

## Original Article

# MicroRNA-203 inhibits proliferation and induces apoptosis of neuroblastoma SH-SY5Y cell via upregulation of TGF- $\beta$ 1/BIM signal

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**Abstract:** Background and Aims: MicroRNAs (miRs) have emerged as critical modulators of carcinogenesis and tumor progression. In the present work, we sought to identify the biological function of miR-203 as well as its underlying mechanism in neuroblastoma (NB) cells. Methods: miR-203 mimics was used to develop miR-203-over-expressed clonal in human neuroblastoma (NB) SH-SY5Y cell line. The cells were used in several in vitro analyses, including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), apoptosis, and signaling assays. A subcutaneously implanted tumor model of SH-SY5Y cells in nude mouse was used to assess the effects of miR-203 on tumorigenesis development. Results: miR-203 overexpression in SH-SY5Y cells showed a 65% decrease in proliferation and 35% increase in apoptosis *in vitro*. Analysis of miR-203 signaling pathways in the clonal derivatives showed an increase in TGF- $\beta$ 1 and BIM expression in SH-SY5Y cells. Interestingly, miR-203 activated BIM by TGF- $\beta$ 1-dependent pathway. Furthermore, miR-203 inhibited proliferation and induced apoptosis via BIM-dependent pathway. Using an orthotopic xenograft mouse model, we found that miR-203 significantly reduced tumor growth in mice. Conclusions: miR-203 can effectively inhibit growth and induce apoptosis of SH-SY5Y cells through activation of TGF- $\beta$ 1/BIM signals.

**Keywords:** Neuroblastoma, microRNAs, apoptosis, TGF- $\beta$ 1, BIM

## Introduction

MicroRNAs (miRNAs) are non-coding short (18-24nt) RNAs, controlling gene expression post transcriptionally, via inhibiting translation or triggering degradation of multiple target mRNAs. They are vitally important regulators of differentiation proliferation and apoptosis. Upregulated and downregulated miRNAs have been found in different malignancies, implicating *miRNAs* as oncogenes or tumor suppressors [1].

*MicroRNA-203* was reported to act as a tumor-suppressive microRNA, and its expression was downregulated in laryngeal carcinoma cells [2] and neuroblastoma [3]. Studies from another group showed that the ectopic expression of *microRNA-203* in prostate cancer cell lines could influence proliferation, apoptosis, and

migration [4, 5], whereas the overexpression of *microRNA-203* in laryngeal carcinoma cells reduced cell viability and led to a cell cycle arrest in G1 phase [3]. Additionally, expression of *microRNA-203* suppressed cell proliferation and migration in human triple-negative breast cancer cells [6]. Other study has found that when the gene encoding *microRNA-203* was deleted in mice, the skin cells proliferated more. These mice also developed more skin tumors than normal mice when they were exposed to cancer-causing chemicals. This indicates that *microRNA-203* could prevent cancerous cells from expanding in number, a key event in the initiation of tumors.

Neuroblastoma (NB) is one of the most common solid cancers of childhood. The disease has a highly varied clinical outcome, some tumours can spontaneously regress without

treatment, while others can progress and lead to the death of the patient in spite of intensive multi-modal chemotherapy [7]. Recently, it has been shown that overexpression of miR-203 inhibited the proliferation, migration and invasion of human SK-N-SH and SH-SY5Y NB cells [3]. However, the molecular mechanism of these effects remains to be elucidated.

Bim is a Bcl-2 homology 3 (BH3)-only protein, it has emerged as an essential pro-apoptotic protein for initiating the intrinsic apoptotic pathway under many physiological and pathophysiological conditions [8]. TGF- $\beta$  is a pleiotropic cytokine that can induce various signal transduction pathways, ultimately leading to cell growth, apoptosis or tumor progression, dependent on the cellular context [9]. TGF- $\beta$ 1 induces apoptosis of normal epithelial cells, osteoclasts and lymphoid cells by a mechanism that depends on Bim [10, 11]. Acquisition of resistance to TGF- $\beta$ -induced apoptosis is a critical step for carcinogenesis in many organs. Recent studies have demonstrated that TGF- $\beta$ 1 signal was directly regulated by miR-203 [12, 13]. We therefore suggested that microRNA-203 inhibits proliferation and induces apoptosis of neuroblastoma SH-SY5Y cell via TGF- $\beta$ 1/BIM signal.

