Methylation-associated Silencing of the Thrombospondin-1 Gene in Human Neuroblastoma

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ABSTRACT

Tumor angiogenesis, a major requirement for tumor outgrowth and metastasis, is regulated by pro- and antiangiogenic factors. Methylation-associated inactivation of the angiogenesis inhibitor thrombospondin-1 (TSP-1) has been observed recently in some adult tumors. To investigate the role of TSP-1 in pediatric cancer, we examined its pattern of expression and mechanisms of regulation in neuroblastoma (NB), TSP-1 was silenced in a subset of undifferentiated, advanced-stage tumors and NB cell lines. In contrast, most localized tumors expressed this angiogenesis inhibitor, and a significant correlation between morphological evidence of neuroblast differentiation and TSP-1 expression was observed. Luciferase assays demonstrated the presence of nuclear factors required for TSP-1 transcription in both TSP-1-positive and -negative cell lines, but no correlation between TSP-1 promoter activity and the level of TSP-1 mRNA expression was seen. Our studies indicate that the transcriptional silencing of TSP-1 was caused by methylation. TSP-1 promoter methylation was detected in all of the NB cell lines lacking TSP-1 mRNA and in 37% of the NB clinical tumors analyzed. Furthermore, treatment with the demethylating agent, 5-Aza-2'-deoxycytidine (5-Aza-dC), restored TSP-1 mRNA and in 37% of the NB clinical tumors analyzed. Disrupting methylation with 5-Aza-dC also led to significant inhibition of NB tumor growth in vivo and expression of TSP-1 in a subset of NB xenografts. These results suggest that 5-Aza-dC inhibits NB growth by augmenting the expression of TSP-1 along with other genes that suppress tumor growth. Demethylating agents may prove to be effective candidates for the treatment of children with NB.

INTRODUCTION

NB, the most common extracranial solid tumor of childhood, is remarkable for its broad spectrum of clinical behavior (1). This clinical diversity correlates closely with numerous clinical and biological factors (1–3), although the biological basis underlying the clinical heterogeneity of NB tumors remains largely unknown. Several recent studies indicate that angiogenesis plays a critical role in the regulation of NB growth. Most clinically aggressive, advanced-stage NB tumors express high levels of angiogenic stimuli and are highly vascular (4–7). In contrast, lower numbers of blood vessels are seen in tumors with a more benign behavior. We and others have reported recently that favorable histology tumors with abundant Schwannian stroma express high levels of angiogenesis inhibitors (8–10). Additional laboratory studies have shown that changes in the balance of angiogenic activators and inhibitors directly impacts NB tumorigenicity. Down-regulation of angiogenesis inhibitors is observed in highly malignant NB cells with exogenous MYCN overexpression (11, 12), whereas angiogenic activators are down-regulated in Trk-A transduced NB cells that display impaired tumorigenicity (13). Furthermore, administration of antiangiogenesis agents effectively inhibits NB tumor growth in vivo (14–18).

TSP-1, a matrix-bound adhesive glycoprotein, is a potent inhibitor of angiogenesis (19). It is able to block endothelial cell migration in response to a wide variety of angiogenic stimuli, induce endothelial cell apoptosis, and prevent neovascularization in the rat cornea and polyvinyl sponge assays (20–22). Transformed cells and tumor cell lines usually express very low levels of TSP-1, and tumorigenicity is suppressed when TSP-1 is re-expressed in these cell lines (23–26). TSP-1 has also been shown to have angiogenic properties in bladder, melanoma, lung, and breast cancer (27, 28). In some cancers, expression of TSP-1 has been reported to inversely correlate with malignant progression (28). Conversely, high plasma levels of TSP-1 are associated with increased angiogenesis in colon cancer (29), suggesting that in some tumors TSP-1 does not have an inhibitory effect.

The mechanisms controlling the expression of TSP-1 are not completely understood. A number of cis-acting transcription elements in the TSP-1 promoter have been identified (30). Additional studies indicate that the tumor suppressor p53 positively regulates the TSP-1 gene (31). Alterations in DNA methylation also appear to play a role in TSP-1 regulation, and promoter-associated CpG island methylation has been implicated recently in the transcriptional inactivation of TSP-1 in glioblastomas (32). Epigenetic mechanisms have similarly been shown to be responsible for the silencing of other tumor suppressor genes in a variety of human cancers (33). Chromatin structure is a dominant force in methylation-associated gene silencing (34). The methyl-CpG-binding protein MeCP2 has been shown to recruit histone deacetylase activity to in vitro methylated promoters (34, 35) indicating that these two processes are linked. Thus, it may be necessary to simultaneously block both DNA methylation and histone deacetylation to achieve maximal reactivation of genes silenced by methylation (36).

