MYCN downregulates integrin α1 to promote invasion of human neuroblastoma cells

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Abstract. Neuroblastoma is a childhood tumor thought to arise through improper differentiation of neural crest cells. *MYCN* amplification is a prognostic factor that indicates a highly malignant disease and poor patient prognosis. Integrins are important regulators of neuroblastoma attachment and migration and participate in many aspects of metastasis. However, the role of integrins in neuroblastoma metastasis, the leading cause of death from this disease, remains less well understood. Screening of neuroblastoma cell lines for integrin mRNA expression showed that integrin α1 expression was higher in lines such as SK-N-SH and NB69 that do not have *MYCN* amplification than in cell lines such as IMR32, NB1, NB9 and NB19 that have MYCN amplification. A knockdown of MYCN in NB1 and NB19 cells resulted in increased expression of integrin α1, which correlated with enhanced attachment to the extracellular matrix and reduced migratory activity. In contrast, the overexpression of MYCN in SK-N-SH and NB69 cells resulted in decreased expression of integrin α1, which correlated with reduced attachment to the extracellular matrix and enhanced migratory activity. These results show that MYCN may limit cell adhesion to the extracellular matrix and promote cell migration by downregulating integrin α1.

Introduction

Neuroblastoma is a pediatric tumor of the peripheral nervous system and arises from primordial neural crest cells (1,2). Neuroblastic tumors account for 8-10% of all pediatric cancers and are second in incidence among solid tumors in children (2,3). Neuroblastoma metastases form through dissemination of transformed cells into the bloodstream and local invasion into bone, leptomeninges and other organs (3-5). Despite a significant improvement in our understanding of the heterogeneous nature of primary neuroblastoma, metastasis, which is the leading cause of death due to this cancer, remains less well understood (6).

Among the prognostic indicators of neuroblastoma, *MYCN* amplification is strongly associated with advanced disease, rapid tumor progression and poor outcome (7). The normal single-copy locus of *MYCN* has been mapped to the short arm of chromosome 2p23-24 (8) and encodes a transcription factor that regulates gene expression during cell differentiation and growth (9,10). We recently reported that MYCN regulates cell growth and differentiation in a neuroblastoma cell line with *MYCN* amplification (11).

Control of cell adhesion is important in a number of biological phenomena, including embryonic development and tumor cell invasion and metastasis (12-14). Integrins are the major metazoan mediators of cell adhesion to extracellular matrix proteins and in vertebrates, play important roles in certain cell-cell adhesions. In addition, integrins make transmembrane connections to the cytoskeleton and activate many intracellular signaling pathways. The mammalian integrin family comprises of 18 α and 8 ß subunits, known to assemble into 24 distinct integrins (12). Previous data suggest that integrins are important regulators of neuroblastoma progression (15-18). Nevertheless, the precise effects of MYCN and integrins on neuroblastoma metastasis remain unclear.

The aim of this study was to elucidate the mechanism of MYCN and integrins in tumor metastasis by investigating whether a reduction of MYCN expression could control tumor attachment and migration in neuroblastoma by regulating integrins. Here, we demonstrate that a knockdown of MYCN in neuroblastoma cells harboring *MYCN* amplifications increases integrin α1 expression, enhances cell attachment and inhibits migration.

Materials and methods

Cell culture. Human neuroblastoma cell lines were obtained from the Health Science Research Resources Bank and the RIKEN Cell Bank. NB1, NB9, NB19 and NB69 cells were maintained in RPMI-1640 and IMR-32 and SK-N-SH cells were maintained in MEM Alpha supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin.

RNA interference. The siRNAs for human *MYCN* and the control (siTrio-negative control siRNA) were obtained from

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B-Bridge International Inc., Sunnyvale, CA, USA. The sequences of the siRNAs targeting human *MYCN* were 5'-CGG AGAUGCUGCUUGAGAA-3', 5'-CGGAGUUGGUAAAG AAUGA-3' and 5'-CAGCAGUUGCUAAAGAAAA-3'. The siTrio-negative control siRNAs were 5'-ATCCGCGCGATA GTACGTA-3', 5'-TTACGCGTAGCGTAATACG-3' and 5'-TATTCGCGCGTATAGCGGT-3'. Transfections were performed with a mixture of all three oligonucleotides, using HiperFect transfection reagent following the manufacturer's instructions (Qiagen Inc., Valencia, CA, USA), 48 h before cell attachment assays or migration assays.

