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MiR-125b Expression Affects the Proliferation and Apoptosis of Human Glioma Cells by Targeting *Bmf*

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Key Words

miR-125b • Human glioma cells • Proliferation • Apoptosis • *Bmf*

Abstract

Background: MicroRNAs (miRNAs) are small noncoding RNAs whose function as modulators of gene expression is crucial for the proper control of cell growth. Although many microRNAs were found to express in central nervous system (CNS), the role of the regulatory networks in which they are involved and their function in the pathological process of nerve cells are only just emerging. In the present study, the possible mechanisms by which one neuronal miRNAs, *miR-125b*, affected the growth of nervous cells were investigated using *in vitro* cell line model. Methods: The expression pattern of *miR-125b* in ATRA-treated human glioma cell lines was detected by Northern blotting and *in situ* localization. The effect of *miR-125b* on the proliferation and apoptosis of human glioma cells was analyzed by MTS assay, TUNEL and Flow cytometry analysis. In addition, the identification of target gene of *miR-125b* was studied by dual-luciferase activity assay and Immunoblot Analysis. Results: We found differential expression of *miR-125b* in 1.0 μM *all-trans*-retinoic acid (ATRA)-treated human glioma cell lines. Up-regulation of *miR-125b* partially restored cell viability and inhibited cell apoptosis in U343 cells treated by ATRA. Down-regulation of *miR-125b* decreased human glioma cells proliferation and enhanced the sensitivity of human glioma cells to ATRA-induced apoptosis. In addition, we found an inverse relationship between the expression of *miR-125b* and the cell apoptosis-related protein Bcl-2 modifying factor (*Bmf*), and *miR-125b* can interact with 3′-untranslated region (UTR) of *Bmf*. Conclusion: These findings indicate that overexpression of *miR-125b* promotes human glioma cell proliferation and inhibits ATRA-induced cell apoptosis and low expression of *miR-125b* sensitizes cells to ATRA-induced apoptosis. BMF may play an important role in the process of *miR-125b* influencing cell apoptosis.

Introduction

The cells of diverse organisms from unicellular organisms to mammal contain many different microRNAs (miRNAs), singlestranded oligoribonucleotides about 22 nucleotides (nt) in length, that function in gene regulation [1-3]. MiRNAs attenuate gene expression by pairing to the 3'-UTR of target transcripts

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inducing RNA cleavage or translational inhibition [4-6]. Only a few mRNAs that are regulated by miRNAs in animals have been verified empirically.

Increasing evidence shows that miRNAs function at all stages of neuronal development, ranging from the initial specification of neuronal cell types to the formation and plasticity of synaptic connections between individual neurons [7-10]. There are many brain-specific (*miR-9, -124a, -124b, -135, -153, -183, -219*) and brain-enriched miRNAs (*miR-9*, -125a, -125b, -128, -132, -137, -139*) to be detected in human and mouse brain [7]. The events that characterize neuronal differentiation and specification are clearly associated with a distinct miRNA profile [11]. For example, *miR-124*, which contributed to the control of neurite outgrowth during neuronal differentiation [12], in adult brain was preferentially expressed in neurons. In contrast, *miR-23*, a miRNA previously implicated in neural specification, was restricted to astrocytes [13].

MiR-125b as a brain-enriched miRNAs was evenly distributed between neurons and astrocytes [13]. High expression of *miR-125b* had been observed in oligodendroglial tumors [14]. *MiR-125b* was also overexpressed in fetal brain specimens from individuals with Down syndrome (DS) when compared with age-matched controls [15]. However, up to now, the available knowledge in the literature about the role of *miR-125b* in neuronal development was mainly to be implicated in neuronal differentiation. In mouse P19 embryonal carcinoma cells induced to differentiate into neurons, *miR-125b* abundance was markedly increased [16]. The depletion of *miR-125b* suppressed the proliferation of differentiated human neuroblastoma cells *in vitro* [17].

To further explore the possible function and molecular mechanisms that *miR-125b* involved in the pathological process of nerve cells, we detected the expression pattern of *miR-125b* in ATRA-treated human glioma cell lines and analyzed the effect of *miR-125b* on the proliferation and apoptosis of human glioma cells. In addition, we also reported the identification of *Bmf* gene as a regulatory target of *miR-125b*. This study may provide new insights into the function of *miR-125b* in the occurrence of nervous system diseases.

Materials and Methods

Plasmid constructs and transfections

The procedure of pre-microRNA plasmid constructs was referred to the method described by Galardi et al [18] and Laneve et al [19]. In brief, The pre-miR-125b-1 and pre-miR-125b-2 sequences were amplified by PCR from human genomic DNA using the following primers: pre-miR-125b-1 forward/*Hin*dIII, 5′-GGC AAG CTT AAC ATT GTT G CGC TCC TCT CA-3′; pre-miR-125b-1 reverse/*Bam*HI, 5′-TAT GGA TCC TTC CAG GAT GCA AAA GCA CGA-3′; pre-miR-125b-2 forward/*Hin*dIII, 5′-GGC AAG CTT CTA CCG C ATC AAA CCA GAC TT-3′; pre-miR-125b-2 reverse/*Bam*HI, 5′-CGA GGA TCC CAG ACA ATC AAT AAG GTC CAA-3′. After being double digested with *Hin*dIII and *Bam*HI, the PCR product was cloned into pCR3.1 vector (Invitrogen, Carlsbad, CA, USA) and the constructs were verified by DNA sequencing. Their expression were detected by RT-PCR and Northern blotting analysis after 48h from transfection into human U343 glioma cells. For transient transfections, The pCR3.1-based plasmid, *miR-125b* inhibitor (2′-*O*-methyl antisense oligonucleotide against a miR-125b) or *anti-miR* control (2′-*O*-methyl scrambled miRNA) was transfected into U343 cells by the lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) essentially as described as the manufacture's instruction. *MiR-125b* inhibitor and *anti-miR* control were synthesized by TaKaLa. These plasmids and synthetic miRNA inhibitor were cotransfected with a reporter plasmid, pEGFP-C1 (Clontech Laboratories Inc., Palo Alto, CA, USA), to monitor transfection efficiency. Cells were analyzed by fluorescence microscopy 48h after transfection to calculate the transfection efficiency.

Cell cultures and treatments

Human U343 and U251 glioma cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 culture medium (Gibco, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 100 IU/mL penicillin, and 10 mg/mL streptomycin. Before detecting cell proliferation and apoptosis, cells were transfected with pCR3.1-miR-125b-1 (p-125b-1), pCR3.1-miR-125b-2 (p-125b-2), combination of both, pCR3.1, *miR-125b* inhibitor or *anti-miR* control (6 wells each treatment). After 48h, half of wells in each treatment group were cultured in the serum-free medium, half of wells were treated with 1.0 μM *all-trans*-retinoic acid (ATRA) (Sigma, St. Louis, MO, USA) that can induce cell apoptosis [20]. Two days later, the cell growth and apoptosis was measured according to the manufacturer's instructions.

