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MiR-223-3p promotes the growth and invasion of neuroblastoma cell *via* targeting FOXO1

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Abstract. – OBJECTIVE: MicroRNAs (miRNAs) have been demonstrated to have crucial roles in cancer development. We investigated the involvement of miR-223-3p in neuroblastoma (NB).

MATERIALS AND METHODS: MiR-223-3p expression in NB cell lines and normal cell line was analyzed with real-time quantitative PCR method. Cell proliferation, cell invasion, and cell apoptosis were assessed by cell counting kit-8 (CCK-8), transwell invasion assay, and flow cytometry assay, respectively. Bioinformatics analysis, Dual-Luciferase reporter assays, and Western blot analysis were conducted to identify the connection of miR-223-3p and forkhead box O1 (FOXO1).

RESULTS: MiR-223-3p level was found highly expressed in NB cell lines compared with normal cell line. Knockdown miR-223-3p expression decreased cell growth and invasion but increased cell apoptosis. MiR-223-3p was able to bind with the 3'-untranslated region of FOXO1, and thereby resulting in a reduction of FOXO1 expression. The knockdown of FOXO1 increased the malignant capacity of NB cells.

CONCLUSIONS: Therefore, given the fact that miR-223-3p suppressed FOXO1 expression to promote NB progression, targeting miR-223-3p may be an effective method for NB treatment.

Key Words:
MiR-223-3p, FOXO1, Neuroblastoma, Growth, Invasion.

Introduction

Neuroblastoma (NB) is a cancer type with high metastasis risk originated from nervous system[1]. The increased understanding of biological and genetical alterations in NB has helped to validate novel diagnostic and treatment methods for NB[2].

Abnormally expressed of forkhead box O1 (FOXO1) has been identified in several human cancers, including bladder cancer, cervical cancer, and prostate cancer[3-5]. For instance, FOXO1 expression was found downregulated in bladder cancer and correlated with worse overall survival of cancer patients[3]. In addition, FOXO1 expression was found reduced in high-grade cervical cancer, indicating loss of FOXO1 is closely associated with late-stage tumors[4]. Besides that, loss of FOXO1 was found to promote prostate cancer cell proliferation and colony formation[5]. Mechanism study showed FOXO1 could be regulated by TMPRSS2-ERG in prostate cancer[5]. These results suggested the tumor suppressive role of FOXO1 in these cancer types.

MicroRNAs (miRNAs) are endogenous non-coding RNAs that can regulate almost all cell behaviors[6]. Galardi et al[7] has suggested miRNA plays crucial roles in the progression of NB. MiR-223-3p was reported to be overexpressed in ovarian cancer and its overexpression promoted cell proliferation, migration, and invasion *in vitro* by regulating sex determining region Y-box 11[8]. Moreover, miR-223-3p expression was also shown to be upregulated in prostate cancer and regulates cell malignancy behaviors by targeting SEPT6[9].

Emerging studies showed that FOXO1 expression could be directly regulated by miRNAs in human cancers. In breast cancer, miR-9 was shown to promote cancer cell proliferation, migration, and invasion by targeting FOXO1[10]. In liver cancer, miR-196a was overexpressed in cancer cell lines and the knockdown of miR-196a in mouse model inhibited liver cancer cell migration and invasion *in vivo*[11]. However, the association of miR-223-3p and FOXO1 was not investigated to date.

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In this study, we investigated the expression and function of miR-223-3p and FOXO1 in NB cell lines. We suggested that FOXO1 was a target of miR-223-3p by bioinformatic analysis. Then, we highlighted a miR-223-3p/FOXO1 axis in regulating NB growth and invasion.

Materials and Methods

Cell Lines and Cell Culture

NB cell lines (SK-N-SH and SHSY5Y) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Human endothelial cell line HUVEC was obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All these cells were incubated at a 37°C humidified incubator containing 5% CO_2 in Roswell Park Memorial Institute-1640 medium (RPMI-1640) supplemented with 10% fetal bovine serum (FBS), and 100 U/ml penicillin/100 µg/ml streptomycin (all purchased from Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Cell Transfection

MiR-223-3p inhibitor, small interfering RNA targeting FOXO1 (si-FOXO1), and their corresponding negative controls (miR-NC or siR-NC) were synthesized by RiboBio (Guangzhou, China). Transfection was conducted using Lipofectamine 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocols.

