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Neuroblastoma cells with overexpressed *MYCN* retain their capacity to undergo neuronal differentiation

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Amplification of *MYCN* in neuroblastoma strongly correlates to unfavorable outcome, but little is known of how the high *MYCN* expression translates into an aggressive tumor phenotype. More aggressive neuroblastomas are generally immature and overexpression of exogenous *MYCN* in cultured neuroblastoma cells and other neuronal cell types has been reported to inhibit induced differentiation, suggesting a link between high *MYCN* expression and an immature phenotype. However, we show here that *MYCN* is expressed in human neuroblasts of sympathetic chain ganglia at fetal week 8.5, a developmental stage at which these neuroblasts express a number of sympathetic neuronal differentiation marker genes. Analyses of 28 neuroblastoma tumor specimens and 27 cell lines for the expression of *MYCN* and a panel of neuronal differentiation marker genes did not reveal any correlation between *MYCN* and marker gene expression levels. Finally, we tested five separate differentiation protocols and show that *MYCN* overexpressing neuroblastoma cells with a neuronal phenotype, derived from the non-*MYCN*-amplified human neuroblastoma cell line SK-N-SH, retain their capacity to differentiate despite constitutive *MYCN* overexpression. Our results show that high *MYCN* expression and sympathetic differentiation are compatible, and indirectly our findings lend support to previously published *MYCN* neuroblastoma tumor data, which suggest that in single *MYCN* copy neuroblastomas there is no direct correlation between a high cellular MYCN protein content and aggressive tumor cell behavior.
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The *MYC* family consists of three closely related transcription factors encoding the proto-oncogenes *MYC*, *MYCN*, and *MYCL* (also known as MYCC or c-*myc*, N-*myc*, and L-*myc*, respectively). *MYC* is the founding member, whose transforming properties were first evidenced by the pioneering studies on avian retroviruses. Subsequently, increased expression of *MYC* oncogenes due to translocations or genomic amplification was detected in various human neoplasms. Over the past 15 years, evidence has accumulated supporting a role for *MYC* in a variety of cellular pathways controlling cell growth, differentiation, cell adhesion, angiogenesis, apoptosis, immortalization, and genome stability.

Amplification of *MYCN* occurs in a number of childhood and adult tumors and is a hallmark of a genetic subgroup (22% of all cases[1–7]) of highly aggressive neuroblastomas, that is, pediatric sympathetic nervous system-derived tumors. Early on, *MYCN* amplification was recognized as an independent prognostic factor for neuroblastoma and was used as one of the first genetic markers in therapy stratification for cancer treatment. In parallel to observations for *MYC*, increased *MYCN* expression was thought to be essential for constituting the malignant phenotype. Consistent with this assumption, overexpression of *MYCN*, targeted to sympathetic precursor cells via the tyrosine hydroxylase promoter in transgenic mice, results in the development of tumors with neuroblastoma characteristics.[8] Recent evidence for a role in perturbed growth control came from the finding that postmitotic

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sympathetic neurons could be induced by *MYCN* to re-enter the S phase of the cell cycle.[9] Furthermore, overexpression of *MYCN* in the epithelial neuroblastoma subclone SH-EP resulted in increased DNA synthesis and growth rate.[10] An extended expression analysis of these cells, in which the *MYCN* gene is under a controllable promoter, revealed that the majority of MYCN-induced genes are regulators of protein synthesis, and many of these genes were also induced by MYC in melanoma cells.[11]

Insights into the role of *MYCN* in differentiation and tumor development might indirectly be deduced from our current knowledge of *MYC*, although evidence indicates separate roles for the *MYC* family members. The identification and, in particular, the validation of MYC and MYCN target genes and the dissection of the cellular network governed by both genes, has, however, proven extremely difficult and our understanding of the exact role of MYC or MYCN in the abovementioned pathways is, as yet, poor. In normal avian and murine development, *MYCN* is expressed preferentially during early embryogenesis with high expression at the time of organogenesis.[12,13] In general, major sites of *MYCN* expression correlate to regions of differentiating cells, rather than to areas with high proliferation rates.[13–15] Of particular relevance for the present study is the high *MYCN* expression in neural crest-derived progenitor cells. In avian cells, a role for MYCN in controlling cell migration and neuronal determination or early differentiation of neural crest-derived cells is suggested.[16] In mice, homozygous elimination of *MYCN* is lethal and the sympathetic nervous system (SNS) has fewer neuroblasts, but those that remained appeared to develop normally.[17] These observations from developmental studies are, however, contrasted by reports of blocked retinoic acid (RA)-induced differentiation and growth arrest by exogenous *MYCN* overexpression in neuroblastoma cell lines carrying an amplified *MYCN* gene.[18,19]

As *MYCN* amplification is closely associated to high-stage neuroblastomas and an inverse correlation between clinical neuroblastoma stage and the expression of differentiation marker genes has been established,[20] a role for MYCN in differentiation arrest can be inferred. However, although *MYCN*-amplified neuroblastoma tumors consistently show increased *MYCN* expression both at the mRNA and the protein level,[21–23] a closer examination reveals that *MYCN* single-copy low-stage neuroblastoma tumors (stages 1, 2, and 4S) also can express high *MYCN* levels, seemingly without affecting either the malignant status of the tumor or patient outcome. In contrast, some, but not all, reports on non-*MYCN*-amplified tumors at higher clinical stages conclude that high *MYCN* expression correlates to poor outcome.[24–26]

In this report, we have addressed the putative role of *MYCN* in blocking neuronal differentiation of human neuroblastoma cells. We have analyzed the expression levels of *MYCN* and correlated them to the expression of established sympathetic neuronal/neuroendocrine marker genes in a panel of neuroblastoma cell lines and tumor specimens. We have further used *MYCN*-transfected clones of SK-N-SH neuroblastoma cells,[27] which are non-*MYCN* amplified and have the rare capacity to differentiate neuronally by several different protocols, to study the effect of overexpression of *MYCN* on induced differentiation. Taken together, our tumor and cell line data strongly suggest that the overall capacity of neuroblastoma cells to undergo neuronal differentiation is not impaired by high *MYCN* expression.

