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MYCN Silencing Induces Differentiation and Apoptosis in Human Neuroblastoma Cells

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Abstract

MYCN amplification strongly correlates with unfavorable outcomes in patients with neuroblastoma. The aim of this study was to investigate the role of *MYCN* in neuroblastoma cell differentiation and apoptosis. We used the technique of RNA interference to inhibit *MYCN* gene expression in neuroblastoma cells with variable expression of *MYCN*. Our results showed that inhibition of *MYCN* gene expression in *MYCN* amplified cells induced apoptosis and suppressed cell growth; neuronal differentiation also occurred after *MYCN* gene silencing. Moreover, N-myc downregulation was associated with decreased Bcl-xL protein levels and caspase-3 activation. These data show that small interfering RNA directed to *MYCN*, which plays a crucial role in neuroblastoma cell survival, may provide a potential novel therapeutic option for aggressive neuroblastomas.

Keywords

MYCN/N-myc; RNAi/siRNA; neuroblastoma; differentiation; apoptosis

Introduction

Neuroblastoma is the most common extracranial solid tumor of childhood and accounts for 15% of cancer-related deaths [1]. Despite recent advances in treatment options, aggressive neuroblastomas remain refractory to current therapy; the overall 5-year survival rate for patients with advanced-stage neuroblastoma is dismal at 30-40%. *MYCN* protooncogene amplification, occurring in up to 25% of neuroblastomas, has been considered the most important prognostic factor, strongly correlating to advanced-stage disease and treatment failure. Targeted overexpression of *MYCN* in transgenic mice results in the spontaneous development of neuroblastomas [2].

Identification of selective inhibitors of N-myc would be important for the development of therapeutic agents for neuroblastomas with *MYCN* amplification. Previously, antisense inhibition of *MYCN* expression in vitro was shown to decrease neuroblastoma proliferation and promote neuronal differentiation [3]. Inhibition has been accomplished either by antisense oligonucleotides targeted to N-myc mRNA or by expression vectors designed to generate N-

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myc antisense RNA [4]. However, a major clinical limitation of conventional antisense oligonucleotides is that they are rapidly degraded by nucleases. Recently, RNA interference (RNAi) to knockdown gene expression has gained significant interest as a potential novel agent for cancer therapy. RNAi silences gene expression through short interfering 21-23-mer double-strand RNA segments that guide mRNA degradation in a sequence-specific fashion [5].

Here, we report targeted inhibition of *MYCN* transcription by RNAi and demonstrate its differential effect in *MYCN* amplified and non-amplified human neuroblastoma cell lines. Selective and specific inhibitory effects on *MYCN* transcription induced growth arrest and apoptosis, which correlated with the level of N-myc expression. Thus, RNAi-mediated post-transcriptional silencing offers a potentially powerful tool to silence *MYCN* gene expression and may provide novel adjuvant treatment of selected neuroblastomas.

Materials and Methods

Materials

N-myc antibody was purchased from EMD Biosciences (San Diego, CA). Anti-Bcl-xL, caspase-3 and cleaved caspase-3 antibodies and cell lysis buffer were obtained from Cell Signaling Technology (Beverly, MA). Anti-neuron specific enolase (NSE) was obtained from Abcam (Cambridge, MA). Anti β-actin monoclonal antibody and fetal bovine serum were from Sigma (St. Louis, MO). NuPAGE Novex 4% to 12% Bis-Tris Gel and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). Horseradish Peroxidase (HRP)-conjugated secondary antibodies against mouse and rabbit IgG were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Cell Death Detection ELISA^{Plus} was purchased from Roche Applied Science (Indianapolis, IN).

Cell culture

Human neuroblastoma cell lines, SK-N-SH, SH-SY5Y, IMR-32 and BE(2)-C were purchased from American Type Culture Collection (Manassas, VA). JF, a primary neuroblastoma cell line, was a gift from Dr. Jason M. Shohet (Baylor College of Medicine, Houston, TX) and LAN-1 was a gift from Dr. Robert C. Seeger (University of Southern California, Los Angeles, CA). Cells were maintained in RPMI 1640 medium with L-glutamine (Cellgro Mediatech, Inc. Herndon, VA) supplemented with 10% FBS. The cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Small interfering (si) RNA transfection

siRNA against *MYCN* (si*MYCN*) and non-targeting control (siNTC) was purchased from Dharmacon, Inc (Lafayette, CO). Cell culture transient transfection was carried out with Lipofectamine 2000 transfection reagent according to the manufacturer's protocol. Cells were seeded on 6-well plates for RNA or protein preparation and 96-well plates for DNA fragmentation or cell growth assays. After 24 h incubation, media was replaced to serum-free RPMI 1640 containing siRNA (150nM) and transfection reagent. Cells were harvested for assays daily for three consecutive days after transfection with the siRNA duplexes. The experiments were repeated on at least 3 separate occasions.

