Knockdown of zinc transporter ZIP8 expression inhibits neuroblastoma progression and metastasis *in vitro*

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Abstract. Neuroblastoma is one of the leading causes of cancer-associated mortality worldwide, particularly in children, partially due to the absence of effective therapeutic targets and diagnostic biomarkers. Therefore, novel molecular targets are critical to the development of therapeutic approaches for neuroblastoma. In the present study, the functions of zinc transporter ZIP8 (Zip8), a member of the zinc transporting protein family, were investigated as novel molecular targets in neuroblastoma cancer cells. The proliferation rates of neuroblastoma cancer cells were significantly decreased when Zip8 was knocked down by lentiviral-mediated RNA interference. Study of the molecular mechanism suggested that Zip8 modulated the expression of key genes involved in the nuclear factor-κB signaling pathway. Furthermore, Zip8 depletion suppressed the migratory potential of neuroblastoma cancer cells by reducing the expression levels of matrix metalloproteinases. In conclusion, the results of the present study suggested that Zip8 was an important regulator of neuroblastoma cell proliferation and migration, indicating that Zip8 may be a potential anticancer therapeutic target and a promising diagnostic biomarker for human neuroblastoma.

Introduction

Neuroblastomas affect 10.2 children <15 years of age in every million in the United States and constitute a major cause of cancer-associated mortality (1,2). A number of factors, including the stage of disease, age at diagnosis, and cellular and genetic features of the tumor, determined whether the tumor will spontaneously regress, or metastasize and become refractory to therapy (3,4). In previous decades, a number of approaches have been developed to treat neuroblastoma, including surgery, radiotherapy, high-dose multi-agent chemotherapy, autologous stem cell transplantation and targeted therapy using anti-disialoganglioside GD2 monoclonal antibodies (5-7). However, there is no effective therapeutic approach for treating neuroblastoma due to its complexity and heterogeneity. Chimeric antigen receptor T cell immunotherapy is one of the most promising therapeutic methods but remains problematic (8). Therefore, it is desirable to further reveal the molecular mechanisms underlying carcinogenesis in order to identify novel prognostic markers or molecular targets, which may facilitate the development of effective therapeutic strategies against neuroblastoma.

There is increasing evidence that a plethora of protein involved in the nuclear factor κ-B (NF-κB) signalling pathway, including NF-κB, inhibitor of NF-κB (IκB), and TRAF family member-associated NF-κB activator (TANK), contribute to the proliferation, apoptosis and metastasis of multiple types of cancer, including neuroblastoma (9,10). Although NF-κB signaling serves an important role in neuroblastoma tumorigenesis and cellular proliferation, its upstream regulators remain to be elucidated. As well as tumor cell proliferation, tumor cell metastasis is a complex phenomenon, which accounts for 90% of cancer-associated mortality worldwide (11). Studies of the molecular mechanism have revealed that matrix metalloproteinases (MMPs) and zinc-dependent endopeptidases (ZDEs) serve vital functions in tumor metastatic progression (12). However, the molecular expression of MMPs and ZDEs, particularly in neuroblastoma, remains to be elucidated.

Zinc is a fundamental dietary element and has a critical role in a range of cellular processes, including cellular proliferation and tumor cell metastasis (13). Zinc metabolism is primarily coordinated by zinc transporters distributed across the cell membrane (14). The zinc transporters in mammals are encoded by two solute-linked carrier (SLC) gene families that include fourteen SLC39 (also known as Zip) family members and ten SLC30 (also known as ZnT) family members (15). Zinc transporter ZIP8 (Zip8) belongs to

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Abbreviations: Zip8, zinc transporter ZIP8; RNAi, RNA interference; MMP, matrix metalloproteinase; NF-κB, nuclear factor κ-B; FBS, fetal bovine serum; RT-qPCR, reverse transcription-quantitative polymerase chain reaction

Key words: Zip8, neuroblastoma, NF-κB, MMP

the SLC39 family and serves an important role in increasing cytosolic zinc content, through the promotion of extracellular uptake or subcellular organelle zinc release (16,17). It has been demonstrated that zinc is involved in tumor regulation and that Zip8is vital to controlling the zinc concentration. Therefore, there is accumulating interest in theinvestigation of Zip8 function in cancer cell progression, proliferation and migration (18-20). In the present study, Zip8 was observed to regulate the proliferation and migration of neuroblastoma cells, and the molecular mechanisms of Zip8 in neuroblastoma cells were further investigated.

Materials and methods

Cell lines and cell culture. The human neuroblastoma SH-SY5Y cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). SH-SY5Y cells were cultured at 37˚C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium-F12 supplemented with 10% fetal bovine serum (FBS), 100 U/ml streptomycin and 100 U/ml penicillin (all Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Cell viability assay. The colorimetric water-soluble tetrazolium salt assay was applied to detect cell proliferation using a Cell Counting kit-8 (CCK8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan), according to the manufacturer's protocol. SH-SY5Y cells were seeded into 96-well plates at a density of $1x10^3$ cells/well, and cell proliferation was evaluated at 12, 24, 48, 72 and 96 h. The number of viable cells was assessed by measuring the absorbance at 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.).