RNA Extraction and QRT-PCR

TRIzol reagent (Thermo Fisher Scientific, Inc, Waltham, MA, USA) was used to extract total RNA from cultured cells. RNA concentration was quantified using Nanodrop-2000 (Thermo Fisher Scientific, Inc, Waltham, MA, USA). RNA was then reverse transcribe into complementary DNA using PrimeScript RT Master Mix (TaKaRa, Dalian, Liaoning, China). QRT-PCR was conducted on ABI 7700 system (Applied Biosystem, Foster City, CA, USA) using SYBR Green Mix (TaKaRa) with the following primers: miR-223-3p: 5'-ACACTCCAGCTGGGTGTCAGTTTGTCAAAT-3' (F) and 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTGGGGTAT-3' (R); U6 snRNA: 5'-CGCTTCGGCAGCACATATAC-3' (F) and 5'-AAATATGGAACGCTTCACGA-3' (R). Relative expression level of miR-223-3p was analyzed using the $2^{-\Delta\Delta Ct}$ method.

Protein Extraction and Western Blot

Protein was extracted from cultured cells using radio immunoprecipitation assay (RIPA) lysis buffer and then quantified using bicinchoninic acid (BCA) kit (Beyotime, Haimen, Jiangsu, China). Equal amount of protein sample was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with fat-free milk and then incubated with primary antibodies (anti-FOXO1: ab58518, anti-GAPDH: ab181602; both from Abcam, Cambridge, MA, USA). Subsequently, membranes were washed, incubated with secondary antibody (ab6721, Abcam, Cambridge, MA, USA), and probed using BeyoECL kit (Beyotime, Shanghai, China).

Cell Proliferation Assay

Cells were seeded into 96-well plate with the density of 3 x 10^3 cells/well. 10 µl of CCK-8 solution (Beyotime, Shanghai, China) was added to the well at the indicated time and incubated for another 4 h. Optical density was measured at 450 nm using microplate reader.

Transwell Invasion Assay

Cell invasion was detected using transwell invasion assay. Cells in serum-free medium were filled in the upper Matrigel pre-coated chamber, and medium supplemented with FBS was added to lower chamber. After 48 h of incubation, invasive cells were fixed in 90% methanol, stained with 0.1% crystal violet, and counted under a microscope.

Flow Cytometry Assay

Cells were suspended in binding buffer, and incubated with Annexin V-FITC, and propidium iodide (PI; Beyotime, Shanghai, China) in a dark room. Cell apoptosis percentage was analyzed using flow cytometry assay (BD Biosciences, San Jose, CA, USA).

Target Predictions

TargetScan (http://www.targetscan.org) and microRNA (http://www.microrna.org) were used to predict the targets of miR-223-3p. Among the predicted targets, FOXO1 was selected for following analyses.

Luciferase Reporter Gene Assay

Wild-type (wt) 3'-untranslated region (3'-UTR) of FOXO1 was amplified by PCR and inserted into luciferase vector to generate wt-FOXO1. Mutant (mt) FOXO1 3'-UTR (mt-FOXO1) was generated using site-direct mutagenesis kit (TaKaRa). Cells were co-transfected with the luciferase plasmids and synthetic miRNAs. Cells were lysed after 48 h of transfection to measure relative luciferase activity using Dual-Luciferase Assay System (Promega, Madison, WI, USA) according to manufacturer's protocol.

Statistical Analysis

Data were presented as mean ±standard deviation after analyzing at SPSS 19.0 (SPSS Inc., Armonk, NY, USA). Paired Student's *t*-test and one-way ANOVA and Tukey post-hoc test were carried out to calculate significance in groups. *p*-value less than 0.05 was regarded as statistically significant.

Results

MiR-223-3p Was Upregulated, While FOXO1 Was Downregulated in NB Cell Lines

As shown in Figure 1A, miR-223-3p expression level was significantly upregulated in NB cell lines compared with normal cell line. On the contrary, we found FOXO1 protein expression was significantly reduced in NB cell lines compared to normal cell line (Figure 1B).