RNA isolation and real time RT-PCR

The total cellular RNA extraction was carried out using RNAqueous kit (Ambion, Inc., Austin, TX) according to manufacturer's instructions. Applied Biosystems assays-on-demand 20× assay mix of primers and TaqMan MGB probes (FAM™ dye-labeled) for target gene, human *MYCN* (NCBI accession no. NM 005378 [Genbank]), and pre-developed 18S rRNA (VIC™-dye labeled probe) TaqMan® assay reagent (P/N 4319413E) for endogenous control were

utilized. The probe sequences of human *MYCN* were ACCCTGAGCGATTCAGATGATGAAG. Singleplex one-step reverse transcription (RT)-PCR was performed with 80ng RNA for both target gene and endogenous control. The reagent used was TaqMan one step RT-PCR master mix.reagent kit (P/N 4309169). The cycling parameters for one-step RT-PCR were as follows: reverse transcription 48° C for 30 min, AmpliTaq activation 95°C for 10min, denaturation 95°C for 15 sec and annealing/extension 60° C for 1 min (repeat 40 times) on ABI7000. Duplicate C_T values were analyzed in Microsoft Excel using the comparative C_T ($\Delta\Delta C_T$) method as described by the manufacturer (Applied Biosystems). The amount of target ($2^{-\Delta\Delta CT}$) was obtained by normalized to endogenous reference (18s) and relative to a calibrator (one of the experimental samples).

Western blot analysis

Whole-cell lysates were prepared using cell lysis buffer with 1mM PMSF and incubated on ice for 30-60 min. Total protein (50 μg/lane) was resolved on NuPAGE Novex 4-12% Bis-Tris gels and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA). Nonspecific binding sites were blocked with 5% milk in TBST (120 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature or overnight at 4°C. Target proteins were detected by using rabbit or mouse anti-human antibodies (1:500 -1,000 dilution) for 3 h at room temperature or overnight at 4°C. The membranes were washed three times and incubated with secondary antibodies (1:5,000 dilution) conjugated with horseradish peroxidase. Immune complexes were visualized using the enhanced chemiluminescence (ECL) system (Amersham Biosciences, Arlington, IL). Equal loading and transfer were confirmed by blotting the same membrane with β-actin antibody (1:5,000 dilution). Data are representative of three independent experiments with nearly identical results.

DNA fragmentation assay

Apoptosis was measured using a DNA fragmentation assay as previously described [6]. Briefly, cells (100 μl; 5-10 $\times 10^3$ cells/well) were plated in triplicate 24 h before transfection. Cells were then treated with control siRNA or *MYCN* smartpool siRNA for 48 and 72 h. Cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) were detected using a Cell Death Detection ELISA ^{plus} kit according to manufacturer's recommended protocol. The experiments were repeated on at least 3 separate occasions.

Cell proliferation assay

Cells were seeded in 96-well plates at a density of 5-10 $\times 10^3$ cells/well in RPMI 1640 culture medium with 10% FBS and grown for up to 3 days after transfection. Cell numbers were assessed by using Cell-Counting Kit-8 (Dojindo Molecular Technologies, Inc., Gaithersburg, MD) daily. Each assay point was performed in triplicate, and the experiment was repeated 3 times for each cell line. The values, corresponding to the number of viable cells, were read at OD450 with EL808 Ultra Microplate Reader (Bio Tek Instrument, Inc., Winooski, VT).

Morphological analysis

MYCN-amplified BE(2)-C, LAN-1 and IMR-32 cells were cultured for up to 48 h in 6-well plates in the presence of siNTC or si*MYCN* (150 nM). Microscopic assessment for cellular differentiation was performed with an ECLIPSE-100 (Nikon instruments Inc., Lewisville, TX). The experiment was repeated twice.

