MicroRNA-143 functions as a tumor suppressor in neuroblastoma by targeting bone morphogenetic protein-7

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Abstract

Introduction: Neuroblastoma (NB) is one of the most common types of extracranial malignant solid tumor in infants and children. MicroRNA-143 (miR-143) was reported to be dysregulated in several human cancers. However, the function and mechanism of miR-143 in NB are still unclear. The purpose of this study was to explore the biological functions of miR-143 in NB progression.

Material and methods: The expression of miR-143 in 32 NB tissue samples and 4 NB cell lines was detected by quantitative real-time PCR. Then, bioinformatics analysis combined with luciferase reporter assay were used to identify the target gene of miR-143 in SK-N-SH cells. Cell proliferation, migration and invasion assay were performed to investigate the roles of miR-143 in NB progression.

Results: Our study revealed that miR-143 was significantly downregulated in NB tissues as well as cell lines. Bone morphogenetic protein-7 (BMP7) was a direct target of miR-143 in NB cells and inversely associated with the expression of miR-143 in NB tissues. Overexpression of miR-143 inhibited NB cell proliferation, migration and invasion through targeting BMP7.

Conclusions: Our data for the first time demonstrated that miR-143 functions as a tumor suppressor in NB by directly targeting BMP7, and also suggest miR-143 as a potential therapeutic target for NB patients in the future.

Key words: miR-143, neuroblastoma, bone morphogenetic protein-7.

Introduction

Neuroblastoma (NB), which is derived from the neural crest, is one of the most common types of extracranial malignant solid tumor in infants and children [1]. Neuroblastoma accounts for about 7–10% of the cases of pediatric cancer and 15% of all pediatric cancer mortality [2, 3]. Neuroblastoma has poor clinical outcome because of its high potential for metastasis, and the 5-year event-free survival rate is less than 50% [4, 5]. Although multiple genetic and molecular factors have been identified to affect the tumorigenesis and metastasis of NB in recent years, the mechanisms underlying the progression of NB remain to be explored.

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules ranging from 22 to 25 nucleotides in length [6]. MiRNAs bind to the 3′-untranslated region (UTR) of target messenger RNAs (mRNAs),

resulting in translation repression or mRNA degradation, and are involved in a variety of biological events, including cellular proliferation, differentiation and apoptosis [7]. Accumulating evidence from previous studies has demonstrated the dysregulated expression of miRNAs in many types of human cancer, including neuroblastoma, and they play important roles in tumor initiation and progression [8–11]. Among these functional miRNAs, miRNA-143 (miR-143) has been demonstrated to significantly decrease in several types of cancer and acts as a tumor suppressor [12–15]. For example, He *et al.* demonstrated that miR-143 acted as a tumor suppressor by regulating cell proliferation, invasion and epithelial-mesenchymal transition by targeting QKI-5 in esophageal squamous cell carcinoma [16]. Yang *et al.* revealed that miR-143 inhibited breast cancer progression and stem-cell properties by targeting CD44 [17]. Although these previous studies indicated that miR-143 plays important roles in carcinogenesis, the mechanism and function of miR-143 in NB have not been elucidated.

This study scrutinized the expression and function of miR-143 in NB tissues and cell lines. Furthermore, we explored the biological mechanism underlying NB progression of miR-143 by ascertaining possible target genes. We hope that this study can enhance possibilities for the development of novel therapeutic and diagnostic strategies for NB.

Material and methods

Human tissue samples

A total of 32 NB tissue samples and matched adjacent non-tumor tissues were obtained from patients who underwent surgical resection at the Department of Pediatrics in Huaihe River Hospital between 2013 and 2015. None of the patients were treated with radiotherapy or chemotherapy before surgery. All specimens were immediately frozen in liquid nitrogen until use. The study was approved by the Institute Research Ethics Committee of Huaihe River Hospital, and all patients provided written informed consent for the use of the samples for research purposes. Total RNA of normal human dorsal ganglia was obtained from Clontech (Mountain View, CA).

