Silencing of *MYCN* by RNA interference induces growth inhibition, apoptotic activity and cell differentiation in a neuroblastoma cell line with *MYCN* amplification

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Abstract. Although it has been suggested that the MYCN oncoprotein functions may influence tumorigenesis and patient survival in neuroblastoma, the mechanism of these functions remains unclear. To elucidate such molecular and biological mechanisms, we performed knock-down of *MYCN* expression using RNA interference (RNAi) method. *MYCN*-siRNAs (*MYCN*-siRNA) were transfected into the *MYCN*-amplified cell line NB-1. To verify the sequence specificity of the siRNA, we prepared three control groups (siRNA control group: siRNAs with no significant homology to any known sequences in human genome, mock control group: reagent and PBS, and the untransfected control group). The cells were analyzed by real-time RT-PCR, Western blotting, immunocytochemistry for gene expression. Cell proliferation activity was measured by WST-1 assay. TUNEL staining was performed to evaluate apoptosis. After the *MYCN*-siRNA transfection, the expression level of the *MYCN* mRNA was significantly reduced to 30% of those of the three control groups ($p<0.05$). Western blotting revealed an obvious reduction in MYCN protein level in the *MYCN*-siRNA group. On immunocytochemistry, intensity of nuclear staining of MYCN was weaker in the *MYCN*-siRNA group than in the three control groups. On WST-1 viability assay, cell proliferation after the *MYCN*-siRNA transfection was significantly suppressed compared to the three control groups ($p<0.05$). The TUNEL positive cells were frequently observed in the *MYCN*-siRNA group. Additionally, after the *MYCN*-siRNA transfection, the morphologic change which was suggestive of neuronal cell differentiation was observed and *TrkA* and *TrkC* expressions were also significantly up-regulated. Using RNAi method, the knock-down of *MYCN* expression induced growth-inhibition, apoptotic activity and cell differentiation in *MYCN*-amplified NB-1 cell line.

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Introduction

Neuroblastoma (NB), a malignant neoplasm of neural crest origin, is the most common solid extracranial tumor in children and is responsible for 15% of pediatric cancer deaths (1-3). The advent of combination of surgery, chemotherapy and radiation therapy, in addition to high dose chemotherapy with stem cell rescue has made significant improvement in terms of survival rates for advanced NBs. However, the prognosis of the advanced NBs, especially tumors with *MYCN* amplification, remains poor (4,5).

MYCN is one of *MYC* family members which are transcription factors that contain to a transcriptional activation domain and a transcriptional regulation domain (6,7). While *MYCN* expression is limited to early stages of embryonic development, the *MYC* gene is expressed in a wide variety of tissues. *MYCN* is normally located on the distal short arm of chromosome 2, but in cells with *MYCN* amplification it also maps to the double minutes or homogeneously staining regions (8). A large region from chromosome 2p24 (including the *MYCN* locus) becomes amplified, presumably because it provides some selective advantage to the cells (7).

In clinical studies *MYCN* amplification has been correlated with advanced stages of disease and rapid tumor progression (9-11). It is generally accepted that amplification of the *MYCN* oncogene is more relevant to prognosis than other prognostic factors such as chromosome 1p deletion, diploid DNA content and *TrkA* expression. In general, there is a correlation between *MYCN* copy number and expression (11,12). Furthermore, a couple of reports have suggested an association between *MYCN* overexpression and patients' prognosis (12). However, it is still controversial whether or not overexpression of *MYCN* mRNA or MYCN protein has prognostic significance in tumors lacking *MYCN* amplification (13-15).

In an experimental model, it was reported that transgenic mice with overexpression of *MYCN* developed NBs (16). Moreover, Manohar *et al* have shown direct evidence that *MYCN* induction in human NB cells resulted in increased *MRP1* mRNA and protein levels, which in turn was

accompanied by increased drug resistance and enhanced MRP1-mediated drug efflux (17). These studies provide evidence, suggesting that *MYCN* overexpression in NB is a possible biochemical pathway that contributes to the malignant behavior.

A recent discovery of RNA interference (RNAi), as a highly efficient method for gene knock-down, has been one of the major breakthroughs in molecular medicine (18,19). RNAi provides a new reliable method to investigate gene function that has many advantages over other nucleic-acid-based approaches such as antisense oligonucleotides, and which is therefore currently the most widely used gene-silencing technique in functional genomics. The previous extensive research on the development of therapeutic antisense nucleic acids should facilitate development of therapeutic siRNAs (20). Although several recent studies have demonstrated high efficiency and versatility of RNAi in cell cultures, the knock-down of *MYCN* in amplified NB cell line with RNAi has not been reported yet.