DNA constructs. A MYCN expression plasmid was constructed by subcloning a human MYCN cDNA into the pCS2+ vector (DNAFORM, Yokohama, Kanagawa, Japan) using *Cla*I and *Xba*I adaptors in the 3'-5' orientation. Primers for cloning MYCN were 5'-ATCCCATCGATCACCATGC CGAGCTGCTCCACGTCC-3' and 5'-TGATCTAGACTAG CAAGTCCGAGCGTGTTCAATTTTC-3'. Transfections of plasmids were performed with Lipofectamine 2000 following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA), 24 h before cell attachment assays or migration assays. Equal amounts of DNA were used for each transfection.

Conventional PCR. Conventional PCR amplification was carried out at 95°C for 1 min, followed by 25 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 1 min. The primers used for PCR were: *MYCN*, 5'-CCACCAGCAGCACAACT ATG-3' (sense), 5'-GTCCGAGCGTGTTCAATTTT-3' (antisense); *integrin α1*, 5'-GCATGACAGTCAGCACT CGT-3' (sense), 5'-TTCAAAAGCTGCTCAGCAAA-3' (antisense); *GAPDH*, 5'-TGAAGGTCGGAGTCAACGGAT-3' (sense), 5'-CATGTGGGCCATGAGGTCCAC-3' (antisense).

Real-time PCR. Real-time PCR was carried out using the ABI Prism 9700 Sequence Detector System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). For quantification, *GAPDH* transcript served as the control and each sample was normalized to its *GAPDH* transcript level. The primer and probe mixtures for *MYCN*, *integrin α1* and *GAPDH* were purchased from Perkin-Elmer Applied Biosystems and the PCR method was as recommended in the manufacturer's protocol.

Western blotting. Cells were homogenized in RIPA lysis buffer (Upstate, Lake Placid, NY, USA) and incubated for 10 min on ice. After centrifugation at 13,000 x g for 10 min at 4°C, supernatants were collected as protein samples. For Western blotting of MYCN and Actin, protein was separated by SDS-PAGE using 10% polyacrylamide gels and electroblotted onto PVDF membranes (Bio-Rad, Hercules, CA, USA). After blocking of non-specific binding for 1 h with 5% non-fat milk in PBS containing 0.1% Tween-20, membranes were incubated for 1 h at room temperature with antihuman MYCN antibody (Santa Cruz, Santa Cruz, CA, USA) or antihuman actin antibody (BD Biosciences, San Jose, CA, USA). Membranes were then washed three times with PBS containing 0.1% Tween-20, incubated with horseradish peroxidase conjugated anti-mouse antibody (Promega

Corporation, Madison, WI, USA) at room temperature and washed three times with PBS containing 0.1% Tween-20. The immunoblots were developed with an enhanced chemi-luminescence system (Amersham Biosciences Inc., Piscataway, NJ, USA). Immunoblotting with antibody to actin provided an internal control for equal protein loading. Densitometry was performed by measuring pixel density in a defined region of each band using Imaging System for Chemiluminescence (AlphaInotech, San Francisco, CA, USA). Blots shown are representative of three independent experiments.

Attachment assay. Attachment assays were carried out using 24-well plates coated with laminin, collagen type IV, or fibronectin (BD Biosciences). Cells ($1x10^4$) were plated on each well, incubated in the presence of 10% FBS for 20 min with or without 3 µg/ml integrin α1 antibody (Chemicon International, Temecula, CA, USA) and rinsed three times with phosphate-buffered saline. Cells that attached to the bottom of the well were fixed with methanol, stained with hematoxylin and eosin and counted under a microscope.

In vitro transwell migration assay. *In vitro* transwell migration assays were performed using 8.0-µm pore size Costar Transwell inserts (Corning Inc., Lowell, MA, USA), according to the manufacturer's protocol. Cells ($5x10^4$) were added to each well and incubated in the presence of 10% FBS for 24 h with or without 3 µg/ml integrin α1 antibody (Chemicon International). Cells that migrated to the bottom of the transwell membrane were fixed with methanol, stained with hematoxylin and eosin and counted under a microscope.

