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Tumorigenesis and Neoplastic Progression

Bmi-1 Is Essential for the Tumorigenicity of Neuroblastoma Cells

Hongjuan Cui,* Bo Hu,† Tai Li,* Jun Ma,* Goleeta Alam,* William T. Gunning,* and Han-Fei Ding*

From the Department of Biochemistry and Cancer Biology, Medical University of Ohio, Toledo, Ohio; and the Department of Neurology,† Union Hospital, Huazhong University of Science and Technology, Wuhan, China*

Activation of oncogenes underlies the pathogenesis of most human cancers. In neuroblastoma, amplification of the oncogene *MYCN* occurs in ~22% of cases and is associated with advanced stages of the disease and poor prognosis. Identification of other oncogenes that are consistently mutated or overexpressed in neuroblastoma is crucial for a molecular understanding of the pathogenic process. Here, we report that the oncogene *Bmi-1* is highly expressed in human neuroblastoma cell lines and primary tumors. Neuroblastoma development in *MYCN* transgenic mice, an animal model for the human disease, was associated with a marked increase in the levels of Bmi-1 expression. Bmi-1 cooperated with MYCN in transformation of benign S-type neuroblastoma cells and avian neural crest cells by inhibiting the apoptotic activity of MYCN. Importantly, down-regulation of Bmi-1 impaired the ability of neuroblastoma cells to grow in soft agar and induce tumors in immunodeficient mice. Moreover, Bmi-1-knockdown neuroblastoma xenografts were characterized by a significant increase in the amount of Schwannian stroma, a histological feature associated with clinically favorable neuroblastomas. These findings suggest a crucial role for Bmi-1 in neuroblastoma pathogenesis and provide insights into the molecular basis of neuroblastoma heterogeneity. ***(Am J Pathol 2007, 170:1370–1378; DOI: 10.2353/ajpath.2007.060754)***

Neuroblastoma is a common childhood malignant tumor of neural crest origin, arising in the sympathetic nervous system. Among the genetic alterations identified in neuroblastoma, amplification of the oncogene *MYCN* is strongly associated with highly malignant behavior and poor prognosis.[1,2] Importantly, transgenic mice with targeted expression of human *MYCN* in neural crest-derived cells develop neuroblastomas that mirror the human disease with respect to anatomical locations, histopathology, and syntenic chromosomal gains and losses,[3] demonstrating directly that MYCN overexpression can be an initiating event in the pathogenesis of neuroblastoma. However, *MYCN* amplification occurs in only ~22% of neuroblastoma cases, and sequential analysis of neuroblastoma samples indicates that patients with no *MYCN* amplification at diagnosis rarely acquire it later during tumor progression.[1] Thus, most neuroblastomas arise from distinct genetic and/or epigenetic alterations. To date, there has been no consistent evidence for activation of any other oncogenes in this disease.[4]

Bmi-1 is a member of the polycomb group family of transcriptional regulators that was originally identified as an oncogenic partner of c-Myc in murine lymphomagenesis.[5,6] Mice lacking Bmi-1 display defects in hematopoiesis and development of the central and peripheral nervous systems.[7,8] Further studies have revealed that Bmi-1 is required for the self-renewal capacity of several types of normal and cancer stem cells, including neural crest stem cells from the peripheral nervous system.[9–12] More recently, it has been shown that Bmi-1 is strongly expressed in primary neuroblastomas.[13] In this study, we examined the functional significance of Bmi-1 expression in neuroblastoma cells.

Materials and Methods

Mice

MYCN transgenic mice[3] and NOD.SCID/NCr mice were maintained under specific pathogen-free conditions at the animal facility of the Medical University of Ohio. All animal experiments were preapproved by the Institutional

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Address reprint requests to Han-Fei Ding, Department of Biochemistry and Cancer Biology, Medical University of Ohio, 3035 Arlington Ave., Toledo, OH 43614. E-mail: hding@meduohio.edu.

Animal Care and Use Committee of the Medical University of Ohio.

Cell Culture

Human neuroblastoma cell lines SHEP1, SK-N-AS, SK-N-F1, SK-N-DZ, and SK-N-SH were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS); IMR32, SK-N-BE(2), BE(2)-C, SY5Y, and LA1–55N in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F12 supplemented with 10% FBS and nonessential amino acids; LAN-6, SMS-KCNR, and SMS-SAN in RPMI 1640 supplemented with 10% FBS. Human glioblastoma cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS. The growth media and FBS were obtained from Invitrogen (Carlsbad, CA). Quail (*Coturnix coturnix japonica*) neural crest cells were isolated from stage 13 or 14 quail embryos as previously described.[14] In brief, fertilized quail eggs (Bear Bayou Quail Farm, Toledo, OH) were incubated at 38°C for ~2 days in a forced draft, humidified incubator. The neural tube and associated somites were surgically dissected from embryos, digested with 0.5% collagenase A (Sigma, St. Louis, MO) for 15 minutes at room temperature, and plated on dishes coated with fibronectin (Sigma). After 16 hours the neural tube explants were removed, and the neural crest cells that had migrated from the neural tube were cultured in Eagle's minimal essential medium supplemented with 15% horse serum and 2% 11-day chick embryo extract.

Retroviral Constructs and Infection

The retroviral constructs pBabe-GFP, pBabe-hygro/MYCN, and pBabe-puro/Bmi-1[15] were used in overexpression studies. For down-regulation of Bmi-1, synthesized 64-bp oligonucleotides containing the human Bmi-1 siRNA sequences (5′-ATGAAGAGAAGAAGGGATT-3′ and 5′-AATGGACATACCTAATACT-3′, positions 269 to 287 bp and 546 to 574 bp relative to the start codon, respectively) were cloned into the *Hind*III and *Bgl*II sites in pSuper-retro to generate pSuper-retro/Bmi-1si no. 1 and no. 2. pSuper-retro-GFPsi [expressing green fluorescent protein (GFP) siRNA] or pSuper-retro (vector) were used as controls. Retroviral production and infection were performed as described previously.[16] One day after the final round of infection, cells were cultured in the presence of puromycin and/or hygromycin B for 3 days, and drug-resistant cells were pooled.