Colony formation assay. SH-SY5Y cells were maintained in complete minimum essential medium supplemented with 10% FBS and 1% penicillin/streptomycin (all Gibco; Thermo Fisher Scientific, Inc.) and the cells were plated in 6-well plates at a density of 1,000 cells/well followed by incubation at 37˚C overnight. The media was replaced every 2 days, and after 2 weeks each well was washed with 1 ml PBS followed by adding 1 ml of crystal violet (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) solution (1% crystal violet and 10% ethanol) into each well. After 10 min incubation at room temperature, the colonies were washed out with PBS three times and were counted by eye.

Cell cycle assay. Cell cycle analysis of SH-SY5Y cells was performed using the BD Cycle assay kit (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's protocol. Different groups of SH-SY5Y cells were seeded into flasks at a density of $7x10^4$ cells (T25 flasks; Thermo Fisher Scientific, Inc.) and incubated at 37˚C for 48 h. Following incubation, the cells were harvested and fixed at 4˚C in 90% methanol for ~30 min. The cells were centrifuged at 4˚C for 5 min at 1,000 x g and washed twice with PBS. The pellets were then resuspended in propidium iodide and incubated at 37˚C for 1 h. A fluorescence-activated cell sorting machine (FACS Calibur flow cytometer; BD Biosciences) was used and data were analyzed using BD CellQuest Pro software version 4.0.2 (BD Biosciences).

Cell migration assay. The culture inserts (ibidi GmbH, Munich, Germany), consisting of 2 reservoirs separated by a 500-μm-thick wall, were placed in a 24-well plate. According to the manufacturer's protocol, an equal amount (70 μl) of SH-SY5Y cell suspension ($1x10^6$ cells/ml) was added to each reservoir followed by incubation at 37˚C. Following complete attachment of the cells (10 h), the culture inserts were gently removed and the medium was replaced with serum-free DMEM-F12 medium containing 0.2% bovine serum albumin (BSA; Beyotime Institute of Biotechnology, Haimen, China). The gap between the two cell layers was observed at 0 and 20 h after Zip8 knockdown under an inverted microscope (Advanced Microscopy Group, Mill Creek, WA, USA).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. RT-qPCR was performed to detect the mRNA expression levels of Zip8, glycogen synthase kinase-3 β (Gsk-3β), β-catenin, NF-κ-B essential modulator (Ikbkg), TANK, MMP2, MMP3, MMP9 and MMP14, according to the manufacturer's protocol. Total cellular RNA was extracted from SH-SY5Y cells by lysing cells with TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) reagent followed by centrifugation at 12,000 x g for 15 min at 4˚C with chloroform (5:1 ratio). The supernatant was centrifuged with isopropanol (1:1 ratio) at 10,000 x g at 4˚C for 10 min. The RNA pellet was washed with 75% ethanol and solubilized with DNase and RNase free water. The RNA was quantified by measuring absorbance at 260 nm using a NanoDrop ND-1000 (Thermo Fisher Scientific, Inc.). Single-stranded cDNA was prepared using a Reverse Transcription system (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. Briefly, 1 μg total RNA was added into the mixture and incubated at 37˚C for 15 min, and then kept at 85˚C for 5 sec. The mRNA expression of the target gene was determined by SYBR-Green assays. The SYBR-Green qPCR kit was purchased from Roche Diagnostics GmbH (Mannheim, Germany). qPCR was performed using an Applied Biosystems 7300 sequence detection system. The thermocycling conditions were as follows: Initial denaturation at 95˚C for 10 min, followed by 45 cycles at 95˚C for 15 sec and at 60˚C for 25 sec. All experiments were performed in triplicate. The relative gene expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (21) and normalized to GAPDH. The following primers were used: Zip8 forward, 5'-ATGCTACCCAAATAACCAGCTC-3' and reverse, 5'-ACAGGAATCCATATCCCCAAACT-3'; Gsk3β forward, 5'-AGACGCTCCCTGTGATTTATGT-3' and reverse, 5'-CCGATGGCAGATTCCAAAGG-3'; β-catenin forward, 5'-CATCTACACAGTTTGATGCTGCT-3' and reverse, 5'-GCAGTTTTGTCAGTTCAGGGA-3'; Ikbkg forward, 5'-CGGCAGAGCAACCAGATTCT-3' and reverse, 5'-CCTGGCATTCCTTAGTGGCAG-3'; TANK forward, 5'-AGCAGAGAATACGTGAACAACAG-3' and reverse, 5'-CAGAAGCAATGTCTACCTTTGGT-3'; MMP2 forward, 5'-CTGCGGTTTTCTCGAATCCATG-3' and reverse, 5'-GTCCTTACCGTCAAAGGGGTATCC-3'; MMP3 forward, 5'-CTGGACTCCGACACTCTGGA-3' and reverse, 5'-CAGGAAAGGTTCTGAAGTGACC-3'; MMP9 forward, 5'-GAGGCGCTCATGTACCCTATGTAC-3' and reverse, 5'-GTTCAGGGCGAGGACCATAGAG-3'; MMP14 forward, 5'-CTTCCGTGGAAACAAGTACTA

CCGT-3' and reverse, 5'-ATCCCTTCCCAGACTTTGATG TTC-3'; and GAPDH forward, 5'-GGAGCGAGATCCCTC CAAAAT-3' and reverse, 5'-GGCTGTTGTCATACTTCT CATGG-3'.