In this study, in order to elucidate the role of *MYCN* molecular and biological mechanisms, we transfected artificially synthesized siRNAs that were designed to target the *MYCN* gene by adopting lipofection method as a siRNA delivery system.

Materials and methods

Cell lines and culture condition. *MYCN*-amplified human NB cell line NB-1 was obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan). NB-1 cells were propagated and maintained in RPMI-1640 medium (Nacalai tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (MP Biomedical, Inc., Eschwege, Germany) and anti-biotic-anti-mycotic solution (Nacalai tesque), and cultured in a 37˚C humidified atmosphere containing 5% CO_2.

SiRNA oligonucleotides. A cocktail of three siRNA oligo-nucleotides targeting human *MYCN* with two thymidine residues (dTdT) at the 3'-end of the sequence was purchased from B-Bridge International Inc. (Sunnyvale, CA). These siRNA oligonucleotides corresponded to nucleotides 536-554, 1526-1544, and 1654-1672 of the human *MYCN* gene (GeneBank Access no. NM 005378). The sequences are as follows: si*MYCN*-1 (sense 5'-CGGAGATGCTGCTT GAGAA-3'), si*MYCN*-2 (sense 5'-CGGAGTTGGTAAAGA ATGA-3'), si*MYCN*-3 (sense 5'-CAGCAGTTGCTAAAGA AAA-3').

Each siRNA oligonucleotide included in the cocktail was separately available and used for preliminary experiments. To verify sequence specific effectiveness of the *MYCN*-siRNAs, we also used negative control siRNAs (NC-siRNA, B-Bridge International Inc.) that have no significant homology with any known sequences in the human genome.

Transfection. Transient transfection of siRNA was carried out using a commercially available transfection reagent (HiPerFect, Qiagen Inc., Valencia, CA), according to the instruction manual. Transfections were performed with a final concentration of 25 or 50 nM of siRNA in serum-free culture media. In this step, we adopted a reverse transfection method, in which cell seeding and transfection were performed simultaneously by adding the mixture of siRNA and the reagent onto the cells as soon as seeding the cells on the plates (21). Transfection efficiency of this transfection condition was estimated by green fluorescent protein (GFP) signals derived from a sham transfection of pEGFP-N1 Vector (Clontech Laboratories, Palo Alto, CA) to the NB-1 cell. Approximately 30% of the cells were fluorescent. To study the specific effect of *MYCN* silencing, we prepared the following four groups including several types of control: group 1 (*MYCN*-siRNA group), transfected siRNA against human MYCN; group 2 (NC-siRNA group), transfected negative control siRNA; group 3 (mock control group), the cells were treated with the reagent and PBS without any siRNAs to verify the influence of the transfection reagent; group 4 (no treatment group), the cells received no treatment.

RNA and cDNA preparation. The reverse transfection treatment was applied to $2.5x10^5$ NB1 cells suspended in 2 ml medium in each well of a 6-well plate. At a later time indicated below, the cells were harvested for RNA extraction and cDNA synthesis. Total cellular RNA of each group was prepared by RNAqueous RNA isolation kit (Ambion, Austin, TX). First-strand cDNA was synthesized from 1 μg of total RNA using MMLV Reverse Transcriptase (Clontech Laboratories) and oligo(dT) primers.

Gene expression assays by real-time RT-PCR. To quantitate the level of mRNA of the *MYCN* and its relating genes Ha-*ras*, *TrkA, TrkB*, and *TrkC* that are associated with differentiation and prognosis of NB, real-time RT-PCR was performed on an ABI PRISM 7700 sequence detection system using Sequence Detector V1.7 software (PE Applied Biosystems Inc., San Jose, CA) (22). Human *GAPDH* was used as an internal control. Primers and TaqMan probes for *MYCN*, Ha-*ras*, *TrkA, TrkB, TrkC* and *GAPDH* were indicated in Table I. The relative expression levels of *MYCN*, Ha-*ras*, *TrkA, TrkB,* and *TrkC* mRNAs were standardized by that of *GAPDH*, and compared to that of no treatment control. *MYCN* mRNA expression was firstly evaluated at post-transfection 48 h, then, time-course of the expression for up to 6 days after transfection was independently investigated. Expressions of the Ha-*ras*, *TrkA, TrkB,* and *TrkC* mRNAs were evaluated at 48 h.