Northern blotting analysis

Total RNA was isolated from cultured cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). 40 μg of total RNA per sample was subjected to electrophoresis on a 15% Urea-PAGE gel, and transferred to a nylon membrane (Hybond N^+; Amersham Pharmacia Biotech, St Albans, Herts, UK). After being UV-cross-linked and baked at 50°C for 30 min, the membrane was prehybridized at 42°C for 4h and then hybridized with 32P-labeled *miR-125b* or *U6* probes at 40°C overnight. Membranes were washed and exposed to PhosphorImager screens (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). The bands were analyzed using Quantity One software (Bio-Rad Hercules, CA, USA). All experiments were repeated at least three times.

Gene name	Primer sequence	Accession number	Size and location	Application
Bmf 3′-UTR (sense)	Forward/*Bam*HI: 5′-TAT GGATCC GAACACCGTCTGGAACAGGA-3′ Reverse/*Xho*I: 5′-TAT CTCGAGCAGGAACCCGTCTTCTTAGC-3′	NM_001003942	1850bp (525-2374)	PCR
Bmf 3′-UTR (deleting putative binding region)	The first part of sequence: Forward/*Bam*HI 5′- TAT GGATCC GAACACCGTCTGGAACAGGA-3′ Reverse: 5′-CAGGAGCTGGCCAGGAATCCTTGA-3′ The second part of sequence: Forward: 5′-TCAAGGATTCCTGGCCAGCTCCTGGGGTTGGGTTTCTCTAAG-3′ Reverse/*Xho*I: 5′-GGCCTCGAGAAGAAGAGGTAATATCCAGA -3′	NM_001003942	1766bp (525-1103 plus 1127-2313)	PCR
Gapdh	Forward: 5′-TGGTATCGTGGAAGGACTCA-3′ Reverse: 5′-GTAGAGGCAGGGATGATGTTC -3′	NM_002046	129bp (606-734)	RT-PCR/Real-Time PCR
Bmf	Forward: 5′-TGATGTGTTCCAACCAGAGGAT-3′ Reverse: 5′-GGTGAGAGGGAAGAGCTGAAGT-3′	NM_001003942	124bp (36-159)	RT-PCR

Table 1. Primer sequences

In situ hybridization of mi-125b with DIG-Labeled LNA Probe

In Situ Hybridization of miRNAs with DIG-Labeled LNA Probes was performed as previously described [21]. Briefly, U343 cells and spinal cord sections (5 μm) were treated with proteinase K (20 g/ml) for 15 min and refixed in 4% PFA for 15 min. After acetylation with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0 for 10 min, sections were prehybridized with hybridization buffer (Roche, Mannheim, Germany) at 40°C for 2h and then hybridized with digoxigenin (DIG)-labeled LNA-miR-125b probe (LNA-miR-125b sequences: 5′-tcA cAa gTt AgG gTc tCa gGg a-3′) at 40°C overnight. The cells were then incubated in buffer containing anti-DIG-antibody (Roche, Mannheim, Germany) 2 h at 37°C, followed by staining with NBT and BCIP (Promega, Madison, WI, USA). The cells and sections were hybridized with DIG-labeled LNA-scrambled probe (LNA-scrambled sequences: 5′-caT taA tgT cGg aCa aCt cAa t-3′) as negative control [22]. Samples were viewed with an Eclipse 80i microscope (NIKON, Tokyo, Japan).

Cell proliferation assay

Cell proliferation was estimated with an MTS assay using the Cell Titer 96 Aqueous One Solution cell proliferation assay (Promega, Madison, WI, USA). U343 cells were seeded in 96-well plates at low density (5000 cells per well) in DMEM/F-12 culture, and allowed to attach overnight. The cell growth was measured according to the manufacturer‘s instructions, Cell Titer 96 Aqueous One Solution was added to each well, the plates were incubated for 4h, then the absorbance was recorded at *A*490nm with a 96-well plate reader (Bio-Rad 3550). There were 3 wells in each treatment group including vehicle control, transfection of p-125b-1, p-125b-2, combination of both, miR-125b inhibitor or anti-miR control. The experiment has been repeated for three times and the results were described as a ratio of transfected pCR3.1, p-125b-1, p-125b-2 or combination of both vs untransfected normal cells or *miR-125b* inhibitor vs *anti-miR* control.

Detection of apoptosis by TUNEL and Hoechst staining

In situ detection of apoptotic cells was performed on adherent cells cultured on chamber slides by using an *in situ* Cell Death Detection Kit (Promega, Madison, WI, USA). Air-dried cell samples were fixed with a freshly prepared fixation solution for 1 h at 15-25°C, and then incubated in permeabilization solution for 2 min on ice, and the TUNEL procedure was conducted according to the manufacturer’s instructions. For the correlation of TUNEL with nuclear morphology, cultures were counterstained with haematoxylin and coverslipped. At least 200 cells for each sample were evaluated for apoptosis in different optical fields (magnification ×400) randomly selected. The results were expressed as ratio of TUNEL-positive cells vs total cells. Each treatment was repeated twice within an experiment. The experiment has been repeated for three times. To confirm the specificity of TUNEL, cultures were treated with 1 μg/mL DNase I (Sigma Chemical Co., St. Louis, MO, USA) at room temperature for 10 min to create positive controls. Terminal deoxynucleotidyl transferase (TdT) was omitted from the labelling reaction mixture in negative controls. Samples were viewed with an Eclipse TE2000-U fluorescence microscope (NIKON, Tokyo, Japan).

Flow cytometry analysis

Cells examined for annexin V expression were washed with PBS and resuspended in 500 μl binding buffer (Annexin V-FITC Kit, Immunotech, Marseille, France), containing 2.5 μl annexin V-fluorescein isothiocyanate (FITC) stock and 5μl 20 μg/ml PI to determine the phosphatidylserine (PS) exposure on the outer plasma membrane. After incubation for 10 min at room temperature in a light-protected area, the specimens were quantified by flow cytometry (BD Biosciences, San Jose, CA, USA), acquiring 8,000 events. Each treatment was repeated twice within an experiment. The experiment has been repeated for three times.

