software program TargetScan. According to the results, the potential binding target site of miR-125b was found in the 3'-UTR of the BAK1 gene (**Figure 2C**). To experimentally confirm that BAK1 is an authentic target of miR-125b in K562 cells, the plasmid pMIR-REPORT-BAK1 wt or pMIR-REPORT-BAK1 mut was transfected into CML cells together with miR-125b mimic or the mimic control. After 48 h of transfection, the results showed that the luciferase activity in the BAK1-wt with miR-125b mimic group was significantly reduced compared with the other three groups (**Figure 2D**). Additionally, BAK1-wt and BAK1-mut luciferase reporter vectors were co-transfected with the miR-125b inhibitor or inhibitor controls into K562 cells. The results showed that the miR-125b inhibitor reversed the reduction in the expression level of luciferase with the wild-type BAK1 3'-UTR in K562 cells (**Figure 2E**). The above data demonstrated that BAK1 is a genuine target of miR-125b.

*miR-125b promotes the progression and proliferation of CML cells*

To further explore the role of miR-125b in CML cells, we examined whether the overexpression or inhibition of miR-125b was capable of affecting cell apoptosis. K562 cells were transfected with the miR-125b mimic, mimic control, miR-125b inhibitor, or inhibitor control separately. Cell apoptosis was evaluated by propidium iodide staining. The miR-125b inhibitor increased the number of apoptotic cells compared with the inhibitor control, and less apoptotic cells were detected in the miR-125b mim-