Histology, Immunohistochemistry, and Immunofluorescence

For histological examination, paraffin-embedded samples were sectioned at 5 μm and stained with hematoxylin and eosin (H&E). For immunohistochemistry, the sections were deparaffinized, rehydrated, and treated with 10 mmol/L citrate buffer (pH 6.0) at 95°C to retrieve antigens. After quenching of endogenous peroxidase activity with H_2O_2 and blocking with normal goat serum, the sections were incubated sequentially with primary antibodies, rabbit anti-Bmi-1 (1:50; Abgent, San Diego, CA) or mouse anti-p16^{Ink4a} (F-12, 1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and secondary antibodies, biotinylated goat anti-rabbit or anti-mouse IgG, and the ABC reagent (Vector Laboratories, Burlingame, CA). The staining with mouse monoclonal antibodies was performed using the M.O.M. kit according to the manufacturer's instructions (Vector Laboratories). The immunostaining was visualized with 3,3′-diaminobenzidine (Sigma). The sections were then counterstained with hematoxylin. For immunofluorescence, tumor sections were incubated sequentially with primary antibodies, rabbit anti-S100 (1:400; DAKO, Carpinteria, CA), rabbit anti-glial fibrillary acidic protein (1:200, DAKO), or anti-GFP (mouse, 1:50, Santa Cruz; rabbit, 1:200, Abcam), and secondary antibodies, fluorescein isothiocyanate-anti-rabbit IgG, fluorescein isothiocyanate-anti-mouse IgG, or Alexa Fluor 594-anti-rabbit IgG (1:500; Molecular Probes, Eugene, OR). Nuclei were stained with 4′,6′-diamidino-2-phenylindole (300 nmol/L; Molecular Probes). Human neuroblastoma specimens were obtained from the Union Hospital, Wuhan, China, with the approval of the Office for Scientific Research of the hospital and the Institutional Review Board of the Medical University of Ohio.

Immunoblotting

Cells were suspended in standard sodium dodecyl sulfate sample buffer. Protein concentrations were determined with a Bio-Rad (Hercules, CA) protein assay kit, using bovine serum albumin as reference. Fifty μg of protein was separated on 10% sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with monoclonal antibodies against either MYCN (AB-1; Oncogene Research, San Diego, CA), Bmi-1 (229F6; Upstate Technology, Lake Placid, NY) or α-tubulin (B-5-1-2; Sigma) or polyclonal rabbit anti-p53 (FL-393, 1:500; Santa Cruz). Horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (ICN, Irvine, CA) was used as the secondary antibody. Proteins were visualized with a SuperSignal West Pico chemiluminescence kit (Pierce, Rockford, IL) and quantified with a Kodak Image Station 440CF (Eastman-Kodak, Rochester, NY).

Soft Agar Clonogenic and in Vivo *Tumorigenic Assays*

For soft agar assays, 1 to 2 $\times$ 10^3 cells were mixed with 0.3% Noble agar in growth medium and plated onto six-well plates containing a solidified bottom layer (0.6% Noble agar in growth medium). Colonies were photographed after 14 to 21 days and scored. For tumorigenic assays, two to three NOD/SCID mice were used for each control and Bmi-1-knockdown cell line, and for each mouse, both flanks were injected subcutaneously with

either 1×10^7 SK-N-AS, 2×10^7 SK-N-DZ, or 5×10^6 BE(2)-C cells in 200 μl of Dulbecco's modified Eagle's medium. Tumor growth was estimated by caliper measurements and tumor volume was calculated with the formula $4/3\pi r^3$, where r is the radius of the tumor.[17] Two to 6 weeks after injection, tumors that formed at the injection sites were removed, weighed, and paraffin-embedded. Tumor sections were subjected to H&E staining for histological examination and immunofluorescent staining for marker expression analyses as described above.

Apoptosis Analysis

Neuroblastoma cells were either untreated or treated with 0.1 μg/ml of TRAIL (Calbiochem, San Diego, CA), 0.5 μg/ml of an agonistic anti-Fas antibody (clone CH-11; Upstate Technology) or 0.5 μg/ml doxorubicin (Ben Venue Laboratories, Bedford, OH). One day after treatment, adherent and floating cells were collected and analyzed for apoptosis by annexin-V staining using a Guava cytometer.

Statistical Analysis

For soft agar colony formation, apoptosis, and *in vivo* tumorigenic assays, values were obtained from three independent experiments (or three to five tumor samples) as described above and loaded into Microsoft Excel data sheets. Means ± SD were calculated, and two-tailed Student's *t*-test was performed for paired samples (eg, control versus Bmi-1 overexpression or down-regulation), using the data analysis tools provided by the software. A P value <0.05 is considered statistically significant.

Results

High-Level Expression of Bmi-1 in Human Neuroblastoma Cell Lines and Primary Tumors

In agreement with a recent study showing strong Bmi-1 expression in primary human neuroblastoma specimens,[13] our immunoblot analysis revealed high-level expression of Bmi-1 in 12 neuroblastoma cell lines examined (Figure 1A, top). Seven of the 12 cell lines also showed significant levels of MYCN expression (Figure 1A, middle). In contrast, Bmi-1 is expressed at very low or undetectable levels in human glioblastoma cell lines (Figure 1B), a brain tumor of glial origin. We also examined Bmi-1 expression in 45 primary human neuroblastoma specimens by immunohistochemistry. A significant number of cells in all of the tumor samples showed strong nuclear Bmi-1 staining (Figure 1C for a representative sample). These data indicate that high-level expression of Bmi-1 is a common feature of neuroblastoma cells.

