

# Restoration of Promyelocytic Leukemia Protein-Nuclear Bodies in Neuroblastoma Cells Enhances Retinoic Acid Responsiveness

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## ABSTRACT

Neuroblastoma is the most common solid tumor of infancy and is believed to result from impaired differentiation of neuronal crest embryonal cells. The promyelocytic leukemia protein (PML)-nuclear body is a cellular structure that is disrupted during the pathogenesis of acute promyelocytic leukemia, a disease characterized by impaired myeloid cell differentiation. During the course of studies to examine the composition and function of PML-nuclear bodies, we observed that the human neuroblastoma cell line SH-SY5Y lacked these structures and that the absence of PML-nuclear bodies was a feature of N- and I-type, but not S-type, neuroblastoma cell lines. Induction of neuroblastoma cell differentiation with 5-bromo-2'-deoxyuridine, all-*trans*-retinoic acid, or IFN- $\gamma$  induced PML-nuclear body formation. PML-nuclear bodies were not detected in tissue sections prepared from undifferentiated neuroblastomas but were present in neuroblasts in differentiating tumors. Expression of PML in neuroblastoma cells restored PML-nuclear bodies, enhanced responsiveness to all-*trans*-retinoic acid, and induced cellular differentiation. Pharmacological therapies that increase PML expression may prove to be important components of combined modalities for the treatment of neuroblastoma.

## INTRODUCTION

Neuroblastoma, a solid tumor of embryonal neural crest origin, is one of the most common pediatric malignancies. Although the causes of neuroblastoma are unknown, the disease is believed to arise from failure of embryonal neuroblasts to undergo differentiation and/or apoptosis (1). In tissue culture, neuroblastoma cell lines may be composed of three distinct cell types (2, 3). N-Type cells are small, round cells that have short neuritic processes, grow as small aggregates, and adhere poorly to tissue culture flasks. *In vitro* induction of N-type neuroblastoma cell differentiation results in a neuronal phenotype characterized by the development of elongated neuritic processes and diminished proliferation in culture. S-Type neuroblastoma cells consist of flattened cells that adhere tightly to the tissue culture substrate. S-Type cells resemble neural crest-derived, nonneuronal cells such as Schwann cells. A third type of neuroblastoma cell, termed I-type, is morphologically intermediate between the first two cell types and can give rise to either neuronal cells [after treatment with all-*trans*-retinoic acid (ATRA)] or S cells [after treatment with 5-bromo-2'-deoxyuridine (BrdUrd)].

The promyelocytic leukemia protein (PML)-nuclear body is a discrete, circumscribed nuclear structure (reviewed in Refs. 4–6). There are 5–20 PML-nuclear bodies in the nuclei of nearly all types of tissue

culture cells. After environmental stress, including heat shock, viral infection, exposure to heavy metals, or exposure to proinflammatory cytokines, the number of PML-nuclear bodies increases to 20–50/cell. PML was identified during the course of studies characterizing the t(15;17) translocation associated with acute promyelocytic leukemia (APL). As a result of the translocation, PML is fused to the retinoic acid receptor (RAR)  $\alpha$ , and expression of the PML-RAR $\alpha$  fusion protein disrupts nuclear bodies and blocks promyelocyte differentiation (reviewed in Ref. 7). Treatment of APL cells with ATRA results in degradation of the fusion protein, reformation of PML-nuclear bodies, and differentiation of promyelocytes.

PML is the central component of the PML-nuclear body. In the absence of PML, other nuclear body components, including Sp100, CBP, Daxx, and SUMO, fail to localize to these structures (8, 9). Using embryonic fibroblasts derived from PML-deficient mice, PML was shown to have an important role in RA-mediated signal transduction (10).

Although PML-nuclear bodies are present in nearly all tissue culture cell lines, two human cell lines have been previously described that lack these structures. The NB4 cell line was derived from a patient with APL; in these cells, PML localizes to hundreds of microspeckles throughout the nucleus (11). The NT2 cell line is a human neuronal precursor cell line; PML-nuclear bodies were reported to be abnormal in (12) or absent from (13) these cells.

During the course of studies to investigate the composition and function of PML-nuclear bodies, we observed that the human neuroblastoma cell line SH-SY5Y lacks immunoreactive and functional PML-nuclear bodies. We report here that N- and I-type neuroblastoma cell lines lack detectable PML-nuclear bodies and that restoration of PML-nuclear bodies in these cells enhances RA responsiveness.

## MATERIALS AND METHODS

**Cell Lines, Cell Culture, and Induction of Differentiation.** Neuroblastoma cell lines SH-SY5Y (N-type) and Be(2)-C (I-type) and the human myelomonocytic cell line HL60 were obtained from American Type Tissue Collection (Manassas, VA). Ten primary cell lines derived from patients with neuroblastoma were provided by A. Thomas Look (Dana-Farber Cancer Institute, Boston, MA, and the Pediatric Oncology Group Neuroblastoma Biology Study, POG 9047). Purified N-type (LA1-N) and S-type (LA1-5S) cell lines, originally subcloned from the primary cell line LA-N-1 (1), were provided by Susan Cohn (Northwestern University Medical School, Chicago, IL).