In this study, we examined the level of TSP-1 expression and its mechanisms of regulation in NB. TSP-1 was silenced in a subset of NB cell lines, and the loss of expression was associated with aberrant 5’ CpG island methylation of the TSP-1 promoter. TSP-1 promoter methylation was also detected in 37% of the NB clinical tumors analyzed. Treatment with the demethylating agent 5-Aza-dC restored TSP-1 transcription in the TSP-1 negative NB cell lines, confirming that the silencing of this gene in the cell lines was because of methylation. Furthermore, disrupting methylation with 5-Aza-dC resulted in inhibited tumor growth in vivo, and TSP-1 expression was restored in a subset of NB xenografts. These results demonstrate that 5-Aza-dC augments the expression of TSP-1 and other genes that impair NB tumor growth. Demethylating agents may prove to be effective candidates for the treatment of children with NB.
**MATERIALS AND METHODS**

**Cell Culture and CM Collection.** The cell lines used in this study have been described previously (37–40), and their biological characteristics are summarized in Table 1 (38–40). The cell lines used in this study have been described previously (37–40), and their biological characteristics are summarized in Table 1 (38–40). Tumors with ≥5% of the tumor cells showing synchronous differentiation of the nucleus and conspicuous, eosinophilic, or amphiphilic cytoplasm (i.e., showing differentiation toward ganglion cells) were considered differentiated, whereas stroma-rich, intermixed tumors were considered undifferentiated. Correlation with survival was analyzed with Kaplan-Meier curves. This study was approved by the Children’s Memorial Medical Center Institutional Review Board.

To highlight endothelial cells in the NB xenografts, immunohistochemical staining with an anti-PECAM-1 (CD31) antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was performed on formalin-fixed and paraffin-embedded tumor sections. MVD was quantified by counting 10 consecutive fields at ×400 magnification, and the average MVD counted in the 10 fields was converted into MVD/mm². The Student test was used to statistically compare the MVD in the 5-Aza-dC-treated and control xenograft tumors.

**Construction of Human TSP-1 Reporter Plasmids.** Fragments of the TSP-1 promoter were amplified using genomic DNA isolated from SH-EP cells as the template. The PCR cycle included denaturation at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s. The PCR products were subjected to 2% agarose gel electrophoresis, and the level of β,-MG was used as an internal control. In some experiments, TSP-1 mRNA expression was also analyzed by real-time quantitative RT-PCR using methods described previously (41). For these studies, the TSP-1 target primer pair 5'-TGGAGCATGAGCGAGAGGAGA-3' and 5'-CAGATGACAAGAGAGGA-3' was used with the probe carboxyfluorescein-5'-CACTGATGCA-3' and 5'-ATCCTCCAATCTCCATGATG-3', respectively, for 35 cycles. Each cycle consisted of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s.

**Immunohistochemical Studies.** Histological sections of human NB tumor tissue and mouse xenografts were fixed in 10% buffered formalin, embedded in paraffin, and immunostained using a mouse anti-TSP-1 monoclonal antibody. Cell pellets, prepared from 1.5 × 10⁷ cells from each NB cell line analyzed, were prepared similarly. Four-μm thick sections were rehydrated in graded alcohols and rinsed in PBS. Antigen retrieval was performed with 0.01 M citrate buffer (pH 6.0) in a boiling steamer for 20 min. Sections were incubated with a 1:100 dilution of primary antibody in a humidity chamber overnight at 4°C, and developed with peroxidase-labeled-dextran polymer followed by diaminobenzidine (DAKO Envision Plus System; DAKO Corporation, Carpinteria, CA). Sections were counterstained with Gill’s Hematoxylin (Fisher, Pittsburgh, PA). For negative controls, primary antibody was omitted. TSP-1 staining above the background in the tumor cytoplasm was scored as positive. The correlation of TSP-1 expression with clinical data including stage, MYCN amplification, and morphological evidence of differentiation was statistically evaluated using Fisher’s Exact Test. Differentiation was evaluated using the published criteria of the International Neuroblastoma Pathology Classification System (42). Tumors with ≥5% of the tumor cells showing synchronous differentiation of the nucleus and conspicuous, eosinophilic, or amphiphilic cytoplasm (i.e., showing differentiation toward ganglion cells) were classified as differentiating, whereas stroma-rich, intermixed tumors were considered undifferentiated. Correlation with survival was analyzed with Kaplan-Meier curves. This study was approved by the Children’s Memorial Medical Center Institutional Review Board.

**Table 1. Biological characteristics of NB cell lines**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Subclone</th>
<th>N or S type</th>
<th>MYCN amplification</th>
<th>Tumorigenic in vivo</th>
<th>TSP-1 mRNA</th>
<th>TSP-1 protein expression</th>
<th>TSP-1 protein secretion</th>
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<tr>
<td>SK-N-SH</td>
<td>No</td>
<td>S</td>
<td>No</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>nd*</td>
</tr>
<tr>
<td>SMS-KCNR</td>
<td>No</td>
<td>S</td>
<td>Yes</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>NMB</td>
<td>No</td>
<td>S</td>
<td>Yes</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>nd</td>
</tr>
<tr>
<td>IMR-5</td>
<td>No</td>
<td>S</td>
<td>Yes</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
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<td>No</td>
<td>S</td>
<td>No</td>
<td>+</td>
<td>+</td>
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<td>nd</td>
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<td>S</td>
<td>Yes</td>
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<td>+</td>
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<td>nd</td>
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<td>S</td>
<td>Yes</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>nd</td>
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<tr>
<td>NBL-W-S</td>
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<td>S</td>
<td>Yes</td>
<td>+</td>
<td>+</td>
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<td>nd</td>
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<td>LA1-55n</td>
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<td>+</td>
<td>+</td>
<td>nd</td>
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<td>LA1-5s</td>
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<td>S</td>
<td>Yes</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>nd</td>
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<tr>
<td>SH-SY</td>
<td>Yes</td>
<td>S</td>
<td>No</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>nd</td>
</tr>
<tr>
<td>SH-EP</td>
<td>S</td>
<td>S</td>
<td>No</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>nd</td>
</tr>
</tbody>
</table>

*nd, not done; un, unknown.