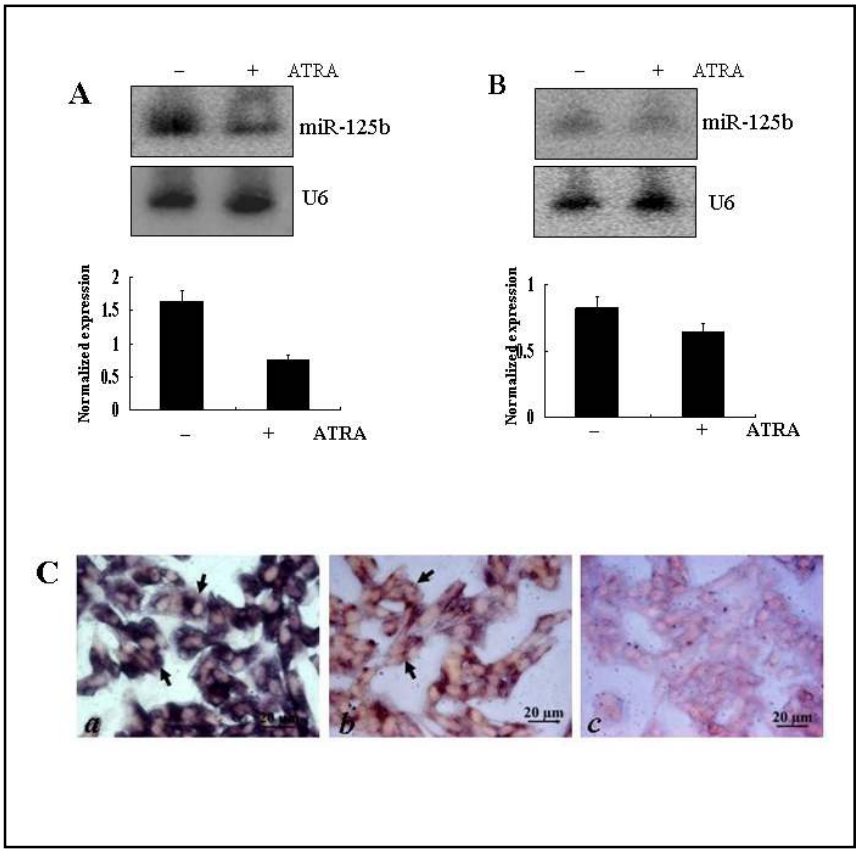

Fig.1. The effects of RA on the expression of *miR-125b*. (A) Northern blotting analysis of the expression of *miR-125b* in U343 cells treated with 1.0 μM ATRA for 48h. Hybridization was done with a 32P-labeled probe for *miR-125b a*nd *U6*. The black histogram represents the optical densities of the signals quantified by densitometric analysis and represented as *miR-125b* intensity/*U6* intensity to normalize for gel loading and transfer. (B) Northern blotting analysis of the expression of *miR-125b* in U251 cells treated with 1.0 μM ATRA for 48h. The black histogram represents the optical densities of the signals quantified by densitometric analysis and represented as *miR-125b* intensity/*U6* intensity to normalize for gel loading and transfer. (C) *In situ* localization of *miR-125b* in U343 cells. Untreated U343 cells (a) and ATRA-treated cells (b) were subjected to *in situ* hybridization using DIG-labeled LNA probes specific to *miR-125b*. The cells were hybridized with DIG-labeled LNA scrambled miRNA probe as a negative control (c). The stain was developed with BCIP/NBT. Black arrows indicate hybridization signal. The scale bar indicated a distance of 20 μM.

Luciferase Activity Assays

Firefly luciferase and *Renilla* luciferase were amplified by PCR from FFO and pRL-TK that were kindly provided by Tian Zhu He (The agricultural University of China, Beijing, China). The PCR products were cloned into pCR3.1 vector (Invitrogen, Carlsbad, CA) and the constructs were verified by DNA sequencing. These vectors were named pCR3-Fluc and pCR3-Rluc, respectively. A 1850-nt-long region, containing the *miR-125b* target sites, of the 3′-UTR of the human Bcl-2 modifying factor (*Bmf*) was PCR-amplified from human genomic DNA and cloned into the downstream of the stop codon in pCR3-Fluc in a sense direction using the primers in Table 1. A 1766-nt-long region (composed of two part of sequence; Table 1), deleting putative *miR-125b* binding region, of the 3′-UTR of *Bmf* was PCR-amplified from *Bmf* 3′-UTR sense-inserted vector and cloned into the downstream of the stop codon in pCR3-Fluc as a negative control. For the luciferase assay, U343 cells (5×104 per well) were seeded into 24-well plates and allowed to attach overnight. The cells were were cotransfected with *Bmf* 3′-UTR sense-inserted vector or control vector and with plasmid expressing *miR-125b-1*, *miR-125b-2*, combination of both or pCR3.1 vector and with *Renilla* luciferase expression vector. Two days later, cells were harvested and assayed with the Dual-Luciferase Assay (Promega, Madison, WI, USA). Each treatment was performed in triplicate in three independent experiments. The *Renilla* luciferase plasmid was used as an internal control. The results were expressed as relative luciferase activity (*Firefly* LUC/*Renilla* LUC).

Reverse transcription-PCR

Total RNAs were extracted from cultured cells with TRIzol reagent, and reverse-transcribed into cDNA with the SuperScript II reserve transcriptase (Invitrogen, Carlsbad, CA, USA). There were not usable primers in coding region of human BMF for Real time PCR, thus the exponential phase of the amplification reaction in *Bmf* was measured by determination of amplification cycle of PCR. The exponential phase of the amplification reaction in *Gapdh* was measured by Real-Time PCR using a SYBR Premix Ex TaqTM kit (TaKaLa DRR041S, Dalian, China) on the ABI prism 7000 sequence detection System (Applied Biosystems, Foster city, CA, USA). All reactions were run in triplicate. Primers used for PCR are listed in Table 1. The human *Gapdh* gene was amplified as a reference gene for normalization. The experiment has been repeated for three times.

Immunoblot Analysis

Protein extracts were boiled in SDS/β-mercaptoethanol sample buffer, and 50 μg samples were loaded into each lane of 15% polyacrylamide gels. The proteins were separated by electrophoresis, and the proteins in the gels were blotted onto PVDF membranes (Amersham Pharmacia Biotech, St Albans, Herts, UK) by electrophoretic transfer. The membrane was incubated with rabbit anti-BMF polyclonal antibody (Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit anti-BCL-2, rabbit anti-BAX and rabbit anti-β-actin ploYclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA)

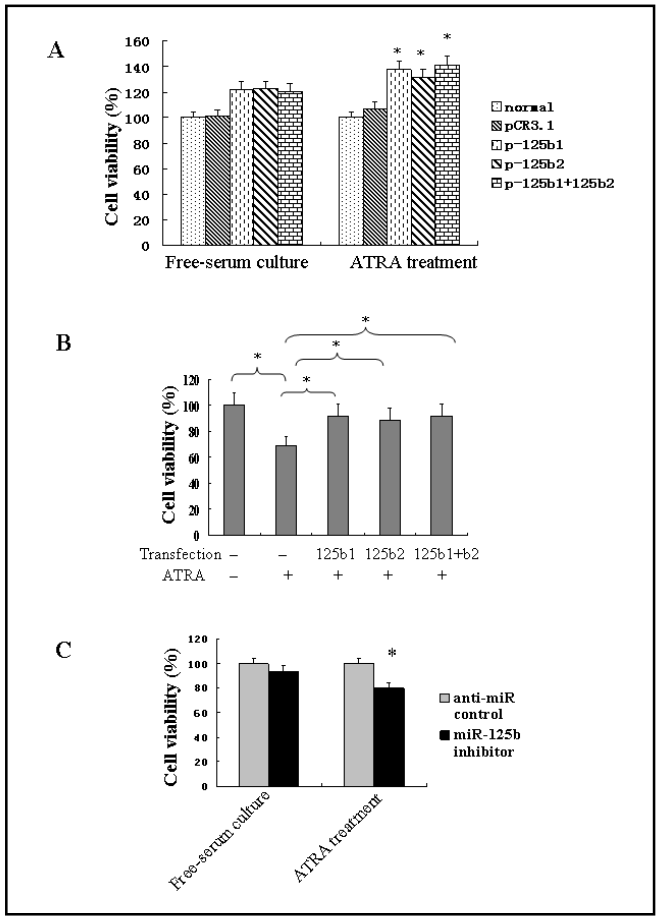

Fig. 2. The effects of *miR-125b* on U343 cells proliferation. (A and B) U343 cells were transfected with p-125b-1, p-125b-2, combination of both or pCR3.1 (6 wells each treatment). (C) U343 cells were transfected with *anti-miR* control or *miR-125b* inhibitor (6 wells each treatment). After 48h, half of wells in each treatment group were cultured in the serum-free medium, half of wells were treated with 1.0 μm ATRA. Two days later, cell proliferation was determined using an MTS assay. The results were expressed at stimulation index (SI) (ratio of *A*490nm with U343 cells expressing with p-125b-1, p-125b-2, combination of both or pCR3.1 vs untreated U343 cells or *miR-125b* inhibitor vs *anti-miR* control). * indicates significant differences (P<0.05).

