Downregulation of N-Myc inhibits neuroblastoma cell growth via the Wnt/β-catenin signaling pathway

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Abstract. Neuroblastoma, one of the most common types of cancer in childhood, is commonly treated with surgery, radiation and chemotherapy. However, prognosis and survival remain poor for children with high-risk neuroblastoma. Therefore, the identification of novel, effective therapeutic targets is necessary. N-Myc, a proto-oncogene protein encoded by the v-myc avial myelocytomatosis viral oncogene neuroblastoma derived homolog (*MYCN*) gene, is associated with tumorigenesis. In the present study, the effect of N-Myc silencing on *MYCN*-amplified CHP134 and BE-2C neuroblastoma cells was evaluated, and the underlying molecular mechanism was investigated. N-Myc was successfully knocked down using an N-Myc-specific small interfering RNA, the efficacy of interference efficiency confirmed by reverse transcription-quantitative polymerase chain reaction and western blotting. Cell viability was evaluated by MTT assay and apoptosis was measured by ELISA assay. The results indicated that *MYCN* silencing significantly decreased cell viability and promoted apoptosis. Subsequently, the expression levels of key Wnt/β-catenin signaling pathway proteins were detected by western blotting, and *MYCN* silencing was demonstrated to inhibit Wnt/β-catenin signaling, decreasing the expression ofanti-apoptosis proteins and increasing the expression of pro-apoptosis protein. This suggested that N-Myc regulated survival and growth of CHP134 and BE-2C neuroblastoma cells, potentially through Wnt/β-catenin signaling. Furthermore, associated proteins, N-Myc and STAT interactor and dickkopf Wnt signaling pathway inhibitor 1, were demonstrated to be involved in this regulation. Therefore, N-Myc and its downstream targets may provide novel therapeutic targets for the treatment of neuroblastoma.

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Introduction

Neuroblastoma, the most common extracranial solid tumor, is the third most common cause of cancer-associated mortality in childhood, causing 7% of all cancer cases in this age group (1). Neuroblastoma develops from undifferentiated neural crest cells in the embryo and is able to spread to other parts of the body through the blood and lymph prior to the manifestation of any apparent symptoms, resulting in a majority of diagnosed cases involving metastasis (2). Current therapies include surgery, radiation and chemotherapy, and their use depends on the cancer stage. Low and intermediate risk neuroblastoma tends to have a favorable outcome and maybe curable with surgery only, whereas high risk neuroblastoma is difficult to treat successfully even using intensive multi-modal therapies (3) which risk severe complications, including cardiac toxicity, infertility, hearing loss and secondary cancers resulting from high-dose chemotherapy (4). As a result, survival and prognoses remain poor for patients with high risk neuroblastoma.

The Wnt/β-catenin signaling pathway, which is critical during embryonic development, is one of the fundamental regulatory mechanisms of cellular proliferation, differentiation, apoptosis, polarity and pluripotency (5,6). Dysregulation of the pathway is associated with birth defects and multiple human diseases, including liver and colon cancer, and certain types of brain cancer (7). The stability of β-catenin is used to evaluate the activity of Wnt/β-catenin pathway. β-catenin is modulated by a destruction complex consisting of glycogen synthase kinase-3, casein kinase 1, adenomatous polyposis

coli and the scaffolding protein axin. In the absence of Wnt stimulation, β-catenin is phosphorylated and degraded by the ubiquitin proteasome pathway mediated by the destruction complex, resulting in the attenuation of Wnt signaling. Wnt stimulation contributes to the accumulation of cytoplasmic β-catenin and its translocation into the nucleus, where β-catenin associates with transcription factor 4/lymphoid enhancer-binding factor to activate the transcription of its target genes, cyclin D1 and c-Myc, which control the G1 to S phase transition in the cell cycle (8-10), resulting in abnormal cellular proliferation. High β-catenin expression levels or activation of its downstream signaling cascades are associated with tumor grade and poor prognosis in glioma (11-15). Blocking Wnt signaling has attracted attention as a therapeutic strategy for the treatment of cancer (16).

N-Myc, a member of the Myc family, is a proto-oncogene protein encoded by the v-myc avial myelocytomatosis viral oncogene neuroblastoma derived homolog (*MYCN*) gene. N-Myc is highly expressed in the fetal brain and is important for normal brain development (17). *MYCN* amplification is presented in ~25% of cases and is hypothesized to be associated with high-risk neuroblastoma and poor prognosis (18). *MYCN* amplification is a genetic marker that is used to stratify grade in neuroblastoma. Previous studies have demonstrated that N-Myc is involved in cell growth, apoptosis, metastasis, angiogenesis and tumorigenesis in neuroblastoma (19). N-Myc primarily functions as a downstream target of Wnt signaling (20). However, it remains unclear if N-Myc regulates Wnt signaling to affect cellular biological functions. N-Myc has previously been reported to directly reduce dickkopf Wnt signaling pathway inhibitor 1(DKK1) expression, and DKK1 regulates neuroblastoma cell proliferation (21). Furthermore, DKK1 was demonstrated to disrupt the Wnt/β-catenin signaling pathway (22,23). Therefore, the present study hypothesized and confirmed that N-Myc regulates neuroblastoma cell growth through affecting the Wnt/β-catenin pathway.