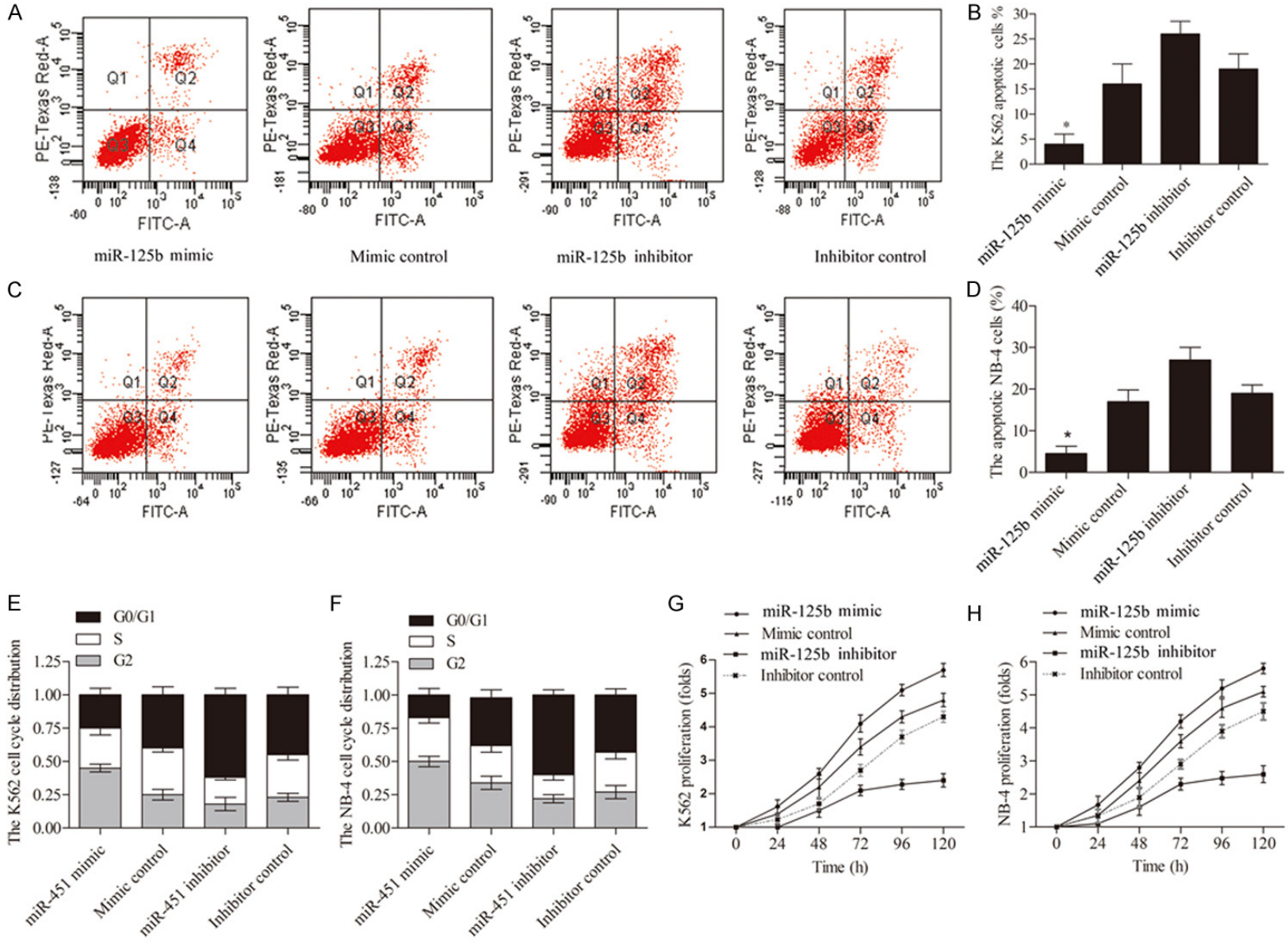



**Figure 3.** Effect of miR-125b on the progression and proliferation of K562 and NB-4 cells. K562 and NB-4 cells were transfected with the miR-125b mimic, mimic control, miR-125b inhibitor or inhibitor control. (A) Apoptotic analysis of K562 cells in each group. (B) Number of apoptotic K562 cells. (C) Apoptotic analysis of NB-4

cells in each group. (D) Number of apoptotic NB-4 cells. (E) Cell cycle analysis of K562 cells with overexpression and inhibition of miR-125b. (F) Cell cycle analysis of NB-4 cells with overexpression and inhibition of miR-125b. The proliferation of K562 (G) and NB-4 (H) cells was determined at the indicated time points by the MTT assay. All experiments were repeated three times with three replicates. (*P < 0.05 versus mimic control; #P < 0.05 versus inhibitor control).