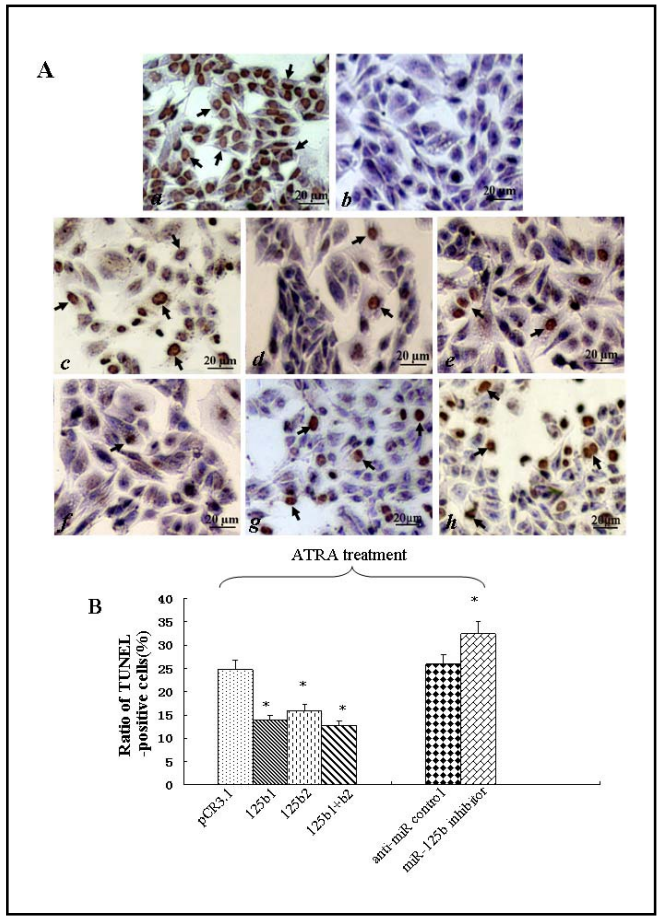

Fig. 3. TUNEL staining of U343 cells. (A) TUNEL staining of U343 cells. (a) Normal U343 cells after treatment with DNAse I (positive control), (b) without terminal deoxynucleotide transferase (negative control), and U343 cells of transfection of pCR3.1 (c), p-125b-1 (d), p-125b-2 (e), combination of both (f), *anti-miR* control (g) or *miR-125b* inhibitor (h) treated by ATRA were stained. Black arrows indicate TUNEL (+) cells. The scale bar indicated a distance of 20 μM. (B) Histogram of ratio of TUNEL-positive cells. The results were expressed as ratio of TUNEL-positive cells vs total cells. * indicates significant differences (P<0.05).

for 2 h at 37°C. The specific protein-antibody complex was detected by using horseradish peroxidase-conjugated (HRP) goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA). Detection by the chemiluminescence reaction was carried using the ECL kit (Pierce, Appleton, WI, USA). The β-actin signal was used as a loading control. The experiment has been repeated for three times. The bands were analysed using Quantity One analyzing system (Bio-Rad, Hercules, CA, USA).

Statistical analysis

The results of Western blotting, cell proliferation and apoptosis were analyzed by one-way ANOVA. All values are reported as the mean±SE. When significant effects of treatments were indicated, Duncan's multiple-range test was used for group comparisons. All statistical analyses were performed using SPSS version 14.0. A value of $P< 0.05$ was considered statistically significant.

Results

Down-regulation of miR-125b by ATRA

The effect of ATRA on the expression of *miR-125b* in human glioma cells was evaluated by Northern blotting

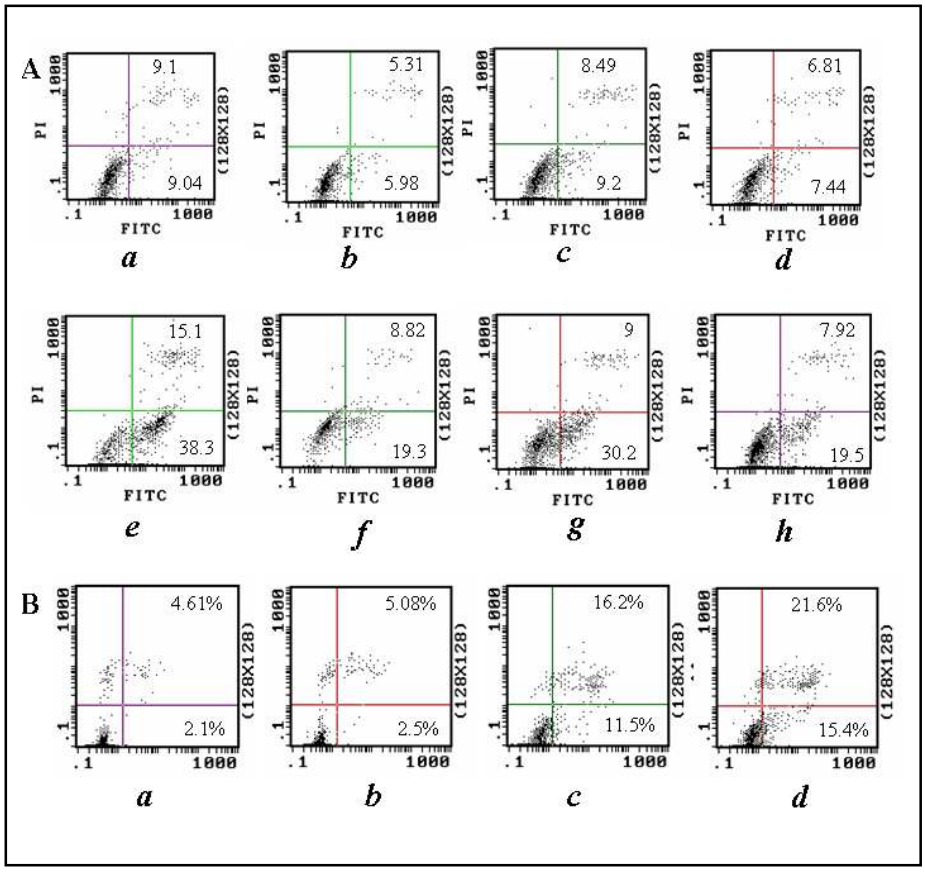

Fig. 4. Apoptosis in U343 cells was detected by flow cytometry. Single cell suspension was prepared and stained with annexin V/ phosphatidylinositol (PI) and subjected to flow cytometry analysis. Cells were transfected with pCR3.1 (A a), p-125b-1 (A b), p-125b-2 (A c), combination of both (A d), *anti-miR* control (B a) or *miR-125b* inhibitor (B b) cultured in the serum-free medium. (A e-h and B c-d) represent respectively the cells transfected with pCR3.1, p-125b-1, p-125b-2, combination of both, *anti-miR* control or *miR-125b* inhibitor then treated with 1.0 μM ATRA for 48h. Lower left quadrant, viable cells (annexin V-FITC and PI negative); lower right quadrant, early apoptotic cells (annexin V-FITC positive and PI negative); upper right quadrant, late apoptosis/necrosis cells (annexin V-FITC and PI positive). The percentage of annexin-V and PI-positive cells (representatives of three separate experiments) is shown in the lower right and upper right panels, respectively.

and *in situ* hybridization. Northern blotting analysis showed that the expression of *miR-125b* in U343 cells was apparently decreased (Fig. 1A), and in U251 cells was moderately reduced after ATRA treatment (Fig. 1B). This result showed that U343 cells was more sensitive to ATRA than U251 cells.