Primary neuroblastoma cell lines and SH-SY5Y, LA1-N, and LA1-5S cell lines were grown in DMEM. Be(2)-C cells were cultured in a 1:1 mixture of DMEM:Ham's F-12. HL60 cells were cultured in RPMI. DMEM, DMEM:Ham's F-12, and RPMI media were supplemented with 10% FCS, L-glutamine (2 mM), penicillin (200 units/ml), and streptomycin (200 mg/ml).

To examine the effect of cellular differentiation on the formation of PML-nuclear bodies, Be(2)-C cells were treated with BrdUrd ( $10^{-5}$  M) for 7 days or with ATRA ( $10^{-5}$  M) for 2 weeks. In addition, LA1-N and HL60 cells were incubated with human IFN  $\gamma$  (1000 units/ml) for 72 h.

**Plasmids and Antisera.** A eukaryotic expression plasmid encoding PML (pSG5-PML; Ref. 14) was provided by Hugues de Thé (Hôpital St. Louis, Paris, France). Rabbit polyclonal anti-Sp100 antiserum and anti-PML antiserum were obtained from Chemicon International, Inc. (Temecula, CA) and

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**Note:** J. H. Yu and H. Nakajima contributed equally to this study.

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MBL International Corporation (Watertown, MA), respectively. Mouse monoclonal anti-PML antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Human serum from patient K142, containing antibodies directed against Sp100, PML, and E2 pyruvate dehydrogenase complex, was described previously (15).

**Construction of E1-Deleted, Recombinant Adenovirus Vectors, and Infection of Neuroblastoma Cells.** An adenovirus encoding Sp100 was prepared by ligating DNA encoding Sp100 into the *NotI* and *BamHI* sites of pAd.RSV4, which contains Rous sarcoma virus long terminal repeat promoter and the SV40 polyadenylation signal. The plasmid encoding Sp100 was cotransfected into 293 cells with pJM17; homologous recombination between the two plasmids resulted in an adenovirus (Ad.Sp100) that contained Sp100 sequences in place of E1 sequences (16). An adenovirus encoding PML and green fluorescent protein (Ad.PML) was generated using the methods described by He *et al.* (17). An adenovirus encoding  $\beta$ -galactosidase and green fluorescent protein was provided by Anthony Rosenzweig (Massachusetts General Hospital, Boston, MA). SH-SY5Y cells and Be(2)-C cells were infected with adenovirus vectors at a multiplicity of infection ranging from 25 to 50 plaque-forming units/cell.

**Immunofluorescence and Immunohistochemical Staining.** For immunofluorescence staining, neuroblastoma cells were grown in tissue culture chambers (Nunc, Inc., Naperville, IL), fixed in 4% paraformaldehyde in PBS, and permeabilized by treatment with 100% methanol. Primary antibodies were incubated with fixed cells for 1 h at room temperature; unbound antibodies were removed using successive washes with PBS. Mouse or rabbit antibodies were detected using species-specific, fluorescein- or Texas Red-conjugated donkey anti-IgG antiserum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), as indicated. Cells were stained with 4',6'-diamidino-2-phenylindole to permit identification of cell nuclei. Cell imaging was performed with a Zeiss Axiophot Microscope; images were processed using Adobe Photoshop 4.0.

Slides containing sections of neuroblastoma tissues prepared from patients with undifferentiated or differentiating neuroblastomas were provided by Paul Thorne (University of Toronto, Toronto, Ontario, Canada, and the Pediatric Oncology Group Neuroblastoma Biology Study, POG 9047). Tissue sections were sequentially incubated with mouse monoclonal anti-PML antibody, biotin-conjugated goat antimouse IgG, and horseradish peroxidase-conjugated avidin using the Vector Elite kit (Vector Laboratories). Exposure of the tissue sections to 3,3'-diaminobenzimide resulted in the formation of a brown precipitate.

**RNA Blot Hybridization.** RNA was extracted from SH-SY5Y and HL60 cells using the guanidinium isothiocyanate/cesium chloride method (18). RNA (10  $\mu$ g/lane) was fractionated in formaldehyde-agarose gels and transferred to nylon membranes. Membranes were hybridized with the radiolabeled *BgIII/KpnI* restriction fragment of human PML cDNA. Membranes were washed and subjected to autoradiography. Equal loading of RNA on the gel was confirmed by staining 28S and 18S rRNA with ethidium bromide.

**SDS-PAGE and Immunoblotting.** SH-SY5Y, LA1-N cells, and HL60 cells were lysed in cold PBS, and cellular extracts were fractionated in 8% SDS polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were incubated in blocking solution (PBS containing 5% nonfat dry milk) and then with human serum from patient K142 diluted 1:1000 in blocking solution. Bound human antibodies were detected using horseradish peroxidase-conjugated protein A (Amersham Pharmacia Biotech) and chemiluminescence.