Materials and methods

Tumor Material, Cell Lines, and Cell Culture Conditions

The clinical material consisted of 13 stage 1, 2, and 4S tumors and 15 stage 3 and 4 tumors. Seven of them had an amplified *MYCN* gene. SK-N-SH, SH-SY5Y, KCN-69n, and SK-N-BE (2) cells were cultured in Eagle's minimum essential medium with 10% fetal calf serum, 100 IU/ml penicillin V, and 100 μg/ml streptomycin added. SH-EP cells were supplied with the same medium and antibiotics, but with 15% fetal calf serum. All other neuroblastoma cell lines were cultured in RPMI 1640 with 10% fetal calf serum and antibiotics as above. The SKMYC2 clone of SK-N-SH cells transfected with human *MYCN* has previously been described.[27] Briefly, *MYCN* cDNA (from pcNMYC3, Dr JM Bishop, University of California, San Francisco, CA, USA) was cloned into an RSV-LTR-driven expression vector in either sense or antisense orientation. Stable transfectants were achieved using calcium-phosphate precipitation and selection for expression of the hygromycin resistance gene also on the pREPcNMYC2 (sense) and pREPcNMYCAS2 (antisense) plasmids. The cells were then cultured as SK-N-SH cells with 200–600 μg/ml Hygromycin B (Calbiochem, San Diego, CA, USA) added. To induce differentiation, the following additives were used: All-*trans*-retinoic acid (RA) (Sigma, St Louis, MO, USA), aphidicolin (Sigma), 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) (Sigma), basic fibroblast growth factor (bFGF), brain-derived neurotrophic factor (BDNF), insulin-like growth factor-I (IGF-I), and nerve growth factor (NGF), all growth factors from PeproTech EC Ltd, London, England. Media and antibiotics were purchased from Life Technologies Inc (Rockville, MD, USA), and fetal calf serum from PAA Laboratories GmbH, Linz, Austria.

Immunohistochemistry and *In situ* Hybridization

Human fetal tissue (ethical approval LU 389-98, Lund University, Sweden) was obtained from

elective abortions and the material was fixed in formaldehyde and paraffin embedded. Abdominal cross-sections (5 μm thick) were secured onto glass slides and deparaffinized. Immunohistochemistry was performed as described,[28] using avidin–biotin–peroxidase conjugated secondary antibodies (ABC-kit, Vectastain, Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine as chromogen. The primary antityrosine hydroxylase (TH) monoclonal antibody (Roche Molecular Biochemicals, Mannheim, Germany) was used at a 1:75 dilution. Slides were counterstained with hematoxylin. For *in situ* hybridizations, sections were pretreated as described.[29] *MYCN* RNA transcripts were detected with an antisense-oligonucleotide mouse sequence probe, with hybridization conditions essentially as described.[30] The following oligonucleotides were used: Antisense probe: 5′-AAG TAG AAG TCA TCT TCG TCC GGG TAG AAG CAG GGC TGC A-3′, sense probe: 5′-CGA TTT CCT CCT CTT CAT CTT CCT CCT CGT CAT CCT CAT C-3′ (Invitrogen AB, Stockholm, Sweden, custom-made primers). The oligonucleotides were 3′-endlabeled with ^{35}S-dATP using terminal deoxyribonucleotidyl transferase. The sections were hybridized at 42°C overnight and washed four times in 1 × SSC at 56°C. The sense probe was used as hybridization control.

Immunofluorescence and Western Blot Analyses

Cells intended for immunofluorescence were plated on glass coverslips, treated as described in the Results section, fixed with 4% paraformaldehyde in PBS, and mounted on object slides with 20 μl PVA-DABCO solution (9.6% polyvinyl alcohol, 24% glycerol, and 2.5% 1,4-diazabicyclooctane in 67 mM Tris-HCl, pH 8.0). TBS with 5% goat serum and 0.3% Triton X-100 was used as blocking and permeabilization buffer, anti-*MYCN* antibody (556438 from BD Pharmingen, San Diego, CA, USA) diluted 1:100 in blocking and permeabilization buffer as primary, and goat anti-mouse IgG-FITC-conjugated antibodies (Alexa Fluor 488, Molecular Probes, Inc., Eugene, OR, USA) diluted 1:100 in TBS as secondary antibodies. The results were documented with or without FITC-filters, using a SONY DKC 5000 camera system (Sony Corporation, Tokyo, Japan). For Western blot analysis, cells were lysed in 10 mM Tris-HCl (pH 7.2), 160 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EGTA, 1 mM EDTA in the presence of complete protease inhibitor (Roche). A measure of 40–60 μg of whole-cell lysate proteins were separated on an SDS-PAGE gel and blotted onto an Immobilon-P membrane (Millipore Corporation, Bedford, MA, USA). The following primary antibodies were used: anti-actin antibody (ICN Biomedicals Inc., Costa Mesa, CA, USA), diluted 1:2500 and anti-MYCN antibody (556438 from BD Pharmingen) diluted 1:500. HRP-coupled secondary antibody fragments were supplied from Bio-Rad Laboratories, Hercules, CA, USA (diluted 1:3000) or Amersham Pharmacia Biotech, Uppsala, Sweden (diluted 1:5000). Rainbow RPN 756 (Amersham) was used as a molecular weight marker.

Northern Blot and Quantitative RT-PCR Analyses

A commercial guanidine–isothiocyanate/phenol–chloroform extraction solution (Trizol, Invitrogen Inc., Carlsbad, CA, USA) was used for isolation of total cellular RNA. For Northern blot analysis, 15 μg of RNA was electrophoretically separated on a 1% agarose–formaldehyde gel and blotted onto a nylon membrane (Hybond-N, Amersham). The following cDNA probes were labeled with [^{32}P]dCTP using an oligonucleotide labeling kit (Amersham Pharmacia Biotech): *GAP43*,[31] *GAPD*,[32] *HES1*,[33] *NPY*,[34] and *MYCN*.[2] Hybridizing probes were visualized by autoradiography (Curix Blue HC-S Plus, Agfa-Gevaert N.V. Mortsel, Belgium). DNase treatment, cDNA synthesis, primer design, and SYBR Green I real-time quantitative RT-PCR were performed as described[35] with minor modifications. After establishment of amplification efficiencies >95% for all primer pairs, the comparative Ct method was used for quantification. PCR reagents were obtained from Eurogentec as SYBR Green I mastermixes and used according to the manufacturer's instructions in a total volume of 25 μl. Reactions were run on an ABI5700 (Applied Biosystems) in duplex (coefficient of variation for calculated quantities always below 10%). Gene expression levels were normalized using the geometric mean of the four most stable internal control genes in neuroblastoma (ie *UBC*, *HPRT1*, *SDHA*, and *GAPD*) as reported previously.[36] Primer sequences for all tested genes are available in the public RTPrimerDB database (http://www.realtimeprimerdatabase.ht.st) (gene (RTPrimerDB-ID): *UBC* (8), *SDHA* (7), *HPRT1* (5), *GAPD* (3), *MYCN* (180), *MYC* (18), *HAND2* (98), *NTRK1* (118), *GAP43* (97), *STMN2* (129), *CHGA* (474), *INHBA* (105)).[37]