Results

High expression of integrin α1 mRNA in neuroblastoma cell lines without MYCN amplifications. To test whether integrins are involved in the metastatic phenotype of neuroblastoma cells, we analyzed integrin (*integrin α1*, *2*, *3*, *4*, *5*, *v*, *integrin β1*, *3 and 5*) mRNA levels in neuroblastoma cell lines with *MYCN* amplifications, which express high levels of MYCN (IMR32, NB1, NB9 and NB19) or without *MYCN* amplifications (SK-N-SH and NB69). Among the integrins we screened, the expression of integrin α1 was relatively high in cell lines lacking *MYCN* amplification (Fig. 1a), as confirmed by real-time PCR (Fig. 1b).

MYCN-siRNA upregulates integrin α1 expression in NB1 and NB19 neuroblastoma cells. We then generated MYCN-knockdown NB1 or NB19 cells using siRNA targeting *MYCN* and analyzed *integrin* (*integrin α1*, *2*, *3*, *4*, *5*, *v*, *integrin β1*, *3 and 5*) expression. RT-PCR and real-time PCR analysis of *MYCN* mRNA in MYCN-siRNA transfected NB1 or NB19 cells showed transcript depletion (Fig. 2a and 2b). RT-PCR and real-time PCR analysis showed that the knockdown of MYCN in neuroblastoma cells harboring *MYCN* amplifications increased *integrin α1* mRNA expression (Fig. 2a and b). In addition, Western blotting and densitometry analysis showed that MYCN protein was depleted in these cells (Fig. 3a and b). These results are consistent with a previous finding that the
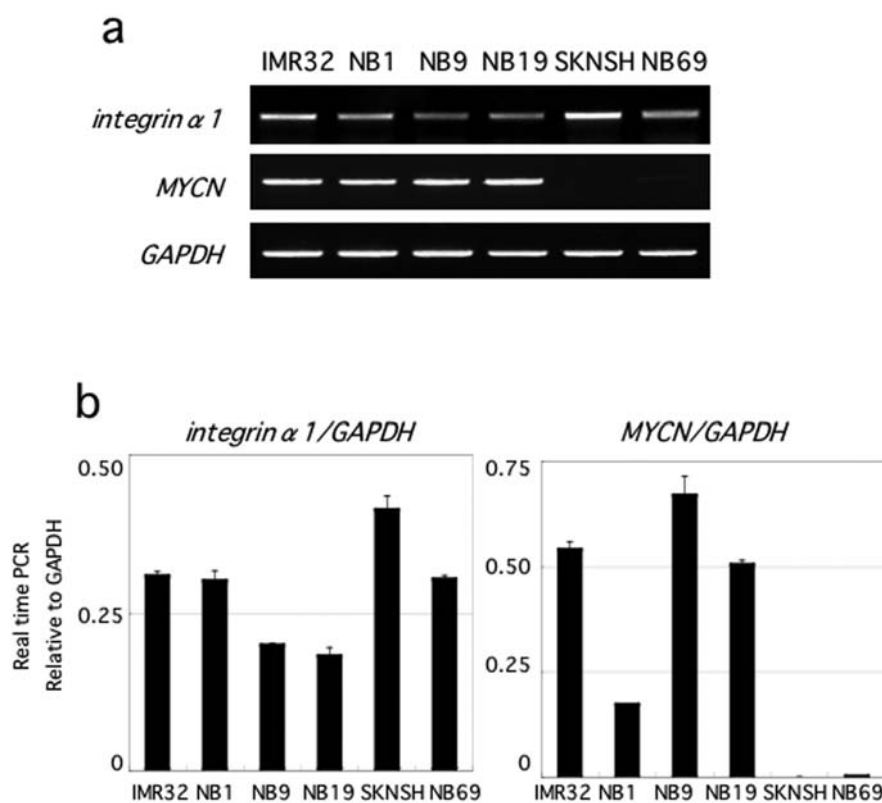
Figure 1. *MYCN* and *integrin α1* mRNA expression in neuroblastoma cell lines. (a) *Integrin α1* and *MYCN* mRNA expression were evaluated by semiquantitative reverse transcription PCR in neuroblastoma cell lines (IMR32, NB1, NB9 and NB19) harboring *MYCN* amplifications and in neuroblastoma cell lines without such amplifications (SK-N-SH and NB69). (b) The relative mRNA expression of *integrin α1* and *MYCN* in these six neuroblastoma cell lines was evaluated by real-time PCR. Error bars represent the standard deviations of three replicates.
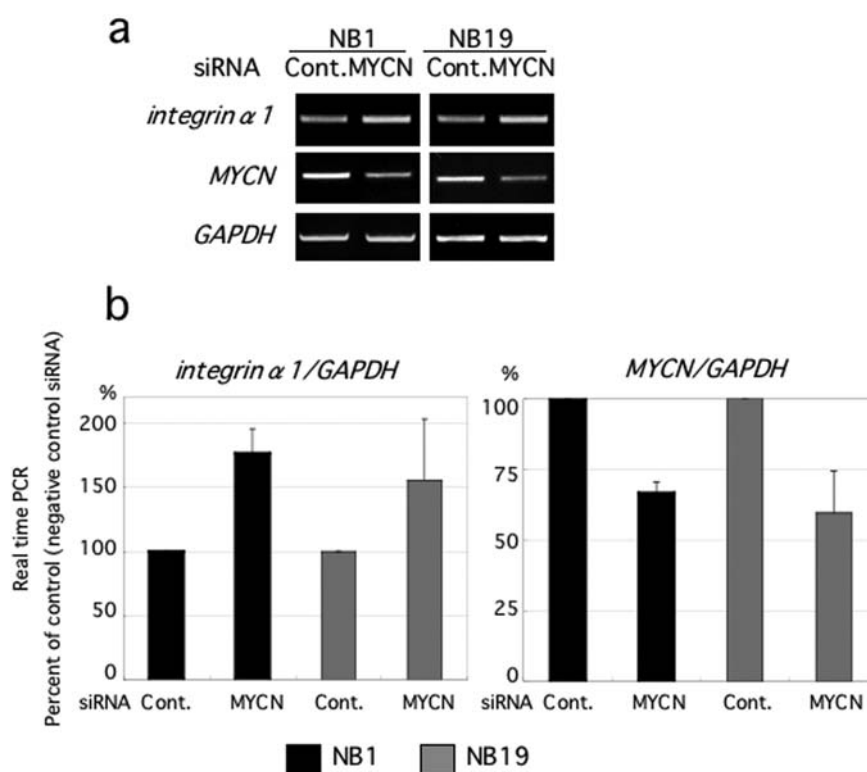