Western blotting. Ninety-six hours after siRNA transfection on $1.5x10^5$ NB1 cells under the similar conditions as the gene expression assay, cells of each group were harvested to quantitate the MYCN protein level using Western blotting (23). Briefly, harvested cells were lysed in RIPA lysis buffer (Upstate, Lake Placid, NY) and protein amounts were measured by BCA protein assay set (Pierce Biotechnology, Inc., Rockford, IL). Then, 25 μg of protein was loaded on a 10% sodium dodecyl sulfate-polyacrylamide gel (Criterion™ XT Precast Gel, Bio-Rad, Hercules, CA) for electrophoresis, and subsequently transferred onto a polyvinyl difluoride membrane. The membrane was soaked in a solvent (Can Get Signal, Toyobo, Osaka, Japan) including anti-MYCN monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a dilution of 2.0 μg/ml, and incubated for 24 h at 4˚C. The membrane was then incubated with a horseradish

Table I. Primers used in real-time quantitative PCR reactions.

Gene		Sequences (5'→3')
MYCN	Forward	GACCACAAGGCCCTCAGTACC
	Reverse	TGACCACGTCGATTTCTTCCT
	TaqMan probe	FAM-CCGGAGAGGACACCCTGAGCGA-TAMRA
GAPDH[a]	Forward	GAAGGTGAAGGTCGGAGTCA
	Reverse	GAAGATGGTGATGGGATTTC
	TaqMan probe	FAM-CAAGCTTCCCGTTCTCAGCC-TAMRA
TrkA	Forward	TTCACCTACGGCAAGCAGC
	Reverse	CCTGCGTGATGCAGTCGAT
	TaqMan probe	FAM-TGGTACCAGCTCTCCAACACGGAGG-TAMRA
TrkB	Forward	GTCTTTGAGTACATGAAGCATGGG
	Reverse	TCAGCACGGCATCAGGG
	TaqMan probe	FAM-ACCTCAACAAGTTCCTCAGGGCACACG-TAMRA
TrkC	Forward	CAAATATGGTCGACGGTCCAA
	Reverse	GAGTCCTCCTCACCACTGATGAC
	TaqMan probe	FAM-TTTGGAATGAAGGGTCCCGTGGC-TAMRA
Ha-*ras*	Forward	CCAGAACCATTTTGTGGACGA
	Reverse	CCCATCAATGACCACCTGC
	TaqMan probe	FAM-CGACCCCACTATAGAGGATTCCTACCGGA-TAMRA

[a]Human glutaraldehydes-3-phosphate dehydrogenase.

peroxidase conjugated anti-mouse antibody for 1 h and developed with an enhanced chemiluminescence system (Amersham Biosciences Inc., Piscataway, NJ).

Immunocytochemistry. For each group, $3.0x10^4$ NB-1 cells were treated and maintained in 0.5 ml medium per chamber on the four-chamber culture slides (BD Falcon™, BD Biosciences, San Jose, CA). At post-transfection 96 h, the cells were fixed in 4% paraformaldehyde, rinsed in PBS, and permeabilized with 1% Triton X-100 for 1 h. Then, they were incubated with anti-MYCN antibody (2.0 μg/ml) at 4˚C overnight after a blockage step of 30 min performed in 10% normal rabbit serum. For visualization, FITC-conjugated rabbit anti-mouse immunoglobulins (6 mg/ml; Dako, Tokyo, Japan) were used as the second antibody, and nuclear staining was done in PBS. The slides were washed and mounted with fluorescence mounting medium (Dako) to be examined with photographs taken by Keyence VB6000 digital photography system (Keyence, Osaka, Japan) attached to Nikon Eclipse C1000 microscope (Nikon, Tokyo, Japan).

Cell viability assay and time-course evaluation. Cell viability was determined by WST-1 assay utilizing a colorimetric detection of mitochondrial dehydrogenase in viable cells (24). NB-1 cells were seeded at a density of $1x10^4$ cells in 100 μl of medium into each well of 96-well plates and maintained without medium change for up to 9 days after transfection. From post-transfection day 1, four wells were devoted for the assay everyday, and time-course of cell viability was monitored. In practice, 10 μl of WST-1 solution (Cell Count Reagent SF, Nacalai tesque) was added to each well, and samples were incubated at 37˚C for 2 h. Then, the absorbency of the treated samples against a blank control was measured by an immunoreader apparatus (Immuno Mini NJ-2300, Nippon InterMed, Tokyo, Japan) under 414 nm as a detection wavelength and 630 nm as a reference wavelength, respectively.

Detection of apoptosis. To further assess influence of *MYCN*-siRNA on the cell survival, apoptotic features of NB-1 cells were evaluated. This was performed semi-quantitatively by using the TUNEL principle (ApopTag Plus Fluorescein *In Situ* Apoptosis Detection Kit, Serologicals Corp., Norcross, GA). Cells were similarly seeded and transfected on the culture slides as the immunocytochemistry study. At post-transfection 96 h, the cells on the slides were fixed and subjected to the assay, according to the manufacturer's instructions. Apoptotic cells were observed and counted under a fluorescence microscope. Moreover, we evaluated the percentage of positive apoptosis cells in each group (25).