**Table 2. TSP-1 promoter constructs and its activity**

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Start</th>
<th>End</th>
<th>Intron1</th>
<th>Distal SPI</th>
<th>Proximal SPI</th>
<th>CAAT box</th>
<th>TATA box</th>
<th>Relative activity (%)</th>
</tr>
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<tbody>
<tr>
<td>pTSP-LUC-1.7</td>
<td>−954</td>
<td>+783</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>52</td>
</tr>
<tr>
<td>pTSP-LUC-1.1</td>
<td>−954</td>
<td>+147</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>93</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>85</td>
</tr>
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<td>+147</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>83</td>
</tr>
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<td>−149</td>
<td>+147</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>100</td>
</tr>
<tr>
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<td>+147</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>11</td>
</tr>
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<td>pTSP-LUC-0.13</td>
<td>+21</td>
<td>+147</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>5</td>
</tr>
<tr>
<td>pTSP-LUC-SP1-Mut</td>
<td>−134</td>
<td>+147</td>
<td>+</td>
<td>+</td>
<td>a</td>
<td>+</td>
<td>+</td>
<td>74</td>
</tr>
<tr>
<td>pTSP-LUC-CAAT-Mut</td>
<td>−75</td>
<td>+147</td>
<td>+</td>
<td>+</td>
<td>a</td>
<td>+</td>
<td>+</td>
<td>15</td>
</tr>
</tbody>
</table>
AGCTCGCGTGGCGCAAGAGTAC-3' pTSP-LUC-0.3: 5'-TTTCGAGCTCG- AAAGTTGCGCGCCAGGC-3'; pTSP-LUC-0.24: 5'-CCCCGAGCTCCCGC- TTCCTGCCCGGC-3'; pTSP-LUC-0.2: 5'-GCCGGAGCTCTCCCCAGGAAT- GCGAG-3'; pTSP-LUC-0.13: 5'-GCCCGAGCTCGCCTGCGAGTTCAG-3'; and pTSP-LUC-SP1-Mut: 5'-TTGCGAGCTCGGCAGCGGGGTTCGGAGAGAG-3'. The antisense primer 5'-CTCCGGTACACCATGGTAAGTCG-CCC-3' was used for all of the constructs except pTSP-LUC-1.7 (5'-CTA- GCGTCCTGTTCCTGATGCAT-3'). The location of primers in the TSP-1 promoter region is shown in Fig. 2 and Table 2.

**Luciferase Assays.** Cells were seeded in a 24-well dish and grown to 90% confluence in growth medium. For each well, 0.5 µg of reporter construct was cotransfected with 25 ng of Renilla luciferase plasmid, pRL-CMV (Promega), into NB cell lines using Lipofectamine2000 (Invitrogen). Cells were harvested 36 h after transfection, and reporter activity was measured using the Dual Luciferase Assay (Promega) according to the manufacturer's instructions. Experimental luciferase activities were normalized for efficiency against control Renilla luciferase readings.

**Analysis of TSP-1 Gene Hypermethylation by MSP and Bisulfite DNA Sequencing.** Genomic DNA was isolated from NB cell lines and tumors by standard techniques (38) and modified by sodium bisulfite using the CpGenome DNA Modification kit (Serologicals, Atlanta, GA). Briefly, 1 µg of genomic DNA was denatured with NaOH and modified by sodium bisulfite, which converts all of the unmethylated cytosines to uracils, whereas methylated cytosines remain unchanged. The modified DNA was desulfonated with NaOH and purified. A pair of primers, 5'-GAATGTGAGTGTTTTTTTA- AATGTG-3' and 5'-CCTAAACTCACAACAACCTCAG-3', was designed for the unmethylated sequence of the TSP-1 promoter region around the transcription start site. Another pair of primers, 5'-TGGAGGGTTTTTTTA- AATGC-3' and 5'-TAAACTCGCAAACCAACTCG-3', was used for the