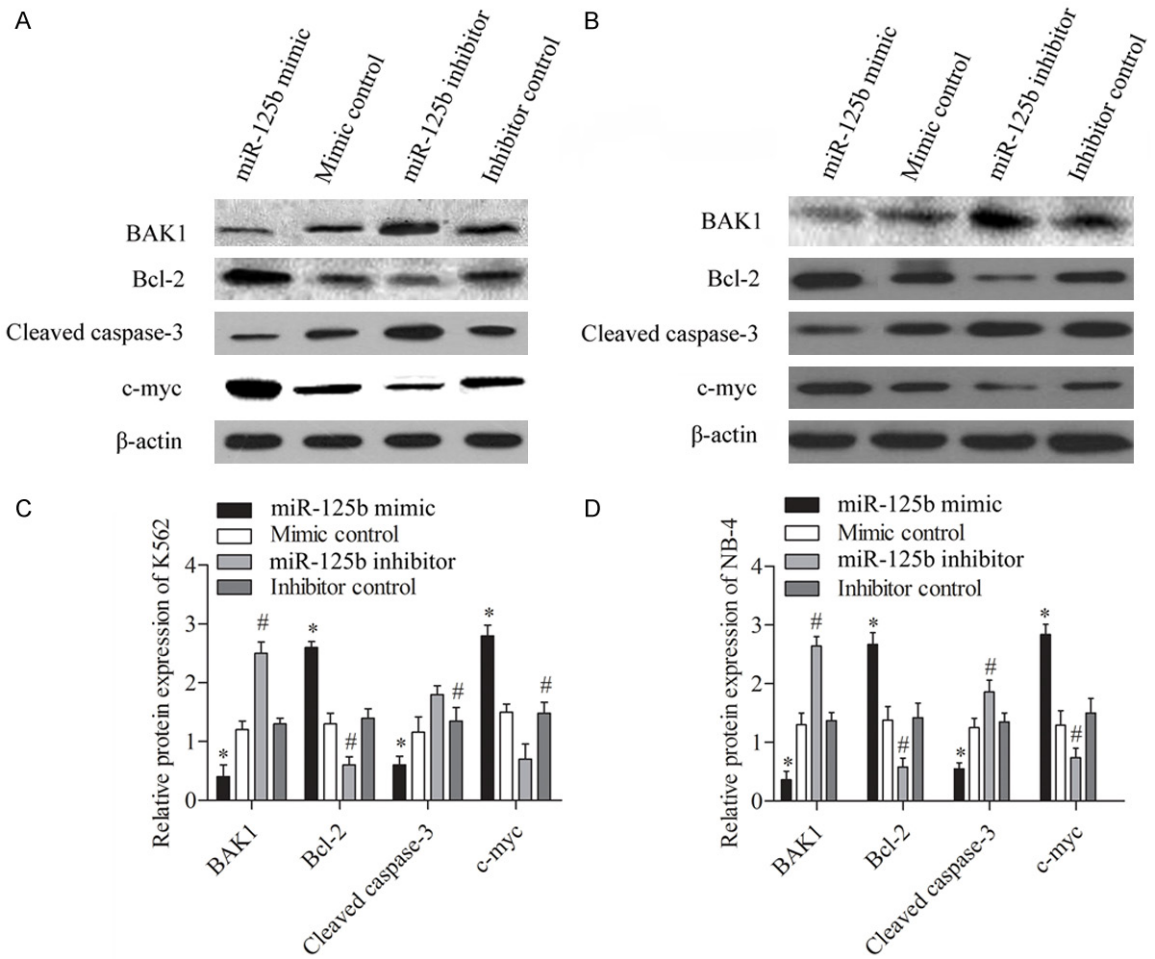



**Figure 4.** miR-125b regulates the mitochondrial apoptotic pathway by targeting BAK1. K562 and NB-4 cells were transfected with the miR-125b mimic, mimic control, miR-125b inhibitor or inhibitor control. A. The protein expression levels of mitochondrial apoptotic pathway members in K562 cells were measured by Western blotting. B. A similar Western blotting assay was performed in NB-4 cells. C. Relative protein expression in K562 cells was quantified using Image-Pro Plus 6.0 software and normalized to β-actin. D. Relative protein expression in NB-4 cells. Data are presented as the mean ± SD of 3 experiments. *P < 0.05 versus mimic control; #P < 0.05 versus inhibitor control.

ic-transfected K562 cells compared with the mimic control cells (**Figure 3A**). Apoptotic cells in each group were quantified in **Figure 3B**. Similar behavior was detected in NB-4 cells; the miR-125b inhibitor promoted cell apoptosis, while the miR-125b mimic suppressed cell apoptosis (**Figure 3C**). NB-4 apoptotic cells in each group were quantified in **Figure 3D**. A cell cycle analysis indicated that miR-125b reduction induced an accumulation of K562 and NB-4 cells in the G0/G1 phase, while the miR-125b mimic shortened the G0/G1 phase compared with the mimic control (**Figure 3E** and **3F**), implying that cell cycle arrest occurred in K562 and NB-4 cells with the change in miR-125b levels. A cell proliferation assay was conducted in K562 cells. The miR-125b inhibitor strongly suppressed the growth of K562 cells compared with the inhibitor control group, and the miR-125b mimic increased the proliferation rate of K562 cells compared with the mimic control group (**Figure 3G**). Similar MTT results were obtained for NB-4 cells. The NB-4 cell proliferation rate was decreased after treatment

with the miR-125b inhibitor, while cell growth was enhanced by overexpression of miR-125b (**Figure 3H**). These findings suggested that miR-125b facilitates cell survival by accelerating the G0/G1 phase and promotes cell proliferation in CML cells.