Figure 2. *MYCN* and *integrin α1* mRNA expression in NB1 and NB19 cells expressing control- or MYCN-siRNA. (a) Semiquantitative reverse transcription PCR and (b) real-time PCR show the expression of *MYCN* and *integrin αl*. Elevation of *integrin α1* and depletion of *MYCN* expression were observed in neuroblastoma cells transfected with MYCN-siRNA compared with cells transfected with control-siRNA. Error bars represent the standard deviations of five replicates.
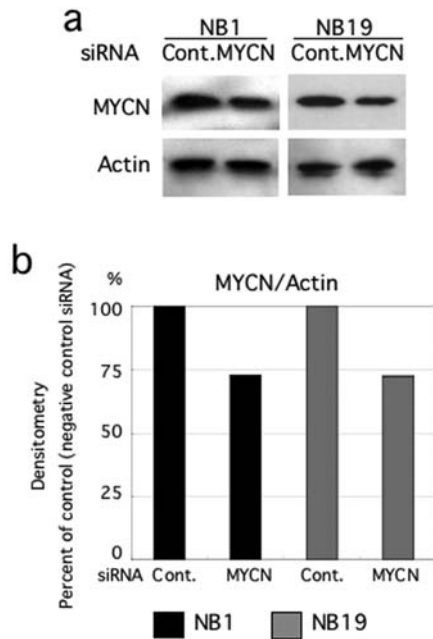
Figure 3. MYCN protein levels in NB1 and NB19 cells expressing control- or MYCN-siRNA. (a) MYCN protein levels were evaluated by Western blot analysis and (b) densitometry. Each figure shows a representative result of at least three experiments.
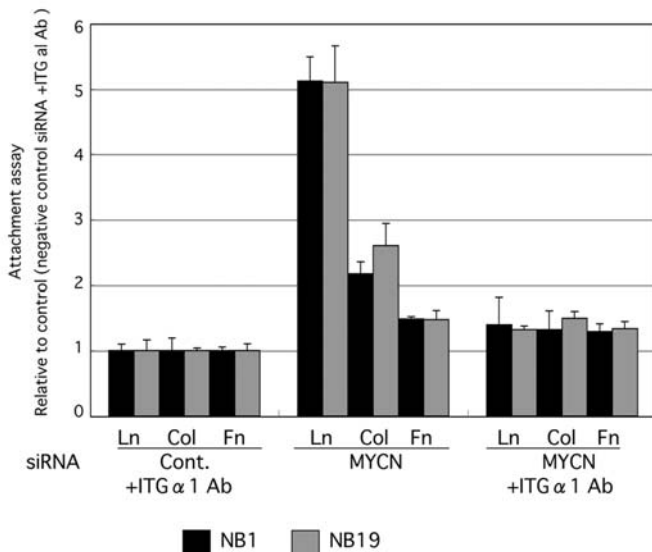