FOXO1 Was a Direct Target of MiR-223-3p

Bioinformatic analysis software predicted FOXO1 contains a putative target for miR-223-3p in its 3'-UTR (Figure 2A). Luciferase activity reporter assay showed miR-223-3p inhibitor transfection increased luciferase activity in cells transfected with wt-FOXO1 (Figure 2B). Western blot results indicated FOXO1 expression could be upregulated by miR-223-3p inhibitor (Figure 2C).

Knockdown of MiR-223-3p Inhibits NB Cell Proliferation and Invasion but Promotes Apoptosis

To evaluate the biological function of miR-223-3p in NB cells, synthetic miRNAs were transfected into NB cells. We found miR-223-3p inhibitor transfection significantly decreased miR-223-3p expression in NB cells compared with the miR-NC (Figure 3A). CCK-8 assay and transwell invasion assay revealed cell proliferation and cell invasion were lower in miR-223-3p inhibitor transfected groups compared with miR-NC transfected groups (Figure 3B and 3C). In addition, we showed that knockdown of miR-223-3p promotes cell apoptosis (Figure 3D).

MiR-223-3p Regulates NB Cell Behaviors Via Targeting FOXO1

We then investigated whether FOXO1 could reverse the effects of miR-223-3p on NB cells. For this purpose, si-FOXO1 and miR-223-3p inhibitor were co-transfected into NB cells. Western blot showed FOXO1 expression was decreased by si-FOXO1 (Figure 4A). As shown

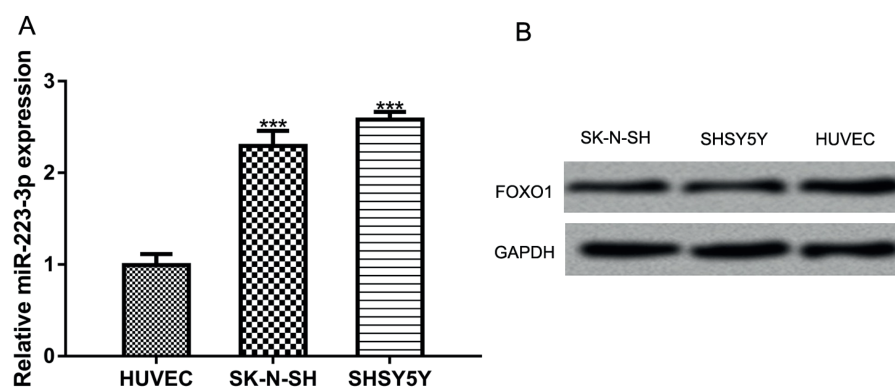

Figure 1. The expression of miR-223-3p and FOXO1 in NB cell lines and normal cell line. **A**, MiR-223-3p expression and **B**, FOXO1 expression in SK-N-SH and SHSY5Y cell lines and HUVEC cell line. MiR-223-3p: microRNA-223-3p; FOXO1: forkhead box O1; NB: neuroblastoma.