In the present study, we assessed the effect of miR-203 on apoptosis and growth of SH-SY5Y cells in vitro and in vivo, and to explore its molecular mechanisms. Our findings demonstrate that miR-203 inhibits growth and induces apoptosis of SH-SY5Y cells at least in part via activation of the TGF- $\beta$ 1/BIM signaling pathway. This study reveals a novel mechanism of miR-203 on growth and apoptosis of SH-SY5Y cells and may also provide a novel therapeutic target.

## Materials and methods

### Cell culture

Neuroblastoma SH-SY5Y cells were obtained from the American Type Culture Collection (ATCC, Shanghai, China) and maintained in DMEM/F-12 medium (1:1, v/v) (Invitrogen, Hangzhou, China) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Hangzhou, China). All cell lines were cultured at 37°C in humidified atmosphere containing 95% air and 5% CO<sub>2</sub> and split twice per week.

### MiRNA and siRNA transfection

Cells cultured in 6-well plates were transfected at 30-50% confluence using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, CA, USA) with Opti-MEM I (Gibco, NY, USA) according to the manufacturer's instructions. Briefly, 25 pmol of miR-203 mimics (miR-203), TGF- $\beta$ 1 siRNA (Santa Cruz, CA, USA), BIM siRNA, or negative control (NC) was used with 7.5  $\mu$ L of the transfection reagent and the medium was replaced with fresh medium 24 h after transfection. siRNA, miR-203 mimics, and NC were chemical synthesized by GenePharma (Shanghai, China) and the sequences were as follows: 5'-GAGCCGGTTCGAAAGTCATATT-3' for TGF- $\beta$ 1 siRNA, 5'-CAGGCTGTGCAAAGGCCTACATT-3' for BIM siRNA, 5'-ACUACCUUGUGUUAGUGUAGGA-3' for miR-203 mimics, and 5'-UUCUCCGAA-CGUUGCACUGT-3' for NC.

### Quantitative RT-PCR

For mRNA, total RNA was prepared using TRIzol reagent (Invitrogen) and genomic DNA was eliminated with TURBO DNA-free Kit (Ambion). One  $\mu$ g of total RNA was reverse transcribed using iScript reverse transcriptase (Bio-Rad). Omission of reverse transcriptase served as a negative control. cDNA was amplified using Platinum PCR SuperMix (Invitrogen). PCR was performed as follows: 5 min at 94°C, 35 cycles of 60 s at 94°C, 60 s at 57-60°C, and 60 s at 72°C, followed 5 min at 72°C. RT-PCR analysis was done using TGF- $\beta$ , BIM, and  $\beta$ -actin primers from Applied Biosystems or Integrated DNA Technologies. For miR-203 expression analysis, the miR-203 was isolated with the RT2 qPCR Grade miRNA Isolation kit (Qiagen), and amplified using real-time RT-PCR techniques and using specific primers for the qRT-PCR miRNA Detection Kit (ABI).

### Western blot analysis

After the treatments, the cells were scraped from the culture dishes, pelleted and gently washed with ice-cold PBS. Cells were lysed by adding pre-warmed (95°C) 125 mM Tris and 1% sodium dodecyl sulfate (SDS; pH 6.8) buffer to the cell pellets. Cell lysates were then centrifuged and the supernatant was used as whole protein cell lysate. After the proteins were electrophoretically separated in 10% SDS polyacrylamide gels and electrotransferred to blotting

PVDF membranes, the unspecific bonds were blocked with 5% skimmed milk in 1X TBST [25 mM Tris (pH 8.0), 125 mM NaCl and 1% Tween 20] for 1 hr at room temperature and then probed with primary antibodies over night at 48°C. After incubation with near-infrared-dye-conjugated secondary antibodies (IRDye; Licor Biotech, CA, USA; 1:20,000, 1 hr, room temperature). Antibody recognition was detected with an Odyssey scanner manufacturer's instructions (Licor Biotech).