Materials and methods

Cell culture. Human neuroblastoma cell lines CHP134 [isolated from a patient at The Children's Hospital of Philadelphia (Philadelphia, PA, USA) and purchased from the European Collection of Authenticated Cell Cultures (Public Health England, Salisbury, UK) and BE-2C (provided by The Children's Hospital of Philadelphia) were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 1% penicillin/streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 2 mM glutamine. Cells were cultured in a 37˚C incubator with 5% CO_2.

RNA interference. *MYCN*-specific small interfering RNA (siRNA; siN-Myc) and control siRNA (siControl) were purchased from Sigma-Aldrich; Merck KGaA. The siN-Myc sequences used were as previously described (24). The si Control sequence used was 5'-ACGTGACACGTTCGGAGA ATT-3', and does not match with any known human cDNA. The synthesized oligonucleotides were annealed and cloned into *Age*I/*Eco*RI (Fermentas; Thermo Fisher Scientific, Inc.) double digested pLKO.1-puro lentivirus plasmids (preserved in our laboratory, The Research Center for Vascular Biology, College of Medicine, Yangzhou University, Yangzhou, China). Green fluorescent protein was used as a reporter gene. Lentiviruses containing pLKO.1-puro vectors were produced by cotransfection with Δ8.2 and vesicular stomatitis virus (VSV-G) plasmids (preserved in our laboratory) into HEK293T packing cells purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Cells were further grown for 48 h after transfection, virus supernatants were collected and used to transfect CHP134 and BE-2C neuroblastoma cells by spin inoculation in the presence of 8 μl/ml Polybrene (Sigma-Aldrich; Merck KGaA) for 72 h. After 48 h of incubation with 2 mg/ml puromycin (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), cells were selected for further analysis. The efficacy of interference was assessed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting.

RT-qPCR. Total RNA was extracted from cells and purified using TRIzol reagent (cat. no. 15596-026; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. The total RNA isolated using TRIzol was free of protein and DNA contamination. The isolated RNA was then treated with amplification grade DNase I (cat. no. 18068-015; Invitrogen; Thermo Fisher Scientific, Inc.). cDNA was obtained by reverse transcription using RevertAid™ First Strand cDNA synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.) and was amplified using TaqMan®Gene Expression assays (Applied Biosystems; Thermo Fisher Scientific, Inc.) with 6-carboxyfluorescein-labeled probes according to the manufacturer's protocols. The primers used for MYCN gene amplification were as follows: Forward 5'-CCCCTGGGT CTGCCCCGTTT-3', reverse, 5'-GCCGAAGTAGAAGTC ATCTT-3'. The sequence of the TaqMan fluorogenic probe was 5'-CCCACCCTCTCCGGTGTGTCTGTCGGTT-3'. For the β-actin gene, the primers were as follows: β-actin, forward 5'-TCACCCACACTGTGCCCATCTACGA-3' and reverse 5'-CAGCGGAACCGCTCATTGCCAATGG-3'; fluorogenic probe, forward 5'-ATGCCCTCCCCCATGCCATCCTGC GT-3'. Both genes were amplified with the following thermocycler protocol: A first step of 120 sec at 95˚C, followed by 45 cycles of 30 sec at 95˚C, 30 sec at 60˚C, and 30 sec at 72˚C. Fluorescence detection was performed using the ABI PRISM 7700 Sequence Detector (PerkinElmer, Inc. Waltham, MA, USA). N-Myc expression was normalized to *β-actin* expression and was calculated using the $2^{-\Delta\Delta Cq}$ formula (25). The relative N-Myc mRNA expression levels were presented as a percentage of the control.

Western blotting. Cells were washed twice with PBS and were lysed in lysis buffer [50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1% NP40, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4] containing a protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). Following centrifugation at 12,000 x g for 10 min, the supernatant was collected and quantified using a bicinchoninic acid quantification kit (Beyotime Institute of Biotechnology, Haimen, China). Proteins (50 μg) were separated