In situ hybridization analysis of *miR-125b* abundance was performed by using a DIG-LNA-miR-125b probe. MiR-125b was mainly located in cytoplasm in U343 cells. Higher cytoplasmic signal was detected in the absence of 1.0 μM ATRA (Fig. 1C a) than in presence of ATRA (Fig. 1C b). This observation was consistent with the Northern blotting results shown in Fig. 1A. No signal was detected in U343 cells that were hybridized with the DIG-labeled LNA-scrambled probe (Fig. 1C c).

The effect of miR-125b on the proliferation of U343 cells

To test the effect of *miR-125b* up-regulation on the *proliferation* of human glioma cells, we made two constructs, p-125b-1, p-125b-2, containing respectively pre-miR-125b-1 and pre-miR-125b-2, under the control of cytomegalovirus promoter. RT-PCR results showed that U343 cells transfected with pre-miR-125b-1 and pre-miR-125b-2, clearly expressed high levels of pre-microRNAs and similar results were obtained with the T47D cell line (data not shown). Northern blotting result showed that U343 cells transfected with pre-miR-125b-1 and pre-miR-125b-2 expressed more the expected mature miR-125b than the control-transfected cells, and cells transfected with p-125b-1 produced more mature *miR-125b* than that transfected with p-125b-2 (Data not shown). The effect of *miR-125b* down-regulation on the *proliferation* of U343 cells was analyzed by transfection of *miR-125b* inhibitor and *anti-miR* control.

Cell proliferation and viability were determined using the MTS assay. As shown in Fig. 2A, transfection of p-125b1, p-125b2 and combination of both can moderately increased cell viability, as compared with cells transfected with the empty vector and normal U343 cells under the condition of serum-free culture. When cells were treated by 1.0 μM ATRA, transfection of p-125b1, p-125b2 and combination of both can significantly promote cell proliferation ($p<0.05$). From Fig. 2B, the proliferation of U343 cells was significantly inhibited by 1.0 μM ATRA ($p<0.05$) and transfection of p-125b-1, p-125b-2 or

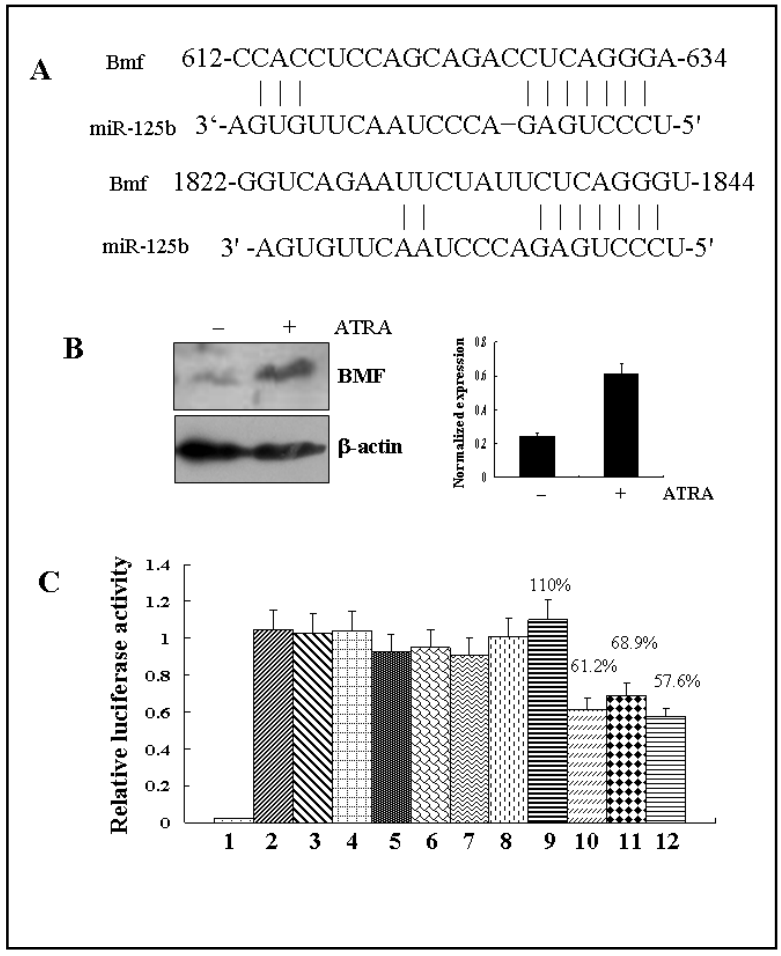

Fig. 5. The prediction and confirmation of the *miR-125b* target. (A) *Bmf* mRNA 3′-UTR putative sites targeted by *miR-125b*. (B) Western blotting analysis of BMF expression in ATRA-treated U343 cells. Total protein was separated by SDS-PAGE, then transferred to PVDF membranes. The membranes were probed with BMF or β-actin antibody, then incubated with anti-rabbit IgG-HRP, and developed using the chemiluminescence (ECL) reagent. The bands were analysed using the Quantity One analyzing system (Bio-Rad Laboratory inc.). The expression of β-actin served as an internal control. The black histogram represents the optical densities of the signals quantified by densitometric analysis and represented as BMF intensity/β-actin intensity to normalize for gel loading and transfer. (C) Luciferase analysis in U343 cells. 1, U343 cell; 2, *Firefly* and *Renilla* luciferase luciferase vector (LUC-V); 3, LUC-V plus pCR3.1; 4, *Bmf* 3′-UTR of deleting putative binding region LUC-V (LUC-V-DS) plus pCR3.1; 5, LUC-V-DS plus p-125b1; 6, LUC-V-DS plus p-125b2; 7. LUC-V-DS plus p-125b1 and 125b2; 8. *Bmf* 3′-UTR sense LUC-V (LUC-V-S) plus pCR3.1; 9. LUC-V-S plus ATRA treatment; 10. LUC-V-S plus p-125b1; 11, LUC-V-S plus p-125b2; 12. LUC-V-S plus p-125b1 and 125b2. The percentages represent enzyme activity in *miR-125b*-treated LUC-V-S transfection relative to pCR3.1-treated LUC-V-S transfection. Each transfection was performed in triplicate. The representative results are shown as relative luciferase activity (*Firefly* LUC/*Renilla* LUC).

combination of both can singnificantly restore the cell viability ($p<0.05$). Transfection of *miR-125b* inhibitor slightly decreased the proliferation of U343 cells compared with *anti-miR* control. When cells were treated by 1.0 μM ATRA, transfection of *miR-125b* inhibitor significantly reduced U343 cell proliferation ($p<0.05$; Fig. 2C). We also analyzed the effect of *mir-125b* on the proliferation of U251 cells, these results was similar with the effect of *mir-125b* on the proliferation of U343 (Data not shown).