## Growth inhibition by MTT assay

Cells were cultured in 96-well plates at a density of 5000 cells/well and left to recover. The quantity of viable cells was estimated by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). MTT (10 µL of 5 mg/mL solution, Sigma Chemical Co., Germany) was added to each well of the titration plate and incubated for 4 h at 37°C. The cells were then treated with DMSO (40 µL/well) and incubated for 60 min at 37°C. The absorbance of each well was determined in an enzyme linked immunosorbent assay (ELISA) plate reader using an activation wavelength of 570 nm and a reference wavelength of 630 nm. The percentage of viable cells was determined by comparison to untreated control cells.

## Apoptosis analysis by flow cytometry

Apoptotic cells were visualized using an Annexin V-FITC apoptosis detection kit (BD Biosciences, San Jose, CA) according to the manufacturer's protocol. Briefly, cells were harvested 48 h after transfection, washed twice with PBS, and re-suspended in 500 µL of Annexin V binding buffer. Two microliters of FITC-conjugated Annexin V was added to the cell solutions, followed by the addition of 5 µL of propidium iodide (PI). After incubation for 5 min at room temperature in the dark, samples were immediately analyzed using a FAC-SCalibur flow cytometer (BD Biosciences, San Jose, USA). Data from approximately  $1 \times 10^4$  cells were analyzed by using the CELLQuest software (BD Biosciences, San Jose, USA).

## Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

TUNEL assay was performed to detect apoptosis using the *In Situ* Cell Death Detection Kit, TMR red (Roche Diagnostics Corp., Indianapolis, IN) following the manufacturer's protocol.

Briefly, cells on coverslips were fixed with 4% paraformaldehyde (PFA) for 1 h, permeabilized in 0.1% citrate buffer containing 0.1% Triton X-100 for 2 min on ice, then incubated in TUNEL reaction mix containing nucleotides and terminal deoxynucleotidyl transferase (TdT) at 37°C for 1 h. Incubation without the TdT enzyme was conducted as negative control. After incubation, the coverslip was mounted onto a slide using mounting medium containing 4'-6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) and observed under an Olympus AX70 microscope (Olympus, Japan).

## Animal model

BALB/c nude mice (4-5 weeks old) were purchased from the Shanghai Laboratory Animal Center (Shanghai, China) and were randomly divided into 3 groups. The animals were maintained in accordance with institutional policies, and all experiments were performed with approval of the affiliated hospital Committee on the Use and Care of Animals of Qingdao University. The animals were injected subcutaneously with untreated SH-SY5Y cells alone, stable miR-203 transfected SH-SY5Y cells (miR-203) and NC (control) transfected SH-SY5Y cells (NC) into the backside of mice for 6 weeks. The tumor volume was calculated with the use of the following formula: tumor volume (in mm<sup>3</sup>) =  $a \times b^2 \times 0.52$ , where 0.52 is a constant to calculate the volume of an ellipsoid.

## Immunohistochemical staining of xenografts

We stained 3 µm sections of paraffin-embedded tumors with an anti-TGF-β1 and BIM antibody to examine TGF-β1 and BIM expression in SH-SY5Y xenograft tumors. TUNEL assays were performed on paraffin sections from SH-SY5Y xenograft tumors using an *in situ* apoptosis detection kit (Hangzhou, China) as the manufacturer's instruction.