Results

MYCN is Expressed in Developing Human Sympathetic Neuroblasts

To verify that *MYCN* is expressed during development of the human SNS, *MYCN* expression was analyzed in abdominal cross-sections of an 8.5-week-old human fetus. At this developmental stage, sympathetic chain ganglia are under formation and TH-positive sympathetic precursor cells are populating the developing adrenal glands (Figure 1c). As shown in Figure 1a and b, strong *MYCN* expression was detected in dorsal root ganglia, sympathetic chain ganglia, and in the spinal cord. Interestingly, the migrating sympathetic adrenal precursor cells

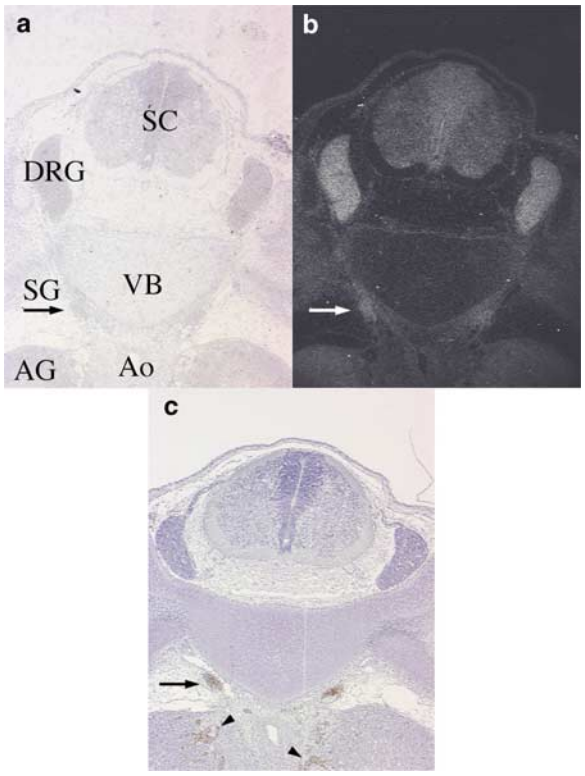

Figure 1 *MYCN* and TH expression in abdominal cross-sections of an 8.5-week human fetus. ((**a**), (**b**)). *MYCN* expression was detected by *in situ* hybridization. A bright-field image is shown in panel (**a**) and a dark-field image in panel (**b**). (**c**). Immunohistochemical analysis of an adjacent section with anti-TH antibodies. The sections were counterstained with hematoxylin. Arrows indicate a TH and *MYCN*-positive sympathetic chain ganglion, and arrowheads in panel (**c**) indicate TH-positive sympathetic cells migrating into the developing adrenal gland. Abbreviations: Ao, aorta; AG, adrenal gland; DRG, dorsal root ganglion; SC, spinal cord; SG, sympathetic ganglion; VB, vertebral body.

appeared not to express *MYCN*, or are at the most expressing *MYCN* modestly (compare TH-positive cells in panel c with the *in situ* signal in the corresponding areas in panels a and b). we conclude that human sympathetic chain ganglion neuroblasts express *MYCN* at a stage when these cells are nonmigrating and when they express a number of sympathetic neuronal marker genes.[38–40]

Expression of *MYCN* and Neuronal Marker Genes in Neuroblastoma Cells and Tumors

The stage of differentiation based on morphological criteria has for a long time been used as a prognostic factor in neuroblastoma, and in line with this, expression levels of differentiation markers such as neuron-specific enolase, synaptophysin, and the neuronal form of $pp60^{c\text{-}src}$ correlate positively to favorable outcome.[20,41] *MYCN* amplification is associated with an aggressive phenotype and when *MYCN* is overexpressed in *MYCN*-amplified cultured neuroblastoma cells, RA differentiation is blocked.[18,19] However, as the link between *MYCN* expression and prognosis or tumor stage in non-*MYCN*-amplified neuroblastomas is contradictory,[25,42] we analyzed a panel of neuroblastoma cell lines and tumor specimens for the expression of *MYCN* and a set of SNS differentiation markers by quantitative real-time RT-PCR (Figure 2). In the 27 analyzed cell lines, the highest *MYCN* expression was found in those derived from *MYCN*-amplified tumors. Similarly, in the panel of 28 investigated tumor specimens, *MYCN* expression was, with one exception, highest in tumors with an amplified gene (Figure 2). The five analyzed SNS neuronal/neuroendocrine differentiation marker genes, *HAND2 (dHAND), NTRK1 (trkA), GAP43, STMN2 (SCG-10)*, and *CHGA (chromogranin A)*, were with few exceptions expressed in all cell lines, which first of all confirms the SNS and neuroblastoma derivation of the analyzed tumor cell lines. However, there was no correlation between *MYCN* expression and the expression levels of the differentiation markers (Table 1),[44] which can be demonstrated visually by ordering the cell lines according to *MYCN* expression levels (Figure 2). A similar result was obtained with tumor specimens when *MYCN* expression was compared to the expression levels of *HAND2*, *NTRK1*, and *GAP43* (Table1 and Figure 2); the mRNA levels of *STMN2* and *CHGA* were not measured. We conclude that *MYCN* expression did not correlate, positively or negatively, to the stage of differentiation in neuroblastoma. As a comparison, PCR data demonstrating a statistically significant negative correlation between *MYC* and *MYCN* expression were included (Table 1, and as previously reported[43,44]).