**Table 3 Clinical and biological characteristics of NB patients**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cases n</th>
<th>TSP-1 positive n (%)</th>
<th>No. TSP-1 negative n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>INSS Tumor Stage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Localized (stages 1 and 2A)</td>
<td>9</td>
<td>7 (78)</td>
<td>2 (22)</td>
</tr>
<tr>
<td>Regional/advanced (stages 2B, 3, and 4)</td>
<td>28</td>
<td>14 (50)</td>
<td>14 (50)</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1 year</td>
<td>13</td>
<td>8 (62)</td>
<td>5 (38)</td>
</tr>
<tr>
<td>≥1 year</td>
<td>24</td>
<td>13 (54)</td>
<td>11 (46)</td>
</tr>
<tr>
<td><strong>MYCN</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplified</td>
<td>7</td>
<td>4 (57)</td>
<td>3 (43)</td>
</tr>
<tr>
<td>Nonamplified</td>
<td>29</td>
<td>15 (52)</td>
<td>14 (48)</td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
<td>1 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><strong>Neuroblast differentiation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>21</td>
<td>18 (86)</td>
<td>3 (14)</td>
</tr>
<tr>
<td>No</td>
<td>16</td>
<td>3 (19)</td>
<td>13 (81)</td>
</tr>
<tr>
<td><strong>Outcome</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alive (NED)</td>
<td>31</td>
<td>19 (61)</td>
<td>12 (39)</td>
</tr>
<tr>
<td>Alive (with disease)</td>
<td>2</td>
<td>1 (50)</td>
<td>1 (50)</td>
</tr>
<tr>
<td>Dead</td>
<td>4</td>
<td>1 (25)</td>
<td>3 (75)</td>
</tr>
<tr>
<td>Total</td>
<td>37</td>
<td>21 (57)</td>
<td>16 (43)</td>
</tr>
</tbody>
</table>

*Median follow-up 69 months (range, 18–140 months). InNSS, International Neuroblastoma Staging System; NED, no evidence of disease.
methylated sequence of the same region. Initial denaturation at 94°C for 3 min was followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for methylated or 54°C for the unmethylated reaction for 30 s, extension at 72°C for 45 s, and final extension at 72°C for 8 min. The PCR products were separated by electrophoresis on a 2.5% agarose gel and visualized under UV illumination using ethidium bromide staining. Universal Methylated DNA (Serologicals), which is enzymatically methylated human genomic DNA, was used as a positive control for MSP. For DNA sequencing, PCR products were gel-purified and cloned into the PCR2.1-TOPO vector (Invitrogen) according to the manufacturer’s protocol. Plasmid DNA was purified with the QIA-prep Spin Mini Prep kit (Qiagen, Chatsworth, CA). Individual plasmids were then sequenced using the ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA).

Fig. 2. Schematic diagram of the deletion and mutant TSP-1-luciferase reporter gene constructs. The numbering indicates the nucleotide position relative to the transcription start site, which is designated +1. Mutation sites are indicated by five-point stars.

Fig. 3. TSP-1 promoter activity in NB cells. A, luciferase activity in a series of deletion TSP-1 reporter/promoter in SMS-KCNR cells. B, luciferase activity of the core TSP-1 promoter region. Studies were performed with SP-1 and CAAT mutant reporter/promoter constructs and a deletion construct (pTSP-LUC-0.25) transiently transfected into SMS-KCNR cells. C, luciferase activity of the TSP-1 reporter/promoter constructs pTSP-LUC-1.7 and pTSP-LUC-1.1 in 4 TSP-1-positive and 3 TSP-1-negative NB cell lines.
Treatment of NB Cells with 5-Aza-dC and TSA. Cells were seeded, allowed to attach over a 24-h period, and treated for times ranging from 6 h to 5 days with the demethylating reagent 5-Aza-dC (Sigma) at a final concentration of 0.1, 1, or 5 μM, or a specific inhibitor of histone deacetylase TSA (Sigma) at 0.25 or 0.5 μM. For the combination 5-Aza-dC/TSA treatment, 1 μM 5-Aza-dC was added for a range of times after which 0.25 or 0.5 μM TSA was added for an additional 24 h. DMSO (Sigma) was used as a control for nonspecific solvent effects on cells. At the end of the treatment period, the medium was removed, and the RNA was extracted or the CM were prepared.

Proliferation Assay. NBL-W-S, NMB, and NBL-W-N cells were seeded into 96-well plates at a density of 5.0 × 10³, 7.5 × 10³, and 7.5 × 10³, respectively. After 24 h, 5-Aza-dC was added at various concentrations to each quadruplicate well. After 72-h treatment, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2H-tetrazolium labeling mixture (Promega) was added, and cells were additionally incubated for 3 h. The absorbance of the samples was measured using a Bio-Kinetics Microplate Reader (Bio-Tek Instruments, Winooski, VT).

Measurement of Cell Cycle Phase Distribution. Cell cycle distribution was determined by flow cytometric analysis. Briefly, NB cell lines NMB,

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**Fig. 4. Methylation status of the TSP-1 gene in NB cell lines.** A, methylation status of TSP-1 was characterized in TSP-1-positive and -negative NB cell lines using MSP. Universal methylated DNA was used as a positive control. B, methylation status of plasmid clones bearing MSP products derived from the genomic DNA of 6 NB cell lines was characterized. TSP-1-MSP products have 5 CpG sites (−18, −16, −7, +5, and +10 from transcription start site). Each row of circles represents a single sequenced plasmid containing cloned MSP product of bisulfite-treated genomic DNA. All cytosines in the 5 CpG islands were methylated in TSP-1-negative cell lines, and cytosines in the 5 CpG islands were unmethylated in TSP-1-positive cell lines. ○ indicates methylated cytosine; □ indicates unmethylated cytosine. C, representative sequencing of the MSP product in NBL-W-N cells. Arrows indicate methylated cytosines.