Cell culture and transfection

Human NB cell lines SK-N-SH, SK-N-AS, SH-SY5Y and SK-N-BE (2) were purchased from American Type Culture Collection (ATCC, Rockville, MD). Cell lines were cultivated in RPMI1640 medium (Life Technologies, Inc., USA) supplemented with 10% fetal bovine se-

rum, penicillin (100 U/ml), and streptomycin (100 µg/ml). Cell lines were cultured in a humidified atmosphere incubator of 95% air and 5% CO_2 at 37°C. The miR-143 mimics and corresponding miRNA negative control were purchased from RiboBio Company (Guangzhou, China). Small interfering RNA (siRNA) for bone morphogenetic protein-7 (BMP7, sequence: 5′-CAAUGAACAAGAUCCUACA-3′) and the corresponding negative control were synthesized and purchased from GenePharma Company (Shanghai, China). Transfection was performed using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. All assays were performed 48 h following transfection.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from NB tissues and cell lines by Trizol Reagent (Invitrogen, USA) according to the manufacturer's protocol. Both mRNA and miRNA were reverse transcribed to cDNA using a reverse transcription kit. The qRT-PCR was performed on the ABI 7500 Fast Real-Time PCR system (Applied Biosystems, USA) using the SYBR Green PCR Kit (Takara, Japan) according to the manufacturer's instructions. U6 and GAPDH were used as internal controls respectively for normalization and quantification of miR-143 and BMP7 expression. All experiments were conducted independently three times. The relative expression of genes was calculated using the $2^{-\Delta\Delta Ct}$ method. The following primers were used: miR-143 forward 5′-CCTGGCCTGAGATGAAGCAC-3′ and reverse 5′-CAGTGCTGGGTCCGAGTGA-3′, BMP7 forward 5′-TCTCATTGATTGGAGCATT-3′ and reverse 5′-GGCAGTGGTAGGATAAAC-3′.

Western blot

Total proteins of NB cells were lysed with RIPA buffer with 1% PMSF, and the concentrations were detected using the BCA Protein Assay Kit (Beyotime, Shanghai, China). Proteins were fractionated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. After probing with primary antibodies against human BMP7 or GAPDH (CST, USA) at 4°C overnight, membranes were incubated with HRP-conjugated secondary antibody. GAPDH was used as an endogenous protein for normalization. After being washed, results were detected using the Odyssey Scanning system (Li-Cor, Lincoln, USA).

Cell proliferation assay

Cell proliferation capacity was measured using Cell Counting Kit-8 (CCK-8) (Beyotime, Shanghai,

China) according to the manufacturer's protocol. Cells (5×10^3) were seeded in each 96-well plate, transfected with the indicated miRNA or siRNA and further incubated for 24, 48, 72 h and 96 h respectively at 37°C in a humidified atmosphere with 5% CO_2. The absorbance in each well was recorded at 450 nm by a microplate reader.

Cell migration and invasion assay

The migratory and invasive potential of NB cells was evaluated using transwell inserts with 8-µm pores (Coring, NY, USA). For the migration assay, at 24 h after transfection, 5×10^4 cells in serum-free medium were added into the upper chamber of an insert. For the invasion assay, 1×10^5 cells in serum-free medium were placed into the upper chamber of an insert coated with Matrigel matrix (BD, NJ, USA). 500 µl of 10% FBS medium were added to the matched lower chamber. After 24 h incubation, cells remaining on the upper membrane were removed with a cotton swab, and cells which had migrated or invaded to the lower membrane were stained with 0.1% crystal violet, photographed, and counted. All experiments were repeated three times independently.

Luciferase reporter assay

A fragment of 3′-UTR of BMP7 containing the wild-type (Wt) or mutant (Mut) miR-143 binding site were cloned into the pGL3 vector (Invitrogen, USA) according to the manufacturer's protocol. Cells (1×10^5) were seeded in 24-well plates and each plate was co-transfected with Wt or Mut 3′-UTR vector and miR-143 mimics using Lipofectamine 2000. Renilla luciferase pGL3 was used as a control. Cells were collected 48 h after transfection. The dual-luciferase activity was examined using the Dual Luciferase Reporter Assay (Promega, USA) following the manufacturer's instructions.