Overexpression of miR-125b inhibits the apoptosis of U343 Cells

To assess the possible role of *miR-125b* in controlling apoptosis, apoptosis in U343 cells was determined by TUNEL and Hoechst staining. *In situ* TUNEL labeling showed that cells apoptosis was slightly observed in cells transfected with p-125b-1, p-125b-2, combination of both, *miR-125b* inhibitor or *anti-miR* control (data not shown). After cells were treated by 1.0 μM ATRA, all treatment groups can apparently observe cell apoptosis (Fig. 3). TUNEL-positive cells constituted approximately 25% of the total cell count in cells expressing pCR3.1. The TUNEL-positive cells were markedly decreased in cells expressing p-125b1, p-125b2 or combination of both, to about 9-12% of total cell number. When cells were transfected with *miR-125b* inhibitor, TUNEL-positive cells were evidently increased, to about 7% of total cell number compared with *anti-miR* control.

In order to futher confirm the role of *miR-125b*, apoptosis in U343 cells was determined by flow cytometry. Flow cytometry assay showed that the early apoptotic cells (annexin V-FITC positive) in U343 cells transfected with pCR3.1, p-125b-1, p-125b-2 or combination of both were about 9.04%, 5.98%, 9.2% or 7.44%, respectively. The late apoptosis/necrosis cells (annexin V-FITC/PI positive) in U343 cells transfected with pCR3.1, p-125b-1, p-125b-2 or combination of both were about 9.1%, 5.31%, 8.49% or 6.81%, respectively. It showed that transfection of *miR-125b-1* precursor can slightly inhibit early and late cell apoptosis under the condition of serum-free culture. After cells were treated by 1.0 μM ATRA, the early apoptotic cells in U343 cells

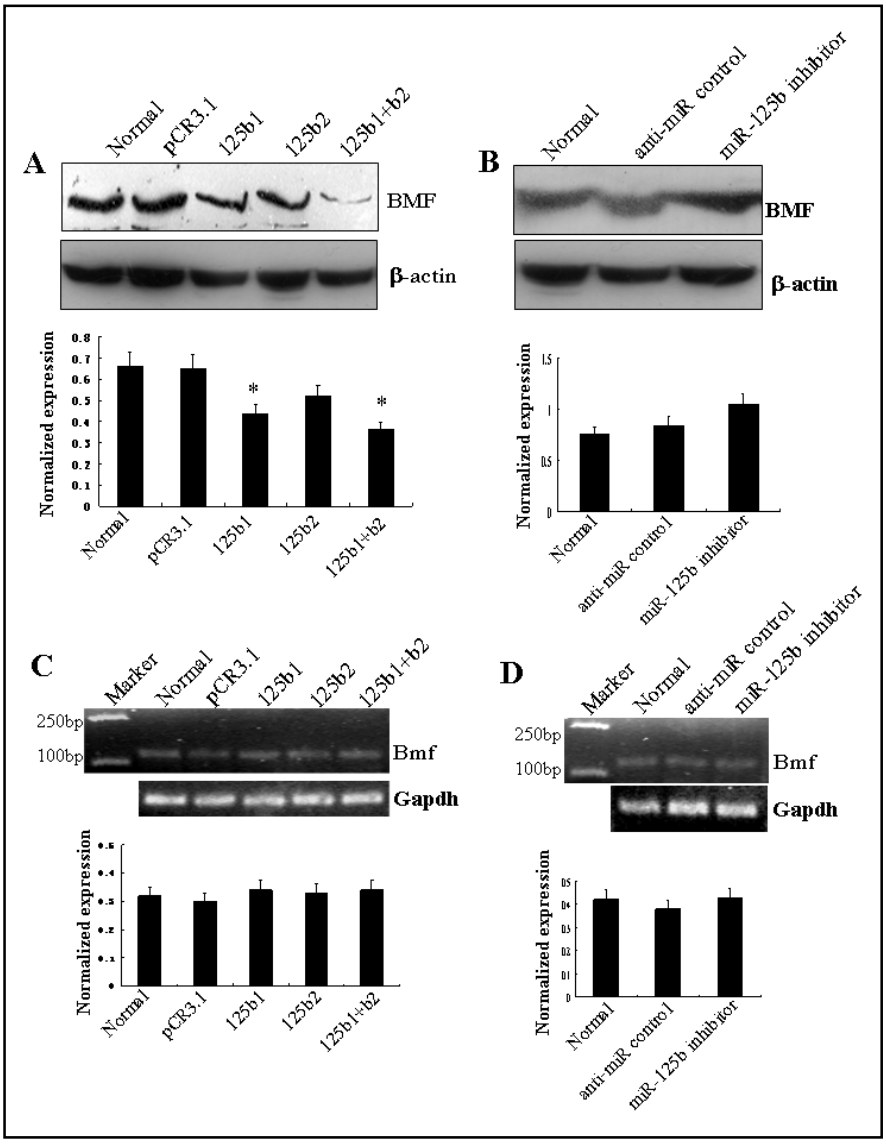

Fig. 6. *MiR-125b* regulates *Bmf* expression. Western blotting analysis of BMF protein expression in *miR-125b* precursor (A) and *miR-125b* inhibitor (B) -treated U343 cells. Total protein was separated by SDS-PAGE, then transferred to PVDF membranes. The membranes were probed with BMF or β-actin antibody, then incubated with anti-rabbit IgG-HRP, and developed using the chemiluminescence (ECL) reagent. The bands were analysed using the Quantity One analyzing system (Bio-Rad Laboratory inc.). The expression of β-actin served as an internal control. The black histogram represents the optical densities of the signals quantified by densitometric analysis and represented as BMF intensity/β-actin intensity to normalize for gel loading and transfer. The expression of *Bmf* mRNA in *miR-125b* precursor (C) and *miR-125b* inhibitor (D) -treated U343 cells was detected by RT-PCR. The bands were analysed using the Quantity One analyzing system (Bio-Rad Laboratory inc.). The expression of *Gapdh* served as an internal control. The black histogram represents the optical densities of the signals quantified by densitometric analysis and represented as *Bmf* intensity/*Gapdh* intensity to normalize for gel loading and transfer.

transfected with pCR3.1, p-125b-1, p-125b-2 or combination of both were about 38.3%, 19.3%, 30.2% or 19.5%, respectively. The late apoptosis/necrosis cells (annexin V/PI positive) in U343 cells were respectively about 15.1%, 8.82%, 9% or 7.92%. It showed that overexpression of *miR-125b-1* precursor can significantly inhibit the early cell apoptosis, and overexpression of *miR-125b-2* precursor only lightly inhibited the early cell apoptosis. All transfected cell, either expressing *miR-125b-1* precursor or *miR-125b-2* precursor, can obviously decrease the late cell apoptosis (Fig. 4 A). These results further indicated that overexpression of *miR-125b* can remarkably inhibit ATRA-induced cell apoptosis. The early apoptotic cells (annexin V-FITC positive) in U343 cells transfected with *anti-miR* control or miR-125b *inhibitor* were about 2.1% or 2.5%, respectively. The late apoptosis/necrosis cells (annexin V-FITC/PI positive) in U343 cells transfected with *anti-miR* control or *miR-125b* inhibitor were about 4.61% or 5.08%, respectively. It showed that transfection of miR-125b inhibitor can slightly increased early and late cell apoptosis under the condition of serum-free culture. After cells were treated by 1.0 μM ATRA, the early apoptotic cells in U343 cells transfected with *anti-miR* control or *miR-125b* inhibitor were 11.5 % or 15.4%, respectively. The late apoptosis/necrosis cells (annexin V/PI positive) in U343 cells transfected with *anti-miR* control or *miR-125b* inhibitor were respectively about 16.2% or 21.6% (Fig. 4 B). It showed that down-regulation of *miR-125b* mainly promoted the late cell apoptosis.