on 10% SDS-PAGE gel (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and transferred to Immobilon-P membranes (Merck KGaA). The membranes were blocked with 5% non-fat dried milk in tris-buffered saline with 0.1% Tween-20 for 1 h, and incubated with specific primary antibodies overnight at 4˚C. Rabbit polyclonal immunoglobulin G (IgG) specific to N-Myc (1:500; cat. no. ab24193; Abcam, Cambridge, UK), mouse monoclonal IgG specific to β-catenin (1:1,000; cat. no. sc7963; Santa Cruz Biotechnology, Inc.), rabbit polyclonal IgG specific to Cyclin D1 (1:1,000; cat no. sc753; Santa Cruz Biotechnology, Inc.), mouse monoclonal IgG specific to c-Myc (1:1,000; cat. no. sc40; Santa Cruz Biotechnology, Inc.), rabbit polyclonal IgG specific to DKK1 (1:1,000; cat. no. 4687S; Cell Signaling Technology, Inc., Danvers, MA, USA), goat polyclonal IgG specific to N-Myc and STAT interactor (Nmi; 1:1,000; cat. no. sc9483; Santa Cruz Biotechnology, Inc.), mouse monoclonal IgG specific to B cell lymphoma 2 (Bcl-2; 1:500; cat. no. sc7382; Santa Cruz Biotechnology, Inc.), rabbit polyclonal IgG specific to Bcl-2 associated X apoptosis regulator (Bax; 1:500; cat. no. sc493; Santa Cruz Biotechnology, Inc.), mouse monoclonal IgG specific to β-actin (1:1,000; cat. no. sc47778; Santa Cruz Biotechnology, Inc.), rabbit polyclonal IgG specific to cleaved-caspase-3 (1:1,000; cat. no. 9661S; Cell Signaling Technology, Inc.) and rabbit monoclonal IgG specific to cleaved-poly ADP ribose polymerase (PARP; 1:1,000; cat. no. 5625P; Cell Signaling Technology, Inc.) were used for detection by an enhanced chemiluminescence detecting reagent (GE Healthcare Life Sciences, Chalfont, UK). Horseradish peroxidase-conjugated secondary antibodies: goat anti-mouse (1:2,000; cat. no. sc-2005; Santa Cruz Biotechnology, Inc.) and goat anti-rabbit IgG (1:2,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc.) were used to incubation for 1 h at room temperature. The protein blots were quantified by densitometry using Quantity One software version 4.6.2 (Bio-Rad, Hercules, CA, USA), and the amounts were expressed relative to the internal reference β-actin.

MTT assay. Cell viability was evaluated by MTT assay. CHP134 and BE-2C cells ($1x10^4$ cells/well) were plated in a 96 well plate, and were cultured for 48 h at 37˚C. MTT (20 μl, 5 mg/ml; Sigma-Aldrich, Merck KGaA) was added to the medium and incubated for 4 h, and the medium was then replaced with 150 μl dimethyl sulfoxide for 10 min at room temperature to dissolve the cells. Cell viability was measured using an enzyme-linked immunosorbent assay (ELISA) spectrophotometer (JK-UVS-760CRT; Shanghai Jingke Scientific Instrument Co., Ltd., Shanghai, China) at 490 nm.

Apoptosis assay. Apoptosis was assessed by ELISA, using the Cell Death Detection ELISA^{PLUS} kit (cat. no. 11774425001; Roche Applied Science, Penzberg, Germany). CHP134 and BE-2C cells ($4x10^3$ cells/well) were plated in a 96-well plate (Thermo Fisher Scientific, Inc.). Following incubation for 9 h, samples were collected and analyzed according to the manufacturer's protocol. Results were expressed as the fold induction relative to control. In addition, caspase-3 activation was detected to verify the effect on apoptosis using the active caspase-3 ELISA kit (cat. no. K106-100; R&D Systems, Inc., Minneapolis, MN, USA). A total of $1x10^6$ cells/well transfected with siN-Myc and siControl were grown in 6-well plates (Thermo Fisher Scientific, Inc.). Following 8 h incubation, the cells were lysed and ELISA was performed according to the manufacturer's protocol.

Morphological observations of neuroblastoma cells. Following transfection, $1x10^5$ cells were cultured in 60 mm culture dishes (Thermo Fisher Scientific, Inc.) for 48 h. The medium was then replaced with fresh medium. Cell morphology was observed and photographed using a light vertical microscope (magnification, x400) (Olympus Corporation, Tokyo, Japan).

Statistical analysis. All experiments were repeated ≥3 times. Statistical analysis was preformed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). Values are expressed as the mean ± standard error of the mean. One-way analysis of variance was used to assess differences between groups. Duncan method was employed for pairwise comparison and followed by Bonferroni correction. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Transfection with siN-Myc silences N-Myc expression in CHP134 and BE-2C neuroblastoma cells. To determine the function of N-Myc in neuroblastoma cells, N-Myc was knocked down by transfection with N-Myc-specific siRNA technology in neuroblastoma cells expressing *MYCN*. N-Myc mRNA and protein expression levels were detected by RT-qPCR and western blotting, respectively. N-Myc mRNA expression levels were significantly decreased in cells transfected with siN-Myc compared with negative control and siControl cells ($P<0.01$; CHP134 cells, Fig. 1A; BE-2C cells, Fig. 1B). Residual N-Myc protein levels in cells transfected with siN-Myc were also visibly inhibited compared with normal control and siControl cells (CHP134 cells, Fig. 1A; BE-2C cells, Fig. 1B). Therefore, transfection with siN-Myc successfully silenced N-Myc expression in CHP134 and BE-2C cells, enabling the identification of the biological function of N-Myc in neuroblastoma cells.