Nuclear protein isolation and western blot analysis. The protein expression levels of ZIP8, NF-κB, IκB, phosphorylated (p)-IκB, and MMP2, 3, 9 and 13, histone H1 and GAPDH in SH-SY5Y cells were evaluated by western blot analysis. Total proteins were extracted using the Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. The cells were washed twice with ice-cold PBS and lysed using a radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology), according to the manufacturer's protocol. Total cell lysates were then centrifuged at 12,000 x g for 15 min at 4˚C and the supernatants were used for further processing. The bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology) was used to determine the protein concentration. Total proteins (20 μg) were separated by 10% SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% BSA for 2 h at room temperature and incubated overnight at 4˚C with different primary antibodies: Anti-Zip8 (cat. no. ab106576; dilution 1:1,000), anti-NF-κB (cat. no. ab32360; dilution 1:1,000), anti-IκB (cat. no. ab32518; dilution 1:1,000), anti-p-IκB (cat. no. ab12135; dilution 1:1,500), anti-MMP2 (cat. no. 37150; dilution 1:1,000), anti-MMP3 (cat. no. 52915; dilution 1:2,000), anti-MMP9 (cat. no. 38898; dilution 1:1,000), anti-MMP14 (cat. no. 51074; dilution 1:5,000), anti-histone H1 (cat. no. 61177; dilution 1:1,000) and anti-GAPDH antibody (cat. no. ab181602; dilution 1:10,000; all Abcam, Cambridge, UK). Following incubation with primary antibodies, membranes were incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibodies (cat. no. 7074; dilution 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) for 1 h at room temperature. Protein bands were visualized using an enhanced chemiluminescent assay kit (Beyotime Institute of Biotechnology) and a luminescent image analyzer.

Construction of recombinant lentivirus and cell infection. The short hairpin (sh)RNA sequences that were used were as follows: Zip8-shRNA1 forward, 5'-GCCAAGTTCATGTAC CTGTTTCTCGAGAAACAGGTACATGAACTTGGCTTT TT-3' and reverse, 5'-AAAAAGCCAAGTTCATGTACCTGT TTCTCGAGAAACAGGTACATGAACTTGGC-3';
Zip8-shRNA2 forward, 5'-CCTTGCTATTCAACTTCCTTT CTCGAGAAAGGAAGTTGAATAGCAAGGTTTTT-3' and reverse, 5'-AAAAACCTTGCTATTCAACTTCCTTTCTCG AGAAAGGAAGTTGAATAGCAAGG-3'; scramble control shRNA forward, 5'-CTCTTCTACTCATCCACTTTTCTC GAGAAAAGTGGATGAGTAGAAGAGTTTTT-3' and reverse, 5'-AAAAACTCTTCTACTCATCCACTTTTCTCG AGAAAAGTGGATGAGTAGAAGAG-3'. The sequences were synthesized (Shanghai GeneChem Co., Ltd., Shanghai, China) and inserted into the lentiviral vector PLVX-IRES-ZsGreen1 by Shanghai GeneChem Co., Ltd., through standard molecular cloning methods and the recombinant plasmids were confirmed by sequencing. The lentiviral plasmids including PLVX-IRES-ZsGreen1 vector, pVSVG, pREV and pRRE were purchased from Takara Biotechnology Co., Ltd. HEK293T cells (Shanghai GeneChem Co., Ltd.) were cultured in 10-cm dishes at a concentration of $6x10^5$ cells/ml for 24 h at 37˚C and 5% CO_2 in a humidified incubator. Recombinant lentiviral particles were then generated by co-transfecting the packaging plasmids (5 μg for each plasmids), including packaging plasmids (pVSVG, pREV and pRRE), and the shRNA1-, shRNA2- or scramble shRNA-containing vector plasmids, into HEK293T cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) as the transfection reagent. Following incubation for 48 h at 37˚C and 5% CO_2 in a humidified incubator, the lentiviral particles were harvested and centrifuged at 50,000 x g for 2 h at 4˚C. SH-SY5Y cells were divided into 3 groups: Scramble control shRNA, Zip8 shRNA1 and Zip8 shRNA2. For the lentiviral infection, the SH-SY5Y cells were seeded onto 6-well plates at a density of $2x10^4$ cells/well. A total of 1 ml of medium containing polybrene (5 μg/ml) and 100 μl lentivirus (virus titer, 10^9 TU/ml) were added into each well. After 24 h, the medium was replaced with fresh medium (DMEM-F12 supplemented with 10% fetal bovine serum, 100 U/ml streptomycin and 100 U/ml penicillin; Gibco; Thermo Fisher Scientific, Inc.) and incubated for another 48 h. The cells were then examined under an inverted fluorescence microscope (IX71; Olympus Corporation, Tokyo, Japan) and the green fluorescence emitted by the green fluorescent protein in successfully infected cells was observed.