Figure 4. The effects of MYCN knockdown on neuroblastoma cell attachment. NB1 and NB19 cells expressing control- or MYCN-siRNA were incubated on 24-well plates coated with laminin, collagen type IV, or fibronectin with or without 3 µg/ml integrin α1 antibody for 20 min during attachment assays. Error bars represent the standard deviations of three replicates (Ln, laminin; Col, collagen type IV and Fn, fibronectin).
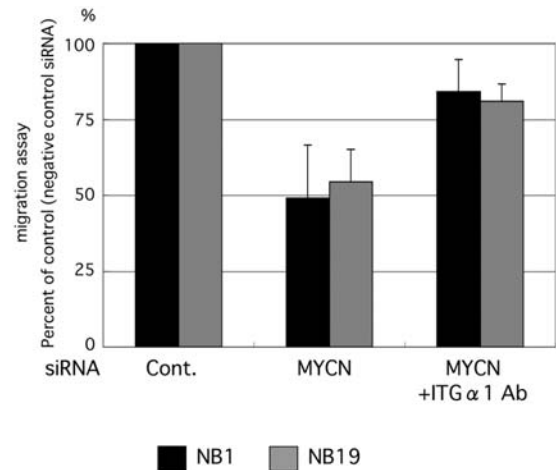

Figure 5. The effects of MYCN knockdown on neuroblastoma cell migration. NB1 and NB19 cells expressing control- or MYCN-siRNA were incubated with or without 3 µg/ml integrin α1 antibody for 24 h during *in vitro* transwell migration assays. Error bars represent the standard deviations of three replicates.

expression of integrin α1 is associated with the morphological differentiation of neuroblastoma cell lines (17,18) and suggests that integrin α1 may be an important regulator of human neuroblastoma progression.

MYCN-siRNA enhances adhesion of NB1 and NB19 cells to the extracellular matrix by upregulating integrin α1. We then investigated whether MYCN influences cellular adhesion to the extracellular matrix by regulating integrin α1. We performed attachment assays using NB1 and NB19 cells transfected with control- and MYCN-siRNA and treated with integrin α1 antibody. An increased number of MYCN-siRNA-treated cells adhered to laminin, collagen type IV, or fibronectin compared to cells transfected with control-siRNA and treated with integrin α1 antibody (Fig. 4). This enhancement of adhesion to laminin and collagen type IV could be abrogated by treatment with integrin α1 antibody (Fig. 4). As fibronectin is not a ligand for integrin α1, the enhancement of adhesion to fibronectin occurs through an unknown mechanism. However, our results show that integrin α1 was the main factor of neuroblastoma adhesion. These results suggest that inhibition of MYCN resulted in increased integrin α1 and cellular adhesion to the extracellular matrix.

MYCN-siRNA inhibits the in vitro invasive behavior of NB1 and NB19 cells by upregulating integrin α1 expression. To examine whether MYCN and integrin α1 are involved in neuroblastoma migration, we performed *in vitro* migration assays using MYCN-knockdown NB1 and NB19 cells. In transwell migration assays, control NB1 and NB19 cells showed active migration, reflecting their invasive properties; in contrast, MYCN-knockdown NB1 and NB19 cells exhibited poor migratory activity (Fig. 5). The defect in migratory activity in MYCN knockdown cells was rescued by integrin α1 antibody (Fig. 5). To exclude the possibility of off-target effects, we used a different siRNA obtained from Qiagen. RT-PCR and real-time PCR analysis of *MYCN* mRNA in MYCN-siRNA transfected NB1 or NB19 cells showed ~30% transcript depletion (data not shown). This partial suppression was similar in migration assays and attachment assays (data not shown). Taken together, these results indicate that MYCN may limit cell adhesion to the extracellular matrix and may promote cell migration by downregulating integrin α1.