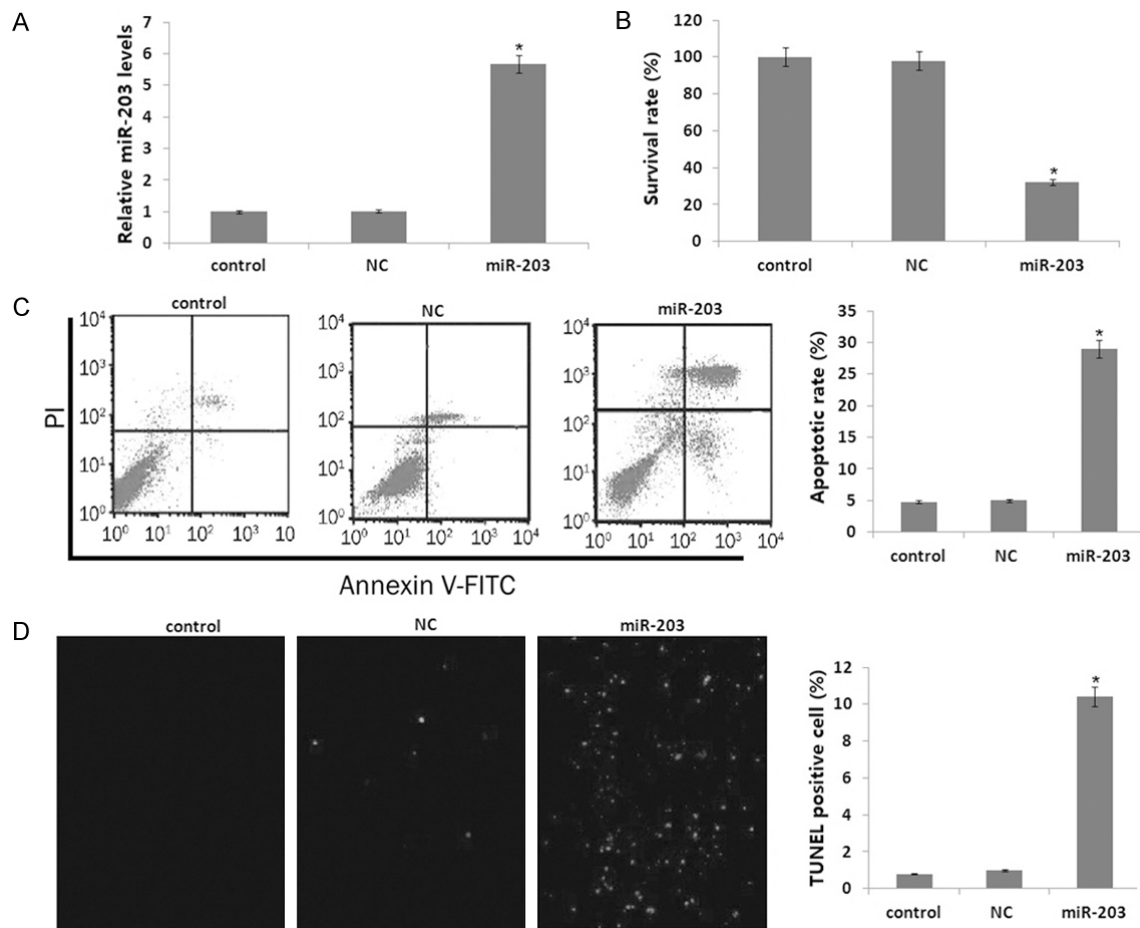
## Statistic assay

Data are presented as mean ± standard deviation. Comparisons were made using Student's *t*-test with significance defined as  $P < 0.05$ .

## Results

### MiR-203 inhibits survival and induces apoptosis of SH-SY5Y cells

To study the function of miR-203 on SH-SY5Y cells, miR-203 mimics (miR-203) was transfected



**Figure 1.** MiR-203 inhibits growth and induces apoptosis of SH-SY5Y cells. A: Relative expression of *miR-203* was detected by qRT-PCR in *miR-203*-transfected SH-SY5Y cells compared with non-mimic-transfected SH-SY5Y cells. B: Effects of *miR-203* or negative control (shown as non-mimic) on the proliferation of SH-SY5Y cells as analyzed by MTT assays; C: Effects of *miR-203* or negative control on cell apoptosis, as analyzed by flow cytometry. D: Effects of *miR-203* or negative control on cell apoptosis, as analyzed by TUNEL assay. Bars represent the mean  $\pm$  SD of three independent experiments. \* $P < 0.01$ .

ed into the SH-SY5Y cells to increase *miR-203* level in the SH-SY5Y cells. The results showed that after 72 h of transfection, expression of *miR-203* was significantly increased compared to the controls by qRT-PCR assay (**Figure 1A**,  $P < 0.01$ ).