Morphologic change evaluation. To evaluate morphologic changes of the NB-1 cells induced by siRNA treatments, each group cells were stained with Phalloidin-Tetramethylrhodamine-B-isothiocyanate (Sigma-Aldrich Corp., St. Louis,

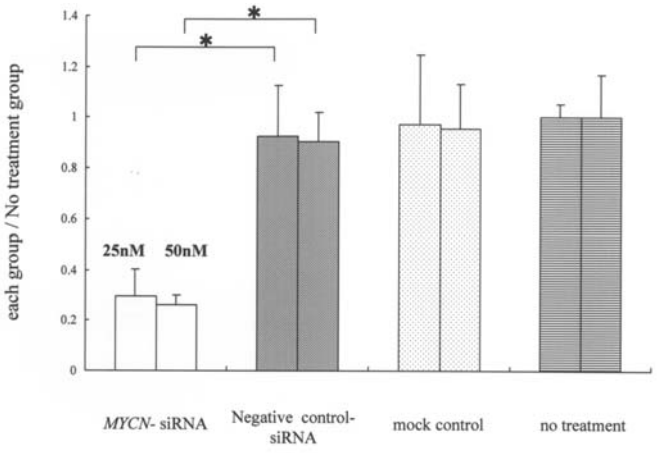

Figure 1. Assessment of relative mRNA expression of *MYCN* by real-time RT-PCR. The expression level of *MYCN* mRNA of the no treatment group defined as 1. The expression level of *MYCN*-siRNA group was significantly reduced to 30% of those of three control groups in NB-1 cell line. No significant change in the NC-siRNA group and mock control group was found compared to the no treatment group. Different *MYCN*-siRNA concentrations (25 and 50 nM) brought similar suppressive effect on the *MYCN* mRNA expression level (*p-value <0.05).

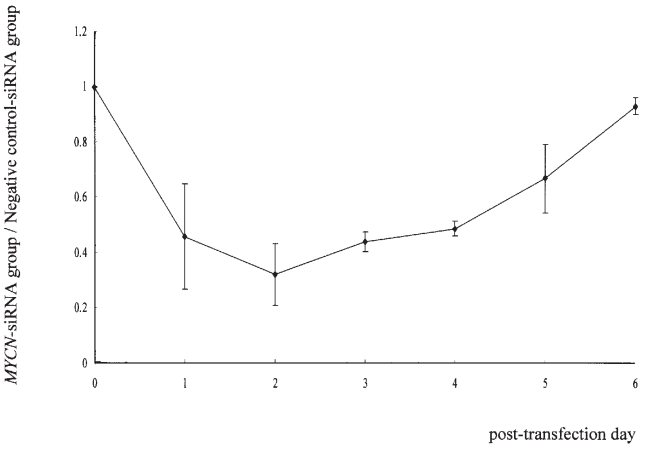

Figure 2. Time-course curve of *MYCN* mRNA expression level after *MYCN*-siRNA treatment. The expression level of the siRNA treated cells relative to that of Negative control-siRNA group on each day was calculated and plotted. Note that the maximum suppression occurred around post-transfection day 2.

MO) at 96 h. This staining highlighted cellular filamentous actin and differentiation appearance of the neuronal cells could be observed under a fluorescence microscope (26).

Statistical analysis. All experiments were performed at least three times and typical results were demonstrated. Data are presented as means together with standard deviation for each parameter. The statistical analysis was performed by an unpaired Student's t-test, and a p-value <0.05 was considered statistically significant.

Results

Effect of siRNA on MYCN mRNA expression. Forty-eight hours after *MYCN*-siRNA transfection, the expression level of *MYCN* mRNA significantly decreased to approximately 30% of group 4 ($p<0.05$) (Fig. 1), whereas the levels observed in groups 2 and 3 were similar to group 4. In these initial experiments, we separately treated the NB-1 cells with different *MYCN*-siRNA concentrations (25 and 50 nM), and found that both concentrations brought similar suppressive effect on the *MYCN* mRNA expression level (Fig. 1). When each of the three *MYCN*-siRNAs (si-*MYCN*-1-3) included in the cocktail was separately used for transfection at 25 nM concentration, we also found that *MYCN* mRNA expression was similarly reduced (data not shown). Then, in our subsequent experiments, we solely used the 25 nM cocktail.