MYCN silencing inhibits cellular growth and promotes apoptosis. The functional effects of N-Myc knockdown were investigated by measuring cell viability and apoptosis. The MTT assay results demonstrated that CHP134 and BE-2C cell viability was significantly inhibited in CHP134 and BE-2C cells transfected with siN-Myc compared with siControl cells ($P<0.01$; Fig. 2A). Apoptosis was analyzed using ELISA to detect histone release, which is an epigenetic marker of early apoptosis. CHP134 and BE-2C cells transfected with siN-Myc demonstrated significantly increased DNA fragmentation compared with siControl cells ($P<0.01$; Fig. 2B), indicating that *MYCN* silencing induced cell apoptosis. Caspase-3 is activated by intrinsic mitochondrial and extrinsic death ligand pathways in apoptotic cells (26,27). Therefore, to confirm the involvement of N-Myc in the regulation of apoptosis, caspase-3 activity was also detected. Caspase-3 activity was significantly higher in CHP134 and BE-2C cells transfected with siN-Myc compared with siControl cells ($P<0.01$ and $P<0.05$, respectively; Fig. 2C), suggesting that *MYCN* silencing promoted

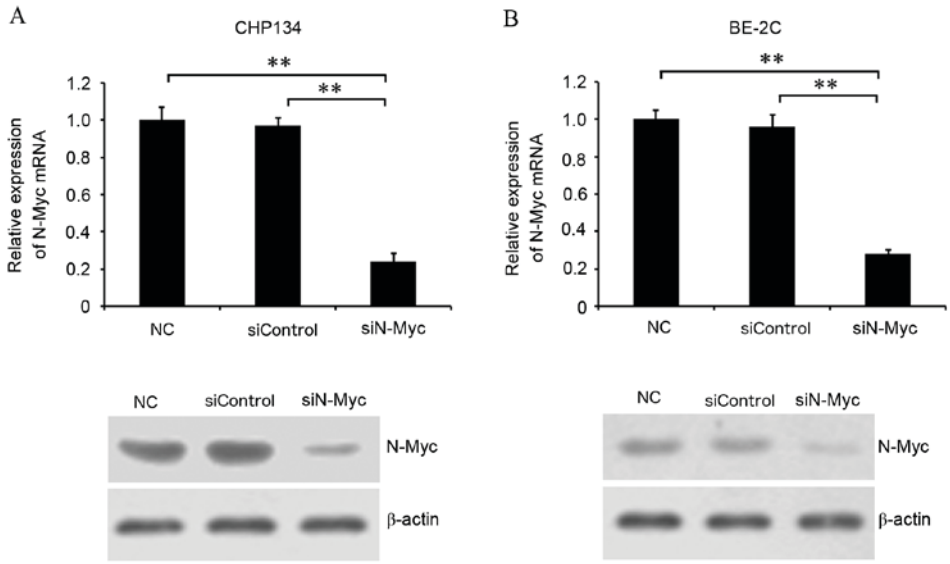

Figure 1. Knockdown of *MYCN* in neuroblastoma CHP134 and BE-2C cells. N-Myc mRNA and protein expression levels in (A) CHP134 and (B) BE-2C cells, determined by reverse transcription-quantitative polymerase chain reaction and western blotting, respectively, following transfection with siN-Myc or siControl. β-actin was used as an internal reference. Data are expressed as the mean ± standard error of the mean. **P<0.01, comparison indicated by brackets. siN-Myc, v-myc avial myelocytomatosis viral oncogene neuroblastoma derived homolog-specific small interfering RNA; siControl, control small interfering RNA.