Statistical analysis

Scoring index, relative DNA fragmentation, cell growth and DNA synthesis were expressed as mean ± SEM; statistical analyses were performed using one-way analysis of variance for comparisons between the treatment groups. A P value of <0.05 was considered significant.

Results

MYCN transcript and N-myc protein depletion by *MYCN* siRNA

To examine the effect of *MYCN* siRNA on *MYCN* expression, we used *MYCN* amplified (LAN-1, IMR-32, JF) and *MYCN* non-amplified (SK-N-SH) human neuroblastoma cell lines. Immunoblotting demonstrated N-myc protein expression was significantly decreased in LAN-1, IMR-32 and JF cell lines. In contrast, no change was observed in *MYCN* non-amplified SK-N-SH cells (Fig. 1A). To examine whether the reduction of N-myc protein was linked to a modification in gene expression, cells were treated with siNTC or with si*MYCN* over a time course (8, 12, 24 h), and total cellular mRNA was extracted for analysis of *MYCN* transcripts by real-time RT-PCR. As shown in Fig. 1B, si*MYCN* resulted in significant *MYCN* mRNA reduction (40-60%) in LAN-1, IMR-32 and JF cell lines after 8 h treatment indicating that *MYCN* siRNA downregulates *MYCN* gene transcription. Comparable results were observed at 12 and 24 h post-treatment (data not shown). However, there was minimal to no *MYCN* mRNA expression noted in SK-N-SH cells.

MYCN silencing inhibits cell growth and induces apoptosis in *MYCN* amplified BE(2)-C cells

We then performed similar experiments with si*MYCN* treatment over a time course on BE(2)-C cells, typically the tumorigenic and aggressive *MYCN* amplified human neuroblastoma cell line [7]. Significant depletion of N-myc protein level was apparent after 8 h of treatment and continued over 72 h (Fig. 2A). This led to decreases in cell growth after 48 and 72 h (16% and 30%, respectively). Consistent with the cell viability results, cell proliferation as measured by BrdU nuclear incorporation was also decreased with si*MYCN* treatment (data not shown). Moreover, a remarkable increase in apoptosis was detected after si*MYCN* treatment for 48 and 72 h (up to 2.5 fold) as measured by levels of DNA fragmentation, a hallmark of apoptosis [8] (Fig. 2C).

si*MYCN*-mediated reduction of Bcl-xL and caspase-3 activation in BE(2)-C cells

Despite the important role of *MYCN* in cellular processes in human neuroblastoma cells, apoptosis target protein levels induced by *MYCN* downregulation have not yet been clearly described [3,9]. Anti-apoptotic Bcl-xL blocks the release of cytochrome c and aborts the apoptotic response [10]. Caspase-3 is one of the key executioners of apoptosis resulting in DNA fragmentation and cell death [11]. Here, we found that N-myc knockdown by siRNA against *MYCN* decreased anti-apoptotic Bcl-xL and induced cleavage of caspase-3 protein in *MYCN* amplified BE(2)-C cells (Fig. 2D).

MYCN siRNA induces apoptosis in *MYCN* amplified neuroblastoma cells

We next examined whether *MYCN* inhibition by si*MYCN* causes comparable apoptotic effects in other neuroblastoma cell lines with differential *MYCN* overexpression. LAN-1, IMR-32, JF, SH-5YSY and SK-N-SH cells were plated and treated with siNTC or si*MYCN* over a time course (24 h to 72 h). As shown in Fig. 3A, significant increases in apoptosis (30-72%) was observed at 48 h (IMR-32) or at 72 h (LAN-1 and JF) post-transfection in si*MYCN* treated groups when compared to siNTC groups as measured by DNA fragmentation. In contrast, there was no significant difference in apoptosis between siNTC treated cells and si*MYCN* treated cells in *MYCN* non-amplified SK-N-SH cells (Fig. 3A); this was further confirmed by Western

blot analysis of SK-N-SH and SH-5YSY, another *MYCN* non-amplified cell line, for caspase-3 cleavage products (Fig.3B).