Time-course curve of relative expression of *MYCN* mRNA is shown in Fig. 2. It appeared that *MYCN* knock-down lasted for 6 days after transfection. The nadir level of reduction occurred around post-transfection days 2, and consistently reached 30% of group 2. On post-transfection day 6, the expression of *MYCN* recovered to the same level observed in group 2.

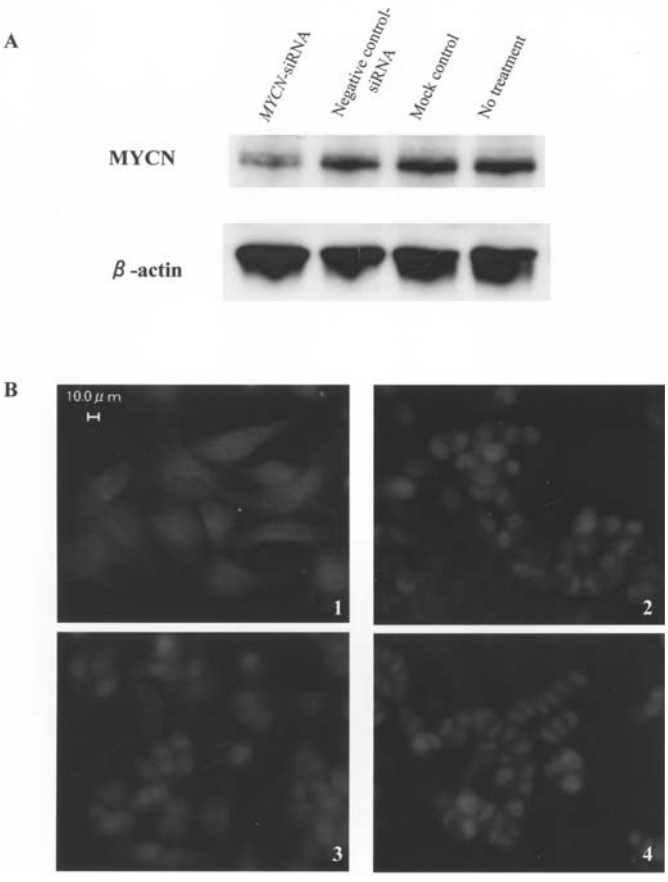

Figure 3. (A), Western blotting of MYCN and ß-actin. The study revealed reduction of cellular MYCN level in NB-1 cells treated by *MYCN*-siRNA. (B), Fluorescent immunocytochemical staining study demonstrating MYCN nuclear staining pattern in NB-1 cells. At 96 h of culture, nuclear staining of MYCN became very faint in the majority of the cells in the *MYCN*-siRNA group, whereas, those in other three control groups, almost all cells showed strong signals. Panel 1, *MYCN*-siRNA group. Panel 2, Negative control-siRNA group. Panel 3, Mock control group. Panel 4, No treatment group.

Western blotting for MYCN. At post-transfection 96 h, Western blot assay revealed a reduction of MYCN protein level in group 1 compared to the other groups. MYCN protein expression did not differ among groups 2, 3 and 4 (Fig. 3A).

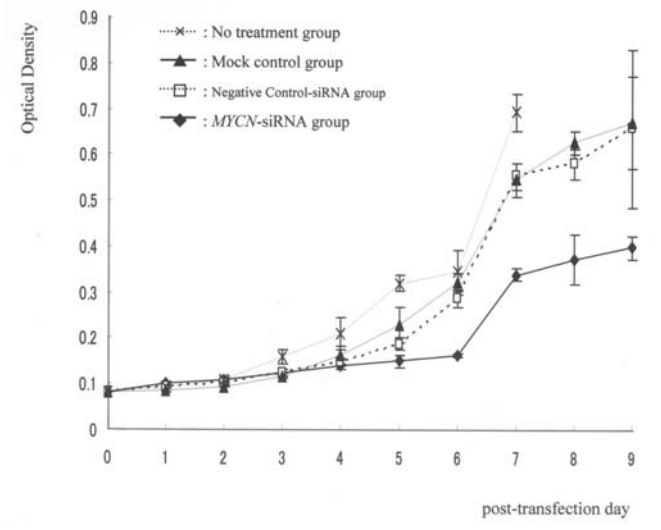

Figure 4. Time-course study assessed by WST-1 method demonstrated a significant reduction of NB-1 cell viability in the *MYCN*-siRNA group from day 5 (p-value <0.05). No significant change was found between the negative control-siRNA group and mock control group.

Immunocytochemistry for MYCN. Immunocytochemistry using anti-MYCN antibody showed intense immunoreactivity of nuclear staining groups 2, 3 and 4. In contrast, 96 h after siRNA treatment, nuclear staining of MYCN became very faint in the majority of the cells in group 1 (Fig. 3B).