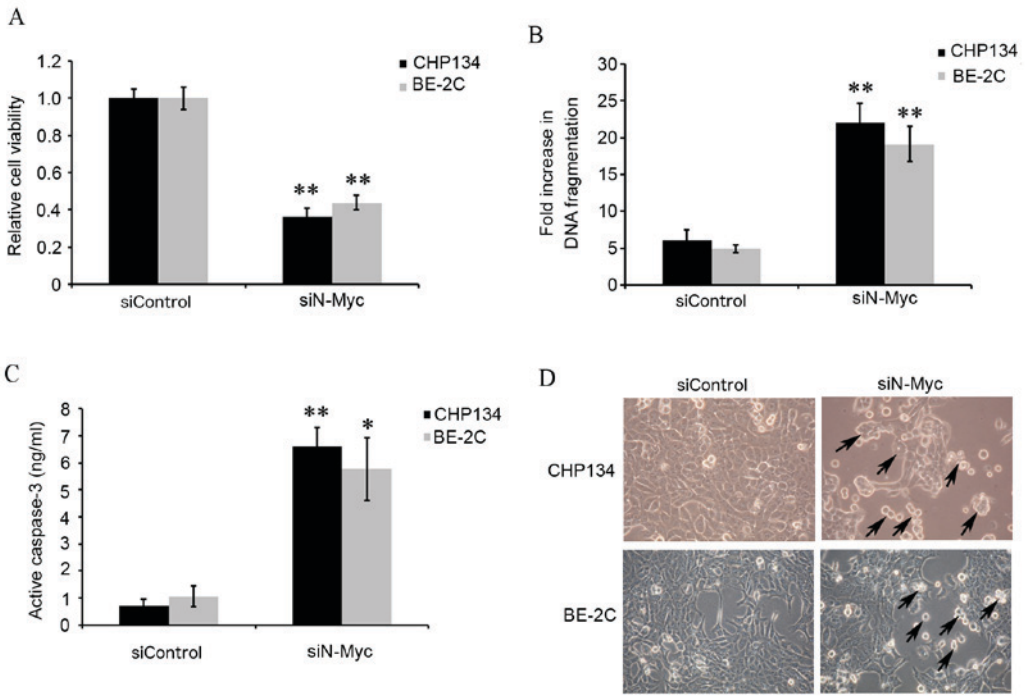

Figure 2. Function of N-Myc in neuroblastoma cells. (A) Cell viability as assessed via MTT assay. Cell apoptosis as evaluated through the detection of (B) histone release and (C) caspase-3 activity in CHP134 and BE-2C cells following transfection with siN-Myc and siControl. (D) Cell morphology was visualized using a vertical microscope (magnification, x400), with arrows indicating membrane-bound apoptotic bodies. Data are expressed as the mean ± standard error of the mean. *P<0.05 and **P<0.01 vs. siControl. siN-Myc, v-myc avial myelocytomatosis viral oncogene neuroblastoma derived homolog-specific small interfering RNA; siControl, control small interfering RNA.

apoptosis. Cellular growth capabilities were also compared by visualizing cell morphology. CHP134 and BE-2C cells transfected with siN-Myc appeared to grow more slowly and exhibit morphological changes, including shrinkage of cell volume and membrane-bound apoptotic bodies in comparison with siControl cells (Fig. 2D).

Effects of N-Myc on apoptosis-associated protein expression levels in neuroblastoma cells. To further confirm the involvement of N-Myc in neuroblastoma cell apoptosis, western blotting was performed to determine the expression levels of the following apoptosis-associated proteins: Bcl-2, Bax, cleaved-caspase-3 and cleaved-PARP (Fig. 3A and B).