MYCN silencing induces differentiation in *MYCN* amplified neuroblastoma cells

We have found that *MYCN* siRNA treatment results in induction of apoptosis in *MYCN* amplified neuroblastoma cells. Based on these findings, we next determined whether *MYCN* inhibition was also associated with cell differentiation. To assess the effects of si*MYCN* on differentiation of *MYCN* amplified neuroblastoma cells (BE(2)-C, LAN-1, IMR-32), morphological changes were evaluated by microscopic analysis performed after 48 h in the presence of siNTC or si*MYCN*. *MYCN* siRNA treated cells demonstrated an increase in neurite-like projections when compared to the control cells (Fig. 5A). We then selected IMR-32 cells, which showed the most significant cellular differentiation after si*MYCN* treatment, to determine expression changes of neuron-specific enolase (NSE), a neuronal marker of cellular differentiation. Consistent with the morphological analysis, immunoblotting demonstrated increased protein expression of NSE in si*MYCN* treated cells at 8 and 12 h post-transfection (Fig. 5B).

Discussion

In this study, we show that specific, selective inhibition of *MYCN* expression by siRNA leads to cell growth inhibition in *MYCN* amplified neuroblastoma cells; cell growth inhibition was strongly associated with increased apoptosis. In contrast, no significant effect was observed in *MYCN* non-amplified neuroblastoma cells. Moreover, *MYCN* inhibition induced neuroblastoma cell differentiation. These findings underscore the significance of *MYCN* amplification and suggest a potential of developing siRNA-based treatment strategy for targeted inhibition of *MYCN* transcription.

MYCN was the first amplified oncogene that was found to be of clinical significance due to its association with aggressive neuroblastoma phenotypes [12,13]. *MYCN* has been proven to be a critical gene for stimulation of neuroblastoma growth; therefore, considerable efforts have been directed to the design of novel molecular therapy targeted at *MYCN*. These efforts have largely met with limited success due to lack of specificity.

RNAi represents an innovative molecular tool for silencing genes at the post-transcriptional level [14]. Synthetic siRNA, introduced into post-mitotic primary neuronal cultures, can effectively reduce the expression of both endogenous and transfected genes [15]. siRNA-directed gene silencing activity has also been demonstrated in human cancer cells [16,17]. RNAi has been touted as potential targeted cancer therapy because of its impressive specificity and efficacy. In comparison to antisense approaches, siRNAs are approximately 1000-fold more active [18]. Recent reports have demonstrated effective *in vivo* delivery of siRNA [19, 20]. We found in this study that si*MYCN* can be delivered efficiently into neuroblastoma cells to produce significant knockdown of mRNA and its protein products.

Transfecting neuroblastoma cells with si*MYCN* produced specific attenuation of *MYCN* gene expression by 8 h which was followed by a decrease in N-myc protein product for up to 72 h. Synthetic 21 bp *MYCN* siRNAs were designed to prevent activation of the interferon (IFN) system (one of the body's defenses against viral infection) [21] as well as other off-target effects. It has been reported that high *MYCN* expression selectively induces S-phase reentry while protecting against apoptosis in postmitotic sympathetic neurons [22]. A comparison of cell growth of *MYCN* amplified BE(2)-C cells (Fig. 2B) and *MYCN* non-amplified SK-N-SH and SH-SY5Y (data not shown), suggest that our si*MYCN* inhibited the proliferation of cells that overexpress N-myc. These results are comparable to those achieved with oligodeoxyribonucleotide [3].

In this study, treatment with si*MYCN* markedly induced apoptosis in *MYCN* amplified neuroblastoma cell lines. Furthermore, we confirmed the induction of apoptosis by determining the reduction of expression of Bcl-xL, an anti-apoptotic member of the Bcl-2 family. Bcl-xL is localized at the mitochondria and in the cytosol; the cytosolic fraction of Bcl-xL is targeted to the mitochondria during induction of apoptosis [23]. In addition, si*MYCN* treatment resulted in an augmentation of caspase-3 cleavage in *MYCN* amplified BE(2)-C cells. In contrast, caspase-3 cleavage was not detected in *MYCN* non-amplified SK-N-SH and SH-SY5Y cell lines after si*MYCN* treatment, further suggesting that *MYCN* inhibition leads to activation of the apoptotic pathway in neuroblastoma cells.

Neuroblastoma cells can differentiate when exposed to various stimuli [24]. Our results show that *MYCN* silencing is an effective inducer of differentiation as noted by the neurite-like extensions (Fig. 4A); further confirmed by expression of neuronal differentiation marker, NSE (8 and 12 h after 150 nM treatment; Fig. 4B). These results are similar to studies by Galderisi et al [25] following antisense-oligodeoxyribonucleotide-inhibition of N-myc expression in human neuroblastoma cells.