**Transfection of DNA Into Mammalian Cells and Reporter Assays.** LA1-N cells were incubated in tissue culture medium containing FCS that was pretreated with charcoal to remove endogenous RA. The cells were transfected with a eukaryotic expression plasmid encoding PML or control vector using the Lipofectamine Transfection System (Invitrogen-Life Technologies, Inc. Carlsbad, CA). A reporter plasmid, (RAR $\beta$ )3-tk-luc, containing the luciferase gene under the control of three copies of the RAR $\alpha$  response element from the RAR $\beta$  promoter (19), was used to measure RA responsiveness. A plasmid encoding *Renilla* luciferase (pRL-TK; Promega Corp., Madison, WI) was used to normalize transfection efficiencies. Cells were incubated with ATRA for 24 h and washed twice with PBS. Cell extracts were prepared and assayed for luciferase activity (20).

**Human Subjects.** Materials from human subjects, including primary neuroblastoma cell lines and neuroblastoma tissue sections, were obtained from the Pediatric Oncology Group Biology Study Specimen Bank. Samples sub-

mitted to the specimen bank were obtained from patients using Institutional Review Board-approved, informed consent.

## RESULTS

**SH-SY5Y Cells Lack Immunoreactive and Functional PML.** mRNA encoding PML was not detected in the human neuroblastoma cell line SH-SY5Y cells by RNA blot hybridization, and immunoreactive PML was not present by immunoblot analysis (Fig. 1) or indirect immunofluorescence (Fig. 2A). To demonstrate that SH-SY5Y cells were capable of forming PML-nuclear bodies, an adenovirus vector (Ad.PML) was used to express PML in these cells. mRNA encoding PML and immunoreactive PML were detected in Ad.PML-infected cells (Fig. 1), and PML localized to nuclear bodies in these cells (Fig. 2C).

To consider the possibility that wild-type SH-SY5Y cells contained endogenous PML but that the protein was below the level of detection by immunoblot analysis or indirect immunofluorescence, the cells were tested for the presence of functional PML. One function of PML is to recruit other proteins, including Sp100, to PML-nuclear bodies (8, 9). Because SH-SY5Y cells lacked endogenous Sp100 (Fig. 1B), an adenovirus vector containing Sp100 (Ad.Sp100) was used to express Sp100 in these cells. Ad.Sp100 infection of SH-SY5Y cells resulted in expression of Sp100 as determined by immunoblot (Fig. 1B) and indirect immunofluorescence (Fig. 2E). Sp100 failed to localize to discrete nuclear bodies in SH-SY5Y cells but was instead detected in a reticular distribution. The failure of Sp100 to localize to PML-nuclear bodies did not simply result from overexpression of the protein because Sp100 failed to localize to nuclear bodies even in those cells with very low levels of Sp100 expression (data not shown). Coexpression of Sp100 and PML in SH-SY5Y cells resulted in localization of Sp100 in discrete nuclear bodies (Fig. 2G).

Localization of CBP to PML-nuclear bodies also requires the presence of PML. In SH-SY5Y cells, endogenous CBP failed to localize to PML-nuclear bodies (Fig. 2I). In contrast, after expression of PML in these cells, endogenous CBP localized to PML-nuclear bodies (Fig. 2K). Taken together, the results of RNA blot, immuno-

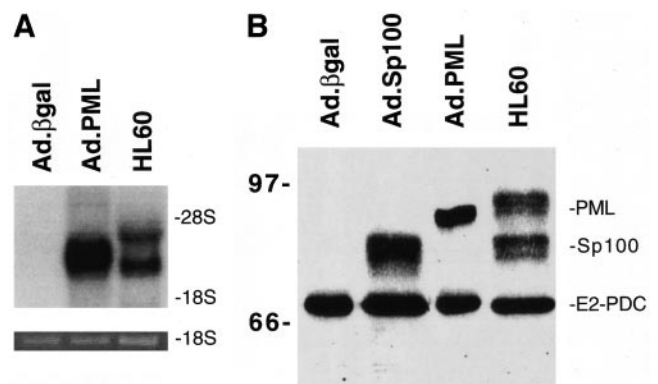
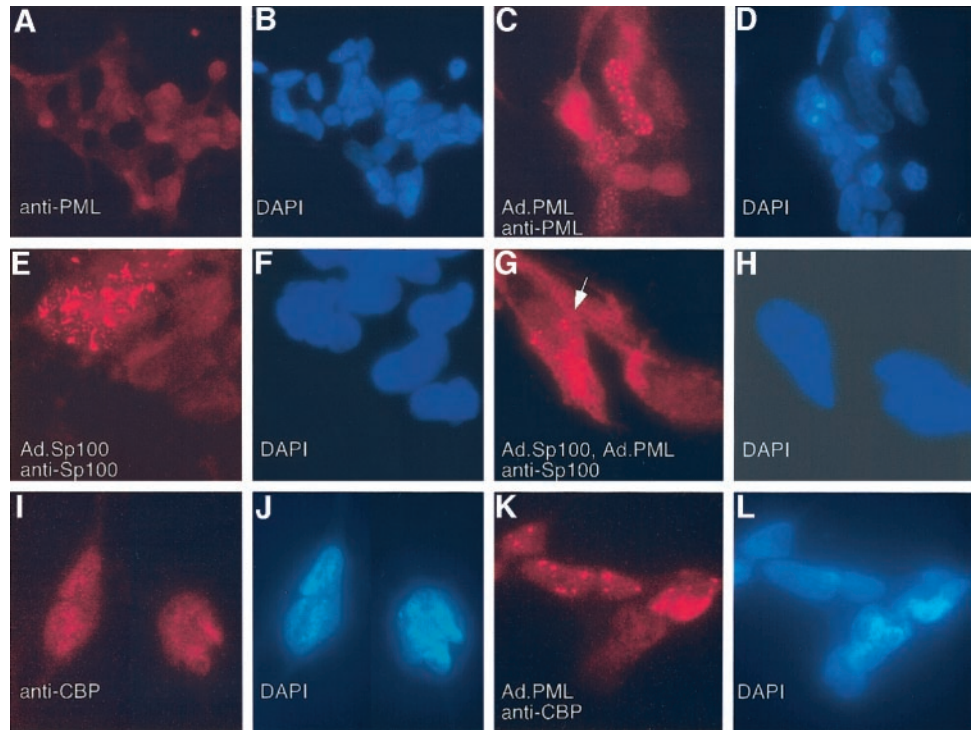