Statistical analysis

All statistical analyses were performed using SPSS version 18.0 software (IBM, USA). Data were presented as the mean ± SD of at least three separate experiments. Differences between groups were analyzed using Student's t test or one-way ANOVA analysis. A value of $p < 0.05$ was considered statistically significant.

Results

MiR-143 is significantly downregulated in NB tissues and cell lines

We detected the miR-143 expression in 32 paired NB tissues and adjacent non-tumor tissues using quantitative real-time PCR. Our date revealed that the expression level of miR-143 was significantly decreased in NB tissues compared with that in adjacent non-tumor tissues (Figure 1 A). Furthermore, we investigated miR-143 expression in four NB cell lines (SK-N-SH, SK-N-AS, SH-SY5Y and SK-N-BE (2)). As shown in Figure 1 B, miR-143 was significantly down-regulated in NB cell lines compared with normal human dorsal ganglia (DG).

MiR-143 suppresses NB cell proliferation, migration and invasion

In order to ascertain the role of miR-143 in the pathogenesis of NB, miR-143 mimics and the corresponding miRNA negative control were transfected into SK-N-SH cells. We then studied the effects of miR-143 on cell proliferation, migration and invasion. As expected, the expression level of miR-143 was obviously upregulated in SK-N-SH cells transfected with miR-143 mimics compared with cells transfected with the negative control (Figure 2 A). The cell proliferation assay revealed that overexpression of miR-143 significantly decreased the proliferation ability of SK-N-SH cells
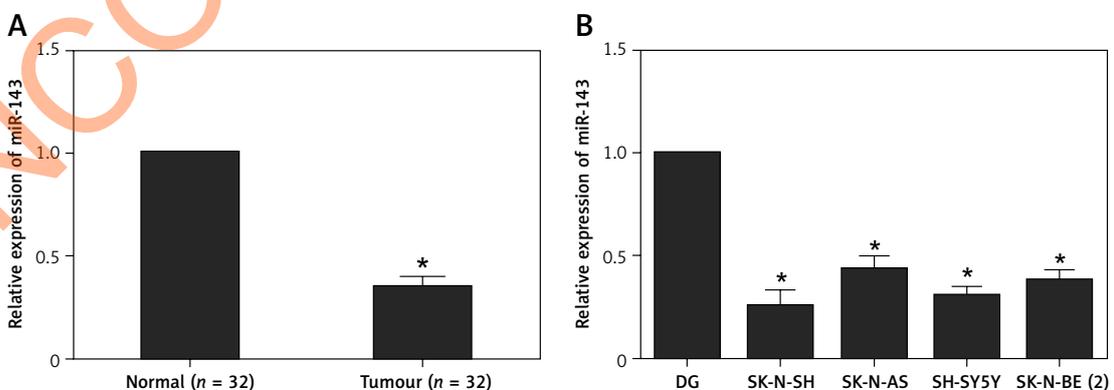