In summary, small interfering RNA appears to be highly specific for amplified *MYCN*, resulting in promotion of apoptosis and differentiation. Compared with other antisense approaches, siRNAs offer a more active and effective treatment option because of resistance to degradation by nucleases and increased specificity [18,26]. Understanding the mechanisms underlying the effects of *MYCN* silencing on differentiation and apoptosis should be further explored to improve treatment responses in patients with *MYCN* overexpressing neuroblastomas.

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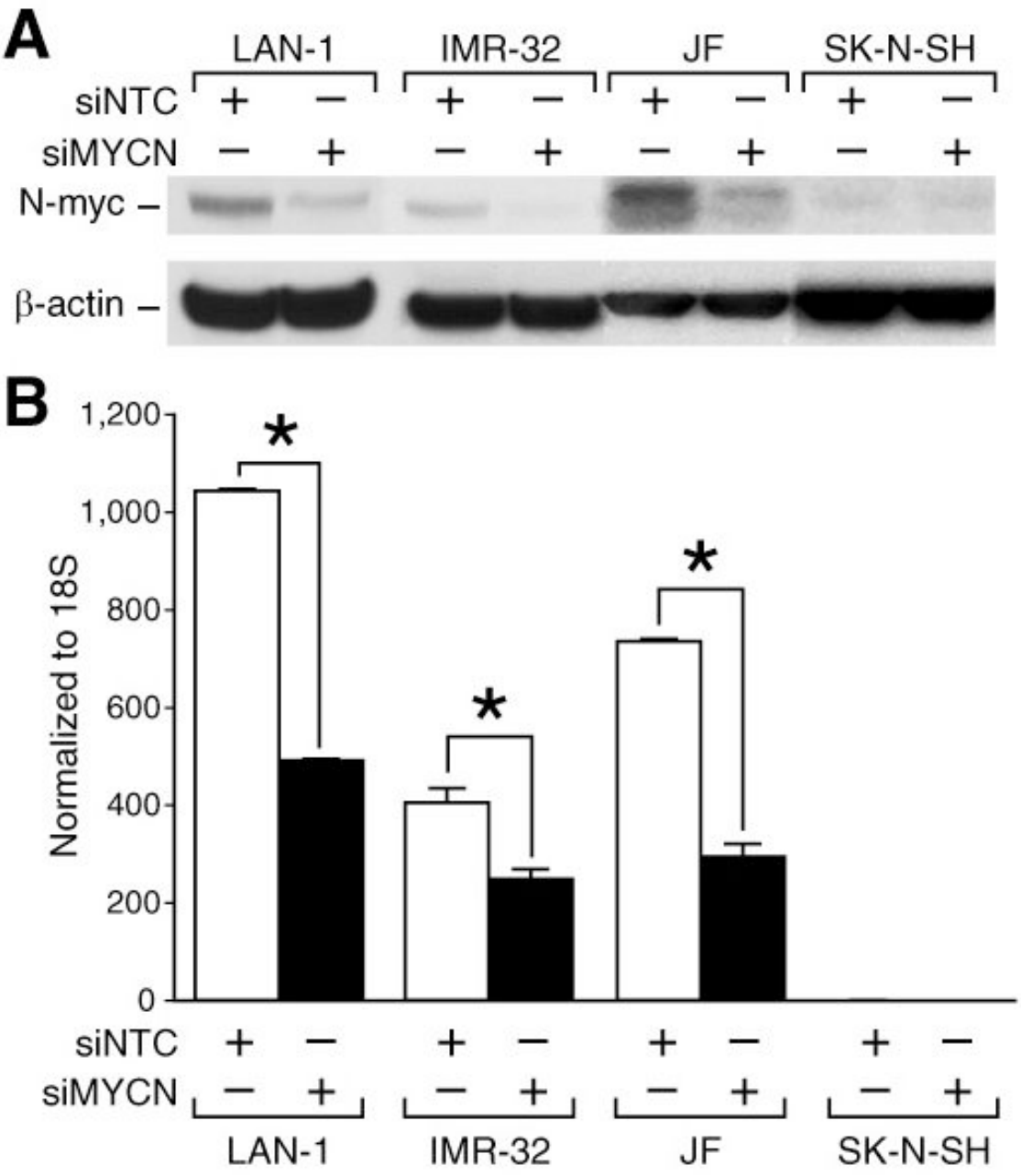