Fig. 1. SH-SY5Y cells lack endogenous PML and Sp100. A, promyelocytic leukemia protein (PML) mRNA was detected in Ad.PML-infected SH-SY5Y cells and uninfected, control HL60 cells, but not Ad.βgal-infected or uninfected (data not shown) SH-SY5Y cells, by RNA blot hybridization. A photograph of 18S rRNA in the ethidium-containing agarose gel is shown to confirm equal loading of RNA samples. B, human serum from patient K142, which contains antibodies directed against PML and Sp100, did not react with either of these proteins in Ad.βgal-infected or uninfected (data not shown) SH-SY5Y cells. Sp100 was detected in extracts prepared from Ad.Sp100-infected SH-SY5Y cells, and PML was detected in extracts prepared from Ad.PML-infected SH-SY5Y cells. Both Sp100 and PML were present in extracts prepared from HL60 cells. Serum K142 also reacted with E2 pyruvate dehydrogenase complex (E2 PDC), which was present in all four lanes.



Fig. 2. SH-SY5Y cells lack immunoreactive and functional promyelocytic leukemia protein (PML). PML was not detected by indirect immunofluorescence in SH-SY5Y cells (A). After infection of SH-SY5Y cells with Ad.PML, the protein was detected in a typical nuclear body distribution (C). Sp100 was also not detected by indirect immunofluorescence in SH-SY5Y cells (data not shown). After exposure to Ad.Sp100, Sp100 was detected in a reticular pattern in the nuclei of infected cells (E). In cells infected with both Ad.Sp100 and Ad.PML (indicated by *white arrow* in G), Sp100 was detected in nuclear bodies and the two proteins colocalized to these domains (data not shown). As with Sp100, CBP also required the presence of PML for localization to nuclear bodies. In the absence of exogenous PML, CBP was diffusely present throughout the nucleus of SH-SY5Y cells (I). After infection with Ad.PML, CBP localized to PML-nuclear bodies (K). 4',6-Diamidino-2-phenylindole (DAPI) staining (B, D, F, H, J, and L) was used to identify the nuclei of SH-SY5Y cells.

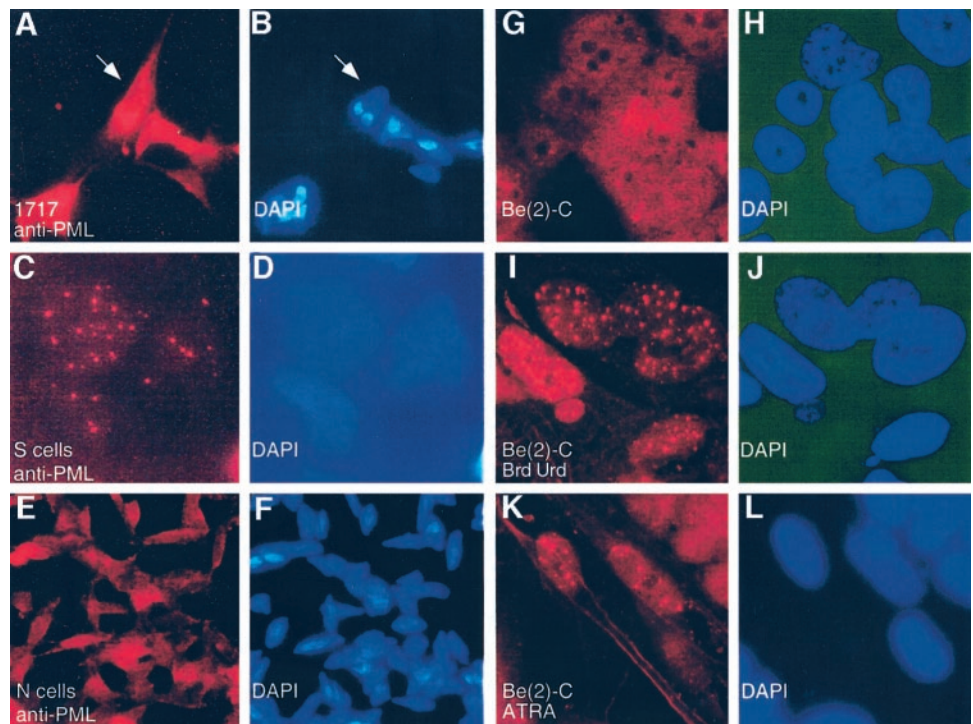


blot, and indirect immunofluorescence demonstrate that SH-SY5Y cells lack immunoreactive and functional PML-nuclear bodies.