*miR-125b inhibits the mitochondrial apoptotic pathway by downregulating BAK1*

The mitochondrial apoptotic pathway is highly correlated with the metastatic progression and survival of CML [18]. BAK1 opposes the function of Bcl-2 [19]. Thus, we detected the effect of miR-125b overexpression and suppression on the expression of BAK1 and the mitochondrial apoptotic pathway, including BAK1, Bcl-2, Caspase-3 and c-myc genes. The protein expression of these factors was detected by Western blotting. The results revealed that the miR-125b inhibitor effectively increased the expression of pro-apoptosis factors (BAK1, Caspase-3) and decreased the expression of anti-apoptosis factors (Bcl-2, c-myc) in K562 cells compared with the inhibitor control cells. Meanwhile, the miR-125b mimic suppressed the expression of pro-apoptosis factors and promoted the expression of anti-apoptosis factors compared with cells transfected with the mimic control (**Figure 4A**) ($P < 0.05$). The Western blotting assay was also performed in NB-4 cells; the miR-125b inhibitor strongly promoted the expression of BAK1 and Caspase-3 and inhibited the expression of Bcl-2 and c-myc compared to the inhibitor control cells. Low expression of BAK1 and Caspase-3 was detected in miR-125b mimic-treated cells compared to mimic control cells (**Figure 4B**). Relative quantification of the protein expression of mitochondrial apoptotic pathway members in K562 and NB-4 cells is summarized in **Figure 4C** and **4D**. The above data suggested that miR-125b suppressed the mitochondrial apoptotic pathway in CML cells by down-regulating pro-apoptosis factors and up-regulating anti-apoptosis factors.

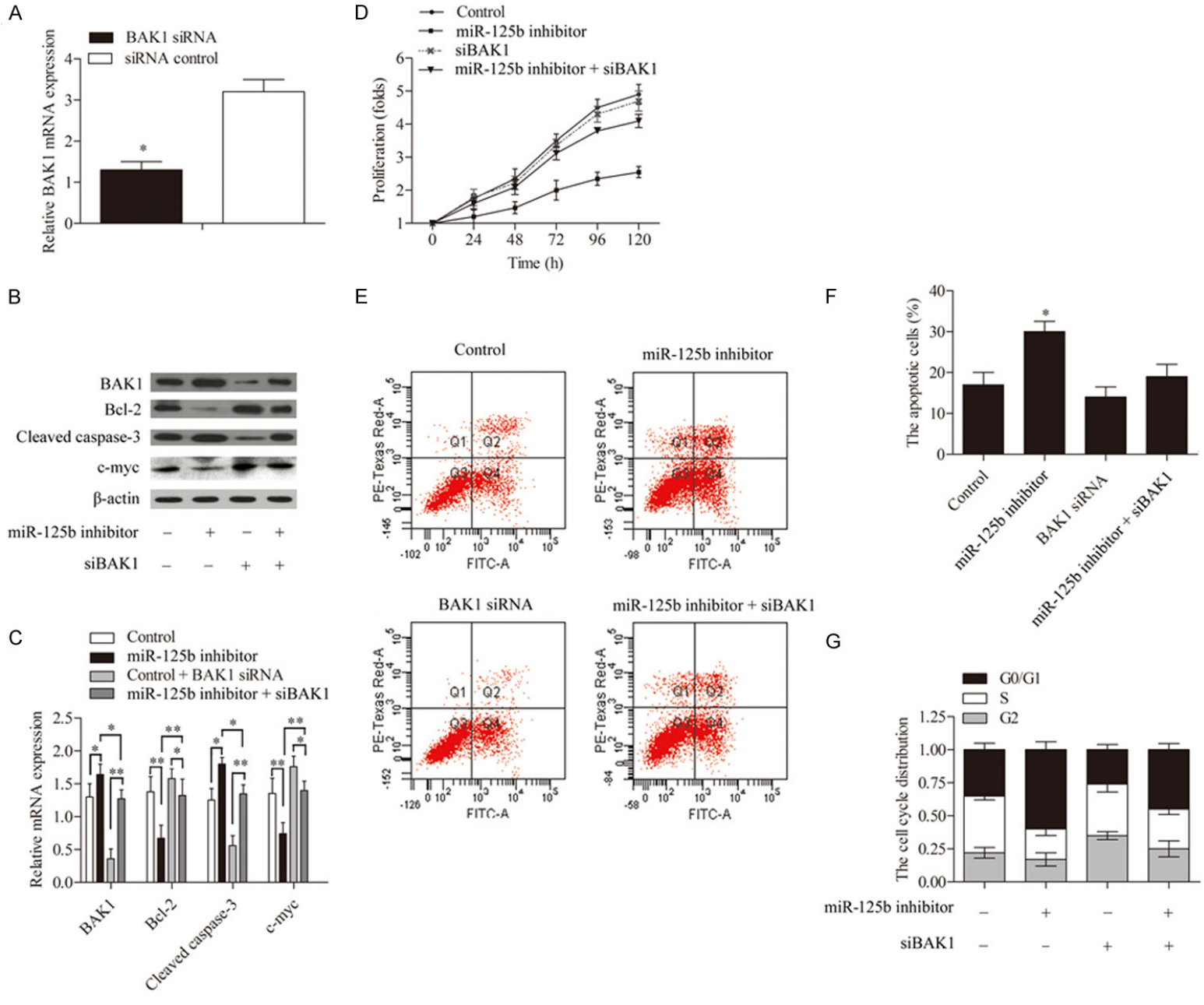
*miR-125b suppression of BAK1 is required for its effect on the mitochondrial apoptotic pathway and CML cells*