Statistical analysis. All the results represented at least three independent experiments and the data are expressed as the mean ± standard deviation. Differences between the control and treatment groups were analyzed using one-way analysis of variance followed by Fisher's least significant difference test and SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Zip8 knockdown inhibits the proliferation of neuroblastoma cancer cells. To investigate the function of Zip8 in neuroblastoma, we knockdown the Zip8 expression in SH-SY5Y cells using lentiviral-mediated RNA interference (RNAi). Zip8 shRNA targets were cloned into lentivirus vectors and the lentivirus was further packaged to infect SH-SY5Y cells. The infecting efficiency was more than 90% as assessed by GFP florescence suggestion of the successful lentivirus infection in neuroblastoma cancer cells (Fig. 1). Quantification analysis by RT-qPCR assay revealed that lentivirus-mediated RNAi obviously reduced the Zip8 mRNA expression levels in SH-SY5Y cells (Fig. 2A). The protein levels of Zip8 were also decreased significantly in SH-SY5Y cells infected by Zip8 shRNA lentivirus (Fig. 2B). Hence, the results demonstrated that the expression of Zip8 was effectively silenced by Zip8 shRNA lentivirus in neuroblastoma cell lines.

The effect of Zip8 on neuroblastoma cell proliferation was assessed by CCK-8 assay. According to the results, the proliferation rates of Zip8 silenced SH-SY5Y cells were both obviously reduced compared with that of the scramble control

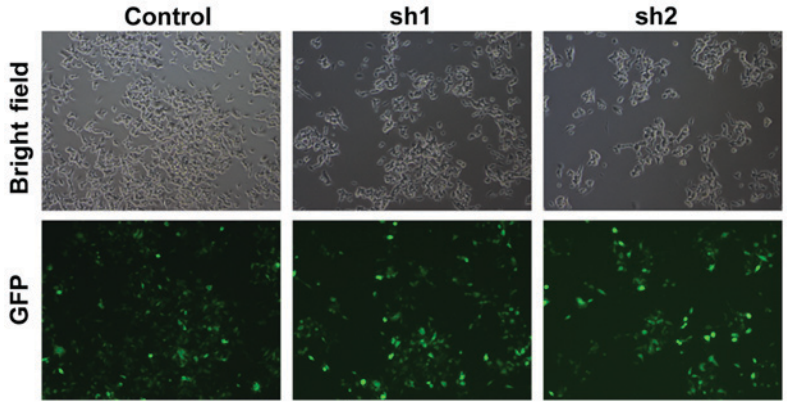

Figure 1. Representative images of SH-SY5Y cells infected by recombinant lentivirus. Magnification, x100. sh, short hairpin RNA; GFP, green fluorescent protein.