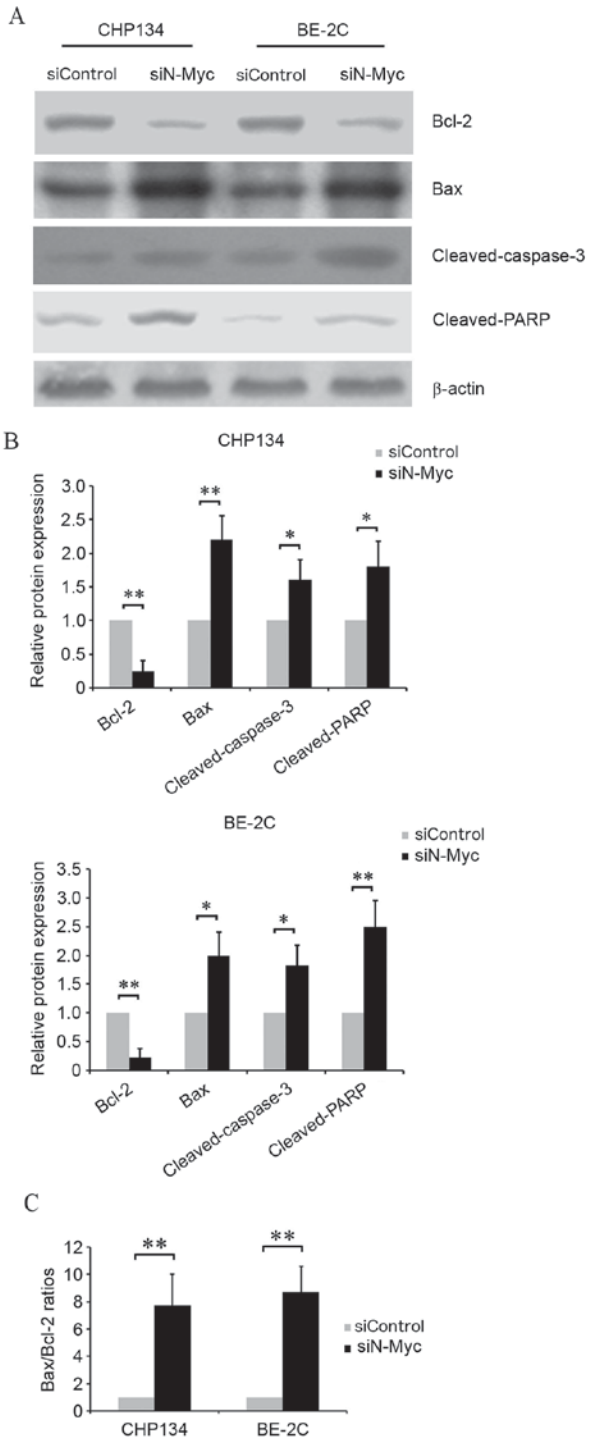

Figure 3. *MYCN* silencing affects apoptosis-associated proteins expression in CHP134 and BE-2C cells. Protein expression levels of Bcl-2, Bax, cleaved-caspase-3 and cleaved-PARP were (A) detected by western blotting and (B) quantified. (C) The Bax/Bcl-2 ratio, determined by Bax protein expression/Bcl-2 protein expression. Data are expressed as the mean ± standard error of the mean. *P<0.05 and **P<0.01 vs. siControl. siN-Myc, v-myc avial myelocytomatosis viral oncogene neuroblastoma derived homolog-specific small interfering RNA; Bcl-2, B cell lymphoma 2; Bax, Bcl-2 associated X apoptosis regulator; PARP, poly ADP ribose polymerase; siControl, control small interfering RNA.

Protein expression levels of Bcl-2, an anti-apoptosis protein, were significantly decreased in CHP134 and BE-2C cells transfected with siN-Myc compared with siControl cells (P<0.01). In addition, transfection with siN-Myc significantly increased pro-apoptosis protein expression levels compared with siControl cells, including Bax (CHP134 cells, P<0.01; BE-2C cells, P<0.05) cleaved-caspase-3 (P<0.05) and cleaved-PARP (CHP134 cells, P<0.05, BE-2C cells, P<0.01). The Bax/Bcl-2 ratio was significantly increased in CHP134 and BE-2C cells transfected with siN-Myc compared with siControl cells (P<0.01; Fig. 3C), and was a key factor in determining the occurrence and level of apoptosis. These data indicated that N-Myc regulated cell apoptosis in CHP134 and BE-2C cells, potentially through modulation of Bcl-2, Bax, cleaved-caspase-3 and cleaved-PARP.

MYCN silencing inhibits the Wnt/β-catenin signaling pathway. To explore the molecular mechanisms underlying N-Myc regulation of neuroblastoma cell viability and apoptosis, the expression levels of key proteins in the Wnt/β-catenin signaling pathway were evaluated. Western blotting revealed that β-catenin (P<0.05), cyclin D1 (P<0.01, CHP134 cells; P<0.05, BE-2C cells) and c-Myc (P<0.01) protein expression levels were significantly decreased in CHP134 and BE-2C cells transfected with siN-Myc compared with siControl cells (Fig. 4A and B), suggesting that N-Myc may regulate cell viability and apoptosis through Wnt signaling. In order to clarify the mechanisms involved in N-Myc induced modulation of the Wnt/β-catenin pathway, DKK1, an inhibitor of the Wnt/β-catenin signaling cascade, and Nmi, an upstream regulator of DKK1, were detected by western blotting. DKK1 and Nmi protein levels were increased in cells transfected with siN-Myc compared with siControl cells (Fig. 4C). This suggested that *MYCN* silencing may affect the Wnt/β-catenin pathway in CHP134 and BE-2C neuroblastoma cells through Nmi-mediated upregulation of DKK1 expression levels.

Discussion

High risk neuroblastoma is an aggressive cancer that is often accompanied by metastasis, resulting in poor prognosis even with coordinated surgical, radiation and chemotherapy treatment (28). Therefore, exploring the molecular mechanisms underlying neuroblastoma is important for the identification of novel therapeutic targets.