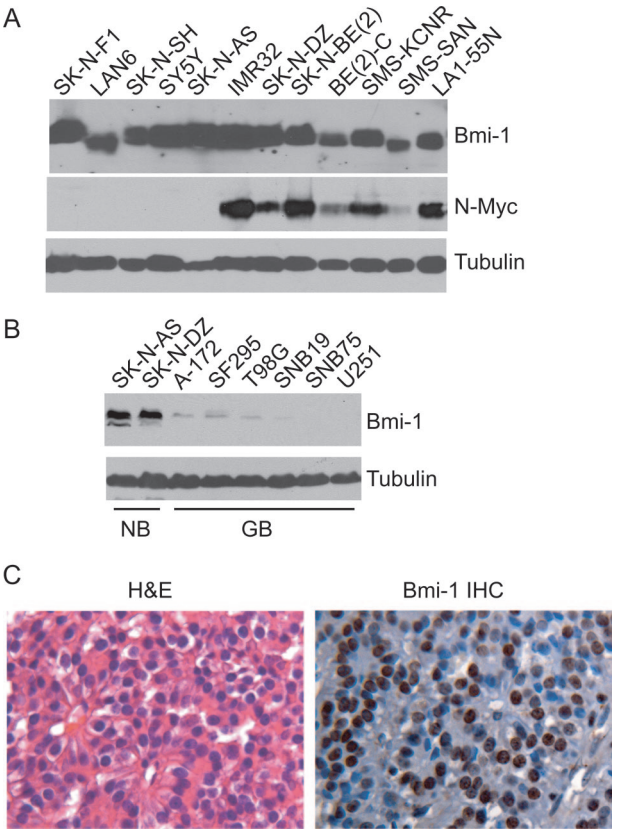


Figure 1. Bmi-1 is highly expressed in human neuroblastoma cell lines and primary tumors. **A** and **B:** Immunoblot analysis of Bmi-1 and/or MYCN expression in neuroblastoma (NB) and glioblastoma (GB) cell lines. α-Tubulin levels are shown as loading control. **C:** Left, H&E staining of a representative primary human neuroblastoma sample; right, immunohistochemical (IHC) staining for Bmi-1 expression in the same tumor sample. A significant number of tumor cells show strong nuclear staining of Bmi-1 (brown). Original magnifications, ×400.

High-Level Expression of Bmi-1 in Primary Neuroblastomas Developed in MYCN Transgenic Mice

MYCN transgenic mice carry human *MYCN* under the control of the tyrosine hydroxylase gene promoter,[3] which is active in migrating neural crest stem cells during early development of the sympathetic system.[18] *MYCN* mice develop neuroblastomas that closely resemble the human disease with respect to the site and morphology of tumors and gain and loss of chromosomal regions. The mouse neuroblastomas are mostly composed of small blue neuroblasts, in contrast to the large mature ganglion cells in normal sympathetic ganglia (Figure 2A). To assess further the role of Bmi-1 in the pathogenesis of neuroblastoma, we examined Bmi-1 expression in primary mouse neuroblastomas and sympathetic ganglia from wild-type littermates by immunohistochemistry. Strong nuclear staining for Bmi-1 was observed in a majority of cells in all of the tumor samples examined (Figure 2B for a representative sample). In contrast, no significant level of Bmi-1 expression was observed in the cells of superior cervical ganglia (SCG) from wild-type mice (Figure 2B). Consistent with the role of Bmi-1 in

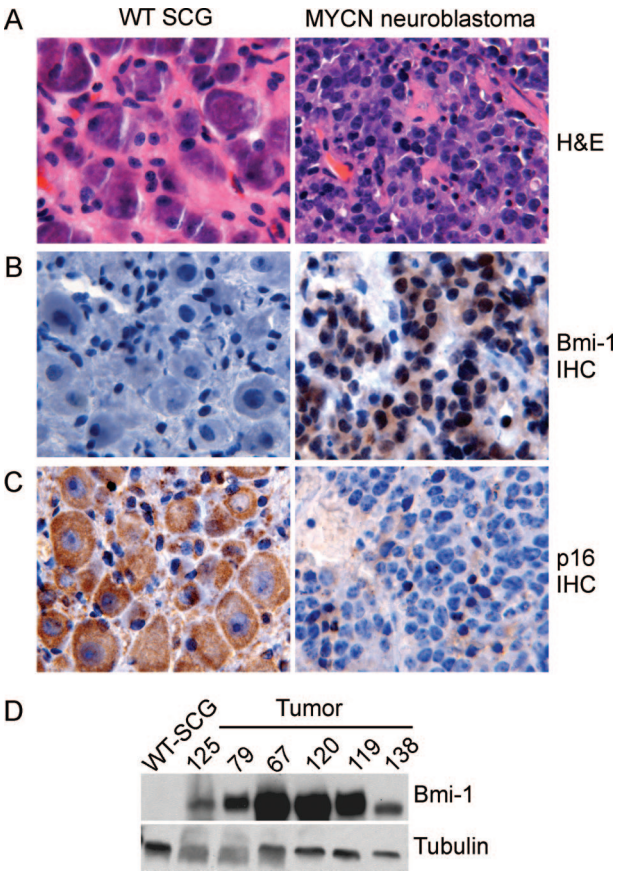


Figure 2. Bmi-1 is highly expressed in primary neuroblastoma tumors from *MYCN* transgenic mice. **A:** H&E staining of an SCG (left) from an 8-week-old wild-type mouse and a neuroblastoma sample (right) from a homozygous *MYCN* mouse of the same age. Note the large mature ganglion cells in the SCG. The neuroblastoma tumor is composed of mostly small blue cells (neuroblasts). **B** and **C:** Immunohistochemical staining of the same SCG and neuroblastoma samples for Bmi-1 (**B**) and $p16^{Ink4a}$ (**C**) expression (brown). Cells were counterstained with hematoxylin. Original magnifications, ×400. **D:** Immunoblot analysis of Bmi-1 expression in wild-type SCG and MYCN neuroblastoma tumors. α-Tubulin levels are shown as loading control.

repression of the Ink4a-ARF locus,[19] wild-type ganglion cells expressed high levels of $p16^{Ink4a}$ in the cytoplasm, whereas tumor cells showed a marked reduction of $p16^{Ink4a}$ expression (Figure 2C). We further confirmed these results by immunoblotting, which revealed no detectable levels of Bmi-1 in normal sympathetic ganglion cells but high-level expression of Bmi-1 in all of the mouse neuroblastoma samples examined (Figure 2D). Together, these data show that neuroblastoma development in *MYCN* transgenic mice is associated with a marked increase in the levels of Bmi-1 expression.