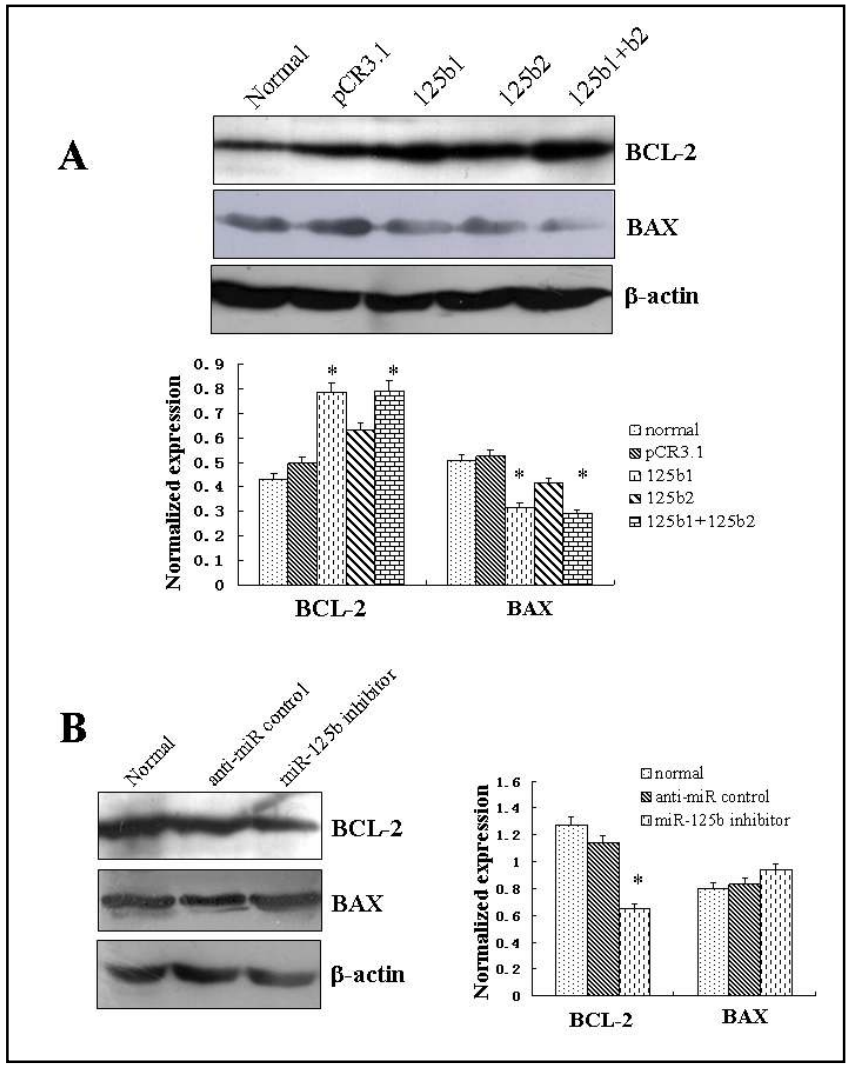

Fig. 7. BCL-2 and BAX expression in U343 cells. Western blotting analysis of BCL-2 and BAX protein expression in *miR-125b* precursor (A) and *miR-125b* inhibitor (B) - treated U343 cells. Total protein was separated by SDS-PAGE, then transferred to PVDF membranes. The membranes were probed with BCL-2, BAX or β-actin antibody, then incubated with anti-rabbit IgG-HRP, and developed using the chemiluminescence (ECL) reagent. The bands were analysed using the Quantity One analyzing system (Bio-Rad Laboratory inc.). The expression of β-actin served as an internal control. The histogram represents the optical densities of the signals quantified by densitometric analysis and represented as BCL-2 or BAX intensity/β-actin intensity to normalize for gel loading and transfer.

Prediction and confirmation of target genes of miR-125b

To analyze the molecular mechanisms in which the *miR-125b* are involved we looked for its target gene. An online search of *miR-125b* targets by miRanda (http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer.pl, [23]) and TargetScan databases (http://genes.mit. edu/targetscan.test/ucsc.html, [24]) provided a large number of putative mRNA targets. Among them, we focused on Bcl-2 modifying factor (*Bmf*) for the following reasons: (*i*) TargetScan and miRanda prediction showed that there were two *miR-125b* responsive elements in 3′-UTR of *Bmf* (Fig. 5A); (*ii*) it was reported that *Bmf* was involved in cell apoptosis [25], and (*iii*) we found that *miR-125b* was down-regulated by ATRA (Fig. 1A), but the level of BMF protein evaluated by Western blotting was up-regulated in ATRA-treated U343 cells (Fig. 5B). Thus it can be seen that the level of BMF protein was inverted with that of *miR-125b*.

To validate BMF as the target of *miR-125b*, we set up a luciferase reporter assay. A portion of the 3′-UTR of BMF, including the *miR-125b* target sites, was cloned into the downstream of the pCR3-Fluc ORF. A portion of the 3′-UTR of BMF, deleting putative *miR-125b* binding region, was used as a negative control. The sense or control reporter vector was cotransfected with pCR3.1, p-125b-1, p-125b-2 or combination of both into U343 cells. The cells transfected with sense reporter vector were also treated with ATRA. Luciferase activity was measured two days after transfection. The histogram in Fig. 5C shows enzyme activity was 61.2%, 68.9% or 57.6% separately in cotransfection of p-125b1, p-125b2 or combination of both and *Bmf* 3′-UTR sense-inserted vector relative to that of pCR3.1 and *Bmf* 3′-UTR sense-inserted vector, and reduced about 31-43%. ATRA treatment measurably increased luciferase activity, to about 9%. Cotransfection of p-125b-1, p-125b-2 or combination of both and *Bmf* 3′-UTR control vector did

not significantly change luciferase activity. These results suggest that the 3′-UTR of *Bmf* transcript may be a *miR-125b* target.

MiR-125b regulation of BMF expression

Although BMF was identified as a target gene for *miR-125b*, it was unknown whether *miR-125b* could regulate endogenous BMF expression. U343 cells were transfected with p-125b-1, p-125b-2 or combination of both to see whether *miR-125b* up-regulation could affect endogenous BMF. Compared with control, BMF protein level was significantly down-regulated by the *miR-125b-1* or combination of both ($P<0.05$; Fig. 6A) and measurably decreased by *miR-125b-2*, and the level of *Bmf* mRNA detected by RT-PCR was not significantly changed (Fig. 6 C). Additionally, U343 cells were transfected with the *miR-125b* inhibitor to see whether *miR-125b* down-regulation could modulate BMF expression (Fig. 6 B). Compared with anti-miR control, BMF protein level in U343 cells was measurably up-regulated by the *miR-125b* inhibitor, and the level of *Bmf* mRNA was also not significantly changed (Fig. 6 D). Based on these data, endogenous BMF protein expression was regulated by *miR-125b*.