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**Fig. 5. Methylation status of TSP-1 in NB patient samples.** A. MSP of TSP-1 in representative samples of NB tumors. B. MSP of TSP-1 in representative samples of GNR and GNB tumors.
NBL-W-N, and NBL-W-S were cultured in RPMI 1640 containing 1 \(\mu\)M of 5-Aza-dC for 48 h. Control cells were cultured in medium lacking 5-Aza-dC. Cells were then washed with PBS, fixed in 70% ethanol, and hypotonically lysed in 1 mL of DNA staining solution [0.05 mg/mL PI (Sigma) and 0.1% Triton X-100]. The cells were incubated, while protected from light, at 4°C overnight before analysis. The cell cycle data were analyzed with an Epics XL-MCL flow cytometer (Beckman Coulter, Miami, FL), with System II (version 3.0) software (Beckman Coulter). Additional analysis of cell cycle distribution was determined by using Modfit LT (Verity Software House, Topsham, ME).

**Annexin V/PI Staining.** To investigate whether treatment with 5-Aza-dC induced NB cells to undergo apoptosis and/or necrosis, two-color flow cytometric analysis using fluorescein-labeled Annexin V and PI was performed (43). Cells were fluorescence-labeled using methods provided by the manufacturers of the Annexin V-FITC Apoptosis Detection kit (Immunotech, Marseille, France). In brief, cultured NB cell lines were treated with 1 \(\mu\)M 5-Aza-dC for the indicated times. Cells were washed twice with PBS, and resuspended at \(1 \times 10^6\) cells/mL in binding buffer with FITC-conjugated Annexin V and PI. Samples were analyzed using the Epics XL-MCL flow cytometer.

**NB Xenograft Studies.** Female 4–6 week old homozygous athymic nude mice (Harlan, Madison, WI) were inoculated s.c. into the right flank with \(1 \times 10^7\) cells from the NBL-W-S, NBL-W-N, or NMB NB cell lines. Once tumors were palpable, mice were treated with three doses of 5-Aza-dC (5 mg/kg/dose) in 1 day with 3-h intervals. For the NBL-W-S cell line, 15 mice were used in the control group, and 25 mice were used for treatment. For the NBL-W-N cell line, 15 mice were used as controls, and 16 mice were used for treatment. For the NMB cell line, 15 mice were used for the control group, and 15 mice were used for treatment. Tumor volumes were measured twice a week for up to 12 days after 5-Aza-dC administration and calculated using the formula: tumor volume = (length \(\times\) width)\(^2\) (44). Mice were sacrificed at 6, 9, or 12 days after 5-Aza-dC treatment to analyze TSP-1 re-expression and MVD at various time points post-treatment. The Student's \(t\) test was used to compare tumor size and MVD in the control and treatment groups. Animals were treated according to NIH guidelines for animal care and use, and protocols were approved by the Animal Care and Use Committee at Northwestern University.

### RESULTS

**Analysis of TSP-1 Expression in NB Cell Lines and Clinical Tumors.** TSP-1 was silenced in 5 of 14 NB cell lines analyzed by RT-PCR (Fig. 1A; Table 1). Three pairs of NB subclones were examined, and higher levels of TSP-1 were seen in the nontumori-}

![Image](https://cancerres.aacrjournals.org)
GNRs, and only 3 expressed TSP-1, indicating that the biology of these benign tumors differs from malignant NB.

NB cells can be induced to differentiate in vitro along N-type and S-type lineages with a number of agents including all-trans-RA or BrdUrd (45–48). To investigate whether in vitro differentiation was also associated with TSP-1 up-regulation, we measured TSP-1 expression by real-time quantitative RT-PCR in NB cells induced to differentiate. We found a 5-fold and 14-fold maximum increase of TSP-1 mRNA expression in NB cells treated with all-trans-RA or BrdUrd, respectively (Fig. 1D).

Human TSP-1 Promoter Activity in NB Cells. To examine the transcriptional regulation of the TSP-1 gene at the molecular level, a series of human TSP-1 luciferase/promoter reporter constructs were generated and transfected into the SMS-KCNR NB cell line (Table 2). pTSP-LUC-1.7 consists of DNA sequences extending from position −954 to position +783, and contains regions within the 5′-flanking sequence and intron 1 that have been shown previously to be necessary for maximal expression of the TSP-1 gene in COS-1 and NIH3T3 cells (Fig. 2; Ref. 30). The same 5′-flanking sequences were present in the pTSP-LUC-1.1 reporter construct, but the intron 1 sequence has been removed. Deletion of flanking sequences 5′ to position −149 had little effect on TSP-1 promoter activity in the SMS-KCNR NB cells (Fig. 3A). However, activity was almost completely abolished with pTSP-LUC-0.2 and pTSP-LUC-0.13, indicating that a positive cis-acting element(s) is located between position −149 and position −52. A proximal SP-1 binding site and CAAT box have been mapped previously to this region (30). To additionally investigate the role of the SP-1 binding site and more finely characterize the core promoter

![Graph](image-url)
elements, an additional deletion construct, pTSP-LUC-0.24, was generated that included the CAAT box but lacked the SP-1 binding site. In addition, two constructs, pTSP-LUC-SP1-Mut and pTSP-LUC-CAAT-Mut containing mutations in the SP1 binding site or CAAT box, respectively, were generated and transfected into SMS-KCNR NB cells. Activity was decreased 50% with construct pTSP-LUC-0.24 compared with pTSP-LUC-0.3. Mutation of the SP1 binding site was associated with a 25% decrease promoter activity compared with pTSP-LUC-0.3, whereas mutation of the CAAT box abolished promoter activity (Fig. 3B). Thus, the CAAT box is required for basal transcription of TSP-1, whereas the adjacent SP-1 site has only a moderate effect on TSP-1 expression in NB cells.