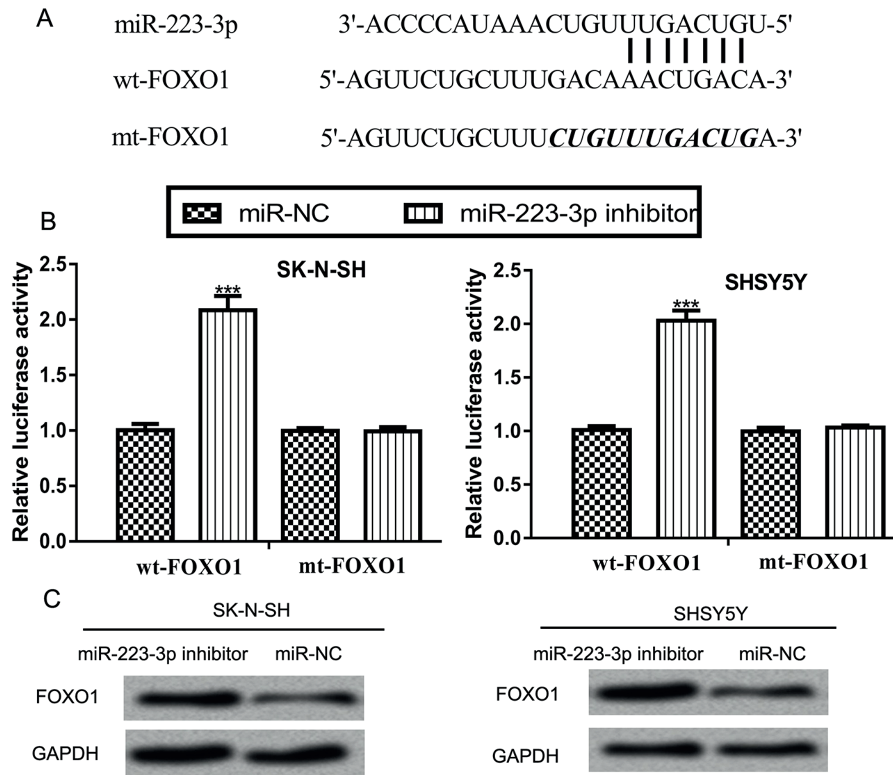

Figure 2. FOXO1 was a target of miR-223-3p. **A**, Putative binding sequence between miR-223-3p and 3'-UTR of FOXO1. **B**, Luciferase activity in NB cells transfected with luciferase activity vectors and synthetic miRNAs. **C**, Western blot assay was performed to detect the expression of FOXO1 after synthetic miRNAs transfection. MiR-223-3p: microRNA-223-3p; FOXO1: forkhead box O1; NB: neuroblastoma; wt: wild-type; mt: mutant; UTR: untranslated region; miR-NC: negative control miRNA.

in Figure 4B-4D, cell proliferation and invasion were increased, while cell apoptosis was decreased in si-FOXO1 transfected groups compared with siR-NC groups. Moreover, we found the transfection of si-FOXO1 could abolish the effects of miR-223-3p on NB cells (Figure 4B-4D).

Discussions

Aberrant expression of miRNA was closely associated with tumor development[12]. Studies[13,14] in the past decades have discovered numerous abnormal expressed miRNAs in cancers. Not surprisingly, multiple miRNAs were found dysregulated in OS[15-17]. For instance, miR-144-3p was downregulated in NB cell lines and regulates cell proliferation via regulating HOXA7[15]. Cheng et al[16] showed miR-34a was reduced expression in NB and correlated with poorer overall survival of NB patients. In addition, miR-34a overexpression inhibits NB cell proliferation, metastasis, autophagy but promotes apoptosis through regulating autophagy-related gene 5[16]. Moreover, Wang et al[17] found miR-129 was able to regulate NB cell growth and chemotherapy sensitivity via targeting MYO10.

In this study, miR-223-3p expression was found significantly upregulated in NB cell lines compared with normal cell line. Knockdown miR-223-3p inhibits NB cell proliferation and invasion but promotes apoptosis. These results implied that miR-223-3p may play an oncogenic role in NB, which is the same as its role in other cancer types, including ovarian cancer and prostate cancer[8,9].

Mechanistically, miRNAs exert their biological roles in human cancer by regulating downstream targets[12]. Therefore, we searched candidate targets of miR-223-3p using publicly available prediction algorithms. Among these predicted targets, FOXO1 was selected for analyses as it was previously reported to function as tumor suppressor in cancers[10,11]. Luciferase activity reporter assay and Western blot assay showed miR-223-3p could directly bind with 3'-UTR of FOXO1, and knockdown of miR-223-3p could elevate FOXO1 level in NB cells. We also found FOXO1 expression was downregulated in NB cell lines, and the knockdown of FOXO1 promotes cancer cell proliferation and invasion but inhibits apoptosis, which validated the tumor suppressive role of FOXO1 in NB. Our data showed that miR-223-3p plays an oncogenic role by promoting cell proliferation and invasion but inhibiting apoptosis via targeting FOXO1 in NB.