Figure 1. MiR-143 is down-regulated in NB tissues and cell lines. **A** – The relative expression levels of miR-143 were significantly down-regulated in NB tissues (tumor) compared with the adjacent non-tumor tissues (normal). **B** – The relative expression levels of miR-143 were significantly decreased in four NB cell lines (SK-N-SH, SK-N-AS, SH-SY5Y and SK-N-BE (2)) compared with normal human dorsal ganglia (DG)
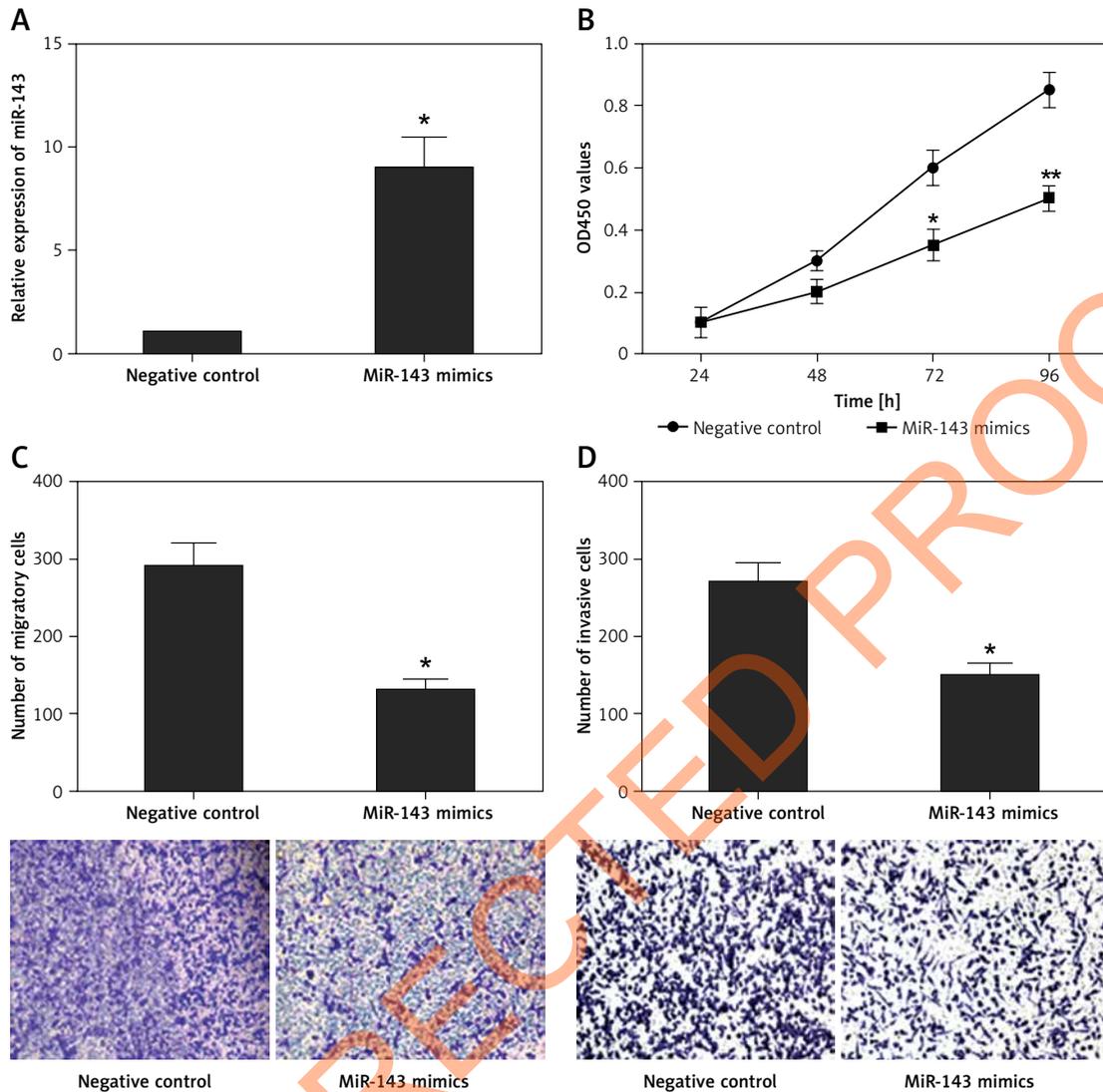
$*P < 0.05$.

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Figure 2. Overexpression of miR-143 constrains NB cell proliferation, migration and invasion. **A** – The expression of miR-143 was obviously upregulated in SK-N-SH cells transfected with miR-143 mimics compared to cells transfected with the negative control. **B** – Overexpression of miR-143 significantly decreased SK-N-SH cells' proliferation ability by CCK-8 assay. **C**, **D** – Overexpression of miR-143 significantly inhibited the migration and invasion ability of SK-N-SH cells

*P < 0.05 and **p < 0.01.

(Figure 2 B). Furthermore, the cell migration and invasion assay indicated that overexpression of miR-143 significantly inhibited the migration and invasion ability of SK-N-SH cells (Figures 2 C and D). Taken together, these data demonstrated that miR-143 may act as a tumor suppressor in NB progression and development.