**N-Type, But Not S-Type, Neuroblastoma Cells Lacked PML-Nuclear Bodies.** To investigate the possibility that all neuroblastoma cell lines lacked PML-nuclear bodies, 10 primary cell lines derived from patients newly diagnosed with neuroblastoma were examined. The median age of the patients at diagnosis was 1.8 years, with a range of 9 months to 7 years. The *MYCN* oncogene was amplified in 4 and not amplified in 5 of these cell lines. In 1 cell line, the *MYCN*

oncogene amplification status was unknown. The cell lines were tested for PML-nuclear bodies by indirect immunofluorescence. PML-nuclear bodies were not detected in 7 of 10 cell lines tested with monoclonal anti-PML antibodies or human serum previously shown to contain anti-PML antibodies. In the remaining 3 cell lines (one with *MYCN* amplification, one without, and one unknown), a subset of cells in the population contained PML-nuclear bodies (Fig. 3, A and B). PML-containing cells tended to have larger nuclei and were flatter and more tightly adherent to tissue culture flasks. These features were

Fig. 3. N-type, but not S-type, neuroblastoma cells lacked promyelocytic leukemia protein (PML)-nuclear bodies. Three of 10 primary neuroblastoma cell lines contained individual cells that expressed PML. Cell line 1717 was representative of these 3 cell lines. The nuclei of PML-positive cells (indicated by *white arrows* in A and B) appeared larger and flatter than PML-negative cells. These features were consistent with the S-type neuroblastoma cell type. Purified populations of N- and S-type cells, derived from the LA-N-1 cell line, were stained for PML. S-Type (C and D), but not N-type (E and F), LA-N-1 cells contained PML-nuclear bodies. Induction of PML-nuclear bodies in neuroblastoma cells. Be(2)-C neuroblastoma cells lacked PML-nuclear bodies as determined by indirect immunofluorescence performed with mouse monoclonal anti-PML antibodies (G). After 1 week of treatment with 5-bromo-2'-deoxyuridine (BrdUrd), Be(2)-C cells expressed high levels of PML (I). After 2 weeks of treatment with all-*trans*-retinoic acid (ATRA), many Be(2)-C cells developed neurite-like processes, and these cells contained PML-nuclear bodies (K). ATRA-resistant cells did not develop neurites, and these cells lacked PML-nuclear bodies. 4',6-Diamidino-2-phenylindole (DAPI) staining is shown in B, D, F, H, J, and L.



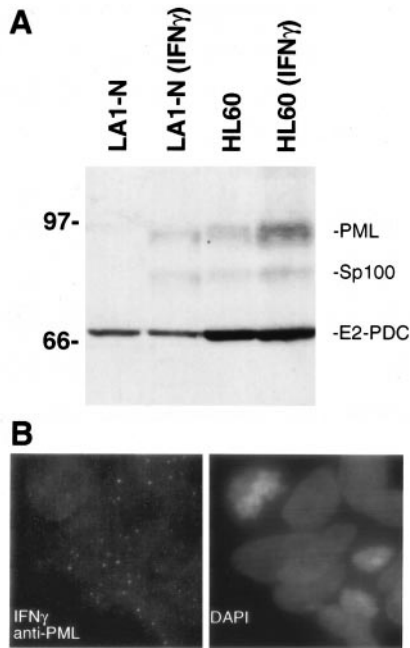


Fig. 4. IFN- $\gamma$  induces expression of promyelocytic leukemia protein (PML) in neuroblastoma cells. **A**, IFN- $\gamma$  induced expression of both PML and Sp100 in LA1-N cells as determined by immunoblot using human serum K142. Protein extracts prepared from HL60 cells, either untreated or exposed to IFN- $\gamma$ , served as a positive control in these studies. **B**, treatment with IFN- $\gamma$  (1000 units/ml) for 72 h induced expression of PML in LA1-N cells, as determined by indirect immunofluorescence using monoclonal anti-PML antibodies.

consistent with the S-type neuroblastoma cell phenotype. To test the possibility that S-type, but not N-type, neuroblastoma cells contained PML-nuclear bodies, homogeneous populations of N cells (LA1-N) and S cells (LA1-5S) derived from the LA-N-1 cell line were stained with anti-PML antibodies. The LA-N-1-derived S cells, but not N cells, contained PML-nuclear bodies (Fig. 3C–F).