Figure 1. Knockdown of N-myc expression using small interfering RNA in *MYCN* amplified and non-amplified neuroblastoma cell lines

(A) Western blot analysis of N-myc protein expression in *MYCN* amplified cell lines; LAN-1, IMR-32, JF and *MYCN* non-amplified SK-N-SH cells at 24 h post-transfection with si*MYCN*. (B) Real time RT-PCR analysis of *MYCN* mRNA expression in LAN-1, IMR-32, JF and SK-N-SH at 6 h post-transfection.

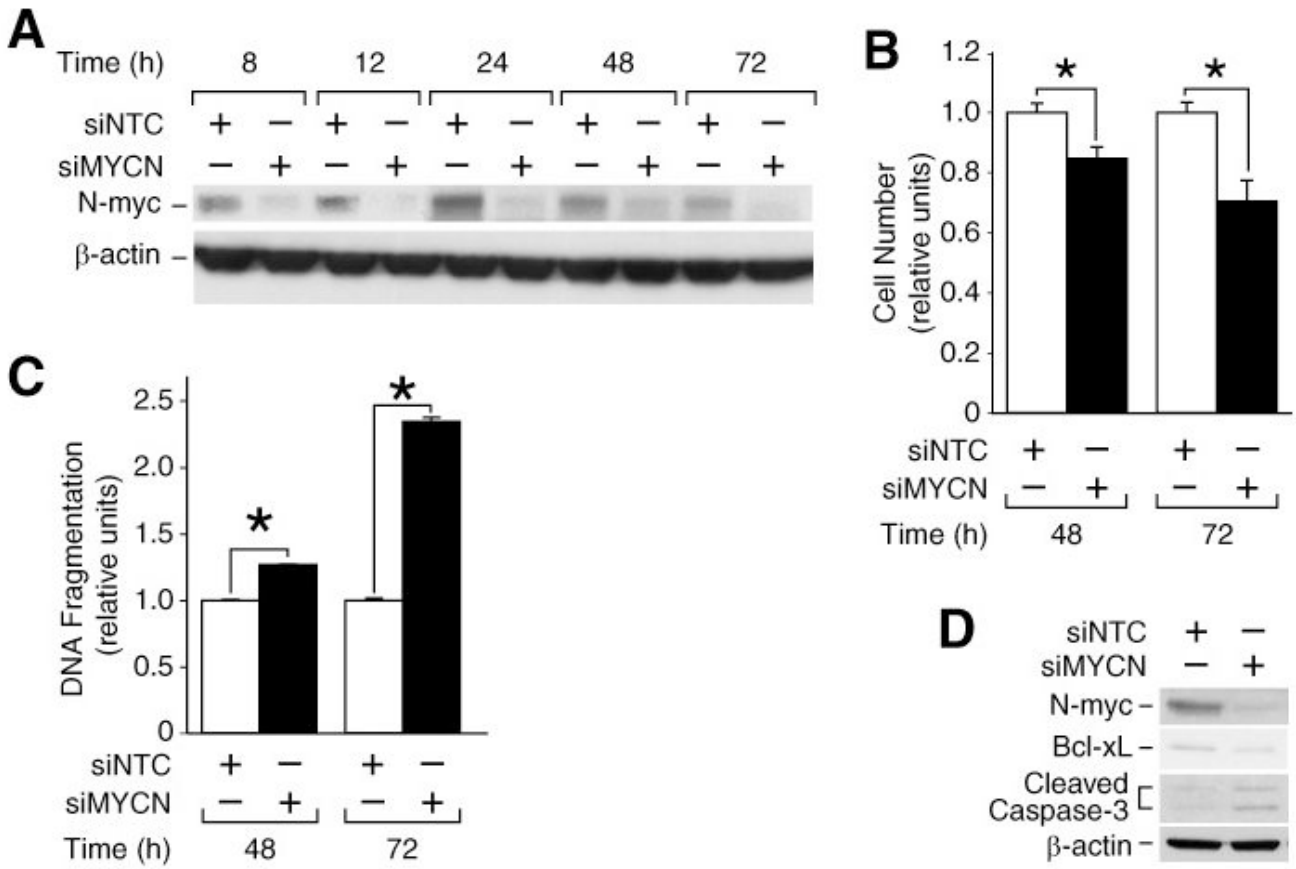

Figure 2. siRNA-mediated inhibition of N-myc expression and expression in aggressive neuroblastoma cell line BE(2)-C