Conclusions

MiR-223-3p expression was upregulated in NB cell lines. Moreover, we demonstrated that knockdown of miR-223-3p suppressed NB cell

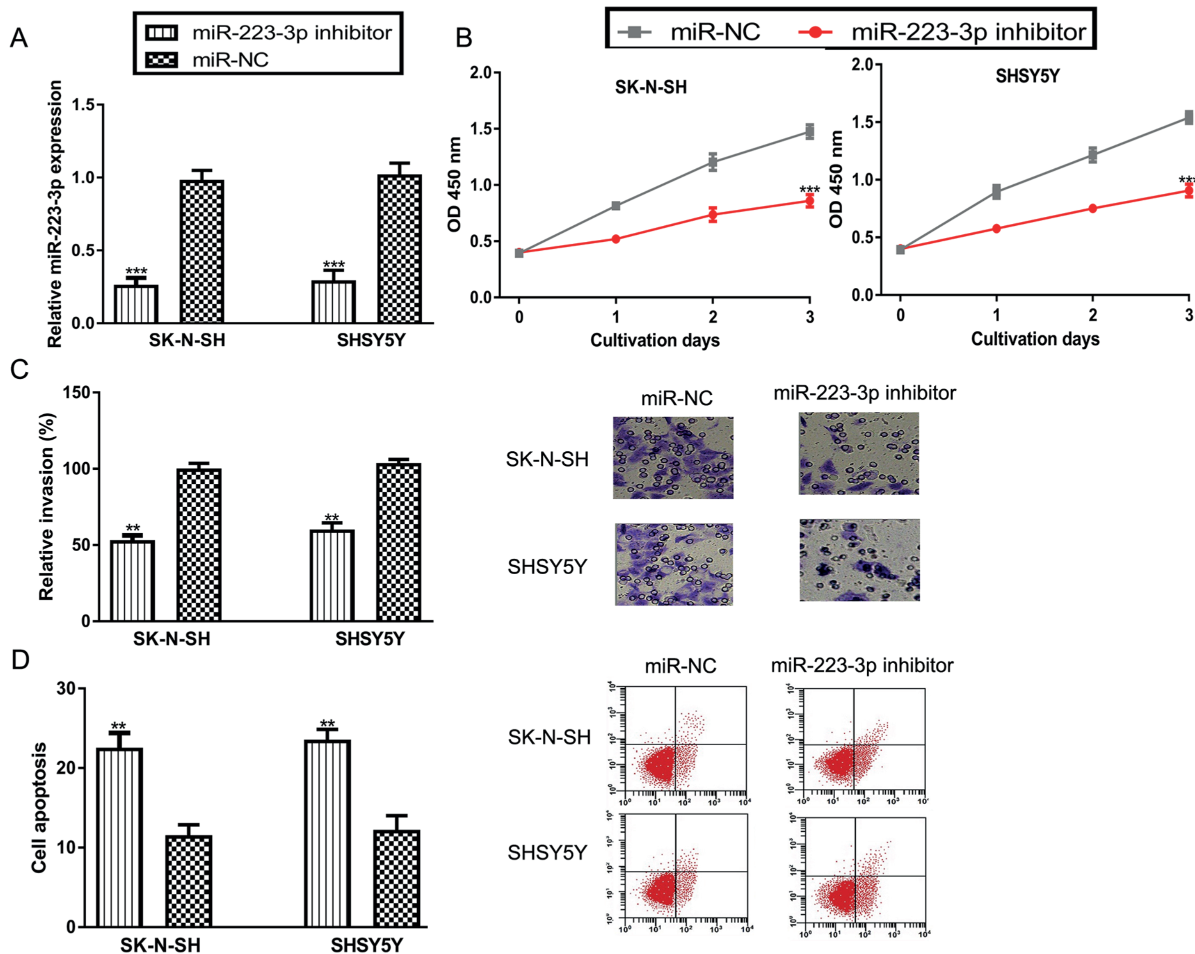

Figure 3. Knockdown of miR-223-3p inhibits NB cell proliferation and invasion. **A**, MiR-223-3p expression, **B**, Cell proliferation, **C**, Cell invasion (magnification 200X), and **D**, Cell apoptosis in NB cells transfected with synthetic miRNAs. miR-223-3p: microRNA-223-3p; NB: neuroblastoma; miR-NC: negative control miRNA.