RNAi suppressed NB-1 cell proliferation. In WST-1 assay, group 1 showed significantly reduced viable cell numbers, compared to groups 2 and 3. This significant suppression in cell proliferation became apparent on post-transfection day 5 and continued until day 9, when cells reached confluent growth in the NC-siRNA and mock groups and the time-course study was terminated ($p<0.05$) (Fig. 4). Cell proliferation modes of groups 2 and 3 were similar, but less propagated than that observed in group 4, probably reflecting some cytotoxic effect of the transfection reagent.

Apoptosis evaluation. Using *in situ* TUNEL assay, we identified significantly higher proportion of TUNEL positive cells in group 1 compared to the other groups ($p<0.0001$) (Fig. 5A). More than 50 cells per 100 were apoptotic in group 1, compared to 13 cells in group 2, and approximately 5 in groups 3 and 4 (Fig. 5B). These findings may indicate that siRNA treatment against MYCN activates an apoptotic process in NB-1 cells.

Morphological evaluation. Original shape of NB-1 is round. In group 1, the cells treated by *MYCN*-siRNA exhibited multidirectional neurite extension. Additionally, size of these cells and nuclei became apparently larger than those observed in the other cell groups (Fig. 6). These morphologic changes were consistent with neural differentiation.

Relative expression of Ha-ras, TrkA, TrkB and TrkC. At forty-eight hours after the siRNA treatment, relative expressions of *TrkA* and *TrkC* mRNA were significantly up-regulated in group 1 compared with other groups ($p<0.05$). The expression

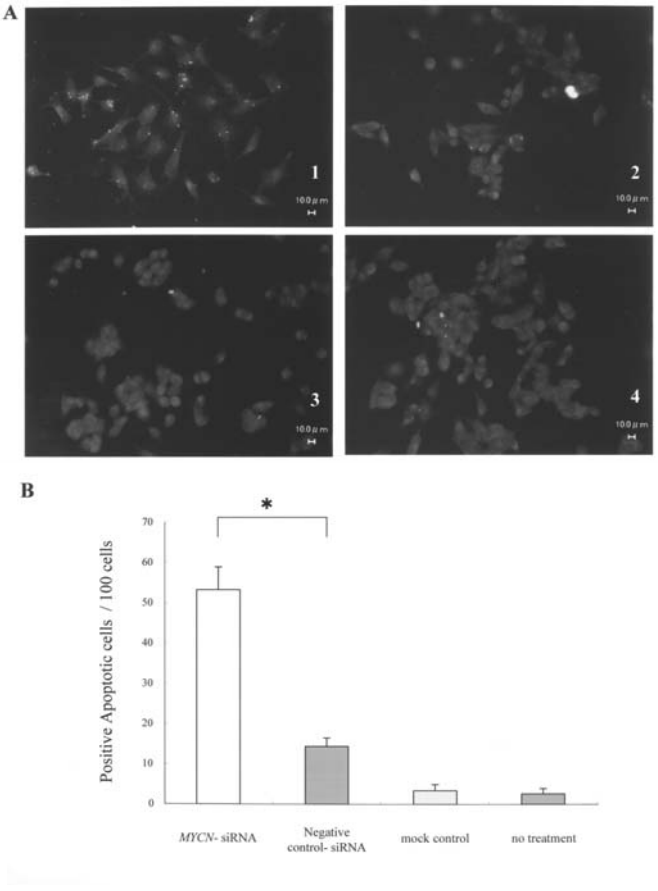

Figure 5. Detection of apoptosis. (A), Effect of *MYCN*-siRNA treatment on NB-1 culture at 96 h. The TUNEL positive cells were observed more frequently in the *MYCN*-siRNA group. Panel 1, *MYCN*-siRNA group. Panel 2, Negative control-siRNA group. Panel 3, Mock control group. Panel 4, No treatment group. (B), Apoptotic cell counting per 100 cells at 96 h. Cells with apoptotic bodies were significantly increased in the *MYCN*-siRNA group (*p-value <0.0001).