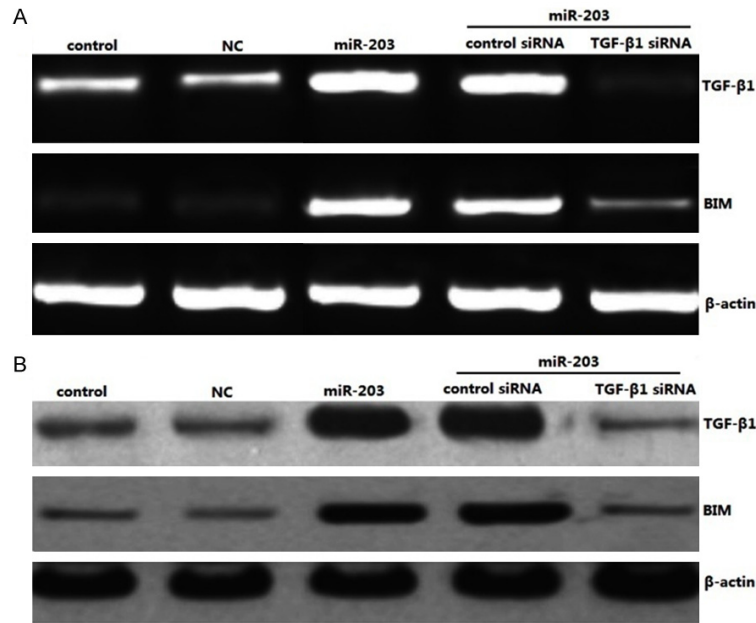
The effect of *miR-203* on viability of SH-SY5Y cells was detected by MTT assay. As shown in **Figure 1B**, cell viability was significantly inhibited in the *miR-203* transfected SH-SY5Y cells compared to the controls ( $P < 0.01$ ). Next, we examined whether the inhibition of cell growth by *miR-203* transfection was also accompanied by the induction of apoptosis. Flow cytometry assay was used to examine the apoptosis of SH-SY5Y cells transfected with *miR-203*. As

shown in **Figure 1C**, *miR-203* transfection induced cell apoptosis compared to the controls ( $P < 0.01$ ). Furthermore, compared to the control treatment, *miR-203* transfection resulted in an increased number of apoptotic cells (TUNEL-positive); the apoptosis index score was significantly higher in *miR-203* transfected SH-SY5Y cells (0.9% for the mock and 1.1% for the control group vs. 9.4% for the *miR-203* transfected group, respectively ( $P < 0.05$ , **Figure 1D**).

#### *MiR-203 increases TGF- $\beta$ 1 and BIM expression*

*MiR-203* mimics (*miR-203*) were transfected into the SH-SY5Y cells for 72 hs. TGF- $\beta$ 1 and





**Figure 2.** MiR-203 upregulated BIM expression by activation of TGF-β1. miR-203 mimics (miR-203) was transfected into the SH-SY5Y cells for 72 hs. miR-203 and TGF-β1 siRNA or BIM siRNA co-transfected into the SH-SY5Y cells for 72 hs. A: MRNA levels of TGF-β1 and BIM were examined by RT-PCR in SH-SY5Y cells; B: Protein levels of TGF-β1 and BIM were examined by western blot assay in SH-SY5Y cells. Bars represent the mean  $\pm$  SD of three independent experiments. \* $P < 0.01$ .

BIM expression was detected by western blot assay and RT-PCR assay. We found that miR-203 significantly increased TGF-β1 and BIM expression in the SH-SY5Y cells (**Figure 2A, 2B**). These results indicated that miR-203 increases TGF-β1 and BIM activity in SH-SY5Y cells.

#### BIM activation by miR-203 is mediated by TGF-β1

RT-PCR and Western blot were performed to investigate the relationship between the expression levels of miR-203, TGF-β1 and BIM. As shown in **Figure 2A, 2B**, targeting TGF-β1 inhibited miR-203-induced upregulation of BIM; by contrast, targeting BIM did not affect miR-203-induced upregulation of TGF-β1 expression (data not shown), suggesting that TGF-β1/BIM signal was upregulated by miR-203.

#### BIM plays an essential role in miR-203-induced apoptosis

In order to investigate the role of BIM in miR-203-induced apoptosis, SH-SY5Y cells were co-transfected with miR-203 and BIM siRNA for 72