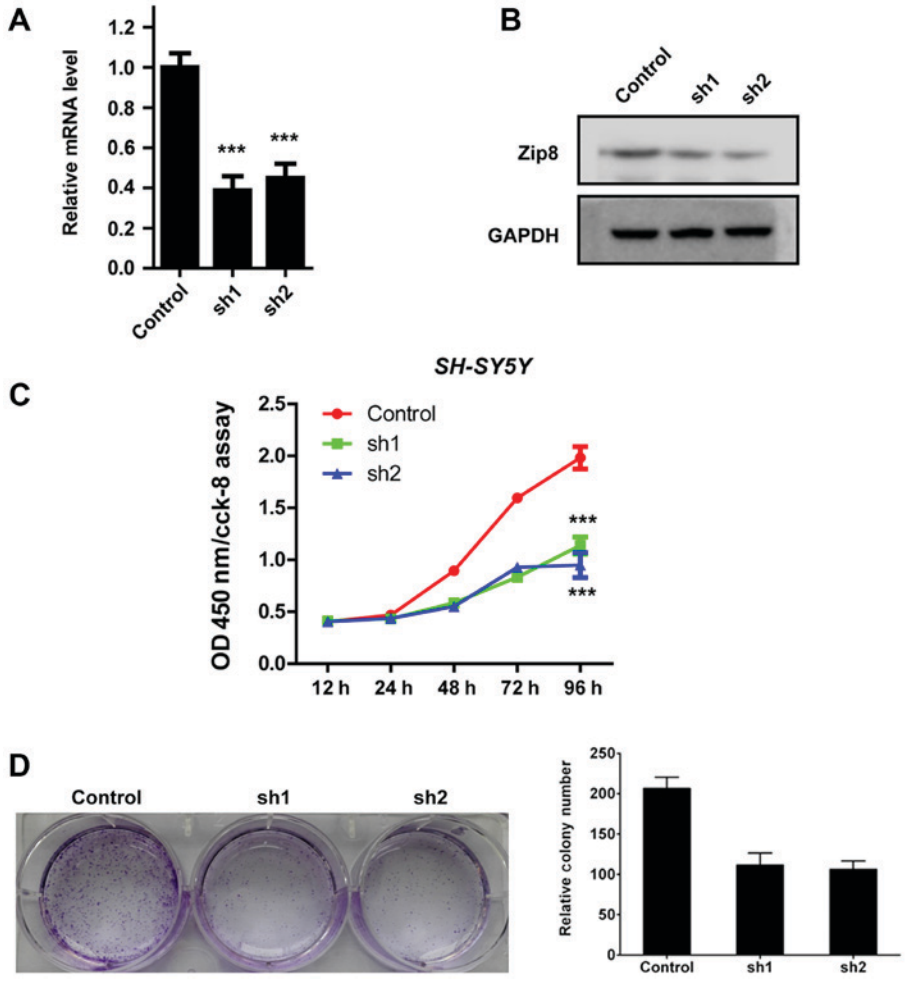

Figure 2. Effect of Zip8 knockdown on the proliferation of SH-SY5Y cells. (A) The knockdown efficiency of Zip8 was determined using the reverse transcription-quantitative polymerase chain reaction (n=3). (B) The knockdown efficiency of Zip8 determined by western blotting. (C) The proliferative rates of SH-SY5Y cells detected by the cck-8 assay (n=3). (D) Knockdown of Zip8 inhibited the colony formation of SH-SY5Y cells. Values are presented as the mean ± standard error of the mean. ***P<0.001 vs. the control group. Zip8, zinc transporter ZIP8; cck-8, Cell Counting kit 8; sh, short hairpin RNA; OD, optical density.

group (P<0.001; Fig. 2C). In addition, the colony formation assay was also performed to evaluate the effect of Zip8 knockdown on the colony formation ability of SH-SY5Y cells. Compared to the scramble control group, the number of cell colonies by crystal violet staining in the Zip8 silenced groups was obviously reduced (Fig. 2D and E). Therefore, these results indicated that Zip8 played an important role in the regulation of neuroblastoma cells proliferation.

Zip8 suppression affects the cell cycle of neuroblastoma cells. The observation that Zip8 knockdown inhibited the proliferation of neuroblastoma cells led to the investigation