Overexpression of Exogenous *MYCN* in SK-N-SH Neuroblastoma Cells

We next wanted to test whether overexpression of *MYCN* could affect induced differentiation of cultured neuroblastoma cells lacking *MYCN* amplification. In order to test this, we had to work in a non-*MYCN*-amplified cell system with the potential to differentiate neuronally, which excludes most, if not all, neuroblastoma cell lines currently available. We chose to work with SKMYC2 cells, which are SK-N-SH cells stably transfected with *MYCN*, for two major reasons: (i) the parental cells have the capacity to differentiate in response to a number of different treatment protocols, and (ii) the differentiated phenotypes are well characterized. To our knowledge, there is no other non-*MYCN*-amplified neuroblastoma cell line that meets these criteria. Furthermore, these transfected cells can be grown and selected in such a way that a majority of the cells retain their neuroblastic features, although the cultures always contain a small fraction of surface adherent, S-type-like cells.[27,45] In Figure 3, the *MYCN* expression in SKMYC2 and in neuroblastoma

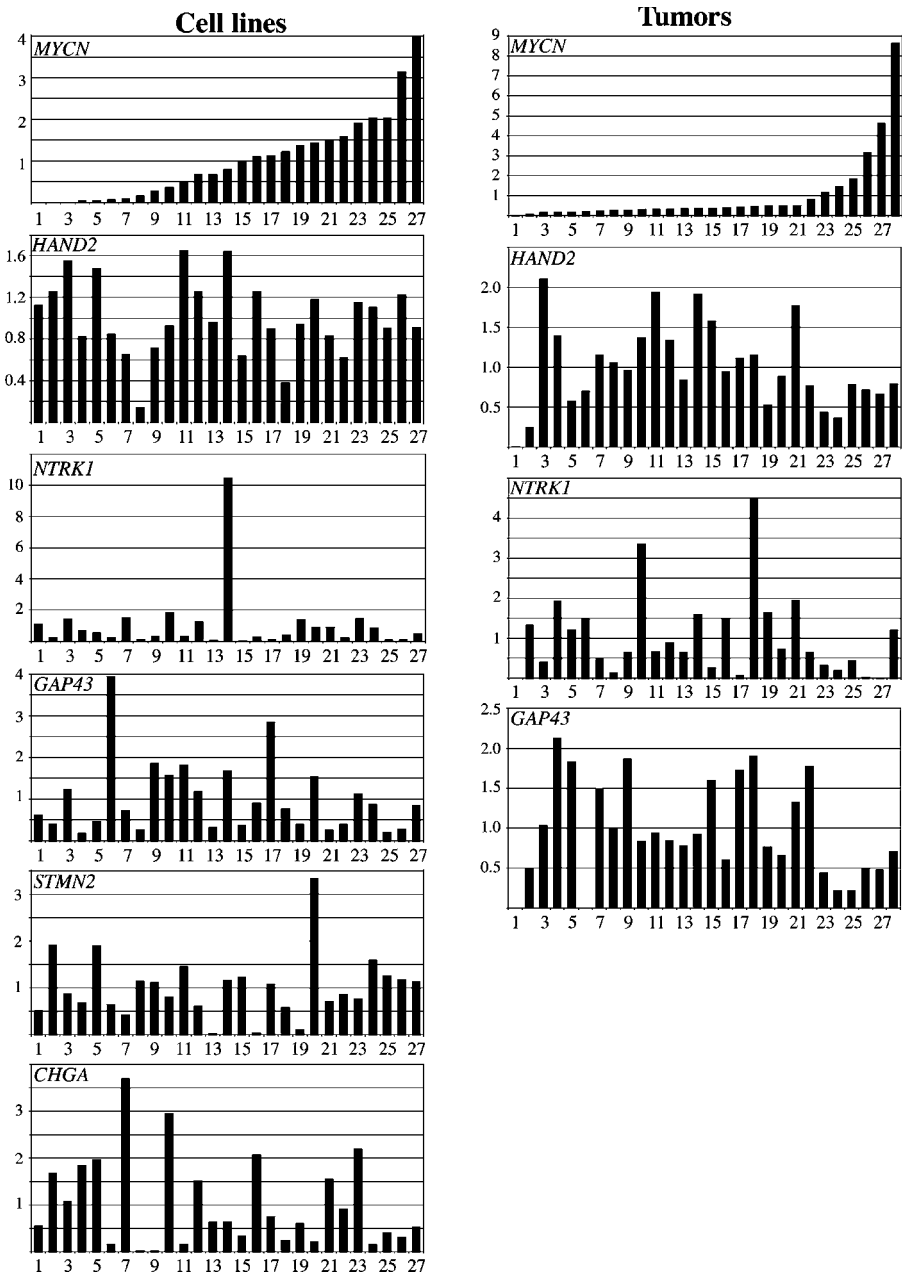

Figure 2 mRNA expression of *MYCN* and a set of marker genes for neuronal differentiation in neuroblastoma cell lines and tumors. In all, 27 neuroblastoma cell lines and 28 tumor specimens were analyzed using quantitative real-time RT-PCR for the expression of *MYCN*, *HAND2*, *NTRK1*, *GAP43*, *STMN2,* and *CHGA*. Of the 27 cell lines analyzed, 20 were amplified (samples 8–27), as were seven of the 28 analyzed tumors (samples 21, 23–28). Cell lines analyzed were (in order of increasing *MYCN* expression) SK-N-AS (1), SK-N-SH (2), LA-N-6 (3), SJNB-1 (4), CLB-GA (5), SK-N-FI (6), NBL-S (7), STA-NB-8 (8), NLF (9), SK-N-BE (10), STA-NB-10 (11), CHP 901 (12), STA-NB-3 (13), LA-N-5 (14), SJNB-6 (15), TR-14 (16), NMB (17), CHP 134 (18), IMR-32 (19), UHG-NP (20), SJNB-10 (21), LA-N-1 (22), N206 (23), SMS-KCNR (24), SJNB-8 (25), SMS-KAN (26), NGP (27).

cells with an amplified *MYCN* gene was compared. At the mRNA level, SKMYC2 cells displayed an *MYCN* expression lower than most *MYCN*-amplified neuroblastoma cell lines except for the LA-N-2 cells (Figure 3a), while the MYCN protein content in the transfected cells was in parity with most of the tested cell lines (Figure 3b). In nontransfected SK-N-SH cells and the derived subclones SH-EP and SH-SY5Y, *MYCN* mRNA or protein was barely detectable. The *MYC* expression is downregulated in neuroblastoma cells with amplified *MYCN*[43] and as expected, the overexpression of *MYCN* in SK-N-SH cells led to a decrease in *MYC* (Figure 3a). Furthermore, SKMYC2 cells downregulate the MYCN responsive gene *INHBA* (*angiogenesis inhibitor activin A*) (Figure 3c and Breit *et al*[46]), demonstrating that the protein resulting from the introduced *MYCN* gene in the SKMYC2 cells used in