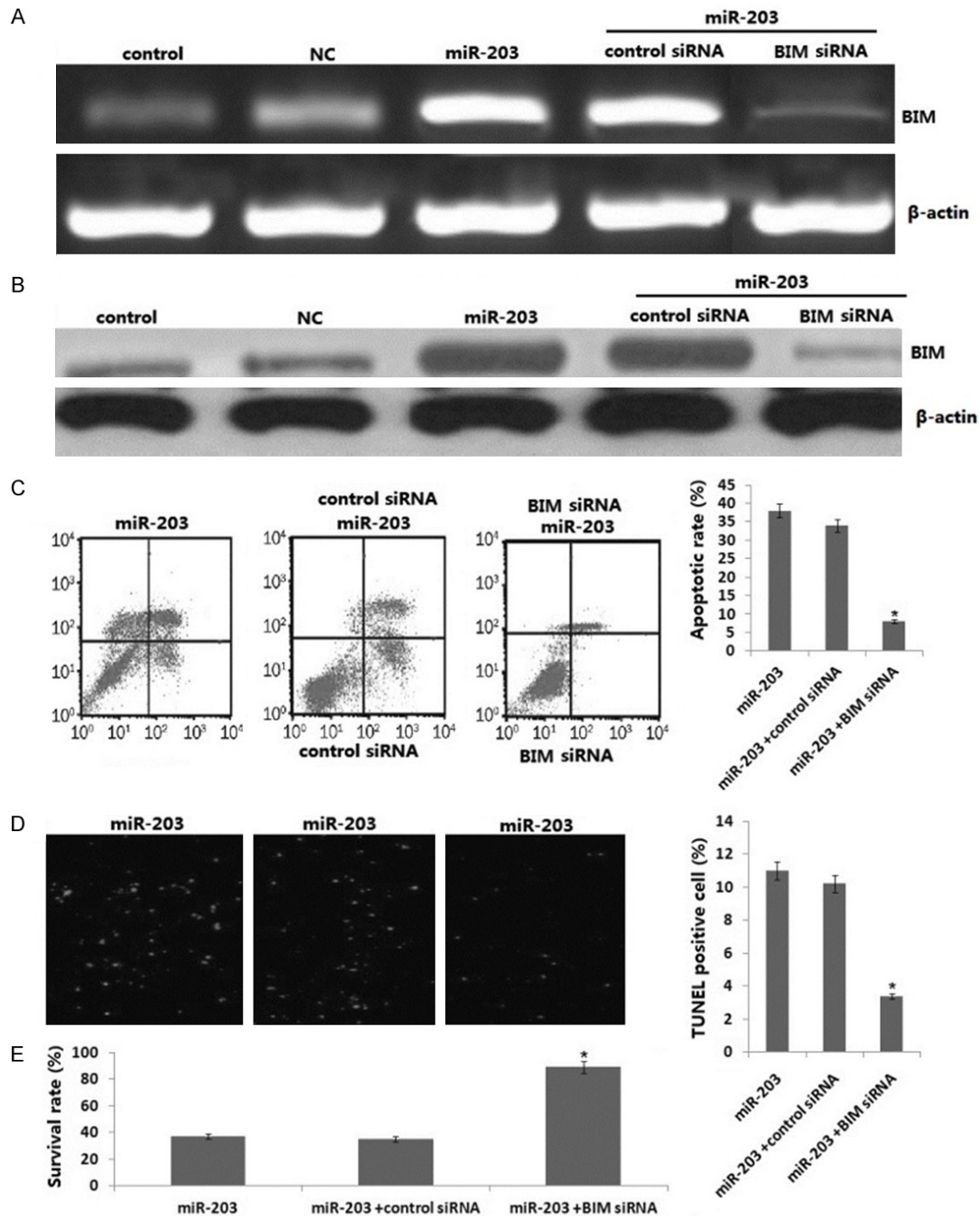
h. As shown in **Figure 3A, 3B**, BIM mRNA and protein was inhibited by BIM siRNA transfection in the miR-203 transfected SH-SY5Y cells. Although miR-203 treatment induced apoptosis in SH-SY5Y cells, miR-203-induced apoptosis was almost completely abolished in BIM siRNA-transfected SH-SY5Y cells by Annexin V/PI staining and TUNEL assay (**Figure 3C, 3D**). In addition, miR-203-induced growth inhibition was almost completely reversed in BIM siRNA-transfected SH-SY5Y cells by MTT assay (**Figure 3E**). Collectively, these results demonstrate that BIM is essential for miR-203-induced apoptosis and growth inhibition in SH-SY5Y cells.