#### Induction of PML-Nuclear Bodies in Neuroblastoma Cells.

Treatment with ATRA ( $10^{-5}$  M) failed to induce neurite formation in the LA1-N and SH-SY5Y cells used in this study, possibly reflecting changes in the cell lines that occurred during *in vitro* culture. To examine the effect of neuroblastoma cell differentiation on PML-nuclear body formation, we therefore used the I-type cell line, Be(2)-C. I-Type cells are morphologically intermediate between N- and S-type cells and can be induced to differentiate along the S-type or neuronal (differentiated N-type) cell pathway using ATRA or BrdUrd, respectively. Untreated Be(2)-C cells lacked PML-nuclear bodies as determined by indirect immunofluorescence (Fig. 3, G and H). One week after treatment with BrdUrd ( $10^{-5}$  M), Be(2)-C cells were enlarged, flattened, and tightly adherent to tissue culture flasks. Staining for PML was strongly positive (Fig. 3, I and J). After 2 weeks of treatment with ATRA ( $10^{-5}$  M), many Be(2)-C cells developed neurite-like processes consistent with differentiated neuronal cells, and PML-nuclear bodies were detected by indirect immunofluorescence in these cells (Fig. 3, K and L). A subset of Be(2)-C cells failed to develop elongated neurites in response to treatment with ATRA, and PML-nuclear bodies were not detected in these cells. These results demonstrate that differentiation of I-type neuroblastoma cells toward either S-type cells or differentiated neuronal cells is associated with induction of PML-nuclear bodies.

Exposure of neuroblastoma cells to IFNs was previously shown to induce expression of biochemical markers of differentiation in LA1-N cells (21). To examine the effects of IFN- $\gamma$  on PML expression, LA1-N cells were incubated with IFN- $\gamma$  (1000 units/ml) for 72 h.

IFN- $\gamma$  induced expression of immunoreactive PML as determined by immunoblot analysis (Fig. 4A). In addition, PML-nuclear bodies were detected by indirect immunofluorescence in IFN- $\gamma$ -treated LA1-N cells (Fig. 4B). Exposure of LA1-N cells to as little as 100 units/ml IFN- $\gamma$  for 72 h was sufficient to induce PML-nuclear body formation as detected by indirect immunofluorescence (data not shown).

Taken together, these results show that differentiation of neuroblastoma cells induced by treatment with BrdUrd, ATRA, or IFN- $\gamma$  is associated with formation of PML-nuclear bodies.

**Immunohistochemical Staining for PML in Neuroblastoma Tissue Sections.** To determine whether PML-nuclear bodies were present in neuroblastoma cells *in vivo*, tissue sections from undifferentiated and differentiating neuroblastomas were stained with monoclonal anti-PML antibodies. Neuroblastoma cells in undifferentiated tumors were identified based on their morphological appearance with large “salt and pepper” nuclei and scant or absent cytoplasm. These cells lacked detectable PML (Fig. 5, A and B). In contrast, PML was present in adjacent Schwannian-type cells. Tissue sections prepared from the differentiating subtype of neuroblastoma contained ganglion-like cells with large, eccentrically located nuclei and abundant cytoplasm. Differentiating neuroblasts stained strongly positive for PML (Fig. 5, C and D). These results demonstrate that, as with N- or I-type neuroblastoma cells in tissue culture, undifferentiated neuroblastoma cells lack PML-nuclear bodies.

**Reconstitution of PML-Nuclear Bodies in Neuroblastoma Cells Enhanced RA Responsiveness and Induced Cellular Differentiation.** Previous studies using cell lines derived from PML-deficient mice demonstrated that PML is an important cofactor for RA-mediated signal transduction (10). The effect of restoring PML in N-type neuroblastoma cells on RA-mediated signal transduction was examined by transfecting PML into LA1-N cells. A reporter plasmid containing the luciferase gene driven by three copies of the RA

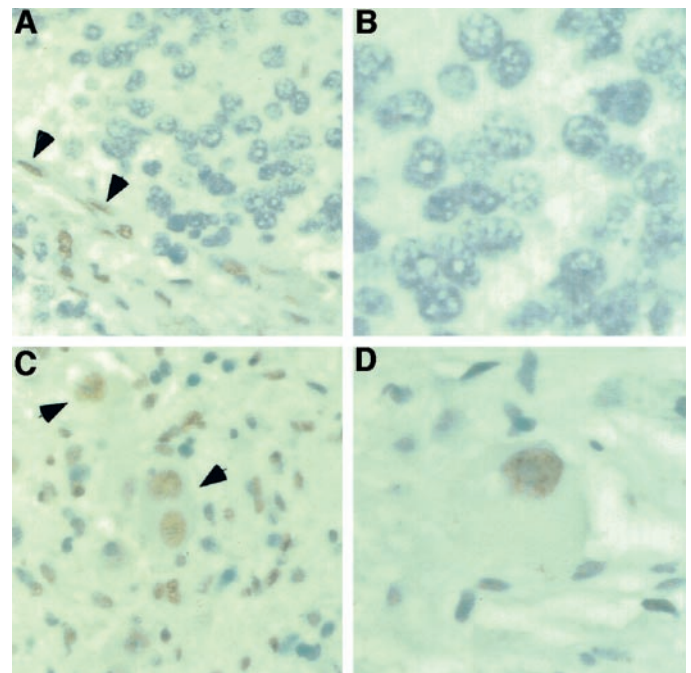


Fig. 5. Immunohistochemical staining for PML in neuroblastoma tissue sections. Neuroblastoma cells in undifferentiated tumors lacked PML-nuclear bodies (A and B,  $\times 40$  and  $\times 60$  magnification, respectively). In contrast, interspersed Schwannian-type cells (indicated by black arrows in A) contained abundant PML. In differentiating neuroblastoma tissue sections (C and D,  $\times 40$  and  $\times 60$  magnification, respectively), ganglion-like cells (indicated by black arrows in C) stained strongly positive for PML. Results are representative of two undifferentiated and two differentiating tumor samples.