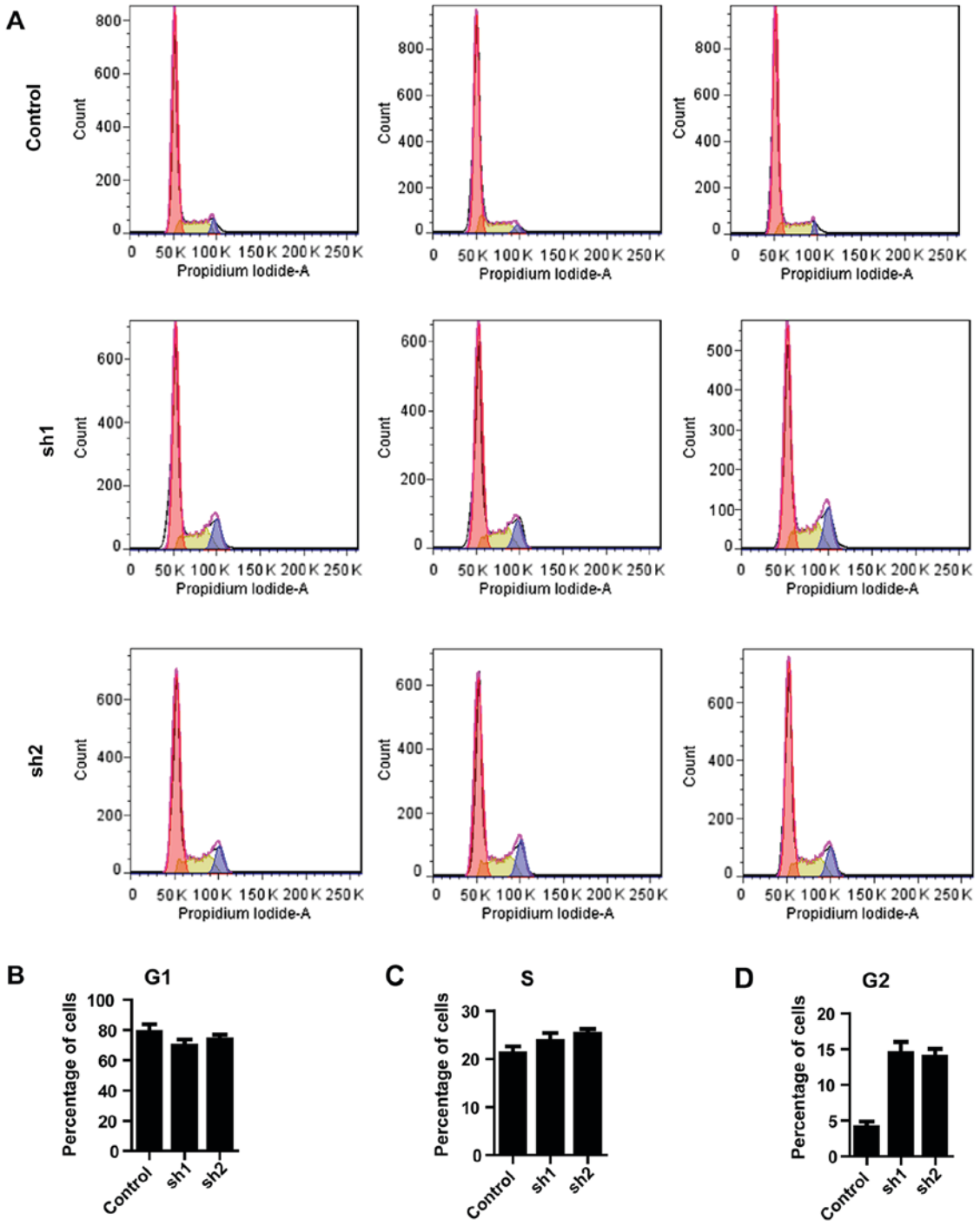

Figure 3. Effect of Zip8 knockdown on cell cycle progression of neuroblastoma cancer cells. (A) Flow cytometric histograms of SH-SY5Y cells in each cell cycle phase. Statistical analysis of (B) G0/G1, (C) S and (D) G2/M phases of cell cycle in SH-SY5Y cells following Zip8 knockdown. Zip8, zinc transporter ZIP8; sh, short hairpin RNA.

of whether Zip8 participates in regulation of the cell cycle. The cell cycle distribution was analyzed by flow cytometry following silencing of Zip8 (Fig. 3A). In the G0/G1 phase of cell cycle, the percentages of SH-SY5Y cells accumulated following Zip8 knockdown by lentiviral-mediated RNAi and the scramble control group presented no difference (Fig. 3B). Similarly, there was no significantly difference between each groups in the S phase (Fig. 3C). In the G2/M phase of cell cycle the percentages of SH-SY5Y cells following silencing Zip8 were increased, suggesting G2/M phase arrest following Zip8 depletion (Fig. 3D). Therefore, the results revealed that knockdown of Zip8 suppressed the growth of neuroblastoma cells possibly through induction of cell cycle arrest.

Zip8 modulates NF-κB signaling pathway. To reveal the essential molecular mechanism involved in the inhibition of neuroblastoma cells proliferation induced by Zip8 silencing, a number of known key genes which serve key roles in regulating cancer cells proliferation, including Gsk-3β, β-catenin, Ikbkg and TANK, were evaluated through RT-qPCR assays

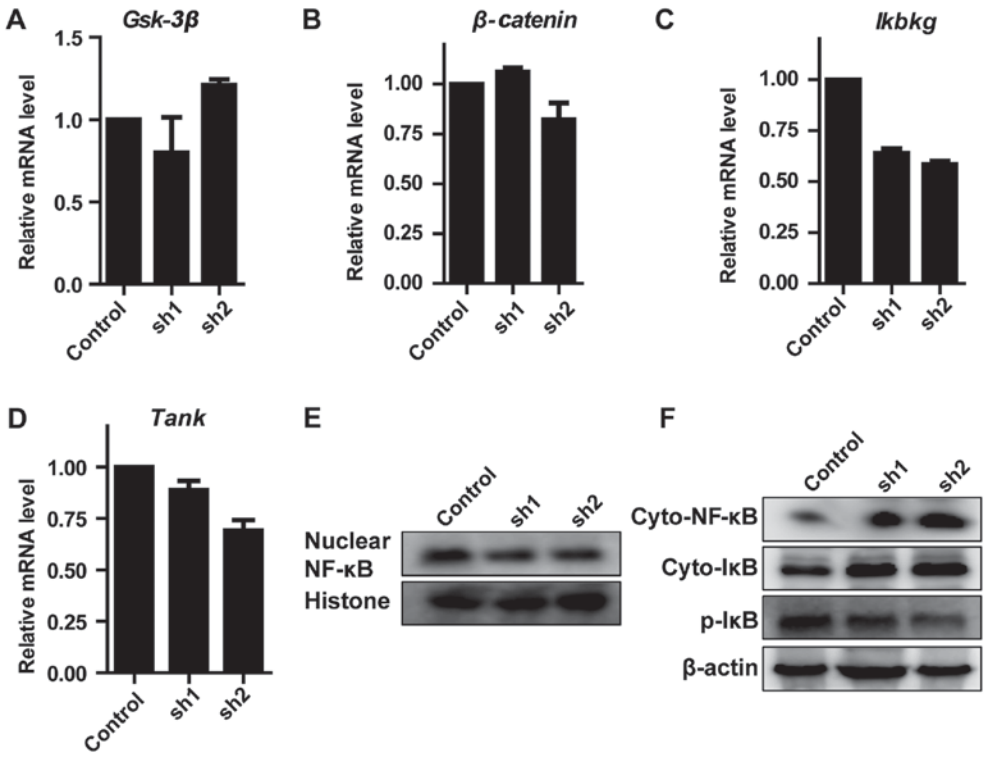

Figure 4. Effect of Zip8 knockdown on NF-κB signaling pathway. The mRNA levels of (A) Gsk-3β, (B) β-Catenin, (C) Ikbkg and (D) Tank in SH-SY5Y cells were detected by the reverse transcription-quantitative polymerase chain reaction (n=3). (E) The nuclear protein levels of NF-κB were detected by western blotting. (F) The cytoplasmic protein levels of NF-κB, IκB and p-IκB were detected by western blotting. Values are presented as the mean ± standard error of the mean. Zip8, zinc transporter ZIP8; NF-κB, nuclear factor κ-B; Gsk-3β, glycogen synthase kinase-3 β; Ikbkg, NF-κ-B essential modulator; Tank, TRAF family member-associated NF-κB activator; IκB, inhibitor of NF-κB; p, phosphorylated; sh, short hairpin RNA; cyto, cytoplasmic.