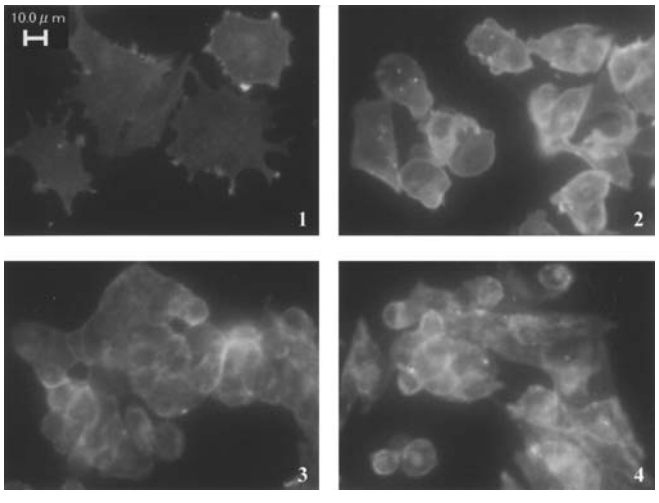

Figure 6. Effect of *MYCN*-siRNA treatment on NB-1 culture at 96 h. Phalloidin-TRTC staining of NB-1 exhibited multidirectional neurite extension in the *MYCN*-siRNA group. Additionally, sizes of the cells and the nucleus in this group became apparently larger than those observed in the other groups. These morphologic changes indicate differentiation effect of *MYCN*-siRNA treatment. Panel 1, *MYCN*-siRNA group. Panel 2, Negative control-siRNA group. Panel 3, Mock control group. Panel 4, No treatment group.

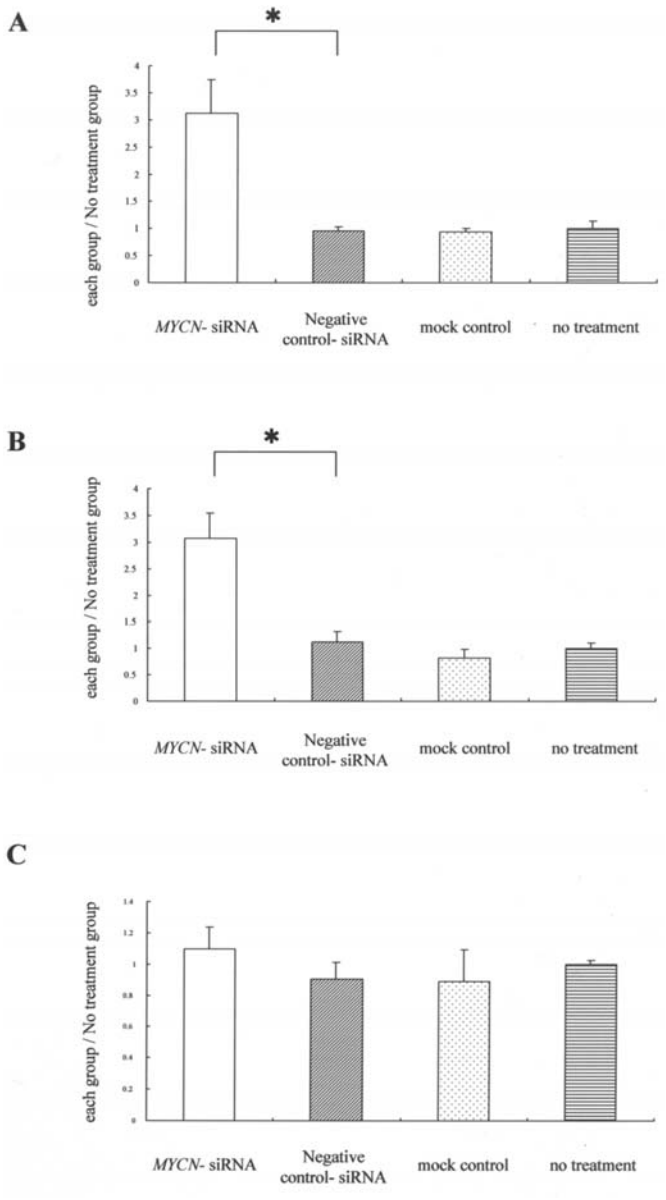

Figure 7. Assessment of relative mRNA expression levels each of *TrkA* (A), *TrkC* (B) and Ha-*ras* (C) by real-time RT-PCR. *TrkA* and *TrkC*, indicators of favorable prognosis, were significantly up-regulated. On the other hand, Ha-*ras* did not show significant change (*p-value <0.05).

of *TrkB* was not detected. The relative expression of Ha-*ras* did not change by the siRNA treatment (Fig. 7).

Discussion

In the present study, by using siRNA treatment, we conducted a knock-down of *MYCN* expression to examine its effect on NB-1 cell line in which the *MYCN* gene is amplified and overexpressed.

siRNA is a synthetic short double-stranded RNA that induces the destruction of homologous single-stranded RNA, when introduced into a cell. Therefore, siRNA has been evaluated as an effective tool for suppressing the target protein by specifically digesting its mRNA (18,27). In our experiment real-time RT-PCR and Western blotting definitely demonstrated that the treatment with siRNA targeted *MYCN* decreased *MYCN* mRNA expression as well as protein expression. Although it was previously reported that knock-down of *MYCN* in NB cells with antisense oligonucleotide against *MYCN* (28-30), siRNA is generally superior to antisense oligonucleotides in terms of efficiency and specificity (31).