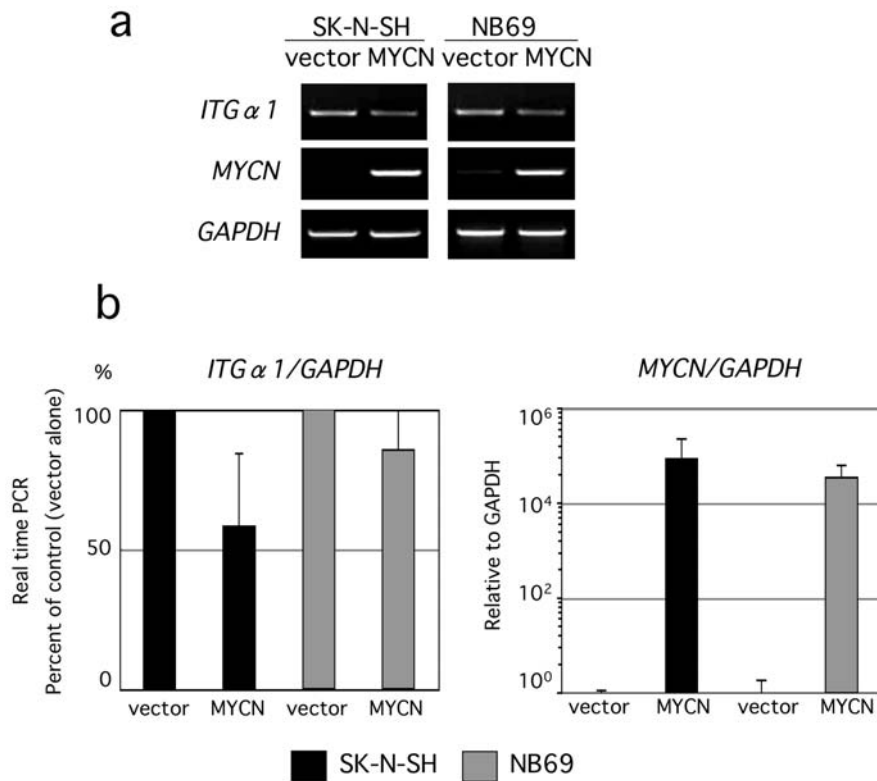*MYCN overexpression downregulates integrin α1 expression in SK-N-SH and NB69 neuroblastoma cells.* To confirm this

Figure 6. MYCN and *integrin α1* mRNA expression in SK-N-SH and NB69 cells expressing pCS2+ or pCS2+MYCN. (a) Semiquantitative reverse transcription PCR and (b) real-time PCR show the expression of *MYCN* and *integrin α1*. Depletion of *integrin α1* and elevation of *MYCN* expression were observed in neuroblastoma cells transfected with pCS2+MYCN compared with cells transfected with vector only. Error bars represent the standard deviations of three replicates.
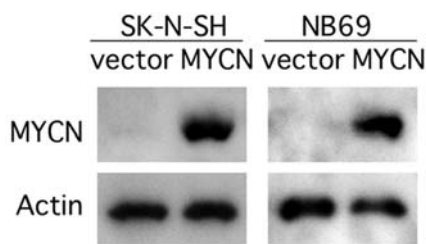