Detection the relationship of BMF with BCL-2 and BAX

Because the BH3 domain of Bmf is required both for binding to prosurvival Bcl-2 proteins and for triggering apoptosis [26], the protein levels of both BCL-2 and BAX was also examined by Western blotting. Compared with pCR3.1 empty vector, the expression level of BCL-2 was significantly higher and the expression level of BAX was slightly lower in U343 cells transfected with p-125b-1 or combination of both ($P<0.05$; Fig. 7A). The expression of BCL-2 was significantly decreased ($P<0.05$) and the expression of BAX was measurably increased when cells were transfected with *miR-125b* inhibitor (Fig. 7B).

Discussion

In this study, we found that the expression of *miR-125b* in U343 cells was apparently decreased after ATRA treatment. Our findings are consistent with our previous observation that the expression of *miR-125b* in spinal cord of rat foetuses was downregulated in ATRA-induced neural tube defects [21]. These results suggest that the expression of *miR-125b* might be implicated in the abnormal development of central nervous system.

To explore the possible function of *miR-125b* in the pathological process of nerve cells, we detected the effect of *miR-125b* expression on the growth of nervous cells using *in vitro* cell lines model. ATRA can induce cell apoptosis [20] and neural tube defects in mouse and rat [21, 27]. Herein we found that ATRA inhibited the proliferation of U343 cells, and transfection of p-125b-1, p-125b-2 or combination of both stimulated the growth of U343 cells and partially recovered cell viability and transfection of *miR-125b* inhibitor restrained cell proliferation. Our findings are consistent with a previous observation that transfection of *miR-125b* into PC3 cells led to cell growth [17]. The depletion of *miR-125b* suppressed the proliferation of differentiated human neuroblastoma cells *in vitro* [17]. These results imply that the expression of *miR-125b* was closely related to the growth of human glioma cells.

To further analyze the role of *miR-125b* in controlling the growth of nervous cells, apoptosis in U343 cells was determined by TUNEL and flow cytometry**.** Transfection of *miR-125b-1* precursor can markedly inhibit the early and late cell apoptosis and transfection of *miR-125b-2* precursor only lightly inhibited the early cell apoptosis and obviously decreased the late cell apoptosis after cells were treated by 1.0 μM ATRA. The discrepancy of the effect of *miR-125b-1* and *miR-125b-2* on cell apoptosis may be caused by transfection of miR-125b-1 precursor producing more mature *miR-125b* than that of miR-125b-2 precursor. Although there was difference in controlling cell apoptosis between *miR-125b-1* and *miR-125b-2*, these results suggest that the overexpression of *miR-125b* can markedly inhibit cell apoptosis. When cells transfected with *miR-125b* inhibitor were treated by ATRA, the late apoptotic cells was visibly increased, indicating that the low expression of *miR-125b* promoted the late cell apoptosis. All these facts further emphasize the important role of *miR-125b* in controlling the growth of nervous cells.

To analyze the possible molecular mechanisms by which *miR-125b* affected cell viability we looked for its target gene. It was well known that the Bcl-2 family proteins were involved in the regulation of cell apoptosis. The Bcl-2 family is divided in two main groups of proteins, antiapoptotic or proapoptotic [28, 29]. The antiapoptotic members display sequence conservation through all four Bcl-2 homology (BH) domains and include *Bcl-2*, *Bcl-XL*, *Mcl-1*, *Bfl-1*(*A1*), and *Bcl-w*. Proapoptotic members are further subdivided in two groups: in the first group are the more conserved multidomain members (eg, *Bax*, *Bak*, and *Bok*) with homology in BH1-3 [30-32] and the

second group, the BH3-only proteins such as *Bmf*, *Bim*, *Noxa*, which contain only the BH3 domain [30]. Some aberrantly expressed miRNAs have been shown to contribute to the pathogenesis of human diseases by targeting Bcl-2 family members. For example, *miR-15* and *miR-16*, which are down-regulated in chronic lymphocytic leukemia had been reported to inhibit *Bcl-2* expression, whereas *miR-29b*, downregulated in cholangiocarcinoma cells, targets *MCL-1*, another anti-apoptotic Bcl-2 family member [33]. *Bcl-w* is a direct target of *miR-122* that functions as an endogenous apoptosis regulator in these hepatocellular carcinoma-derived cell lines [34]. *MiR-125b* down-regulated the expression of *Bak1* in prostate cancer cells [35]. In this study, we had identified *Bmf* as a target of *miR-125b* and three lines of evidence supported this finding. First, there are two *miR-125b* binding site in the 3′-UTR of *Bmf* predicted by TargetScan and miRanda. Second, overexpression of *miR-125b* reduced BMF protein levels and low expression of *miR-125b* promoted the expression of BMF. Third, *Bmf* 3′-UTR-mediated luciferase activity is specifically responsive to transfection of p-125b-1 and p-125b-2. In previous studies, *Bmf* can acts as a sentinel in sensing intracellular damage on the main cytoskeletal structures [26, 36]. The apoptotic activity of *Bmf* is post-translationally regulated through interaction with the myosin Vactin motor complex by its binding to dynein light chain 2 (DLC2). In response to stress stimuli, such as detachment of cells adhesion of the substratum (anoikis) or exposure to ultraviolet irradiation, *Bmf* is released and binds to prosurvival Bcl-2 proteins [26], which is thought to function as a barrier against mis-localisation of cells and metastatic spread of tumours [37], it may be regarded as a candidate tumour suppressor. The silencing of *Bmf* significantly protected cells from arsenic trioxide-induced apoptosis [25]. Therefore, downregulation of BMF by *miR-125b* may contribute to resist ATRA treatment in human glioma cells and abnormal development of nerve cells.

The BH3 domain of Bmf is required both for binding to prosurvival Bcl-2 proteins and for triggering apoptosis [26]. Our study found that the protein levels of BCL-2 in U343 cells transfected with *miR-125b* precursor was apparently higher than that transfected with pCR3.1 empty vector, and the expression of BCL-2 was significantly decreased when cells were transfected with *miR-125b* inhibitor. Thus it could be seen that the expression of proteins that interacted with BMF was also changed when *miR-125b* regulated BMF expression. All these facts suggest that there is a complicated regulation network between microRNA and mRNA.

In summary, overexpression of *miR-125b* suppressed ATRA-induced human glioma cells apoptosis and low expression of *miR-125b* enhanced the sensitivity of cells to ATRA-induced apoptosis, at least in part by blocking *Bmf* mRNA translation. Indeed, a search of the Sanger miRNA target database reveals many target candidates for *miR-125b*. Additional studies will be needed to validate those true targets of *miR-125b* in glioma cells. With identification of more *miR-125b* targets in nervous cells, we believe that this study will lead to a better understanding of the molecular mechanisms involved in *miR-125b* mediated abnormal development of nervous system.

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