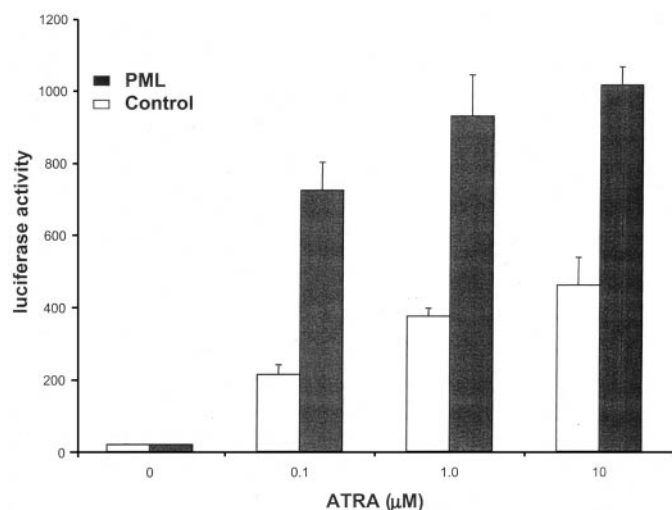


Fig. 6. Expression of PML in neuroblastoma cells enhances retinoic acid (RA) responsiveness. A luciferase reporter plasmid with three upstream copies of a RA response element was cotransfected into LA1-N cells together with PML or control vector. Expression of PML enhanced the RA responsiveness of the reporter plasmid at all *trans*-retinoic acid (ATRA) concentrations ranging from  $10^{-8}$  to  $10^{-5}$  M. Transfections were performed in triplicate, and results are presented as mean  $\pm$  SE of the means. Results are representative of three separate experiments.

response element derived from the RAR $\beta$  gene was cotransfected with either PML or control plasmid. LA1-N cells were grown in tissue culture medium supplemented with charcoal-treated FCS to remove endogenous RA. In the absence of ATRA, PML expression did not increase luciferase activity in LA1-N cells. At ATRA concentrations ranging from  $10^{-8}$  to  $10^{-5}$  M, PML enhanced luciferase activity (Fig. 6). These results demonstrate that expression of PML in N-type neuroblastoma cells enhances RA responsiveness.

To examine the effect of PML on neuroblastoma cell differentiation, Be(2)-C cells were infected with Ad.PML or Ad. $\beta$ gal. Both adenovirus vectors also encoded green fluorescent protein. At a multiplicity of infection of 50 plaque-forming units/cell,  $\sim 75\%$  of the cells expressed green fluorescent protein after 24 h in culture. The cells were grown for 10 days in tissue culture medium containing FCS

that was not pretreated with charcoal. Be(2)-C cells infected with Ad.PML developed neurites, consistent with neuronal differentiation (Fig. 7, A and B). In contrast, the morphology of Be(2)-C cells was unaffected by infection with Ad. $\beta$ gal (Fig. 7, C and D). These results show that expression of PML in Be(2)-C cells is sufficient to induce cellular differentiation in the absence of exogenous ATRA.

## DISCUSSION

Neuroblastoma and APL are malignancies that result from blocked differentiation of immature precursor cells and both diseases respond, at least in part, to differentiation therapy with RA. In patients with APL, RA is included in a multidrug regimen to induce and consolidate disease remission (reviewed in Refs. 22, 23). In patients with advanced stage neuroblastoma who have achieved clinical remission, treatment of minimal residual disease with RA reduces the risk of disease relapse (24).

In APL cells, the PML-RAR $\alpha$  fusion protein disrupts PML-nuclear bodies. RA induces degradation of the fusion protein, reformation of PML-nuclear bodies, and cellular differentiation. In this study, we showed that N- and I-type neuroblastoma cells *in vitro* and undifferentiated neuroblastomas *in vivo* also lacked PML-nuclear bodies. As with APL cells, differentiation of neuroblastoma cells was associated with restoration of PML-nuclear bodies.

Recent studies have demonstrated the important role of PML in nuclear hormone receptor-mediated signal transduction. Doucas *et al.* (25) showed that PML recruits CBP to PML-nuclear bodies and enhances glucocorticoid and RA-mediated signal transduction. Zhong *et al.* (26) described a coactivator complex containing PML and CBP that is recruited to RA response elements; they suggested that PML functions as a transcriptional coactivator. Wang *et al.* (10) showed that cell lines derived from PML-deficient mice have impaired RA responsiveness compared with cells lines from PML wild-type littermates. In this study, we demonstrated that restoration of PML-nuclear bodies in neuroblastoma cells recruited CBP to PML-nuclear bodies. In addition, transient expression of PML in neuroblastoma cells enhanced the responsiveness of these cells to RA. The degree of enhanced RA-mediated signal transduction in PML-transfected cells,