Bmi-1 Overexpression Cooperates with MYCN in Cellular Transformation

The observation that Bmi-1 expression is significantly up-regulated in neuroblastomas developed in *MYCN* transgenic mice suggests that the two oncogenes may cooperate in driving the tumorigenic process. We investigated this possibility in cellular transformation studies. The human neuroblastoma cell line SHEP1 is a subclone of the neuroblastoma cell line SK-N-SH[20] that contains no *MYCN* amplification.[21] SHEP1 cells express low levels of MYCN and Bmi-1 (Figure 3A, GFP) and have a benign phenotype, being unable to grow in soft agar (Figure 3B, GFP) or to induce tumors in immunodeficient mice (data not shown). The human *MYCN* and *Bmi-1* genes were introduced, individually or sequentially, into SHEP1 cells by retroviral infection (Figure 3A). SHEP1 cells infected with GFP-, MYCN-, or Bmi-1-expressing retroviruses failed to grow in soft agar (Figure 3B). However, the cells infected with both MYCN- and Bmi-1-expressing retroviruses were able to grow into large colonies ($^{**}P < 0.01$), demonstrating that the two oncogenes cooperate in transformation of SHEP1 cells.

As an established cell line, SHEP1 cells may harbor genetic lesions necessary for the transformation by MYCN and Bmi-1. We therefore asked whether the two oncogenes could also cooperate to transform normal cells of neural crest origin. Neural crest cells were isolated from quail embryos and infected, either individually or sequentially, with MYCN- or Bmi-1-expressing retroviruses. Again, only the neural crest cells infected with both MYCN- and Bmi-1-expressing retroviruses were able to grow into colonies in soft agar (Figure 3C; $^{**}P < 0.01$), demonstrating that the two oncogenes also cooperate in transformation of normal neural crest cells.

Bmi-1 Inhibits the Apoptotic Activity of MYCN

Amplification of *MYCN* is associated with aggressively growing neuroblastomas.[1,2] However, MYCN also sensitizes cells to apoptosis,[22–24] suggesting that inactivation of apoptotic pathways through cooperating oncogenes may be necessary for the pathogenesis of neuroblastoma with *MYCN* amplification. Notably, it has been reported that Bmi-1 cooperates with c-Myc in lymphomagenesis by blocking apoptosis.[25] Thus, inhibition of the apoptotic activity of MYCN could be one mechanism for the cooperative oncogenic activity of Bmi-1. We examined this possibility by assessing the ability of Bmi-1 to inhibit the apoptotic activity of MYCN in SHEP1 cells. As reported previously,[22] SHEP1 cells overexpressing MYCN showed a marked increase in their sensitivity to apoptosis induced by a variety of agents (Figure 3D; $^{*}P < 0.05$; $^{**}P < 0.01$). In contrast, SHEP1 cells with overexpression of both MYCN and Bmi-1 were highly resistant to all of these apoptosis-inducing agents (Figure 3D; $^{**}P < 0.01$), demonstrating that Bmi-1 inhibits the apoptotic activity of MYCN.

Bmi-1 represses the expression of $p14^{ARF}$, an inhibitor of MDM2 that targets p53 for ubiquitin-dependent degradation.[19,26] Consistent with the notion, SHEP1 cells overexpressing Bmi-1 showed marked repression of p53 expression (Figure 3E). Because p53 up-regulates a large group of apoptotic genes critical for DNA damage- and death receptor-induced apoptosis,[27,28] its repression probably contributes to the anti-apoptotic activity of Bmi-1.