Given that BAK1 is a target of miR-125b, we investigated whether BAK1 mediates the effect of miR-125b on the mitochondrial apoptotic pathway and CML cells. A synthetic siRNA targeting BAK1 was employed to silence the expression level of BAK1 in K562 cells (**Figure 5A**). Western blotting was used to measure the expression of BAK1, Bcl-2, Caspase-3 and c-myc (**Figure 5B**). The bands were quantified and are shown in **Figure 5C**. In the miR-125b inhibitor plus siBAK1 group, the protein expression of BAK1 and Caspase-3 was effectively increased compared with the siBAK1 group ($P < 0.01$) and decreased compared with the miR-125b inhibitor group ($P < 0.05$). The protein expression of Bcl-2 and c-myc were decreased compared with the siBAK1 group ($P < 0.05$), and the expression of these proteins was increased compared with the miR-125b inhibitor group ($P < 0.01$). These results illustrated that the suppression of BAK1 abrogated the effect of miR-125b silencing on pro-apoptosis factors (BAK1, Caspase-3) and the effect of miR-125b activating anti-apoptosis factors (Bcl-2, c-myc). As shown by MTT assay, a decreased BAK1 level restored the proliferation rates of CML cells treated with miR-125b inhibitors (**Figure 5D**).

To further confirm whether BAK1 overexpression counteracts the effect of miR-125b in K562 cells, we co-transfected the miR-125b inhibitor or inhibitor control with or without the siBAK1 vector into K562 cells. A cell apoptosis analysis indicated that the siBAK1 group exhibited the lowest apoptotic cell numbers. Compared to the inhibitor control, miR-125b inhibitor transfection effectively increased the apoptotic cell numbers. The apoptotic cell numbers in K562 cells transfected with the miR-125b inhibitor plus siBAK1 were effectively decreased compared with the miR-125b inhibitor transfection group (**Figure 5E**). Apoptotic K562 cells were quantified and are shown in **Figure 5F**. Flow cytometric analysis of cell cycle progression demonstrated that G0/G1 arrest was prevented in miR-125b-downregulated CML cells by BAK1-targeting siRNA (**Figure 5G**).