Table 1 Correlation between MYCN mRNA levels and differentiation marker genes

	Cell lines (27)		*Tumors (28)*	
MYCN vs	*Spearman*	p	*Spearman*	p
MYC	−0.701[a]	4.67E-5[a]	−0.254[a]	0.192[a]
HAND2	−0.109	0.590	−0.180	0.359
NTRK1	−0.165	0.411	−0.153	0.438
GAP43	−0.134	0.506	−0.347	0.076
STMN2	0.140	0.487	n.t.	n.t.
CHGA	−0.200	0.317	n.t.	n.t.

Spearman's rank correlation coefficients between *MYCN* and *MYC*, *HAND2*, *NTRK1*, *GAP43 STMN2*, and *CHGA* mRNA levels in 27 neuroblastoma cell lines and 28 primary tumors (significance level is indicated; n.t.: not tested).
[a]Data taken from Vandesompele *et al*.[44]

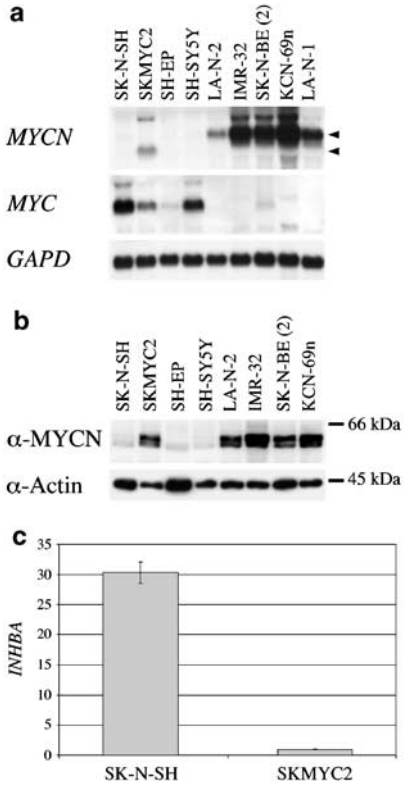

Figure 3 Expression of *MYCN* and *MYC* in neuroblastoma cell lines ((**a**), (**b**)) and downregulation of *INHBA* in SKMYC2 cells (**c**). (**a**) Northern blot analysis of the expression of *MYCN* and *MYC* in indicated cell lines. Total RNA (15 μg) was analyzed with *GAPD* mRNA levels (1.5 kb) as a loading control. The endogenous (2.7 kb) and exogenous (2.2 kb) *MYCN* transcripts are indicated by arrowheads. (**b**) Western blot analysis of MYCN protein levels in indicated cell lines, using actin protein levels as a loading control. A measure of 30 μg of whole cell protein lysate was loaded in each lane. The SKMYC2 cells analyzed in (**a**) and (**b**) were selected in 200 μg/ml of Hygromycin B. (**c**) Quantitative real-time RT-PCR analysis of *INHBA* expression in SK-N-SH and SKMYC2 cells. The relative expression levels are presented as mean expression $\pm$ s.d. of two independent cultures, with the expression in SKMYC2 cells set to one.

this study was functional (Figure 3a and c). The functionality of the transfected *MYCN* construct has further been demonstrated in previous studies showing altered β1 integrin subunit and NCAM levels, indicating that the normal integrin-dependent growth regulation had been short-circuited, a phenomenon not observed in mock-transfected cells.[27,47,48]

The SKMYC2 parental cell line, SK-N-SH, contains three morphologically distinct and interconvertible cell types, the N-type with neuroblastic properties (represented by the SH-SY5Y subclone), the surface-adherent S-type (represented by the SH-EP subclone), and intermediate forms, I-type cells.[49] In SK-N-SH cultures, N-type cells dominate and have the capacity to differentiate neuronally.[50] S-type cells virtually lack neuronal properties and the capacity to differentiate neuronally. In general, SKMYC2 cell cultures contained a smaller proportion of N-type cells than did cultures of untransfected SK-N-SH cells (data not shown), but the proportion of N-type cells increased as the SKMYC2 cultures grew denser (compare Figure 4a and c). To be able to correlate morphology and *MYCN* expression at a cellular level in *MYCN*-transfected and nontransfected cell clones, an immunofluorescence assay for MYCN in cultured cells was developed. All morphological subpopulations of SKMYC2 cells displayed a nuclear MYCN staining pattern (Figure 4a–d), in contrast to the weaker, probably non-specific, uniform fluorescence detected in the non-*MYCN*-transfected SK-N-SH and SH-EP cells (Figure 4e–h).

RA- and TPA-induced Differentiation of SKMYC2 Cells

To investigate if *MYCN* overexpression affects induced neuronal differentiation of neuroblastoma cells, SKMYC2, SK-N-SH, SH-SY5Y, and SH-EP cells were treated with 1 μM RA or 16 nM TPA, treatments previously shown to differentiate SH-SY5Y and SK-N-SH cells.[50,51] Addition of either TPA or RA (Figure 5a-f) to SKMYC2 cultures led to morphological differentiation of *MYCN* expressing N-type cells, seen as an extension of neurites with growth cones and varicosities, while S-type cells appeared unaffected. In line with these and previous