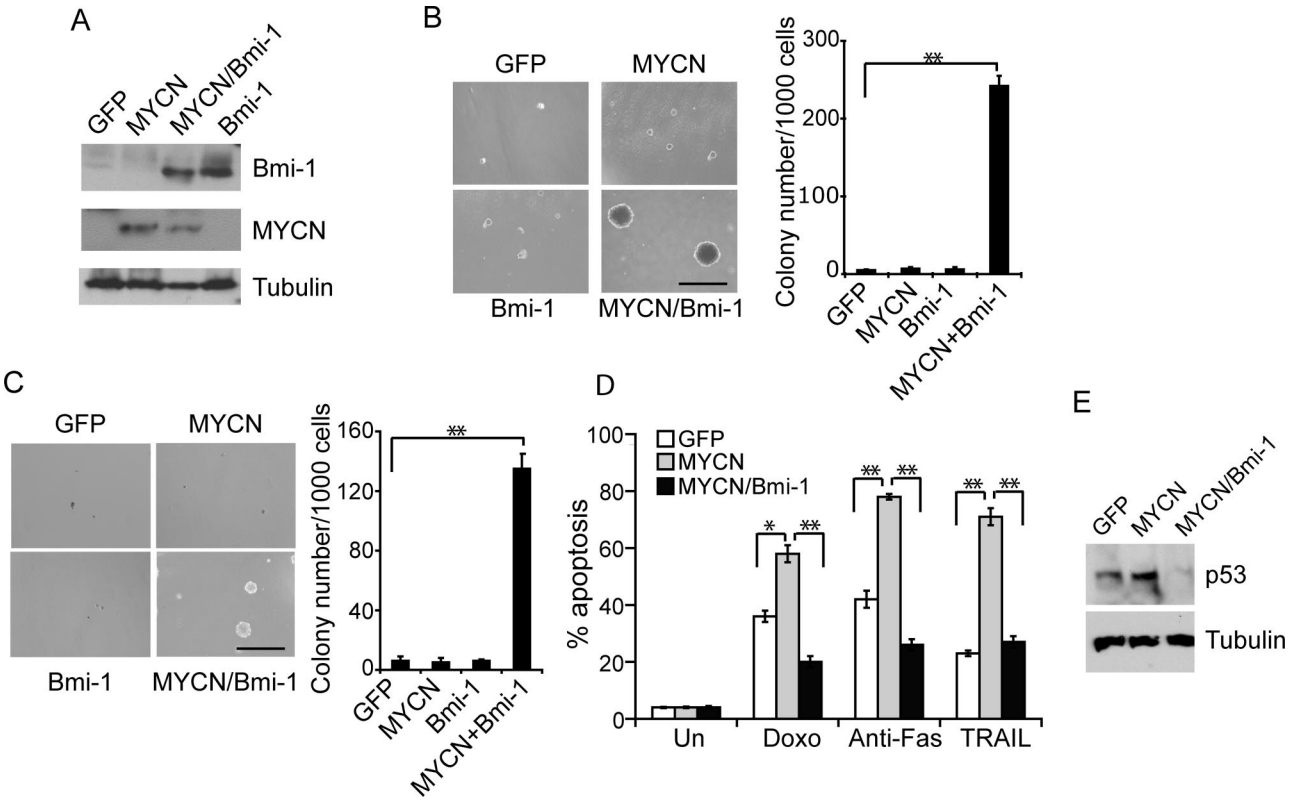


Figure 3. Bmi-1 cooperates with MYCN in transformation. **A:** Immunoblot analysis of Bmi-1 and MYCN overexpression in SHEP1 cells. α-Tubulin levels are shown as loading control. **B** and **C:** Soft agar colony growth by the indicated SHEP1 cell lines and quail neural crest cells after 14 to 21 days of culture. Also shown are numbers of colonies per 1000 plating cells. Colonies that contained more than 50 cells or were larger than 0.5 mm were scored. Each bar represents the average ± SD of three independent experiments. Scale bars = 2 mm. **D:** Bmi-1 abrogates MYCN-induced sensitization of SHEP1 cells to apoptosis induced by the DNA damage drug doxorubicin (0.5 μg/ml), agonistic anti-Fas antibody (0.5 μg/ml), or TRAIL (0.1 μg/ml). Cells were collected 24 hours after treatment and analyzed for apoptosis by annexin-V staining. Each bar represents the average ± SD of three independent experiments. Statistical analyses (**B–D**) were performed using two-tailed Student's *t*-test; $^{*}P < 0.05$; $^{**}P < 0.01$. **E:** Immunoblot analysis of p53 expression in SHEP1 cells expressing GFP, MYCN, or MYCN and Bmi-1. α-Tubulin levels are shown as loading control.

Bmi-1 Is Essential for the Tumorigenicity of Human Neuroblastoma Cells

The observation that Bmi-1 is also highly expressed in neuroblastoma cell lines without *MYCN* amplification (Figure 1A) implicates a more general role for Bmi-1 in the development of neuroblastoma. Recent studies suggest that the self-renewal capacity of cancer stem cells drives tumorigenesis.[29,30] Because Bmi-1 is required for the self-renewal of neural crest stem cells[11] and the human neuroblastoma BE(2)-C cells,[31] we speculated that Bmi-1 might be important for the tumorigenicity of neuroblastoma cells. We first investigated the possibility in soft-agar clonogenic assays, based on the report that the ability of a cancer cell line to grow in soft agar correlates closely with its tumorigenicity in immunodeficient mice.[32] We generated two Bmi-1 siRNA-expressing retroviral constructs that target different regions of the Bmi-1-coding sequence, and retroviruses produced from either construct were able to down-regulate Bmi-1 expression in all of the neuroblastoma cell lines tested (data not shown). SK-N-DZ and BE(2)-C (with *MYCN* amplification) and SK-N-AS (without *MYCN* amplification) neuroblastoma cells infected with Bmi-1 siRNA retroviruses showed down-regulation of Bmi-1 by 70 to 80% (Figure 4A). No such effect was observed in the same cell lines infected with retroviruses expressing siRNA against either *GFP*, human *MYCN*, or *p53* (data not shown), demonstrating the specificity of the Bmi-1 siRNA constructs. We detected no significant effect of Bmi-1 knockdown on the cell-cycle status of neuroblastoma cells under standard culture conditions,[31] in agreement with results reported for hematopoietic stem cells from Bmi-$1^{-/-}$ mice.[9] However, the neuroblastoma cells with reduced levels of Bmi-1 gave rise to significantly fewer and smaller colonies in soft agar than did control cells (Figure 4, B and C; $^{*}P < 0.05$, $^{**}P < 0.01$). Similar results were also obtained with several other neuroblastoma cell lines, including SK-N-F1, SK-N-BE(2), SMS-SAN, and SMS-KCNR (data not shown). Together, these results indicate that down-regulation of Bmi-1 inhibits the clonogenic activity of neuroblastoma cells with or without *MYCN* amplification.

We next examined the effect of Bmi-1 knockdown on the ability of neuroblastoma cells to induce tumors in immunodeficient mice. SK-N-DZ, SK-N-AS, and BE(2)-C neuroblastoma cells infected with either control (pSuper-retro or pSuper-retro/GFPsi) or Bmi-1 siRNA-expressing retroviruses were injected subcutaneously into the flanks of NOD/SCID mice. The mice injected with SK-N-DZ/pSuper cells developed large tumor masses at three of