#### MiR-203 attenuates the growth of SH-SY5Y cells *in vivo*

We next investigated the efficacy of miR-203 against tumor growth *in vivo*. miR-203 was overexpressed in stable miR-203 transfected subcutaneous xenograft tumors (**Figure 4A**). Stable transfection of miR-203 mimics into SH-SY5Y cells resulted in decreased growth volume of subcutaneous xenograft tumors in athymic nude mice, when compared to those stably transfected with empty vector (mock) (**Figure 4B**). TUNEL assay showed the apoptotic cells was significantly increased in miR-203 transfected xenograft tumors (**Figure 4C**). In addition, the miR-203 levels within tumors were increased, and the intratumoral expression of TGF-β1 and BIM was also increased by stable transfection of miR-203 (**Figure 4D**). These results suggested that miR-203 could inhibit the growth of SH-SY5Y cells *in vivo*.

#### Discussion

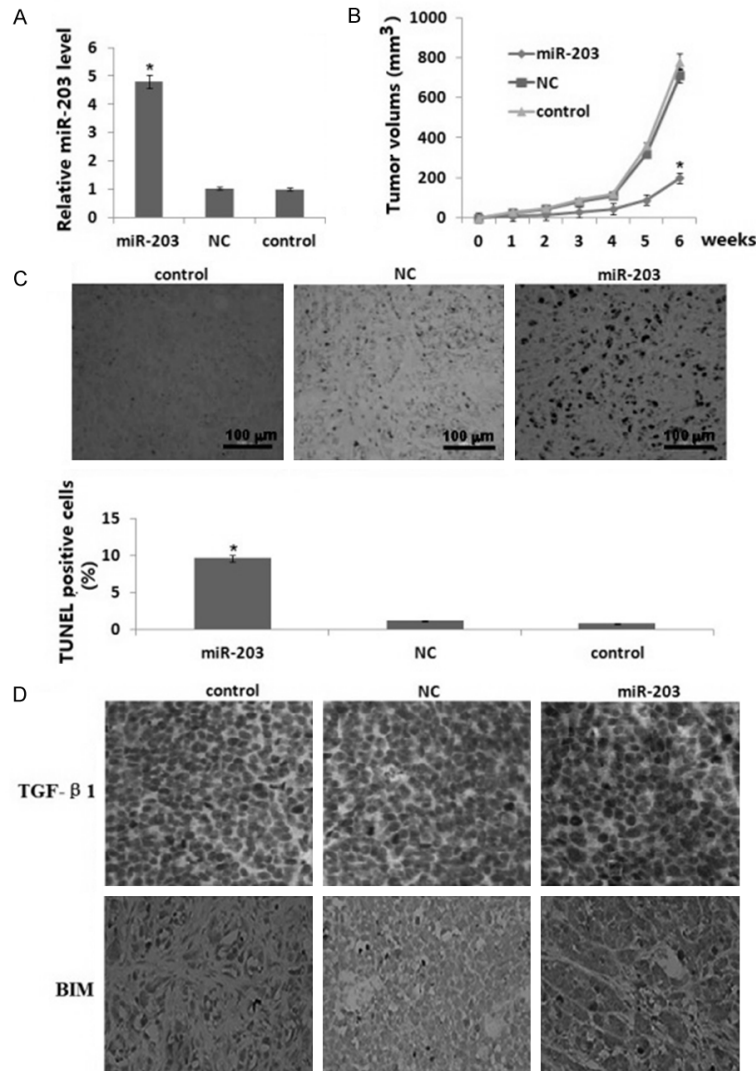
MicroRNAs regulate mRNA stability and protein expression, and certain miRNAs have been demonstrated to act either as oncogenes or tumor suppressors [14]. Since miRNAs regulate multiple target genes simultaneously, they function as the critical control nodes in the



**Figure 3.** MiR-203 induces BIM-dependent apoptosis and growth inhibition in SH-SY5Y cells. miR-203 and BIM siRNA co-transfected into the SH-SY5Y cells for 72 hs. A: BIM mRNA were examined by RT-PCR in SH-SY5Y cells; B: BIM protein were examined by Western blot in SH-SY5Y cells; C: Apoptosis was determined by flow cytometry assay; D: Apoptosis was determined by TUNEL assay. E: The survival of SH-SY5Y cells as analyzed by MTT assays. Bars represent the mean  $\pm$  SD of three independent experiments. \* $P < 0.01$ .

existing tumor signaling network, making them a promising target for cancer treatment [15]. A potential role for miRNAs in malignancy has

been suggested by the location of the genes for several miRNAs at sites of translocation break-points or deletions linked to a specific neoplas-