(A) N-myc protein in BE(2)-C cells transfected with siRNA against *MYCN* or NTC at 8, 12, 24, 48 and 72 h. β-actin was used to check for equal protein loading of cell lysates. (B) WST-8 assay was used to compare the growth of si*MYCN* and siNTC transfected BE(2)-C cells. (C) DNA fragmentation was measured at 48, 72 h post-transfection with either si*MYCN* or siNTC. Data represent mean ± SEM. *P < 0.05. (D) BE(2)-C cells were transfected with siRNA against *MYCN* and protein levels for N-myc, Bcl-xL and cleaved caspase-3 were detected at 48 h post-transfection. β-actin was used to check for equal protein loading.

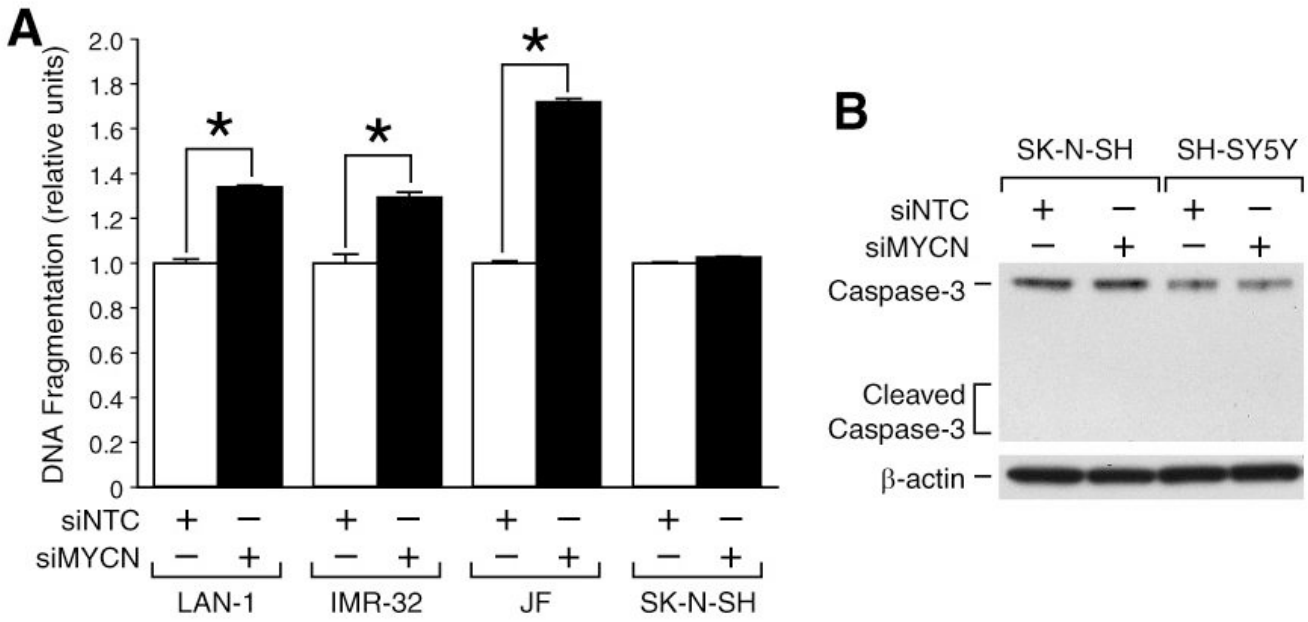

Figure 3. *MYCN* downregulation by RNAi induces apoptosis in *MYCN* amplified neuroblastoma cells

(A) Cells were transfected with si*MYCN* or siNTC and DNA fragmentation was analyzed at 48, 72 h post-transfection. Data represent mean ± SE. *P < 0.05. (B) SK-N-SH and SH-SY5Y cells were transfected with si*MYCN* and siNTC. After 72 h, the cells were analyzed by Western blot for caspase-3 and cleaved caspase-3. β-actin was used to check for equal protein loading.