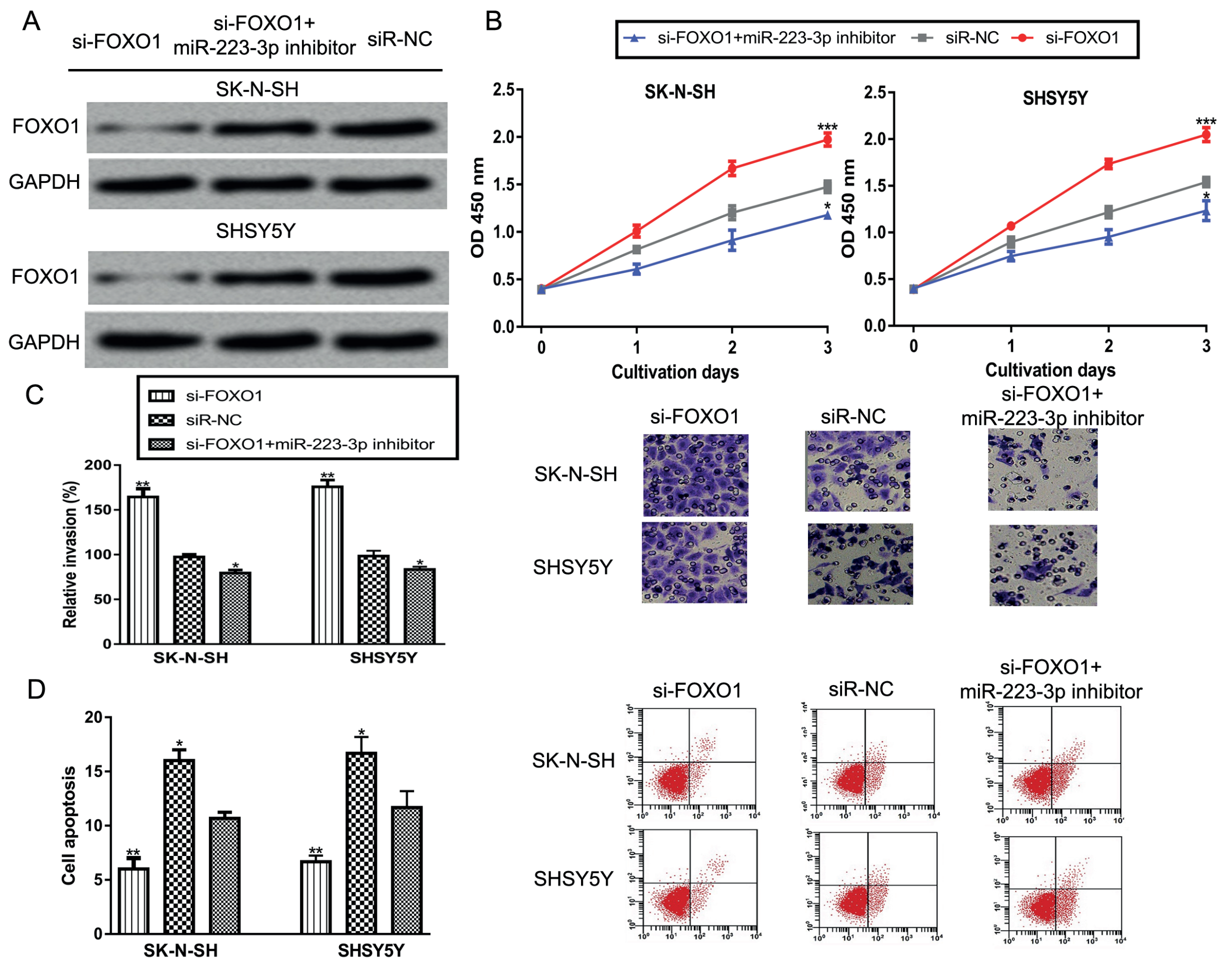

Figure 4. Knockdown of FOXO1 partially reversed the effects of miR-223-3p. **A**, FOXO1 expression, **B**, Cell proliferation, **C**, Cell invasion (magnification 200X), and **D**, Cell apoptosis in NB cells transfected with si-FOXO1; siR-NC; or si-FOXO1 and miR-223-3p inhibitor. MiR-223-3p: microRNA-223-3p; NB: neuroblastoma; FOXO1: forkhead box O1; siR-NC: negative control small interfering RNA.

proliferation and invasion but promoted apoptosis in part via regulating FOXO1. The finding of this result may provide novel therapeutic strategies against NB.

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Conflicts of interest

The authors declare no conflicts of interest.

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