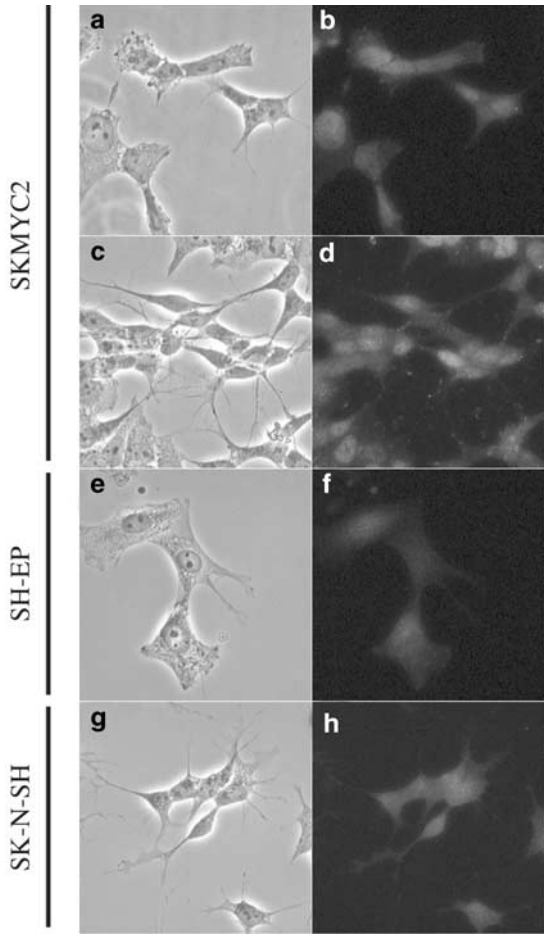

Figure 4 Morphology and MYCN expression in neuroblastoma cell lines. SKMYC2 cells grown in serum-containing medium and analyzed at a low cell density, day 1 ((**a**), (**b**)), and at a higher cell density at day 6 ((**c**), (**d**)). Cell morphology was assessed using phase-contrast microscopy ((**a**), (**c**)) and nuclear MYCN expression was detected by immunofluorescence ((**b**), (**d**). SH-EP ((**e**), (**f**)) and SK-N-SH ((**g**), (**h**)) cells grown for 3 days in serum-containing medium and analyzed by phase-contrast microscopy ((**e**), (**g**)) and for MYCN expression by immunofluorescence ((**f**), (**h**)).

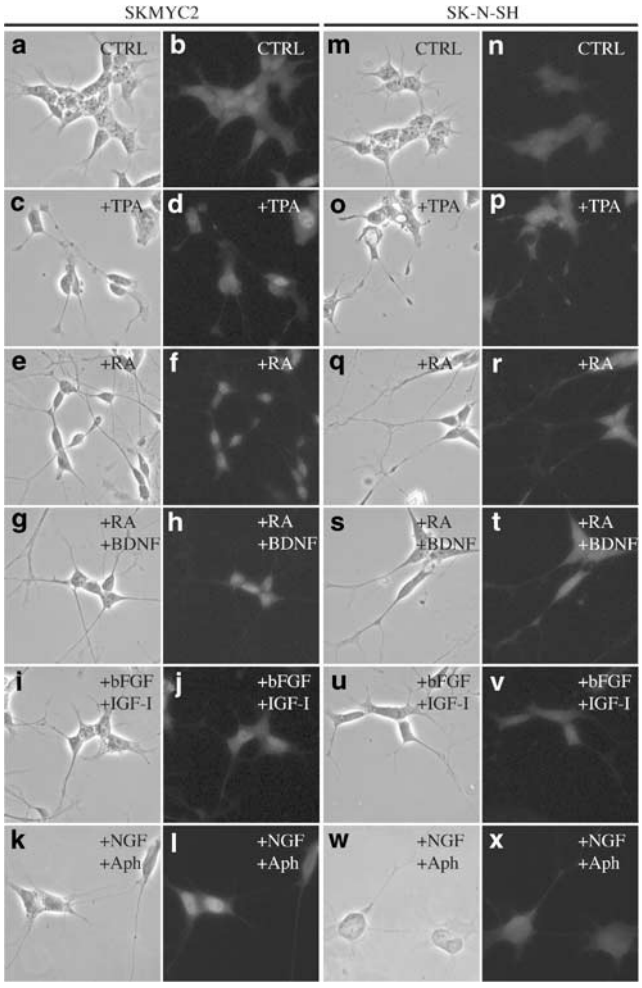

Figure 5 Morphological differentiation and MYCN expression in SKMYC2 ((**a**)–(**l**)) and SK-N-SH ((**m**)–(**x**)) cells treated with various differentiation-inducing agents. ((**a**)–(**d**)). SKMYC2 cells grown in serum-containing medium for 3 days with no additives ((**a**), (**b**)), or in the presence of 16 nM TPA ((**c**), (**d**)). ((**e**)-(**j**)). SKMYC2 cells grown in serum-containing medium for 6 days in the presence of 1 μM RA ((**e**), (**f**)), 1 μM RA + 100 ng/ml BDNF ((**g**), (**h**)), or 3 nM bFGF and 5 nM IGF-I ((**i**), (**j**)). ((**k**)–(**l**)). SKMYC2 cells grown in serum-containing medium for 4 days in the presence of 0.3 μM aphidicolin and 100 ng/ml NGF. ((**m**)-(**x**)). SK-N-SH cells grown for the same amount of time and with the same additives as the SKMYC2 analyzed in ((**a**)–(**l**)). Cell morphology was assessed using phase-contrast microscopy ((**a**), (**c**), (**e**), (**g**), (**i**), (**k**), (**m**), (**o**), (**q**), (**s**), (**u**), (**w**)) and nuclear MYCN expression detected by immunofluorescence ((**b**), (**d**), (**f**), (**h**), (**j**), (**l**), (**n**), (**p**), (**r**), (**t**), (**v**), (**x**)). Note the diffuse, uniform fluorescent staining in the SK-N-SH cells, contrasted to the distinct nuclear MYCN staining in the SKMYC2 cells.

results, virtually no cells in the SH-EP cultures responded to either RA or TPA (not shown), while morphologically differentiated cells in RA- and TPA-treated SK-N-SH cultures were abundant (Figure 5m–r).