To determine whether the disparity in the levels of TSP-1 expression in the NB cell lines was consequent to differences in activity of the TSP-1 promoter, TSP-1 reporter constructs were transfected into 7 NB cell lines expressing a range of TSP-1 mRNA levels, and luciferase assays were performed. We found no correlation between promoter activity and TSP-1 mRNA expression in the 7 NB cell lines analyzed (Fig. 3C). Relatively low levels of TSP-1 promoter activity were seen in SH-EP cells, which express high levels of TSP-1 mRNA, and high levels of TSP-1 promoter activity were seen in IMR-5 cells, which express very low to undetectable levels of TSP-1 mRNA. For each cell line examined, similar levels of promoter activity were seen with pTSP-LUC-1.7 and pTSP-LUC-1.1 indicating that cis-acting elements within intron-1 do not play a major regulatory role in the promoter activity in NB cells (Fig. 3C).

Methylation and Silencing of the TSP-1 Promoter in NB Cell Lines and Tumors. The luciferase assays demonstrated that nuclear factors required for TSP-1 transcription were present in both TSP-1-positive and -negative cell lines, indicating that the silencing of TSP-1 in NB was not because of an absence of positive trans-acting factors. Therefore, we next examined the methylation status of the TSP-1 promoter. Methylation of promoter-associated CpG islands has been implicated in the transcriptional inactivation of a number of genes including TSP-1 in various types of cancer (32). Genomic DNA was isolated from 4 TSP-1 negative NB cell lines (IMR-5, NMB, NBL-W-S, and NBL-W-N) and 2 NB cell lines that express TSP-1 (LA1-5s and SH-EP). Bisulfite-PCR analysis indicated that the TSP-1 CpG islands were methylated in all 4 of the TSP-1 negative cell lines. In contrast, TSP-1 was completely unmethylated in the TSP-1 positive NB cell lines (Fig. 4A).

To confirm that the hypermethylation occurred around the transcription start site, MSP PCR products were purified, cloned into the pCR2.1-TOPO vector, and sequenced. The results show that all 5 of the CpG sites around the transcription start site were completely methylated in the 4 NB cell lines with silenced TSP-1. On the other hand, CpG sites are completely unmethylated in the 2 TSP-1-positive NB cell lines (Fig. 4B). Cytosines at non-CpG sites were converted to thymine, excluding the possibility that successful amplification could be attributable to incomplete bisulfite conversion (Fig. 4C).

To investigate whether aberrant methylation also occurred in vivo, the methylation status of the TSP-1 promoter was analyzed in 60 NB tumor samples using MSP. Methylation of the TSP-1 promoter was detected in 37% of the NB tumor samples (Fig. 5, A and B), but was not observed in 5 benign GNRs. Only 1 of 5 GNBs had TSP-1 promoter methylation. Evaluation of TSP-1 protein expression in 12 NB cases by immunohistochemistry did not show a correlation between methylation status of the promoter and TSP-1 protein expression. Methylated promoters were detected in 2 of 4 TSP-1 positive tumors and 1 of 8 TSP-1 negative NB tumors. The methylation studies were performed with whole tumor DNA, whereas only a subset of tumor cells express TSP-1. Thus, the lack of association between promoter methylation and TSP-1 expression in these studies may reflect the heterogeneous nature of NB tumor tissues.

Restoration of TSP-1 Expression in TSP-1 Negative Cell Lines by 5-Aza-dC Alone and in Combination with TSA. The demethylating agent 5-Aza-dC, a methyltransferase inhibitor, was used to investigate whether TSP-1 expression could be restored in TSP-1 negative cell lines. Four TSP-1 negative cell lines were treated with 1 \( \mu M \) and 5 \( \mu M \) 5-Aza-dC for 60 h, and then TSP-1 mRNA expression levels were analyzed. Although treatment with 5-Aza-dC had little or no effect on TSP-1 expression in NBL-W-N, NMB, and IMR-5 cell lines at this time point (Fig. 6A), expression was induced after longer exposure to 5-Aza-dC in all 3 of the cell lines (Fig. 7A). The NBL-W-S cells responded more rapidly to treatment. In these cells, TSP-1 was up-regulated within 24 h of treatment with the demethylating agent, and maximal levels were observed between 72 h and 96 h of treatment (Fig. 6B). Furthermore, Western blot analysis revealed the presence of TSP-1 protein in CM collected from NBL-W-S cells after 96 h and 114 h of treatment with 5-Aza-dC (Fig. 6C).