Figure 7. MYCN protein levels in SK-N-SH and NB69 cells expressing pCS2+ or pCS2+MYCN. MYCN protein levels were evaluated by Western blot analysis. Each figure shows a representative result of at least three experiments.
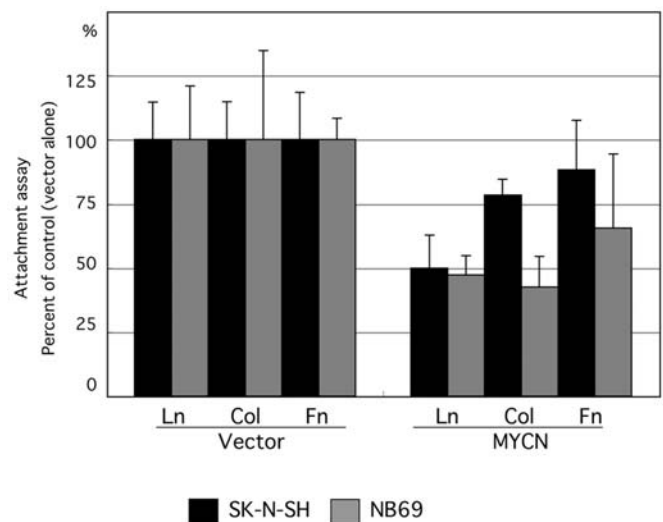

Figure 8. The effects of MYCN overexpression on neuroblastoma cell attachment. SK-N-SH and NB69 cells expressing pCS2+ or pCS2+MYCN were incubated on 24-well plates coated with laminin, collagen type IV, or fibronectin for 20 min during attachment assays. Error bars represent the standard deviations of three replicates (Ln, laminin; Col, collagen type IV and Fn, fibronectin).

hypothesis, we generated MYCN-overexpressing SK-N-SH and NB69 cells using a MYCN expression vector. RT-PCR and real-time PCR analysis of *MYCN* mRNA in pCS2+MYCN transfected SK-N-SH and NB69 cells showed transcript enhancement (Fig. 6a and b). RT-PCR and real-time PCR analysis showed that overexpression of MYCN in SK-N-SH and NB69 cells decreased *integrin α1* mRNA expression (Fig. 6a and b). In addition, Western blot analysis showed that the MYCN protein was elevated in these cells (Fig. 7).

MYCN overexpression inhibits adhesion and enhances the in vitro invasive behavior of SK-N-SH and NB69 cells by downregulating integrin α1. To determine whether MYCN overexpression can induce opposite effects on cell adhesion and motility, we performed attachment assays and *in vitro*

migration assays using SK-N-SH and NB69 cells transfected with pCS2+ and pCS2+MYCN. A decreased number of pCS2+MYCN transfected cells adhered to laminin or collagen type IV compared to cells transfected with pCS2+ (Fig. 8). A decreased number of pCS2+MYCN-transfected cells adhered
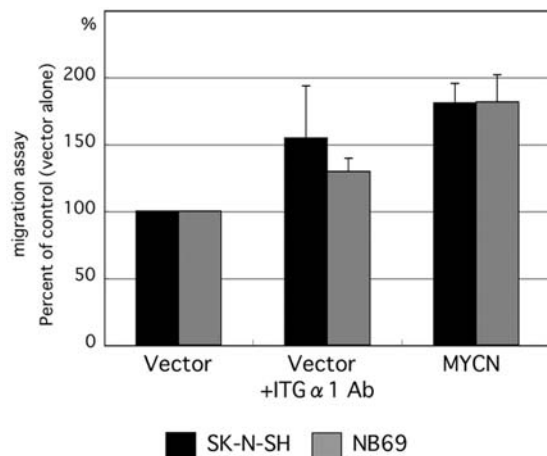
Figure 9. The effects of MYCN overexpression on neuroblastoma cell migration. SK-N-SH and NB69 cells expressing pCS2+ or pCS2+MYCN were incubated with or without 3 μg/ml integrin α1 antibody for 24 h during *in vitro* transwell migration assays. Error bars represent the standard deviations of three replicates.

to fibronectin, through an unknown mechanism. In transwell migration assays, increased migration of cells treated with integrin α1 antibody and pCS2+MYCN were observed compared to the control cells (Fig. 9). Thus, downregulation of integrin α1 by MYCN overexpression inhibits adhesion and enhances the *in vitro* invasive behavior of SK-N-SH and NB69 cells.

Taken together, our results show that MYCN limits cell adhesion to the extracellular matrix and promotes cell migration by downregulating integrin α1.