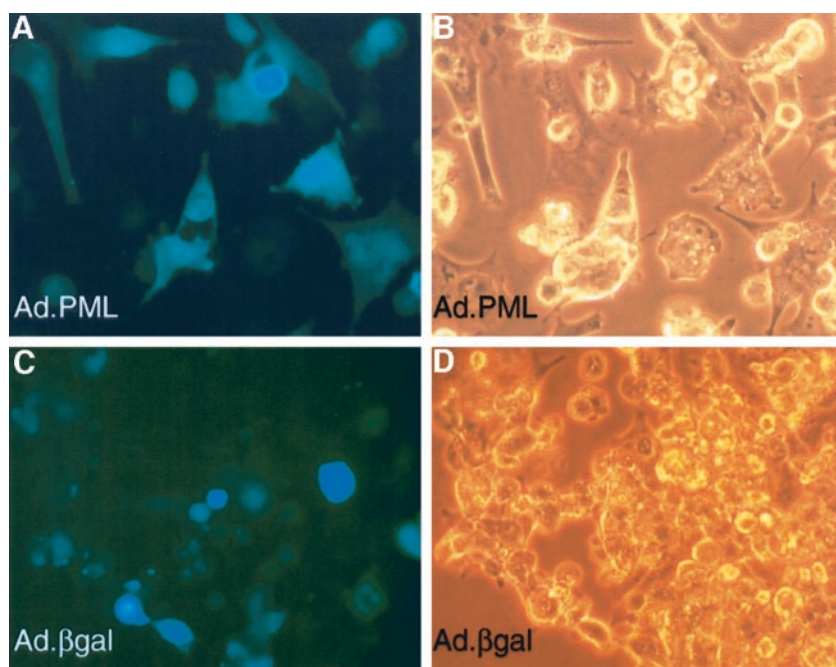


Fig. 7. PML expression in neuroblastoma cells induces neuronal differentiation. Be(2)-C cells were infected with Ad.PML or Ad. $\beta$ gal and incubated for 10 days in culture. Both adenovirus vectors contained green fluorescent protein, and cells were examined by indirect immunofluorescence (A and C) and phase contrast (B and D) microscopy. Cells infected with Ad.PML developed neurites consistent with neuronal differentiation (A and B). In contrast, the morphology of Ad. $\beta$ gal-infected Be(2)-C cells was unchanged (C and D).

compared with control neuroblastoma cells, was comparable with that observed by Wang *et al.* (10) in PML wild-type, compared with PML-deficient cell lines.

Treatment of neuroblastoma cells with IFN- $\gamma$  induced PML-nuclear body formation. Furthermore, expression of PML in neuroblastoma cells enhanced RA-mediated signal transduction and induced cellular differentiation. On the basis of these observations, the combination of IFN- $\gamma$  and RA would be expected to enhance neuroblastoma differentiation to a greater extent than either agent alone. Wuarin *et al.* (27) demonstrated synergistic effects of IFN- $\gamma$  and RA on differentiation and growth inhibition in LA1-N cells. Guzhova *et al.* (28) extended these observations to include four additional neuroblastoma cell lines. It seems likely that IFN- $\gamma$ -induced expression of PML underlies the enhanced RA responsiveness seen in cell lines treated with both RA and IFN- $\gamma$ .

Two practical consequences of the lack of PML in neuroblastoma cells should be noted. First, because undifferentiated, but not differentiating, neuroblastomas lacked PML, staining for this protein may assist in assessing the degree of differentiation in individual tumors. Second, the absence of PML (and Sp100) from N-type neuroblastoma cell lines may facilitate studies of the function of PML-nuclear bodies. Reconstitution of PML and Sp100, individually and in combination, in these cells may reveal important interactions among nuclear body components. Neuroblastoma cells may represent a unique opportunity to examine the function of nuclear body components in human cell lines.

Although we demonstrated in this study that expression of PML enhanced RA-mediated signal transduction, it is unlikely that PML is required for neuroblast differentiation. Wang *et al.* (10) observed that PML-deficient mice were at increased risk of lymphoma and skin malignancies, but these animals did not develop neuroblastoma. While this article was in preparation, Yu *et al.* (29) reported that undifferentiated neuroblastoma cells lacked PML and that neuroblastoma differentiation was associated with increased levels of PML. The findings in this study support the observations of Yu *et al.* (29) and suggest a possible mechanism by which PML may induce neuroblastoma differentiation.

In summary, N- and I-type neuroblastoma cells *in vitro* and undifferentiated neuroblastomas *in vivo* lack PML-nuclear bodies. Differentiation of neuroblastoma cells is associated with increased expression of PML. Restoration of PML in neuroblastoma cells enhances RA responsiveness and can induce neuroblastoma differentiation. In view of the emerging role of PML as a cofactor for RA-mediated signal transduction, the results of this study suggest that the addition of IFN- $\gamma$  to RA-containing therapeutic regimens may be beneficial for patients with neuroblastoma.

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## Restoration of Promyelocytic Leukemia Protein-Nuclear Bodies in Neuroblastoma Cells Enhances Retinoic Acid Responsiveness

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