MiR-143 directly targets BMP7 in NB cells

Through bioinformatics analyses using TargetScan, we found that bone morphogenetic protein-7 (BMP7) was predicted to be a target of miR-143 (Figure 3 A). To further determine whether BMP7 is a direct target of miR-143, we performed a luciferase reporter assay in SK-N-SH cells. As shown in Figure 3 B, overexpression of miR-143 signifi-

cantly decreased the relative luciferase activity of wild type 3'-UTR of BMP7 in SK-N-SH cells, but it had no effect on luciferase activity of the mutant 3'-UTR of BMP7 (Figure 3 B). Moreover, the expression levels of BMP7 protein and mRNA were consistently and significantly reduced in SK-N-SH cells transfected with miR-143 mimics by using western blot and quantitative real-time PCR analyses (Figures 3 C and D). Taken together, these data suggested that BMP7 was a direct target of miR-143 in NB cells.

Inverse relationship between BMP7 and miR-143 expression in NB tissues

Additionally, we detected the BMP7 expression in 32 paired NB tissues and adjacent non-tumor
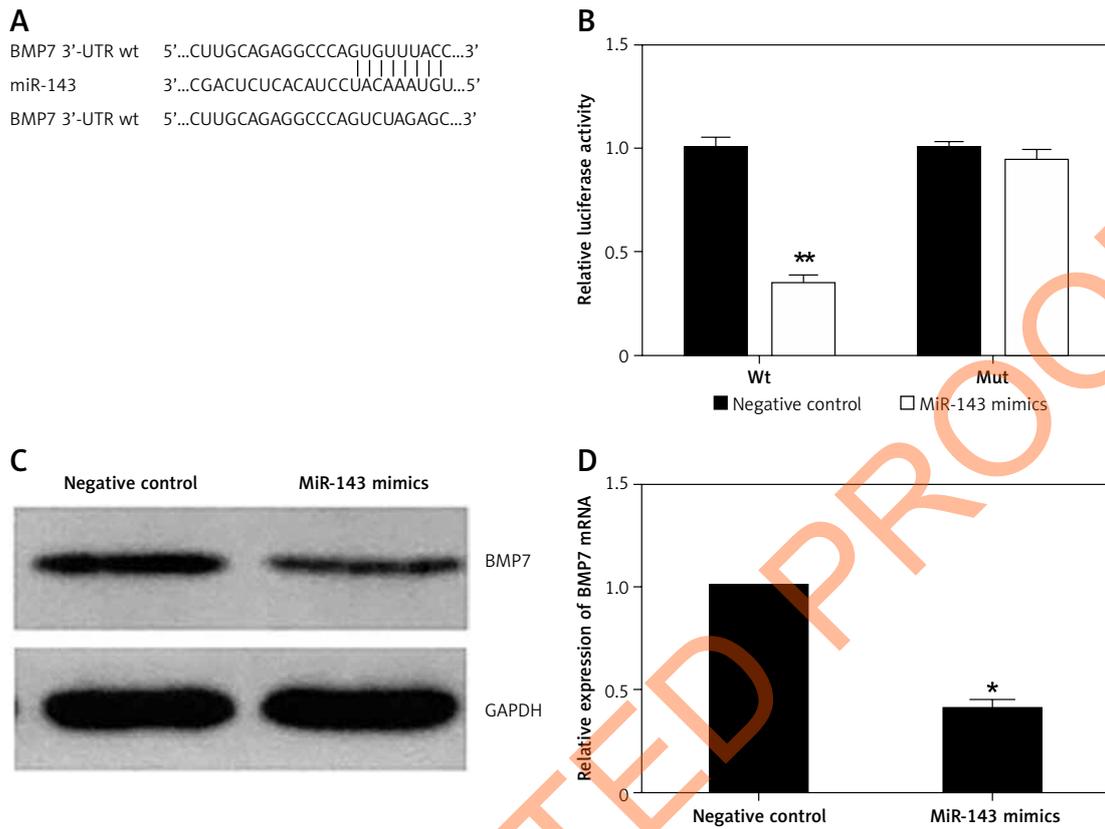
Figure 3. BMP7 is a direct target of miR-143 in NB cells. **A** – Potential miR-143 binding sites of BMP7 3′-UTR and the mutant. **B** – Luciferase activity assay was performed with co-transfection of wild-type or mutant BMP7 and miR-143 mimics or negative control in SK-N-SH cells. **C**, **D** – Expression levels of BMP7 protein and mRNA were significantly reduced in SK-N-SH cells transfected with miR-143 mimics in western blot and quantitative real-time PCR analyses