*miR-125b inhibitor suppresses the growth of xenografts in vivo*

Given that miR-125b plays an important role in the proliferation of CML cells, we investigated whether this miRNA also contributes to CML growth in vivo. Thus, the sizes of CML tumors established in mice were determined with a caliper after miR-125b was suppressed. The results revealed that the tumor volumes were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BAK1 siRNA
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miR-125b inhibitor
BAK1 siRNA
miR-125b inhibitor + siBAK1
G
G0/G1
S
G2
The cell cycle distribution
miR-125b inhibitor
siBAK1

**Figure 5.** BAK1 restoration is required for the effect of miR-125b suppression on CML cells. Cells were treated with the miR-125b mimic or mimic control with or without the siBAK1 vector. A. The relative mRNA expression of BAK1 treated with siRNA or negative control in K562 cells was measured by qPCR. B. Relative BAK1, Bcl-2, Caspase-3 and c-myc expression was detected by Western blotting. C. Relative protein expression in CML cells was quantified using Image-Pro Plus 6.0 software and normalized to β-actin. D. The MTT assay was employed to examine the proliferation rates of K562 cells treated with the miR-125b mimic or mimic control with or without the siBAK1 vector. E. Apoptotic K562 cells in each group were measured by flow cytometry. F. Cell cycle progression was measured by flow cytometric analysis. The data represent the means ± SD of three independent experiments.

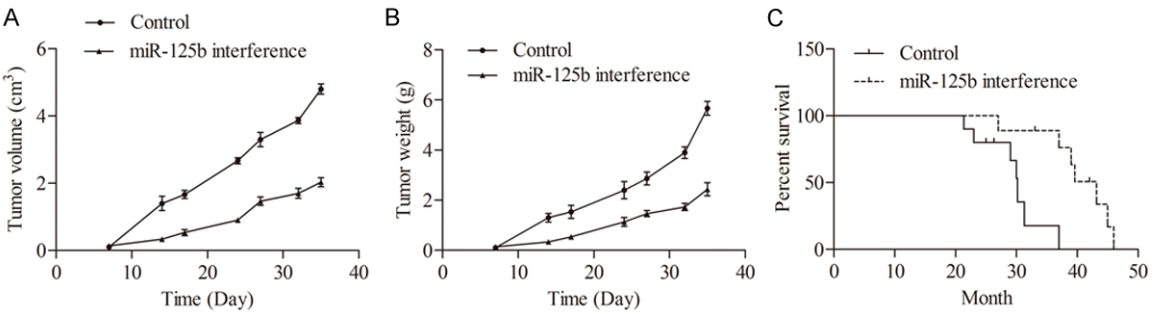


**Figure 6.** miR-125b accelerates the growth of CML in mice. CML cells were transfected with the miR-125b inhibitor and subcutaneously injected into the flanks of nude mice. The diameter of the CML tumor was periodically measured. A. Tumor volume was calculated and is shown as the mean ± SD (n = 6). B. Tumor weights. C. The survival of mice was detected when the miR-125b inhibitor was used (n = 10).

significantly smaller in the group treated with the miR-125b inhibitor than the controls (**Figure 6A** and **6B**). To further confirm the role of miR-125b in the growth of CML, we performed a survival curve analysis. The data indicated that miR-125b downregulation suppressed the growth of xenografts and prolonged the survival time of CML-bearing mice (**Figure 6C**).

## Discussion

CML remains incurable possibly due to the treatment resistance of leukemic stem cells, resulting in rapid disease relapse after the discontinuation of therapy. Many studies aim to recognize molecular signals in CML stem cells. miRNAs are noncoding RNAs that can suppress the expression of protein-coding genes by binding to the target sequence at the 3'-UTR of the target gene [20]. miR-125b has been reported to cause leukemia [21].

The strikingly increased expression level of miR-125b has been reported in many cancers, including breast cancer [22], skin tumors [23], prostate cancer [24] and leukemogenesis [25]. Marina et al. suggested that miR-125b plays an important role in early hematopoiesis by transforming cells and inducing leukemia that affects both the myeloid and lymphoid lineages. Their report also indicated that miR-125b is involved in leukemia as a second event to accelerate the tumorigenicity of the BCR-ABL fusion protein [21]. Accumulating evidence has indicated that BAK1 is a susceptible gene in CML [16, 17, 26]. Thus, we identified BAK1 as a miR-125b-targeted protein. In our study, the results showed that miR-125b was increased significantly in CML cells and CML patients compared with healthy controls, which was consistent with a former study [21]. Furthermore, we detected a strong negative correlation between the expression level of miR-125b and the mRNA expression level of BAK1 in CML patients. To verify the targeting reaction between miR-125b and BAK1, luciferase reporter vectors of wild-type and mutant BAK1 were constructed. The results showed that the overexpression of miR-125b inhibited luciferase expression when cells were transfected with the wt-BAK1 luciferase reporter system, but these results were not observed in the mut-BAK1 groups. Moreover, the inhibition of miR-125b increased luciferase activity in the wt-BAK1 transfection group compared with the mut-BAK1 group. These results demonstrated that BAK1 is a target gene for miR-125b.