To examine the role histone deacetylase activity plays in the silencing of the TSP-1 gene in NB, cells were treated with the histone deacetylase inhibitor TSA alone and in combination with 5-Aza-dC. As shown in Fig. 7A, treatment with TSA alone failed to reactivate...
transcription of TSP-1 in any of the NB cell lines. Furthermore, treatment with TSA after 5-Aza-dC treatment did not result in enhanced levels of TSP-1 mRNA expression in 3 of the cell lines. Although higher levels of TSP-1 mRNA were detected with the combination treatment in NBL-W-S cells (Fig. 7B), histone deacetylation does not appear to play a significant role in the silencing of TSP-1 in NB.

5-Aza-dC Inhibits NB Growth in Vitro. We next examined the effect of 5-Aza-dC treatment on NB growth in vitro. As shown in Fig. 8A, growth of NMB and NBL-W-N cells was significantly suppressed, with concentrations of 5-Aza-dC as low as 0.1 μM. Flow cytometric analysis revealed a marked accumulation of NMB and NBL-W-N cells in the G2-M phase of the cell cycle after 5-Aza-dC treatment (Fig. 8, B and C). Treatment with 5-Aza-dC also induced NB cell apoptosis and necrosis. As shown in Fig. 8E, double staining for Annexin-V and PI revealed that a large fraction of NMB and NBL-W-N cells progressed to late apoptosis/secondary necrosis (positive for Annexin-V and positive for PI) or necrosis (negative for Annexin-V and positive for PI) after 48 h of treatment. In contrast, even at high concentrations of the demethylating agent (100 μM), little change in proliferation of NBL-W-S cells was seen (Fig. 8A). Furthermore, G2-M phase arrest was not detected in these cells after treatment (Fig. 8D). A low percentage of NBL-W-S cells also underwent apoptosis after treatment with 5-Aza-dC, although an increase in necrosis was not observed. Thus, although 5-Aza-dC restored TSP-1 expression in the NBL-W-S cells, this drug had limited effect on the cell cycle, cell viability, and in vitro proliferation.

5-Aza-dC Suppresses Growth of NB Xenografts in Vivo and Restores TSP-1 Expression in a Subset of NB Xenografts. To investigate whether NB growth in vivo could be suppressed with 5-Aza-dC treatment, we generated xenografts using NMB, NBL-W-N, and NBL-W-S cell lines in nude mice. Treatment with 5-Aza-dC resulted in significant inhibition in the growth of the NB xenografts derived from the cell lines that also displayed suppression of in vitro growth with treatment (NMB and NBL-W-N; P < 0.01; Fig. 9, A and B). Interestingly, growth of the NBL-W-S xenografts was also significantly inhibited (P < 0.01; Fig. 9C), although in vitro proliferation of these cells was largely not effected by treatment.

Histological analyses of the treated NBL-W-S xenografts revealed morphological evidence of neuroblast differentiation with increased amounts of cytoplasm and vesicular nuclei. Immunohistochemical studies demonstrated that 5-Aza-dC restored TSP-1 expression in many of the treated tumors (Fig. 10, A–F). We found that the percentage of TSP-1 positive tumors increased with prolonged treatment. Tumors from 5 of 10 animals sacrificed 6 days after treatment showed restoration of TSP-1. All of the 7 tumors that were harvested 9 days after treatment re-expressed TSP-1, and 5 of 7 tumors harvested 12 days after treatment were TSP-1 positive. To investigate whether the
restoration of TSP-1 expression in the treated tumors was associated with inhibited angiogenesis, tumor vascularity was examined in the control and 5-Aza-dC-treated xenografts by immunohistochemistry (Fig. 11, A–F). The MVD was significantly lower in the treated xenografts (82 ± 34) compared with controls (120 ± 33; P < 0.003; Fig. 11G). Although 5-Aza-dC treatment also inhibited the growth of all of the NB xenografts, TSP-1 was detected in only 1 of 8 NBL-W-N and 0 of 11 NMB 5-Aza-dC-treated xenografts. Thus, in these NB xenografts, the impaired tumor growth does not appear to be because of re-expression of TSP-1.

**DISCUSSION**

TSP-1 is a potent inhibitor of angiogenesis, and several studies have indicated that this glycoprotein has tumor suppressive properties in vivo (25, 49). In this study, we examined TSP-1 expression and its mechanisms of regulation in NB. We found that TSP-1 was silenced in a subset of NB tumors and cell lines. The frequency of TSP-1 expression was higher in localized tumors compared with regional and widely disseminated NBs, and a strong correlation between morphological evidence of differentiation and TSP-1 expression was observed in the NB tumors. However, only 3 of 12 mature GNRs expressed TSP-1, indicating that whereas this protein may play a role in inhibiting angiogenesis in a subset of NB tumors, other factors are likely to be responsible for the low vascularity observed in benign GNRs (4). Schwann cells, which are present in GNRs, also produce a spectrum of angiogenesis inhibitors, including tissue inhibitor of metalloproteinase-2, pigment epithelium-derived factor, and secreted protein acidic and rich in cysteine, which appear to contribute to the more benign nature of tumors with abundant Schwannian stroma (8–10).
Enhanced levels of TSP-1 expression were seen in NB cell lines induced to differentiate with either RA or BrdUrd. Furthermore, consistent with the tumor-suppressive activity of TSP-1 reported in other types of cancer cell lines, both of the NB subclones that were not capable of forming tumors in nude mice expressed TSP-1 mRNA and secreted TSP-1 protein. In contrast, Western blot analysis did not reveal TSP-1 protein secretion into CM collected from any of the tumorigenic NB cell lines analyzed, although TSP-1 mRNA and protein expression were detected in a subset of these cell lines. These observations emphasize the complex nature of TSP-1 regulation, and indicate that transcriptional and post-transcriptional mechanisms are involved.