*P < 0.05 and **p < 0.01.

tissues using quantitative real-time PCR. The expression level of BMP7 was significantly upregulated in NB tissues compared with that in adjacent non-tumor tissues (Figure 4 A). Furthermore, the expression of BMP7 was inversely associated with the expression of miR-143 in NB tissues (Figure 4 B).

Knockdown of BMP7 expression inhibits NB cell proliferation, migration and invasion

To further investigate the potential relationship between miR-143 and the downstream gene BMP7, we tested cell proliferation, migration and
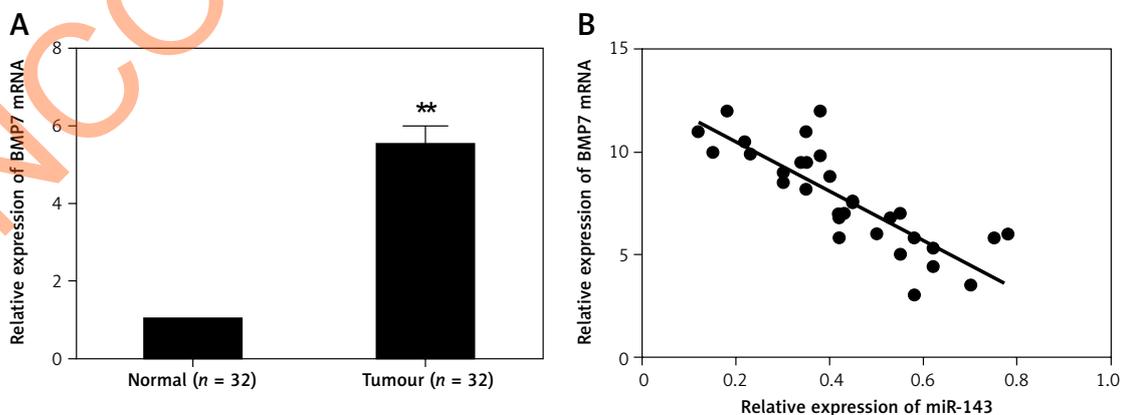