The Myc proto-oncogene family is associated with the establishment of multiple types of human cancer, functioning as transcription factors that trigger cell immortalization, continued cell-cycle progression and inhibition of differentiation in various cell lines. N-Myc is a member of the Myc family that has a basic helix-loop-helix (bHLH) domain. N-Myc is located in the nucleus and binds DNA by dimerizing with another bHLH protein (29). N-Myc expression has been previously reported to be elevated in several types of cancer, including prostate, colorectal, lung and breast cancer, and is associated with malignancy and a poor prognosis (30-34). In addition, amplification of *MYCN* has previously been observed in human neuroblastoma and is correlated with the malignant progression of neuroblastoma (35,36). Therefore, N-Myc may be a potential therapeutic target for the treatment of malignant neuroblastoma that overexpresses *MYCN*. In the present study, downregulation of *MYCN* by siN-Myc inhibited the cellular growth of CHP134 and BE-2C *MYCN*-amplified

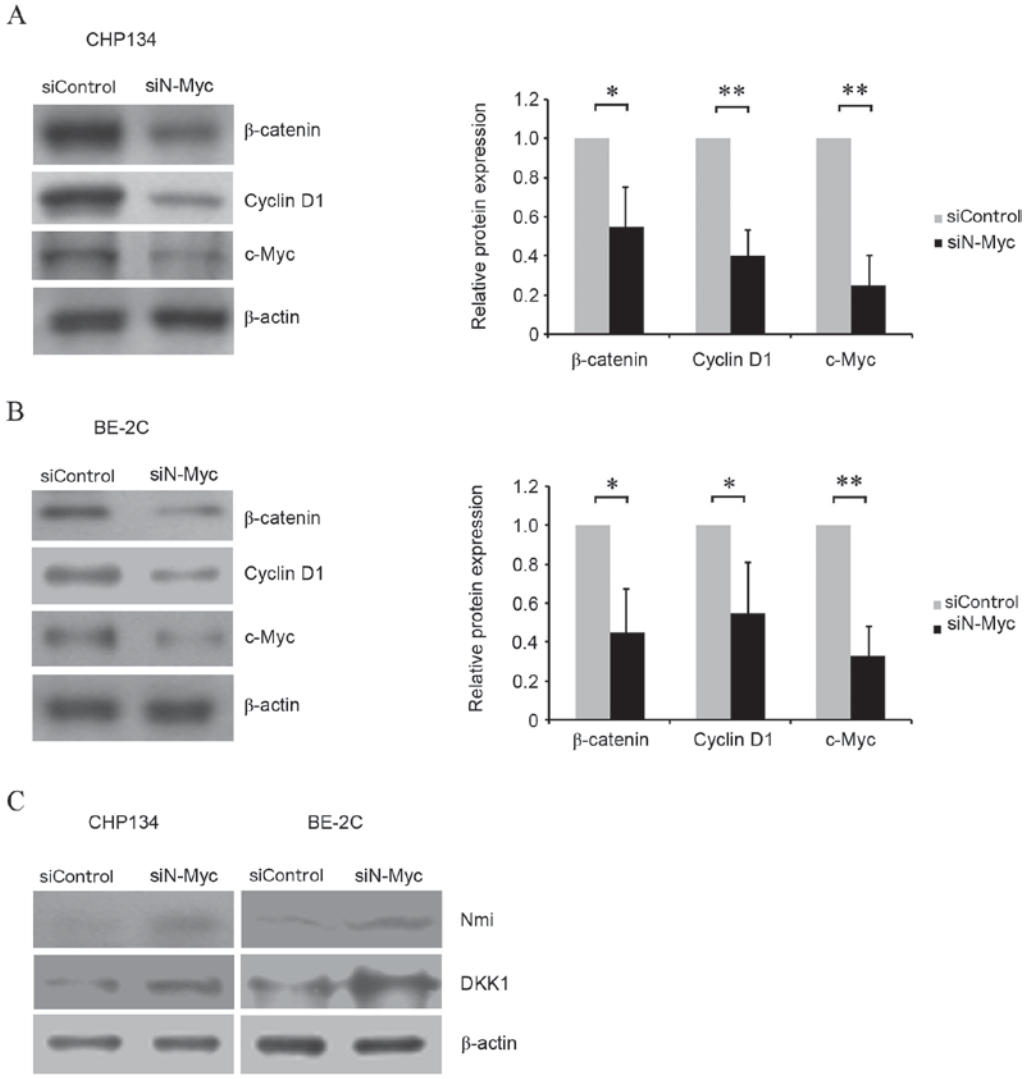

Figure 4. Transfection with siN-Myc inhibits the Wnt/β-catenin pathway. β-catenin, Cyclin D1 and c-Myc protein expression levels in (A) CHP134 and (B) BE-2C cells. β-actin was detected as an internal reference and quantification was normalized to β-actin expression. (C) DKK1 and Nmi protein expression levels as detected by western blotting. Data are expressed as the mean ± standard error of the mean. *P<0.05 and **P<0.01 vs. siControl. siN-Myc, v-myc avial myelocytomatosis viral oncogene neuroblastoma derived homolog-specific small interfering RNA; Nmi, N-myc and STAT interactor; DKK1, dickkopf Wnt signaling pathway inhibitor 1; siControl, control small interfering RNA.

neuroblastoma cell lines, potentially through the induction of apoptosis.