in SH-SY5Y cells. As the results demonstrated, the alterations in the mRNA levels of Gsk-3β and β-catenin in each group were not significant (Fig. 4A and B). The mRNA levels of Ikbkg and TANK involved in the NF-κB signaling pathway decreased markedly following Zip8 silencing (Fig. 4C and D). Therefore, western blotting was subsequently performed to evaluate the effect of Zip8 silencing on the NF-κB signaling pathway in each group. The protein levels of nuclear NF-κB were reduced following Zip8 knockdown (Fig. 4E). The NF-κB and IκB levels of Zip8-silenced groups in the cytoplasm were increased; however, p-IκB levels were decreased in cytoplasm (Fig 4F). These results suggested that silencing of Zip8 had an essential effect on the role of the NF-κB signaling pathway in neuroblastoma cell proliferation.

Zip8 silencing decreases the migration of neuroblastoma cells. To examine whether Zip8 serves a role in regulating the migration of SH-SY5Y cells, a cell migration assay was performed. The gap between 2 cell layers was observed and recorded at 0 h and 20 h, respectively. The control group of SH-SY5Y cells migrated towards to the empty area after culturing for 20 h. However, the cells of the Zip8-silenced groups demonstrated a marked reduction in their migratory ability, as the gaps between the two cell layers were obviously increased compared with that of the control group after 20 h, suggesting that Zip8 knockdown reduced cell migration (Fig. 5). These data indicated that Zip8 could affect the migration of neuroblastoma cells.

Zip8 regulates the expression of MMPs. Based on the above data demonstrating that knockdown of Zip8 inhibited the migratory ability of neuroblastoma cells, the key molecular mechanism involved in the phenomenon was investigated. The gene expression levels of migratory-associated proteins, including MMP2, 3, 9 and 14, were detected by RT-qPCR. The results suggested that, the expression levels of all the four MMPs decreased markedly (Fig. 6A-D). The western blot analysis further reconfirmed that the expression levels of MMP2, 3, 9 and 14 were reduced following Zip8 knockdown in SH-SY5Y cells (Fig. 6E). The results demonstrated that Zip8 could regulate the expression of MMPs to affect the migratory ability of neuroblastoma cells.

Discussion

Carcinogenesis is a complex and multifactorial process, resulting from a number of environmental effects. Neuroblastoma is commonly lethal due to its aggressive metastasis, migration and invasion. However, the molecular mechanism underlying neuroblastoma metastasis remains unclear. Zinc, which participates in multiple enzymatic and metabolic functions, is required for the activity of >300 enzymes (13). A total of >2,000 transcription factors involved in gene expression require zinc for maintaining structural integrity and DNA binding properties (22,23). Therefore, zinc serves an important role in protein function and regulation of zinc metabolism affects a range of cellular progress, including cellular proliferation. There

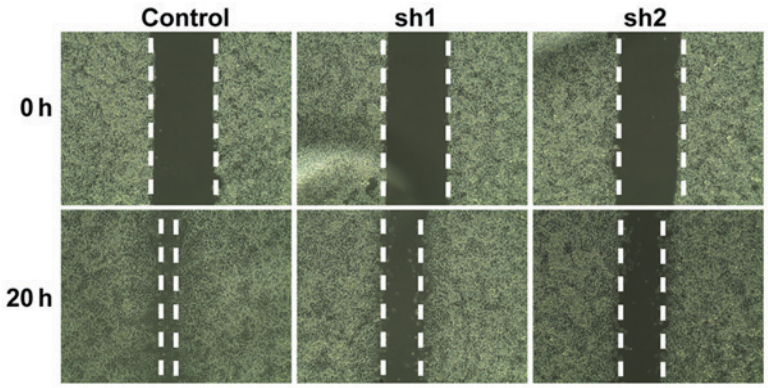

Figure 5. Zip8 knockdown suppresses the migratory potential of SH-SY5Y cells. Phase contrast images taken at 0 h and 20 h. Magnification, x100. sh, short hairpin RNA.