Gene transfection into neuronal cells has been generally considered difficult. Biewenga *et al* mentioned that of all mammalian cells, neurons are probably the most difficult to transfect (32). Especially with conventional techniques like calcium-phosphate transfection or lipofection, very poor efficiency is commonly achieved. Biewenga *et al* recommended the biolistic gene transfer technique in which the plasmid DNAs of interest were coated onto small particles, which were accelerated by a particular driving force. However, they were able to achieve only up to 2% of transfection efficacy for gene transfer into NB cell lines (32).

To solve this problem, we adopted a reverse transfection method, in which cell seeding and transfection were performed simultaneously by adding the mixture of siRNA and the reagent onto the cells as soon as the cells had been seeded on the plates (21). Although we employed modified reagent for lipofection, reverse transfection method successfully achieved transfection efficacy around 30% and resulted in 70% reduction of *MYCN* expression. Our results show that reduction of *MYCN* mRNA expression induce growth inhibition of NB-1 cells.

After reduction of MYCN protein expression, the expression of target genes of *MYCN*, such as *ODC, MCM7* and *MRP1*, was probably suppressed. Those genes usually lead to cell progression through the G1 phase of the cell cycle (17,33,34). Thus, reduction of those gene expressions may lead to G1 cell cycle arrest and results in the suppression of cell proliferation.

To investigate whether an apoptotic pathway was involved in suppression of cell proliferation after silencing of *MYCN*, we conducted TUNEL assay and confirmed that significantly higher proportion of TUNEL positive cells were observed in siRNA treated NB-1 cells. Galderisi *et al* showed that antisense oligonucleotide treatment of substrate adherent NB cells (S cells) resulted in a clear increase of the proapoptic Bax and Bak gene expression, along with a drastic decrease in the level of anti-apoptic Bcl-2 mRNA (35). Thus, these genes may have important roles in cell death after *MYCN* gene inhibition. Galderisi *et al* also found that the differentiation and apoptosis that followed antisense treatment persisted after the end of *MYCN* gene inhibition, indicating that a lasting *MYCN* downregulation is not required to induce these processes (35).

Moreover, after transfection with *MYCN*-siRNA we also observed that a pattern of the outgrowth was mostly multidirectional neurite extension of cell processes, and gradual long neurite elongation. Additionally, sizes of the cells and the nucleus in this group became larger than those observed in the other groups. These morphologic changes indicate differentiation effect of *MYCN*-siRNA treatment. Similarly previous studies had reported that *MYCN* suppression using antisense oligonucleotides resulted in cell differentiation in NB (28,30,35,36). These studies suggested that alterations in the regulation of *MYCN* expression can modulate the differentiation process of NB cells (28).

After silencing of *MYCN* expression by RNA interfering, relative expressions of *TrkA* and *TrkC* mRNA were significantly up-regulated. At the same time, morphological change corresponded to tendency of differentiation was observed. Evidence from several independent studies suggests that high expression of *TrkA* is an indicator of favorable outcome, and there is an inverse correlation between *TrkA* expression and *MYCN* amplification. *TrkC* is expressed in favorable NBs, essentially all of which also express *TrkA* (37-39). These findings suggest that suppression of *MYCN* up-regulated *TrkA* which activate specific signaling pathways linking to differentiation and survival.

Ha-*ras* genes are also closely associated with the growth, differentiation, and survival of neuronal tissues. Several observations suggest a role of Ha-*ras* p21 in promoting cellular differentiation and suppression of the proliferation activity of PC12, a tumor cell line originating from rat sympathetic nerve tissue (40). Thus, we examined Ha-*ras* expression after *MYCN* silencing. Consequently, expression of Ha-*ras* mRNA did not increase. Therefore, the differentiation after silencing *MYCN* may have a relation not to Ha-*ras* but rather to *TrkA* cascade.

In conclusion, the expression level of the *MYCN* mRNA was significantly reduced using the RNAi method. As a result, the knock-down of *MYCN* expression induced growth-inhibition, apoptotic activity and cell differentiation in *MYCN*-amplified NB-1 cell line. These data indicate that *MYCN* might be the key factor in the tumorigenesis and prognosis of NBs. Thus, silencing the *MYCN* gene by the RNAi method could be a potential tool for the treatment of NBs with *MYCN* amplification in the future.

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