BCL-2 was previously shown to cooperate with PML-RARα to block neutrophil differentiation

and initiate APL [27]. As a BCL-2 antagonist or killer, BAK1 is associated with apoptosis and autophagy in CML, according to the expression profile reported by Wang et al. [26]. This result implies that BAK1 represses apoptotic cell death in CML. From the above finding, targeting BAK1 with certain small RNAs provides a novel strategy to prevent the growth of CML cells. Thus, to investigate the role of miR-125b in CML cell growth and apoptosis via targeting BAK1, we detected the effect of a miR-125b inhibitor and miR-125b mimic on the expression of BAK1 and the proliferation and apoptosis of K562 and NB-4 cells. The results showed that miR-125b inhibition led to a significant increase in BAK1, induction of cell apoptosis with cell cycle arrest at the G0/G1 phase, and prohibition of cell growth in K562 and NB-4 cells. Furthermore, the miR-125b mimic promoted the expression of BAK1, which is similar to the physiological feature in CML. The overexpression of miR-125b resulted in a decrease in the number of apoptotic cells with a shortened G0/G1 phase and an increase in cell proliferation.

The Bax and BAK proteins play critical roles in the mitochondrial apoptosis pathway and in determining cell fate [28]. Defects in cell death regulation represent an essential characteristic of acute myeloid leukemia (AML), promoting the accumulation of leukemia cells by conferring tolerance to oncogene activation, cell cycle checkpoint defects, and genetic instability. Bcl-2 family proteins are central regulators of cell life and death, affecting both apoptotic and non-apoptotic cell death. The Bcl-2 family includes both cell survival- and death-promoting members, with the relative levels and activities of these proteins becoming imbalanced in favor of cell survival in AML and most other malignancies [29]. Our study was consistent with previous studies. The overexpression of pro-apoptosis factors (BAK1, Caspase-3) and down regulation of anti-apoptosis factors (Bcl-2, c-myc) were detected in K562 and NB-4 cells after addition of the miR-125b inhibitor, while the miR-125b mimic led to the opposite results. These results suggested that the inhibition of miR-125b promoted the expression of pro-apoptosis factors of the mitochondrial apoptosis pathway by increasing BAK1 expression, which may promote CML cell apoptosis.

To further confirm the regulatory effect of miR-125b on the expression of mitochondrial apoptosis pathway proteins and on the apoptosis and proliferation of CML cells via targeting BAK1, BAK1 was interfered in K562 cells. The results showed that siBAK1 restored the miR-125b inhibition of the protein expression of the mitochondrial apoptosis pathway. The miR-125b inhibitor plus siBAK1 group exhibited a faster growth rate and less apoptotic cells with a shorter G0/G1 phase compared to the miR-125b inhibitor group. This result agreed with the report of Zhang Lei et al.; simultaneous knockdown of BAX and BAK significantly rescued BI 6727/VCR-induced apoptosis in Ewing's sarcoma [30]. Finally, the in vivo experiment indicated that miR-125b downregulation suppressed the growth of CML and prolonged the survival of CML-bearing mice.

In conclusion, our results demonstrated that the down-regulation of miR-125b affects the expression of BAK1, leading to the overexpression of pro-apoptosis factors with suppressed expression of anti-apoptosis factors in the mitochondrial apoptosis pathway. These effects lead to an increase in apoptotic cells with cell cycle arrest at the G0/G1 phase and a decreased proliferation rate in CML. This study provides an important clue to help elucidate the pathogenesis of CML and suggests that miR-125b is a potential therapeutic target for CML.

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