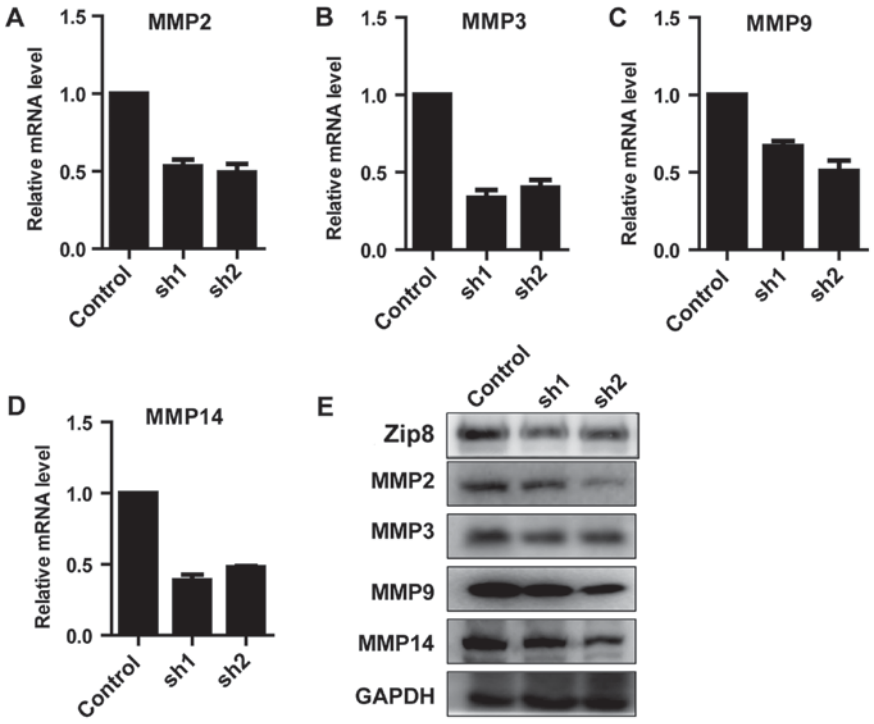

Figure 6. Effects of Zip8 knockdown on the expression of MMPs. The mRNA levels of (A) MMP2, (B) 3, (C) 9 and (D) 14 were detected using the reverse transcription-quantitative polymerase chain reaction (n=3). (E) The protein levels of MMP2, 3, 9 and 14 were detected by western blotting. Values are presented as the mean ± standard error of the mean. Zip8, zinc transporter ZIP8; MMP, matrix metalloproteinase; sh, short hairpin RNA.

is evidence suggesting that zinc concentrations maybe associated with the risk of cancer (24-26). Conversely, zinc transport proteins, including Zip8, are known to control intracellular zinc concentrations and metabolic homeostasis in mammalian cells, including cancer cells (27). Therefore, it was hypothesized that inhibiting the metabolic homeostasis of zinc by silencing Zip8 in neuroblastoma cancer cells may affect cell progression, including proliferation and migration. The results of the present study revealed that Zip8 knockdown could suppress the proliferation of neuroblastoma cells *in vitro* and reduce the migratory ability of neuroblastoma cells. These results suggested that Zip8 may be a potential therapeutic target of neuroblastoma. There are studies demonstrating that the expression levels of Zip8 are high in several types of cancer cells (28-30).

Cellular proliferation assays in the present study demonstrated that Zip8 could effectively regulate the proliferation of neuroblastoma cells. However, the molecular mechanism of Zip8 modulation remained unclear and a further study to elucidate the Zip8 modulation mechanism is required. The NF-κB signaling pathway is involved in several aspects of tumorigenesis, including cancer cell survival and proliferation, the prevention of apoptosis, and an increase in the metastatic potential of tumor cells (31). In addition, activation of the NF-κB signaling pathway serves an essential role in the modulation of apoptosis and migration in neuroblastoma cells (32,33). Therefore, whether Zip8 modulated the proliferation of neuroblastoma cancer cells through the NF-κB signaling pathway requires investigation. As demonstrated in the present study, Zip8 silencing in neuroblastoma cancer cells

exhibited an essential effect on the expression of key genes involved in the NF-κB signaling pathway.

Metastasis, a complex phenomenon mediated by a large number of signaling cascades, accounted for >90% of cancer-associated mortalities worldwide (34). The progression of metastasis involves cellular migration from the primary tumor site to secondary sites through the blood vascular system. In the present study, it was observed that Zip8 silencing decreased the migratory potential of neuroblastoma cells. MMPs, including the key members MMP2, 3, 9 and 14, perform vital functions in the regulation the cancer cell metastasis by modifying the cell microenvironment (35). Therefore, whether Zip8 regulates the expression of MMPs in neuroblastoma cells was investigated in the present study. The gene expression levels of migratory-associated proteins, including MMP2, 3, 9 and 14, decreased following Zip8 knockdown, suggesting that Zip8 could regulate the expression of MMPs to further affect the migratory ability of neuroblastoma cells.

In conclusion, the present study identified an important protein, Zip8, which serves an important role in the regulation of neuroblastoma cell proliferation. Further study revealed that Zip8 depletion inhibited cancer cell proliferation by modulating the expression of key genes involving in the NF-κB signaling pathway. Additionally, Zip8 depletion decreased the migratory potential of neuroblastoma cancer cells by modulating the expression of MMP2, 3, 9 and 14. The present study demonstrated that Zip8 serves important roles in the regulation of the proliferation of neuroblastoma cells and the modulation of the migration of neuroblastoma cells.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

ZM and FH designed the experiments. YW and SL conducted experiments. PY analyzed and interpreted data.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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