**Figure 4.** MiR-203 suppressed SH-SY5Y cell tumor growth in vivo. The untreated SH-SY5Y cells, stable miR-203 transfected SH-SY5Y cells (miR-203) and NC (control) transfected SH-SY5Y cells (NC) into the backside of mice respectively for 6 weeks. A: Relative expression of *miR-203* was detected by qRT-PCR in xenograft tumors. B: The growth of the xenograft tumors was referred to the measurement of the long and short dimensions of the tumors, and the calculation of the tumor size were described in the “Materials and methods” section. C: Cell apoptosis was detected by TUNEL assay in xenograft tumors; D: Tumor tissues were examined by immunohistochemical staining with anti-TGF-β1 and anti-anti-BIM antibody. Original magnifications: 200 ×. \*P<0.01.

tic state [16]. There is increasing experimental evidence supporting the role of miRNAs in the regulation of a range of physiological responses, including development [17], cellular apoptosis [18], differentiation [19], proliferation [20], and cancer [21].

Studies have found that miR-203 was significantly down-regulated in some tumors, such as

laryngeal carcinoma cells [2], head and neck squamous cell carcinomas [22], hematopoietic malignancy [23], colon cancer [24] and neuroblastoma [3]. Moreover, it was reported that inhibition of miR-203 expression could significantly increase the proliferation of Hela cells [25], whilst re-expression of miR-203 could inhibit the proliferative capacity of cells in human head and neck squamous cell carcinoma [22], hepatocellular carcinoma [26], chronic myelogenous leukemia and B cell leukemia [23]. These findings suggest that miR-203 might function as a tumor suppressor gene in a variety of tumors. In the present study, we further evaluated the functions and targeted genes of miR-203 in SH-SY5Y cells and found that miR-203 overexpression inhibited cell proliferation and induced apoptosis in vitro and in vivo, indicating miR-203 as a tumor suppressor in NB. However, the underlying molecular mechanisms remain largely uninvestigated.

The Transforming Growth Factor-β (TGF-β) signaling pathway is instrumental in mammalian development as well as in tumor suppression through inhibition of proliferation and induction of apoptosis in multiple cell types. TGF-β regulates cell proliferation mainly by inhibiting cell cycle progression through G1-arrest. TGF-β can both induce and suppress

apoptosis depending on cellular and extracellular factors [27]. Unlike the TGF-β cytostatic program, there is not a unique TGF-β-induced apoptotic program. *In vitro* studies have shown some Smad-dependent and -independent mechanisms, e.g., TGF-β increases the expression of death-associated protein kinase (DAPK) in HCC cell-lines [28], but it induces the expression of SH2-domain-containing inositol-

5-phosphate (SHIP) in hematopoietic cell-lines, which in turn inhibits the survival signals from the PI3K-AKT pathway. Other apoptotic related genes affected by TGF- $\beta$  pathway are DAXX (that normally activates p38MAPK), FAS and BIM (in gastric cancer cell lines) and GADD45b (in hepatocytes) [29]. The final targets in TGF- $\beta$ -induced apoptosis are the proapoptotic caspases and several members of the BCL2 family [30].

Significant relation was reported between TGF- $\beta$ 1 and MicroRNAs. Martin et al. [31] identified multiple binding sites for miR-744 located in the proximal TGF- $\beta$ 1 3'-UTR. MiR-744 transfection increased endogenous TGF- $\beta$ 1, which given the pleiotropic nature of cellular responses to TGF- $\beta$ 1 is potentially significant. Other study demonstrated that TGF- $\beta$  increases the expression of miR-203 to promote EMT and tumor metastasis. In addition, miR-203 could also induce TGF- $\beta$  expression and promote EMT and tumor metastasis. In the present study, we found that miR-203 activated TGF- $\beta$ 1 dependent BIM upregulation, followed by tumor suppressor in vitro and vivo. Targeting BIM reversed miR-203-induced tumor suppressor. Furthermore, our data also revealed that miR-203-induced tumor suppressor was dependent on the BIM-dependent cell apoptosis.

In conclusion, the present study suggests that therapies of miR-203 overexpression in tumor cells may be effective in decreasing tumorigenesis in vitro and vivo, which may be related to activating TGF- $\beta$ 1/BIM signaling.

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#### Disclosure of conflict of interest

None.

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