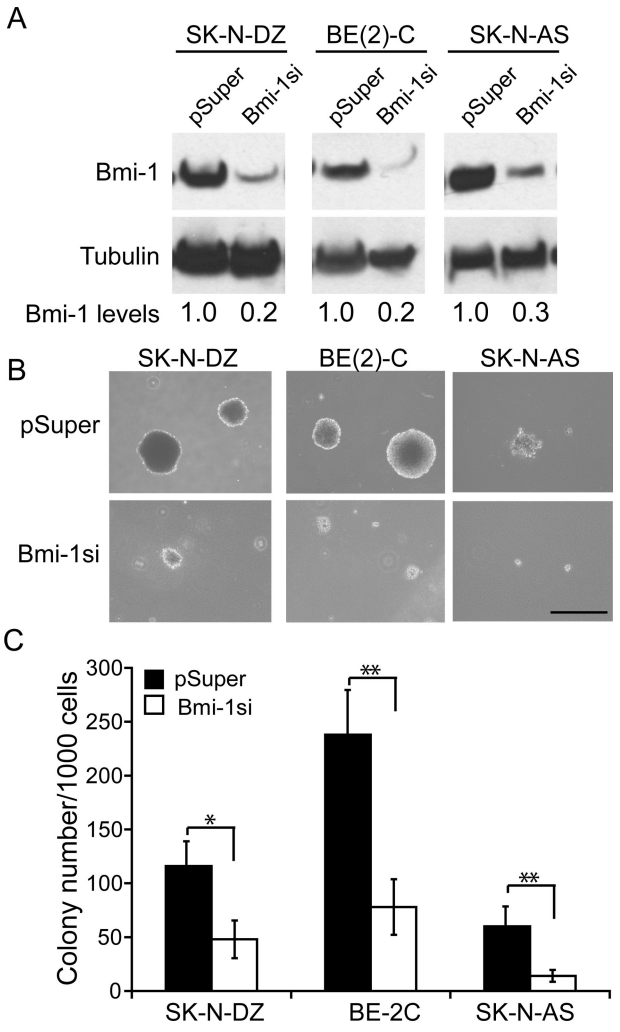


Figure 4. Down-regulation of Bmi-1 inhibits the clonogenic activity of neuroblastoma cells. **A:** Immunoblot analysis of the indicated neuroblastoma cell lines for Bmi-1 knockdown by siRNA. Relative levels of Bmi-1 are indicated. α-Tubulin levels are shown as loading control. **B:** Soft agar colony growth by the indicated neuroblastoma cell lines after 14 to 21 days of culture. Scale bar = 2 mm. **C:** Numbers of colonies per 1000 plating cells. Colonies that contained more than 50 cells or were larger than 0.5 mm were scored. Each bar represents the average ± SD of three independent experiments. Statistical analysis was performed using two-tailed Student's *t*-test; $*P < 0.05$; $**P < 0.01$.

the four injection sites after 6 weeks, whereas SK-N-DZ/Bmi-1si cells failed to produce tumors in NOD/SCID mice during the same time period (Figure 5, A and B). For SK-N-AS and BE(2)-C cells, Bmi-1 knockdown had a modest effect on tumor incidence (17 to 25% reduction, Figure 5A). However, these cells showed a significantly diminished tumorigenic activity, producing tumors that, on average, were approximately five times smaller than those produced by the control cells (Figure 5A; $*P < 0.05$). Immunoblot analysis confirmed that the tumors derived from SK-N-AS/Bmi-1si and BE(2)-C/Bmi-1si cells expressed significantly lower levels of Bmi-1 than the xenografts derived from the control cells (Figure 5C). Together, these results demonstrate that Bmi-1 is required for the tumorigenicity of neuroblastoma cells with or without *MYCN* amplification.

Bmi-1 Knockdown Promotes the Development of Schwannian Stroma in Neuroblastoma Xenografts

To gain insights into the cellular basis for the role of Bmi-1 in the tumorigenicity of neuroblastoma cells, we performed histological examination of the tumor xenografts from NOD/SCID mice. The examination revealed that four of the five tumors produced by SK-N-AS/pSuper cells were composed of almost entirely small blue neuroblasts with a minimal amount of fibrous stromal tissue (Figure 6A; SK-N-AS/pSuper, H&E). In contrast, all of the tumors derived from SK-N-AS/Bmi-1si cells were characterized by a marked increase in the amount of stromal tissue, and neuroblasts in these tumors were arranged in nests or nodules surrounded by dense stromal tissue (Figure 6B; SK-N-AS/Bmi-1si, H&E). Moreover, immunofluorescent staining using antibodies against S-100 and glial fibrillary acidic protein (markers for Schwann cells) showed a significant increase in the number of Schwann cells in the tumors formed by SK-N-AS/Bmi-1si cells, in comparison with the tumors formed by the control SK-N-AS/pSuper cells (Figure 6, A and B; S100 and GFAP). Similar results were also obtained with BE(2)-C/Bmi-1si cells (data not shown). Thus, down-regulation of Bmi-1 promotes the development of Schwannian stroma in neuroblastoma xenografts. The presence of abundant Schwannian stroma in neuroblastoma is a histological feature that correlates with a better clinical prognosis.[33]

We next determined the origin of the tumor-associated Schwann cells. SK-N-AS/Bmi-1si cells were transfected with pcDNA3-GFP and selected in medium containing G418. Individual cell clones that expressed GFP were pooled and injected into the flanks of NOD/SCID mice. Examination of tumor sections using an antibody against GFP revealed that in addition to tumor cells, most of the stromal cells also expressed GFP (Figure 6C, GFP). Dual-immunofluorescent staining for both GFP and S100 showed that all S100-positive cells expressed GFP (Figure 6C, S100 and GFP), suggesting that the tumor-associated Schwann cells were derived from the injected SK-N-AS/Bmi-1si/GFP cells.

Discussion

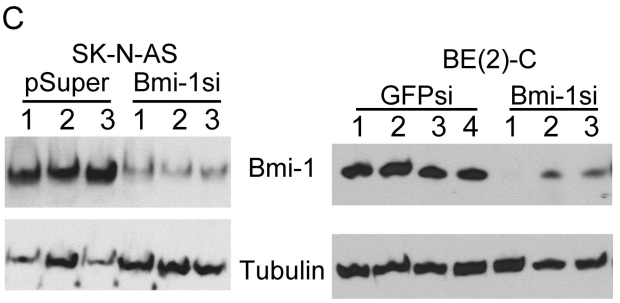
In this study, we present several lines of evidence implicating a crucial role of Bmi-1 in the pathogenesis of neuroblastoma, a pediatric malignant tumor of the sympathetic nervous system. First, Bmi-1 is highly expressed in all of the 12 human neuroblastoma cell lines and 45 primary tumor samples examined in this study. In addition, neuroblastoma development in sympathetic ganglia of MYCN transgenic mice is associated with a marked increase in Bmi-1 expression. Second, Bmi-1 cooperates with MYCN in the oncogenic transformation of benign S-type neuroblastoma cells and avian neural crest cells, most likely by inhibiting the apoptotic activity of MYCN. Third, down-regulation of Bmi-1 significantly impairs the ability of neuroblastoma cells to grow in soft agar or to induce tumors in immunodeficient mice, suggesting an