To explore the molecular mechanisms underlying the function of N-Myc in neuroblastoma cells, the present study detected apoptosis-associated protein (Bcl-2, Bax, cleaved-caspase-3 and cleaved-PARP) expression levels by western blotting. The anti-apoptosis protein Bcl-2 and the pro-apoptosis protein Bax are members of the Bcl-2 protein family, and regulate mitochondrial permeability and apoptosis through the intrinsic pathway (37). N-Myc inhibition significantly decreased Bcl-2 protein expression levels and significantly increased Bax protein expression levels in CHP134 and BE-2C cells, and significantly elevated the Bax/Bcl-2 ratio, which is a standard used to measure the occurrence and level of apoptosis. Cleaved-caspase-3 is an activated form of caspase-3 that is an important mediator of cell apoptosis (38). All caspases require cleavage adjacent to aspartates to liberate one large and one small subunit, which associate into an a2b2 tetramer to form the active enzyme. Caspase-3 cleaves PARP to create the specific 85 kDa form observed during apoptosis. In the present study, inhibition of N-Myc increased cleaved-caspase-3 and cleaved-PARP protein expression levels, which was consistent with the promotion of apoptosis.

Wnt/β-catenin signaling has previously been demonstrated to regulate cellular proliferation and apoptosis during embryonic development, and is involved in advanced disease stages of several human cancers, including liver, colon and brain cancer (4,5,39). It has previously been reported that the Wnt/β-catenin signaling is involved in neuroblastoma proliferation (40). β-catenin stability may reflect the activity levels of this pathway. In the present study, *MYCN* silencing induced a decrease of β-catenin protein levels in CHP134 and BE-2C cells, suggesting that N-Myc inhibition contributes to the degradation of β-catenin. The present study also demonstrated that protein expression levels of cyclin D1 and c-Myc, the downstream target proteins of β-catenin, were reduced following *MYCN* silencing, indicating that the Wnt/β-catenin pathway was inhibited. β-catenin has been demonstrated to bind T-cell factor (TCF) to stimulate cellular growth and proliferation

in tumorigenesis by triggering the cell-cycle regulator cyclin D1 (41), and furthermore, c-Myc is involved in neuroblastoma prognosis (8). In addition, N-Myc has previously been demonstrated to mediate Wnt signaling functions as the downstream target. Wnt signaling has been demonstrated to promote neuronal fate commitment and the proliferation of neural precursor cells via N-Myc (19). Wnt/β-catenin signaling is a key upstream regulator of N-Myc and modulates proximal-distal patterning in the lung, in part, through the interaction of β-catenin and lymphoid enhancer binding factor/TCF transcription factors to activate the promoter of *MYCN* (42). However, the present study revealed that N-Myc was an upstream modulator of Wnt signaling. These results appear inconsistent but they were not contradictory, which presents the cross-talk among intracellular signal pathways. Additionally, *MYCN* silencing was demonstrated to upregulate DKK1 protein expression levels, revealing the potential mechanism underlying *MYCN* silencing-induced Wnt signaling downregulation. DKK1 has previously been reported to be a secreted protein that interacts with the Wnt receptor LDL receptor related protein (LRP)5/6, resulting in the rapid removal of the receptor through transmembrane protein Kremen 1/2-mediated endocytosis, followed by blocking of Wnt signaling (43). However, there are several mechanisms involved in the modulation of N-Myc on the Wnt/β-catenin pathway. N-Myc downstream regulated gene 1 (NDRG1) has been observed to modulate Wnt/β-catenin signaling via interaction with the Wnt receptor LRP6, and pleiotropically suppresses metastasis in prostate and breast cancers (44). Nmi has also been previously demonstrated to inhibit Wnt/β-catenin signaling by upregulating DKK1 expression in MDA-MB-231 breast cancer and MDA-MB-435 melanoma cells, and retard tumor growth (45). In the present study, Nmi protein expression levels were demonstrated to increase in *MYCN*-silenced cells. The interaction of Nmi with N-Myc has also been observed in neuroblastoma cells (46). Therefore, *MYCN* silencing may inhibit Wnt signaling through Nmi-mediated DKK1 upregulation in neuroblastoma cells. However, the effect of NDRG1 or other molecules on the modulation of N-Myc on Wnt signaling in neuroblastoma remains to be established.

In conclusion, these data indicated that N-Myc downregulation suppressed cell viability and promoted apoptosis in CHP134 and BE-2C neuroblastoma cells, potentially via the Wnt/β-catenin signaling pathway. However, these results require further validation *in vivo* and *in vitro* to improve understanding of the mechanism and the potential relevance of N-Myc for neuroblastoma therapy. The present study lays a theoretical foundation for novel, targeted neuroblastoma therapies.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JL and SG made substantial contributions to the conception and design of the present study. YW, WW and YX made substantial contributions of data analysis and performed the experiments. YW and JL were major contributors in drafting the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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