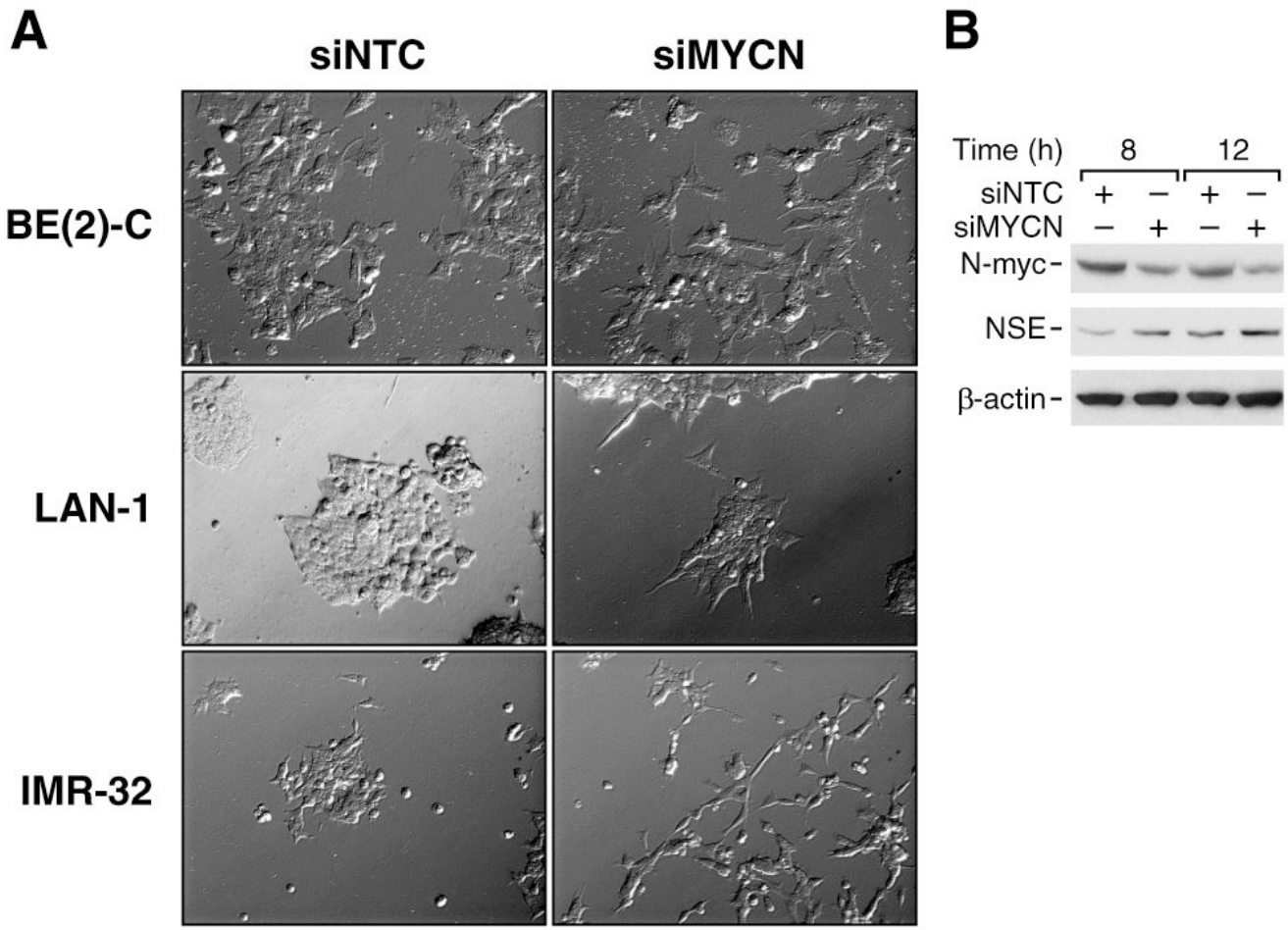

Figure 4. *MYCN* downregulation by RNAi induces differentiation in *MYCN* amplified neuroblastoma cells

(A) BE(2)-C, LAN-1 and IMR-32 cells were transfected with si*MYCN* or siNTC. Cell morphology was analyzed by microscopy with representative images shown. (B) IMR-32 cells were transfected with si*MYCN* or siNTC and the protein was analyzed by Western blot for N-myc and NSE at 8 and 12 h post-transfection. β-actin was used to check for equal protein loading.