A

Tumorigenicity in NOD/SCID mice

	cell lines		incidence	weight (g)
1	SK-N-DZ	pSuper	3/4	2.8±2.3
		Bmi-1si	0/4	0
2	SK-N-AS	pSuper	5/6	0.89±0.57
		Bmi-1si	4/6	0.17±0.09*
3	BE(2)-C	GFPsi	4/4	2.60±0.85
		Bmi-1si	3/4	0.50±0.43*

* Student's *t*-test, p<0.05



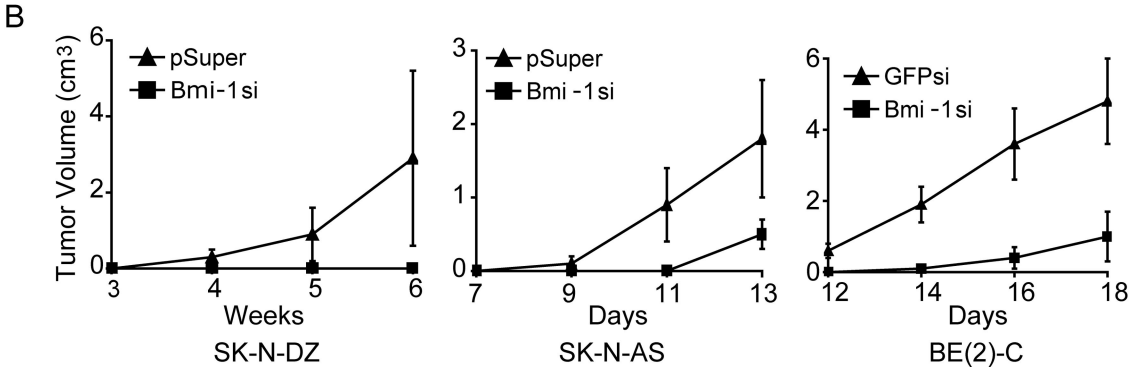


Figure 5. Down-regulation of Bmi-1 inhibits the tumorigenicity of neuroblastoma cells. **A:** Tumorigenic activity exhibited by the indicated neuroblastoma cell lines. **B:** Tumor growth in NOD/SCID mice injected with indicated human neuroblastoma cell lines, as estimated by caliper measurements. **C:** Immunoblot analysis of Bmi-1 expression in mouse neuroblastoma xenografts derived from indicated human neuroblastoma cell lines. α-Tubulin levels are shown as loading control.

essential role for Bmi-1 in maintenance of the tumorigenic capacity of neuroblastoma cells. Finally, tumor xenografts produced by neuroblastoma cells with down-regulation of Bmi-1 display a significantly higher content of Schwannian stroma, suggesting a key role for Bmi-1 in the generation of neuroblastoma heterogeneity.

Our findings have a number of implications in understanding the pathogenesis of neuroblastoma. Amplification of *MYCN* is associated with aggressively growing neuroblastomas.[1,2] However, MYCN also sensitizes cells to apoptosis,[22–24] suggesting that inactivation of apoptotic pathways through cooperating oncogenes may be necessary for the development of neuroblastoma with *MYCN* amplification. In addition, neuroblastoma development in *MYCN* transgenic mice is characterized by a prolonged latent period, partial penetrance of the tumor phenotype, and chromosomal gains and losses, providing further evidence that the tumor formation requires genetic lesions in addition to *MYCN* overexpression.[3] In this study, we found that Bmi-1 is an oncogenic partner of MYCN in cellular transformation and a potent inhibitor of its apoptotic activity, probably by inhibiting the ARF-p53 signaling pathway through which Myc proteins sensitize cells to apoptosis.[25,26] The overexpression of Bmi-1 in neuroblastomas developed in *MYCN* transgenic mice and the presence of abundant Bmi-1 in human neuroblastoma cell lines with *MYCN* amplification suggest that this oncogenic cooperation could be a relevant mechanism in the pathogenesis of neuroblastoma.

In addition, our results suggest a more general role for Bmi-1 in the development of neuroblastoma. We show here that Bmi-1 is required for neuroblastoma cells, with or without *MYCN* amplification, to initiate new tumor growth in immunodeficient mice, which is attributed to the self-renewal capacity of cancer stem cells.[29] Thus, Bmi-1 may play an essential role in the maintenance of neuroblastoma stem cells. Interestingly, Bmi-1 is also required for the self-renewal of neural crest stem cells.[11] Together, these observations suggest that neuroblastoma development depends on inappropriate activation of pathways that normally regulate the self-renewal of neural crest stem cells. We speculate that aberrant regulation of Bmi-1 expression during early development of the sympathetic nervous system may lead to expansion of the pool of neural crest stem cells, and some of these cells may sustain additional genetic mutations, such as *MYCN* amplification, leading to neuroblastoma genesis. This model suggests a molecular link between the development of the sympathetic nervous system and the pathogenesis of neuroblastoma.

Clinically, neuroblastoma is a heterogeneous group of tumors, displaying histological features that range from tumors with predominantly undifferentiated neuroblasts to those primarily consisting of fully differentiated ganglion cells surrounded by a dense stroma of Schwann cells.[33] Abundant Schwannian stroma is one of the histological features in neuroblastoma associated with a clinically favorable prognosis.[33] The genes and signaling pathways that regulate the abundance of Schwannian stroma in neuroblastoma are primarily unknown. We found that down-regulation of Bmi-1 promotes Schwannian stromal development in neuroblastoma xenografts.