Luciferase assays, performed with TSP-1 promoter-reporter constructs, demonstrated no correlation between TSP-1 promoter activity and the level of TSP-1 expression in NB cell lines. Therefore, we examined the methylation status of CpG residues in the TSP-1 promoter to determine whether epigenetic mechanisms were responsible for the silencing of TSP-1 in NB. TSP-1 was detected in NB cell lines that lacked promoter methylation, whereas loss of TSP-1 transcription was associated with hypermethylation of the promoter. Treatment with the demethylating agent 5-Aza-dC restored TSP-1 expression in TSP-1 negative cell lines, confirming that the transcriptional silencing of TSP-1 was caused by methylation. Although DNA methylation and histone deacetylation have been reported to synergistically silence genes in cancer (36), combinations of the histone deacetylase inhibitor TSA with 5-Aza-dC only increased TSP-1 expression levels in 1 of the 4 NB cell lines studied.

TSP-1 CpG island methylation also occurred in 37% of the clinical NB tumors studied. A few of these NB tumors were available for TSP-1 expression studies, and no clear correlation between methylation of the TSP-1 promoter and silencing of this gene was seen. Yan et al. (50) have examined recently the mechanisms of regulation of CD44, an adhesion receptor that is silenced in highly malignant NB with MYCN amplification. Hypermethylation of the CD44 gene promoter was seen in CD44-negative NB cell lines, whereas CD44-expressing cell lines were unmethylated. However, similar to our results, the level of expression of CD44 did not correspond to the methylation status of the promoter in NB tumors. These results emphasize the difficulty of studying gene regulation in clinical tumors that contain heterogeneous cell populations. However, it is also possible that whereas the status of promoter methylation is a critical determinant of TSP-1 and CD44 expression in NB cell lines, silencing of these genes in NB tumors may involve methylation-dependent as well as methylation-independent mechanisms.

Two independent groups have reported recently that the promoter region of RASSF1A, a newly described 3p21.3 tumor suppressor gene, is methylated in NB tumors and cell lines (51, 52). Although RASSF1A expression was not evaluated in primary NB tumors in either study, treatment with 5-Aza-dC restored RASSF1A mRNA in cell lines. CASP8, a gene encoding a component of the Fas apoptotic pathway, has also been shown to be inactivated in NB tumors and cell lines by DNA methylation (53). Interestingly, Astiti et al. (52) demonstrated a strong association between RASSF1A and CASP8 methylation in NB, indicating that >1 gene is likely to be aberrantly methylated in the same tumor. Thus, the phenotype of an individual NB tumor is likely to be influenced by the silencing of several genes. 5-Aza-dC treatment significantly inhibited the growth of the NBL-W-N and NMB NB cell lines both in vitro and in vivo. In addition to arresting cell cycle, tumor cell apoptosis and necrosis were induced by 5-Aza-dC. In contrast, in vitro growth of the NBL-W-S cell line was largely unaffected by 5-Aza-dC. Nevertheless, the in vivo growth of NBL-W-S xenografts was significantly impaired after treatment with this demethylating agent. Restored TSP-1 expression and decreased tumor vascularity was seen in almost all of the NBL-W-S xenografts after 5-Aza-dC treatment, suggesting that the impaired growth was attributable, at least in part, to angiogenesis inhibition. Other mechanisms are likely to be involved in the inhibition of tumor growth observed in the NBL-W-N and NMB-xenografts after treatment, as TSP-1 re-expression was only rarely detected in these tumors. It is possible that other growth-suppressive genes that are known to be aberrantly hypermethylated in cancer (54, 55) may be restored in these cells after treatment, or that 5-Aza-dC may directly inhibit tumor growth by arresting cell cycle or by inducing apoptosis and necrosis.

Our studies demonstrate that 5-Aza-dC treatment results in significant inhibition of NB growth. In a subset of NB xenografts, TSP-1 expression was restored, and these tumors displayed inhibited angiogenesis. However, 5-Aza-dC is known to globally affect gene expression, and the level of expression of other genes that are capable of suppressing NB tumor growth is also likely to be affected by this demethylating agent. We are currently conducting microarray studies in an effort to identify additional genes that are up-regulated in NB after treatment with 5-Aza-dC. Hopefully, these experiments will enhance our understanding of the molecular mechanisms by which demethylating agents inhibit tumor growth and provide insight into how this treatment strategy can most effectively be used to treat children with NB.

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Methylation-associated Silencing of the *Thrombospondin-1* Gene in Human Neuroblastoma

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