Discussion

Neuroblastoma is the most common solid tumor in children (5) and children with metastasis fail to respond to medical intervention (5,6). The only available therapeutic strategies for advanced neuroblastoma include surgical resection, radiation and myeloablative chemotherapy with bone marrow transplantation (5,19). Even with the most intensive treatment, the 2-year relapse-free survival of stage IV neuroblastoma with remote metastasis is only ~40% (5,19). Neuroblastoma typically spreads to regional lymph nodes, leptomeninges, bone and bone marrow (3-5). Whereas our knowledge of the heterogeneous nature of primary neuroblastoma has significantly improved during recent years, metastasis, the leading cause of death, remains poorly understood (6). Improvements in neuroblastoma patient survival require the identification of molecular targets for treatment based on a thorough understanding of the metastatic process.

Metastasis is a complex and organized process that consists of multiple but interrelated steps, during which tumor cells leave the primary tumor, gain access to the circulatory system, are carried to a distant site, exit the circulation and eventually grow in a different microenvironment (6). Local invasion, the first step of metastasis, requires complex interactions, including recognition and attachment of tumor cells to extracellular matrix binding sites, proteolytic dissolution of the extracellular matrix and tumor cell migration into the surrounding tissue (6,20). Control of cell attachment is

therefore important for tumor cell invasion (12,21,22). Integrins are the major metazoan receptors for cell adhesion to extracellular matrix proteins and, in vertebrates, also play important roles in cell-cell adhesion (12). Integrins and their ligands play key roles in development, immune responses, leukocyte traffic, hemostasis and cancer (12). The involvement of some integrins in neuroblastoma progression has also been reported (15-18).

MYCN amplification was identified in neuroblastoma by Schwab *et al* over 20 years ago (8). Soon after this discovery, *MYCN* amplification was confirmed as the most significant prognostic indicator of adverse disease outcome in neuroblastoma (7). However, the association of *MYCN* amplification and a more aggressive phenotype is still unclear (5). MYCN is a transcription factor and oncoprotein known to repress several adhesion-related genes, including integrins (21,22). MYCN may modulate neuroblastoma tumorigenicity through interference with integrin-mediated cell adhesion. Our experiments have established that MYCN is essential for the invasive behavior of neuroblastoma cells by downregulating integrin α1. Four lines of evidence support this idea. i) The knockdown of MYCN in neuroblastoma harboring *MYCN* amplifications resulted in increased integrin α1 expression. ii), High expression of endogenous integrin α1 mRNA was observed in neuroblastoma cell lines that did not harbor *MYCN* amplifications. iii), MYCN depletion in these cells enhanced attachment and inhibited migratory activity. iv), The enhanced adhesion and defective migration were both abrogated by treatment with integrin α1 antibody. We confirmed these observations by overexpression of MYCN in neuroblastoma cells without MYCN amplifications.

Previous reports have indicated increased tumorigenicity associated with reduced levels of integrin α2, 3 and ß1 caused by MYCN overexpression in neuroblastoma (17,18,23,24). In this study, to elucidate the MYCN-dependent mechanism regulating cell adhesion and motility, we investigated various cell adhesion molecules, including N-cadherin, neural cell adhesion molecule, integrins α1-5, V and ß1, 3 and 5 (data not shown). We found a significant influence of MYCN-knockdown on integrin α1; in contrast, integrins α2, 3 and ß1 were unaffected. Although previous reports showed that integrin α1 was associated with neuroblastoma cell differentiation (17,18), this is the first report to demonstrate an invasive role for integrin α1 regulated by MYCN. As fibronectin is not a ligand for integrin α1, the enhancement of adhesion to fibronectin occurred through an unknown mechanism. However, our results suggest that integrin α1 might be key for adhesion and motility of neuroblastoma cells harboring *MYCN* amplifications.

Our results suggest that MYCN may be exerting a negative regulatory effect on transcription of integrin α1, especially since MYCN is a DNA binding protein. Previous reports have shown that several integrins are influenced by increased levels of MYCN (23,25). However, it is not known whether any of these genes is directly regulated by MYCN binding to the promoter region (25). Thus, more direct transcriptional analyses will be required to test these hypotheses.

Our findings suggest that MYCN promotes metastasis by downregulating integrin α1, representing one mechanism that promotes increased neuroblastoma metastasis. Integrin α1

therefore may be a promising molecular target for therapeutic drugs that target neuroblastoma metastasis. Future studies will fully explore the interaction between MYCN and integrins during neuroblastoma cell invasion.

Acknowledgements

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