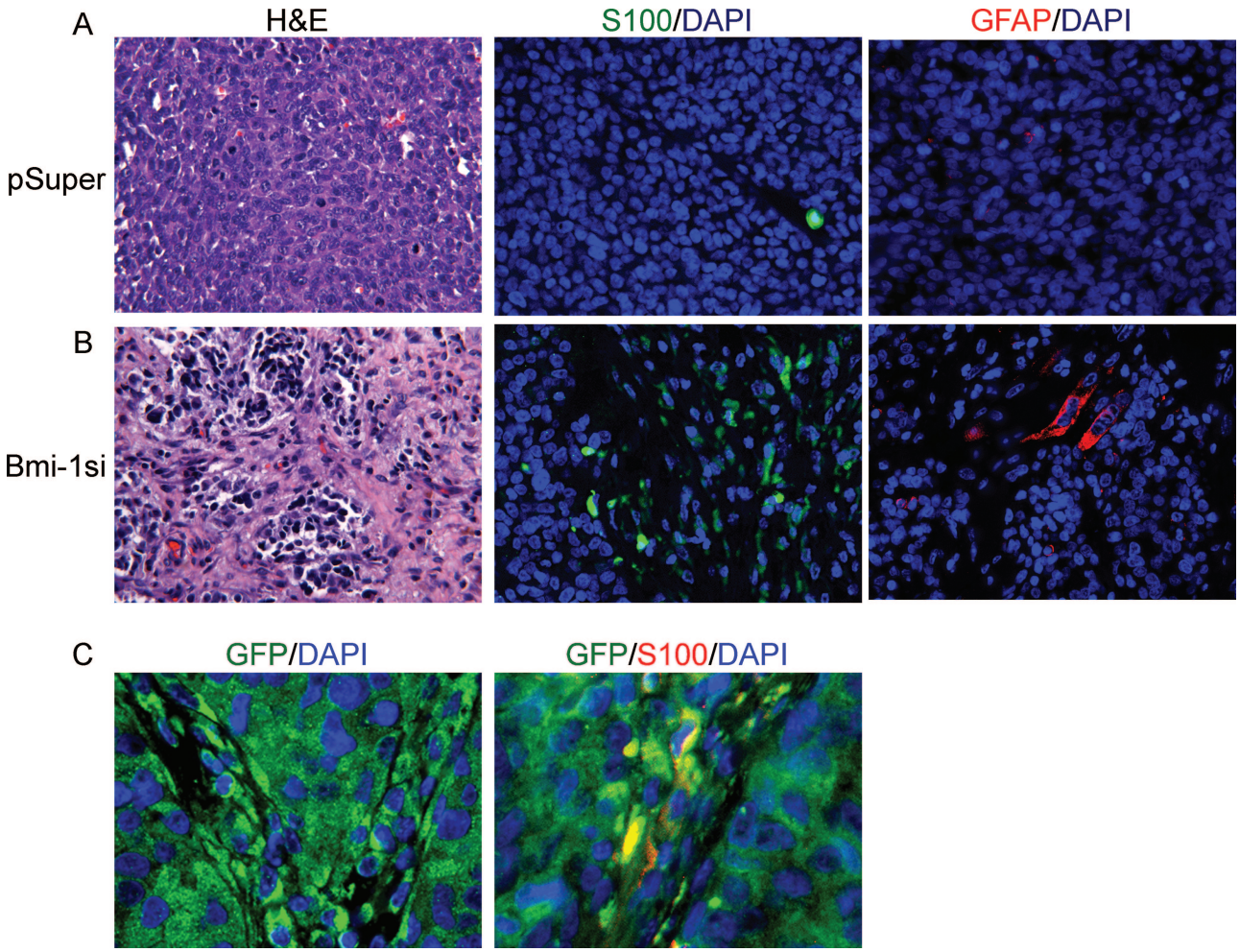


Figure 6. Bmi-1 knockdown promotes the development of Schwannian stroma in neuroblastoma xenografts. **A** and **B:** Histological and immunofluorescent examination of tumor xenografts derived from SK-N-AS/pSuper (**A**) and SK-N-AS/Bmi-1si cells (**B**). Left: H&E staining for small blue neuroblasts and fibrous stromal tissue; middle: immunofluorescent staining for S100 expression (green); and right: immunofluorescent staining for glial fibrillary acidic protein (GFAP) expression (red). **C:** Immunofluorescent staining for GFP (green) or GFP and S100 (yellow) expression in tumor xenografts derived from SK-N-AS/Bmi-1si/GFP cells. Nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI). Original magnifications: ×200 (**A**, **B**); ×600 (**C**).

The observation suggests that a normal function of Bmi-1 is to inhibit the differentiation of Schwann/glial cells. In support of this model, our recent study demonstrates that down-regulation of Bmi-1 promotes the human neuroblastoma BE(2)-C cells to differentiate along the Schwann/glial pathway.[31] In addition, Bmi-1-deficient mice display a significant increase in the number of astroglial cells in the central nervous system, and neural stem cells from these mice preferentially generate astroglial cells in culture.[34]

Bmi-1 is a negative regulator of the *Ink4a-ARF* locus that encodes two tumor suppressors, $p16^{Ink4a}$ and $p19^{ARF}$ ($p14^{ARF}$ in the human).[19] As expected, we found a close correlation between the up-regulation of Bmi-1 and down-regulation of $p16^{Ink4a}$ in neuroblastoma tumors from *MYCN* transgenic mice. $p16^{Ink4a}$ and $p19^{ARF}$ promote cell cycle arrest and apoptosis mainly through the activation of pRb and p53 pathways, respectively. Therefore, overexpression of Bmi-1 may potentially inactivate both pRb and p53 in promoting neuroblastoma development, which may explain why loss of these two major tumor suppressors rarely occurs during neuroblastoma pathogenesis.[35–38]

In summary, our study suggests a general role for Bmi-1 in neuroblastoma development. The finding that neuroblastoma cells require Bmi-1 for their tumorigenic activity suggests that interference with Bmi-1 activity could be a therapeutic strategy for neuroblastoma.

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