SKMYC2 cells treated with RA and TPA were analyzed for the expression of the neuronal marker genes *NPY* and *GAP43*, using SH-SY5Y and SH-EP cells as references. TPA treatment led to an increased expression of *NPY* in SH-SY5Y as well as in SKMYC2 cells. Treatment with RA resulted in a downregulation of *NPY* expression in both cell systems (Figure 6a), consistent with the development of a cholinergic phenotype, as demonstrated previously.[52,53] The SH-SY5Y response was more pronounced and mimicked that of the SK-N-SH cells (Figure 6a and b). SH-EP did not express *NPY* regardless of treatment. Treatment with RA resulted in a slight upregulation of the *GAP43* expression in SH-SY5Y, SKMYC2, and SK-N-SH cells, while TPA clearly upregulated *GAP43* in these cells (Figure 6a and b), as previously reported in SH-SY5Y cells.[53,54]

The *NPY* expression in the TPA-treated SKMYC2 cells was low compared to the expression in the correspondingly treated SK-N-SH and SH-SY5Y cells (Figure 6a and b). This difference could be explained by the comparatively high proportion of S-type cells, expressing low amounts of *NPY*, present in the SKMYC2 cultures. To estimate the

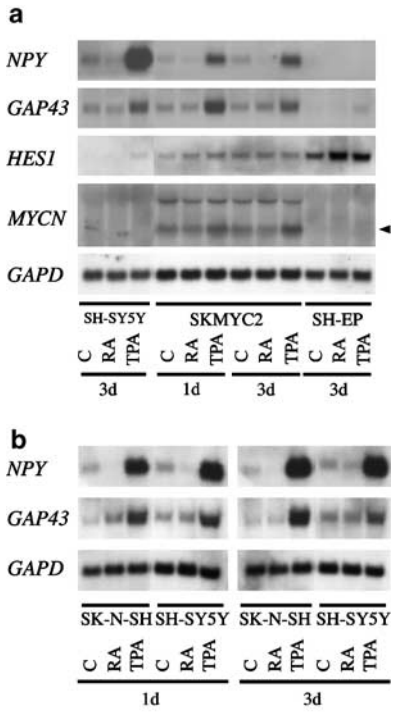

Figure 6 Neuronal marker gene expression in RA- and TPA-treated SKMYC2, SH-SY5Y, SK-N-SH, and SH-EP cells. (**a**) Northern blot analysis of the expression of the differentiation marker genes *NPY* (0.8 kb mRNA) and *GAP43* (1.4 kb mRNA), epithelial marker gene *HES1* (1.7 kb mRNA), and *MYCN* (2.2 kb mRNA) in SH-SY5Y, SKMYC2, and SH-EP cells. Cells were grown for 1 day or 3 days in serum-containing medium with the addition of 1 μM RA and 16 nM TPA, where indicated. Total RNA (15 μg) from all samples was analyzed on one gel for expression of the genes above with *GAPD* mRNA levels (1.5 kb mRNA) as a loading control. (**b**) Northern blot analysis of the expression of the differentiation marker genes *NPY* and *GAP43* in SK-N-SH and SH-SY5Y cells, grown for 1 day or 3 days in serum-containing medium. The exogenous (2.2 kb) *MYCN* transcript is indicated by the arrowhead. Total RNA (15 μg) from all samples was analyzed on one gel with *GAPD* mRNA levels as a loading control.

mRNA contribution from S-type cells, *HES1* expression was analyzed as *HES1* mRNA is abundant in SH-EP cells, but no or little mRNA can be detected in SH-SY5Y cells (Figure 6a and Grynfeld *et al*[33]). As shown in Figure 6a, the SKMYC2 cells had higher *HES1* mRNA levels than the SH-SY5Y cells, confirming a substantial contribution of S-type cell mRNA in these assays.

Growth Factor-induced Differentiation of SKMYC2 Cells

To extend the analysis of the capacity of the SKMYC2 cells to differentiate, we utilized additional protocols known to induce differentiation of SH-SY5Y cells, including protocols employing growth factors of importance in normal sympathetic development.[55–57] SKMYC2 and SK-N-SH cells were treated with 1 μM RA or a combination of 1 μM RA and 100 ng/ml BDNF, which induced neurite outgrowth of neuroblastic but not S-type cells in both SK-N-SH and SKMYC2 cultures (Figure 5g, h, s and t). Notably, the differentiated SKMYC2 cells retained a nuclear MYCN staining, suggesting that they differentiated despite a high MYCN expression (Figure 5h). Following another published differentiation protocol, the cells were treated with 3 nM bFGF and 5 nM IGF-I.[56] Both SKMYC2 and SK-N-SH cells differentiated morphologically in response to these growth factors, and nuclear staining of MYCN was again seen in SKMYC2 cells with long neurites (Figure 5i,j,u and v). Finally, the cells were differentiated with NGF (100 ng/ml) in combination with aphidicolin (0.3 μM), a cell cycle blocking agent, which induces *NTRK1* expression and NGF responsiveness in SH-SY5Y cells.[57–59] As aphidicolin blocks cell division, the number of cells in these cultures was comparatively low and N-type neuroblastoma cells were few. However, the cells present extended long neurites in the presence of NGF (Figure 5k,l,w and x), and the differentiated SKMYC2 cells retained a nuclear MYCN staining pattern (Figure 5l). Interestingly, the aphidicolin/NGF-treated SKMYC2 cells had considerably longer neurites than SK-N-SH cells treated in the same way (data not shown).

Differentiating SKMYC2 Cells Retain a High *MYCN* Expression

Neuroblastoma cells endogenously overexpressing *MYCN* as a result of gene amplification downregulate

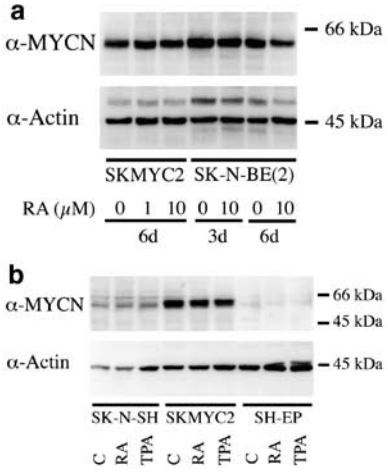

Figure 7 Expression of MYCN in differentiating neuroblastoma cells, as determined by Western blot analysis, using actin protein levels as a loading control. (**a**) MYCN protein levels in 30 μg of whole cell lysate from SK-N-BE (2) and SKMYC2 cells cultured for 3 or 6 days in serum-containing medium in the presence of 1 or 10 μM RA as indicated. (**b**) MYCN protein levels in 60 μg of whole cell lysate from SK-N-SH, SKMYC2, and SH-EP cells cultured in serum-containing medium for 3 days in the presence of 1 μM RA or 16 nM TPA, where indicated. Molecular weight marker positions are indicated in the right-hand margin.