Figure 4. Inverse relationship between BMP7 and miR-143 expression in NB tissues. **A** – Expression of BMP7 was upregulated significantly in NB tissues compared to adjacent non-tumor tissues. **B** – Expression of BMP7 was inversely associated with expression of miR-143 in NB tissues ($r = -0.58$, $p < 0.05$)
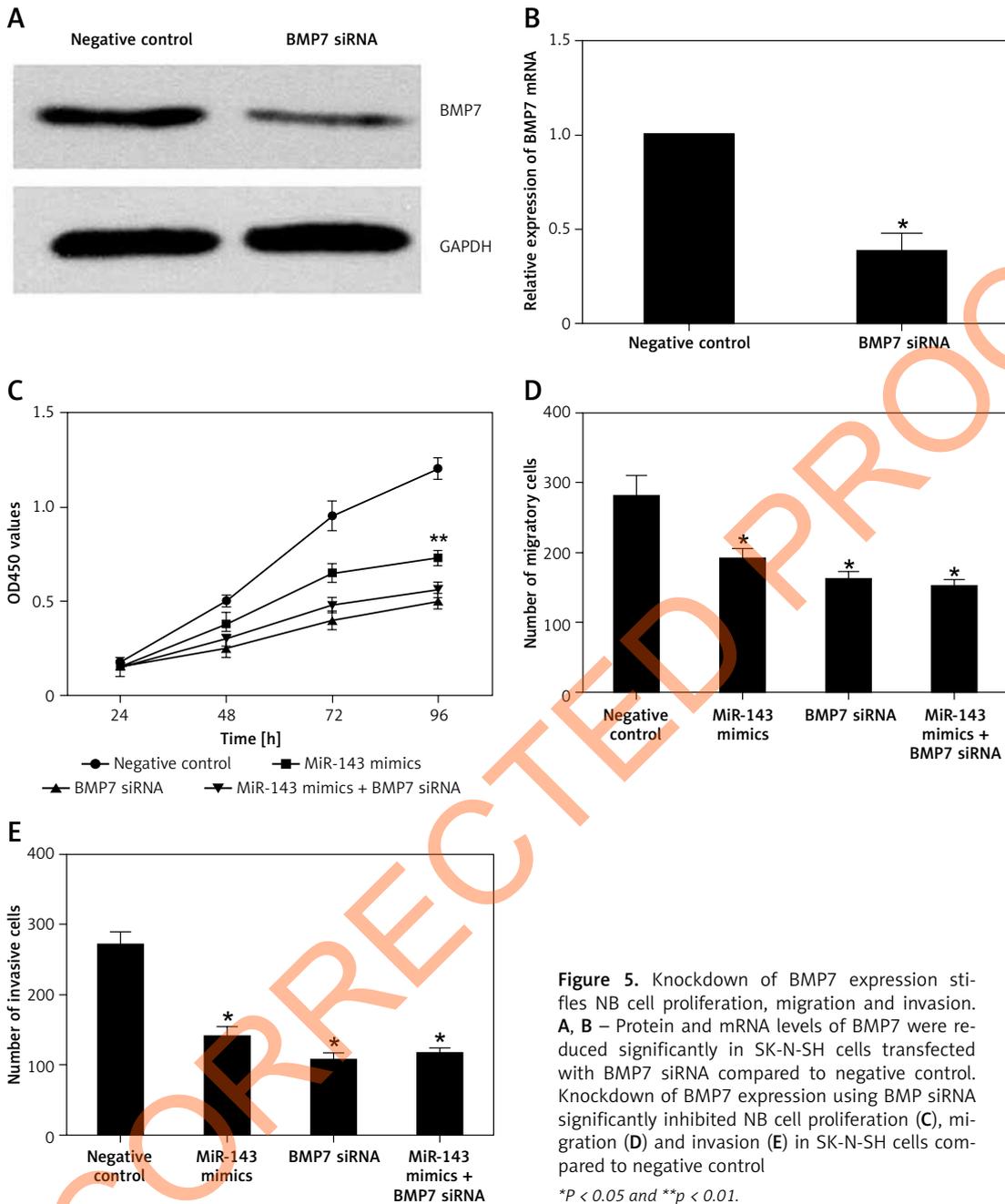
*P < 0.05 and **p < 0.01.

Figure 5. Knockdown of BMP7 expression stifles NB cell proliferation, migration and invasion. **A**, **B** – Protein and mRNA levels of BMP7 were reduced significantly in SK-N-SH cells transfected with BMP7 siRNA compared to negative control. Knockdown of BMP7 expression using BMP siRNA significantly inhibited NB cell proliferation (**C**), migration (**D**) and invasion (**E**) in SK-N-SH cells compared to negative control

*P < 0.05 and **p < 0.01.*

invasion under the condition of siRNA-mediated knockdown of the BMP7 gene. The protein and mRNA levels of BMP7 were markedly decreased in SK-N-SH cells transfected with BMP7 siRNA compared to the negative control (Figures 5 A and B). Knockdown of BMP7 expression significantly inhibited NB cell proliferation, migration and invasion in SK-N-SH cells (Figures 5 C–E), which was in keeping with the inhibitory effects induced by overexpression of miR-143. However, overexpression of miR-143 did not have further suppressive effects on cell proliferation, migration and invasion in SK-N-SH cells transfected with BMP7 siRNA. These results demonstrated that

miR-143 suppressed NB cell growth and metastasis by targeting BMP7.