MYCN during induced differentiation.[55–57] To exclude the possibility that the effect of exogenous *MYCN* was lost due to downregulation of *MYCN* in differentiating SKMYC2 cells, *MYCN* expression levels were analyzed in more detail. The *MYCN*-amplified SK-N-BE (2) cells, which differentiate with RA, served as a positive control. In cells treated for 3 and 6 days, MYCN protein levels decreased slightly, as expected (Figure 7a), while the expression in SKMYC2 cells was virtually unaffected when treated with 1 or 10 μM RA. Furthermore, SK-N-SH, SKMYC2, and SH-EP cells treated with TPA or RA for 3 days were analyzed for MYCN protein content. Neither of the treatments substantially changed the MYCN protein levels (Figure 7a and b), in agreement with mRNA expression and immunofluorescence data in Figures 5 and 6.

Discussion

The important role of *MYCN* in early embryonal development is illustrated by profound disturbances and early embryonic death of homozygous *MYCN* knockout mice. However, the role of *MYCN* in cellular differentiation in relation to neuroblastoma development has not yet been fully addressed. Detailed *MYCN* expression studies performed in mice show that *MYCN* is preferentially expressed at sites with ongoing cell differentiation, tissue maturation, and organogenesis.[13–15] In agreement with these results, we recently observed that in human neuroblastoma cells the loss of a differentiated phenotype induced by hypoxia was paralleled by *MYCN* downregulation in all studied *MYCN*-amplified cell lines.[60] We have found that *MYCN* is expressed in human sympathetic neuroblasts at a comparatively late developmental stage (week 8.5). Clearly, neuroblasts are at this stage committed to sympathetic neuronal development and express several sympathetic marker genes such as *TH*, *HAND2*, *GAP43*, *NPY*, *BCL2*, and *STMN2*.[38–40] Unlike most neuroblastoma cells, these neuroblasts have passed the stage of *ASCL1* (*HASH-1)* expression.[40] *ASCL1* is involved in the determination of the sympathetic ganglionic lineage,[61] suggesting that normal sympathetic neuronal differentiation beyond the stage at which most neuroblastoma cells appear to be arrested proceeds in the presence of a significant *MYCN* expression.

To further investigate the putative role of *MYCN* in controlling cellular differentiation, we analyzed the phenotypic response of non-*MYCN*-amplified neuroblastoma cells with constitutive exogenous overexpression of *MYCN* to several established differentiation protocols. Under all conditions, these cells were shown to retain their capacity to differentiate morphologically. In none of these settings, the differentiation was accompanied by any substantial reduction of *MYCN* mRNA or protein levels, clearly indicating that differentiation could proceed in the presence of high *MYCN* expression. We further studied the expression levels of well-established sympathetic nervous system neuronal/neuroendocrine differentiation markers in neuroblastoma cell lines and tumors. Our results clearly demonstrate an absence of correlation between the expression levels of differentiation markers and *MYCN*, suggesting the results from the SKMYC2 system to be relevant also *in vivo*.

These findings appear to contradict *in vitro* data demonstrating that overexpression of *MYCN* in human neuroblastoma cells and rat cells derived from the brain blocks induced differentiation.[18,19,62] A role for the maintenance of the immature phenotype is also suggested by the high incidence of *MYCN* amplification and overexpression in a subset of high stage, less-differentiated neuroblastomas. Overexpression of *MYCN* in non-*MYCN*-amplified neuroblastoma cells and the impact on differentiation have not been studied in a neuronal context, as previous *MYCN* overexpression studies in non-*MYCN*-amplified neuroblastoma cells have mainly utilized SH-EP cells.[10,11,19] These cells virtually lack neuroblastic properties (Ross *et al*,[49] Rettig *et al* [63] and this report) and cannot be induced to differentiate neuronally by existing differentiation protocols (this report and unpublished observations). Thus, this cell system would not be expected to be informative when studying putative effects of *MYCN* on differentiation into a neuronal sympathetic neuroblastoma phenotype. Nor does the recently published study in which *MYCN* overexpression in rat postmitotic sympathetic neurons elicited the capacity of these cells to re-enter cell cycle (but not enhanced survival) necessarily exclude a specific role for *MYCN* during differentiation.[9] It is possible that the differences in outcome between the present study and the studies using *MYCN*-amplified cell lines[18,19] result from differences in the total *MYCN* expression levels and that extreme levels of *MYCN* expression will antagonize a neuronal sympathetic phenotype, thus assuming a functional threshold for this effect of *MYCN*.[6] This would be in line with a recent report showing that overexpression of *MYCN* can block terminal differentiation of sensory neurons.[64] However, the MYCN protein content in the SKMYC2 cells is in parity with that of some neuroblastoma cell lines with amplified *MYCN* and, more importantly, no signs of a correlation between *MYCN* expression and expression of differentiation marker genes in neuroblastoma tumors and cell lines could be detected. This was true even when only those tumors with the highest *MYCN* expression were considered. *In vivo*, not only the absolute level of expression, but also the regulation of expression, for example, in response to stress has to be considered. In this respect, the behavior of SKMYC2 cells might be expected to be closer to that of *MYCN*-amplified cells than that of high *MYCN* expressing cells without *MYCN* amplification.

If the link between *MYCN* amplification and poor prognosis cannot be explained by blocked differentiation, what are the alternative explanations? It has been shown that *MYCN* can functionally replace *MYC* in murine development[65] and we can assume that *MYCN*, like *MYC*, is involved in a variety of pathways that, when disturbed, can contribute to cancer development. Presumably, the pathways affected vary depending on the differentiation status and the presence of additional genetic changes. Furthermore, *MYCN* amplification is a result of genetic instability, which in itself is associated with poor prognosis and tumor progression. The picture is further obscured by the fact that the varying MYCN expression seen in low-stage neuroblastomas might instead reflect the differentiation and proliferation status of the tumor cells. In view of this limited understanding of the molecular basis of *MYCN* gene regulation and the possibility that *MYCN* forms an integral part of a subtle transcriptional network, dependent on cell type and differentiation status, we can only speculate on the exact nature of the role of *MYCN* in neuroblast differentiation. Thus, further experiments using different overexpressing systems in parallel with identification of *bona fide MYCN* target genes are needed. However, the data presented here strongly suggest that expression of *MYCN* is compatible with normal sympathetic neuronal development and that neuronal differentiation of neuroblastoma cells is not blocked by high *MYCN* levels.

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