Discussion

In this study, we investigated the function of miR-143 in NB progression and development. Our results indicated that miR-143 was significantly downregulated in NB tissues and cell lines. Overexpression of miR-143 in SK-N-SH cells transfected with miR-143 mimics significantly inhibited NB cell proliferation, migration and invasion. In addition, we found that BMP7 was a direct and functional target of miR-143 in NB cells. The expression of BMP7 was inversely associated with the

expression of miR-143 in NB tissues. Moreover, knockdown of BMP7 expression transfected with BMP7 siRNA significantly inhibited NB cell proliferation, migration and invasion in SK-N-SH cells. These data demonstrated that miR-143 played an important role in NB progression and development, and miR-143 suppressed NB cell growth and metastasis by targeting BMP7.

Neuroblastoma carcinogenesis is a complex multistep process and is characterized by clinical features including spontaneous regression, maturation or aggressive progression. Mounting evidence has indicated that deregulation in the expression of miRNAs may be involved in cancer initiation and progression, including NB [18]. For example, Li et al. reported that miR-21 was significantly increased in NB tissues compared with adjacent non-tumor tissues, and miR-21 promoted NB cell growth and motility partially by targeting CHL1 [19]. Liu et al. found that the expression of miR-451 was significantly reduced in NB tissue, and miR-451 inhibited NB cell proliferation, invasion and migration by targeting macrophage migration inhibitory factor [20]. Das and Bhattacharyya showed that miR-432 contributed to the dopamine cocktail and retinoic acid induced neuronal differentiation of human neuroblastoma cells by targeting NESTIN and RCOR1 genes [21].

Different miRNAs may play different roles in NB, which is why there is a need to further clarify the clinical significance and function of certain specific miRNAs. Recently studies have revealed that miR-143 plays essential roles in tumorigenesis. MiR-143 acted as a tumor suppressor in several types of cancer, but the expression and function of miR-143 in NB have not been fully explicated. Herein, we found that miR-143 was significantly downregulated in NB tissues and cell lines, and overexpression of miR-143 in SK-N-SH cells significantly inhibited NB cell proliferation, migration and invasion. MiR-143 functioned as a tumor suppressor in NB progression.

Until now, hundreds of miRNAs have been demonstrated to participate in the initiation and progression of cancer though the regulation of oncogenes or suppressors. There is still a need to explore the molecular mechanism underlying NB progression of miR-143 through identification of the possible target gene. We found that bone morphogenetic protein-7 (BMP7) was a direct target of miR-143 by using bioinformatics analyses and the luciferase reporter assay. Bone morphogenetic proteins (BMPs) are firstly known as important signaling molecules in promoting osteogenesis [22]. Furthermore, BMPs are multi-functional growth factors belonging to the transforming growth factor-β superfamily and are involved in the regulation of cell proliferation, survival, differentiation and apoptosis [23].

However, recent studies have indicated that BMP7 plays important roles in tumor progression and development. Zhang et al. found overexpression of BMP7 in colon cancer tissues in its advanced stage; moreover, upregulation of BMP7 was closely associated with nodal metastasis [24]. Camara-Clayette et al. demonstrated that BMP7 expression was significantly increased at relapse in patients who developed secondary resistance, incubation of BMP7 increased mantle cell lymphoma cell lines' resistance to bortezomib and cytarabine, and inhibition of BMP7 correlated with the increased cell death linked to drug application [25]. Li et al. observed that the expression of BMP7 in hepatocellular carcinoma cells was significantly higher than in normal hepatic cells, and a high expression level of BMP7 resulted in a significantly poor prognosis of hepatocellular carcinoma patients [26]. In our study, BMP7 was a direct target of miR-143 in NB cells, and the expression of BMP7 was inversely associated with the expression of miR-143 in NB tissues. In addition, knockdown of BMP7 expression significantly inhibited NB cell proliferation, migration and invasion. Taken together, these results indicated that BMP7 is involved in NB progression.

In conclusion, our study revealed that miR-143 was significantly downregulated in NB tissues and cell lines. BMP7 was a direct target of miR-143 in NB cells. Overexpression of miR-143 inhibited NB cell proliferation, migration and invasion through targeting BMP7. This novel miR-143/BMP7 pathway provides further insight into the molecular mechanisms underlying NB progression and indicates new therapeutic targets for the treatment of NB patients.

Conflict of interest

The authors declare no conflict of interest.

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