PML Suppresses Oncogenic Transformation of NIH/3T3 Cells by Activated *neu*

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Summary

The chromosomal translocation t(15;17)(q22;q12) is a consistent feature of acute promyelocytic leukemia (APL) that results in the disruption of genes for the zinc finger transcription factor PML and the retinoic acid receptor α (RARα). We have previously shown that PML is a growth suppressor and is able to suppress transformation of NIH/3T3 by activated *neu* oncogene. In the study presented here, the full-length PML cDNA was transfected into B104-1-1 cells (NIH/3T3 cells transformed by the activated *neu* oncogene) by retrovirally mediated gene transfer. We found that expression of PML could reverse phenotypes of B104-1-1 including morphology, contact-limiting properties, and growth rate in both transient-expression and stable transfectants. We also demonstrated that PML is able to suppress clonogenicity of B104-1-1 in soft agar assay and tumorigenicity in nude mice. These results strongly support our previous finding that PML is a transformation or growth suppressor. Our results further demonstrate that expression of PML in B104-1-1 cells has little effect on cell cycle distribution. Western blot analysis demonstrated that suppression of *neu* expression in B104-1-1 by PML was insignificant in the transient transfection experiment but significant in the PML stable transfectants. This study suggests that PML may suppress *neu* expression and block signaling events associated with activated *neu*. This study supports our hypothesis that disruption of the normal function of PML, a growth or transformation suppressor, is a critical event in APL leukemogenesis.

Acute promyelocytic leukemia (APL)[1] represents a clonal expansion of the leukemia blast at the promyelocyte stage of myeloid differentiation. The nonrandom chromosomal translocation t(15;17)(q22;q12) is a consistent cytogenetic hallmark for APL (1, 2). This translocation event results in the disruption of the PML gene on chromosome 15 and the retinoic acid receptor α (RARα) gene on chromosome 17 (3–8). In addition, the translocation results in formation of the PML-RARα and RARα-PML fusion genes, which are transcriptionally active and encode PML-RARα and RARα-PML fusion proteins (3–8). The PML-RARα fusion protein consists of the entire functional domains of the PML and RARα genes, and it may play an important role in APL leukemogenesis (9).

The involvement of the RARα gene in the t(15;17) breakpoint of APL has been an interesting subject to many investigators, especially for the remarkable responsiveness of APL to all-*trans*-retinoic acid therapy (10–12). However, disruption of the RARα gene in APL is insufficient for the development of APL. This is illustrated in HL-60 cells, which lose RA sensitivity because of a mutation that disrupts the normal function of RARα: sensitivity can be restored by increased expression of other forms of RAR or retinoid X receptor (RXR) (13). It is now clear that expression of the PML-RARα protein in myeloid precursor cells can induce RA sensitivity (9). Furthermore, APL-derived NB₄ cells (14) that lose their RA inducibility also lose the expression of PML-RARα protein (15).

Although RARα biologic function as a RA-dependent transcription factor has been well characterized, the functional role of PML remains relatively unknown. PML belongs to a novel family of proteins corresponding to a subgroup of a large family that possess a "ring finger" (16). Nuclear proteins in this family share common structural features: a cysteine/histidine-rich motif at the NH₂ terminus followed by a predicted coiled-coil structure (7, 17, 18). The COOH terminus of the predicted PML protein consists of a proline/serine-rich region, which suggests that PML may be a phosphoprotein and a substrate for serine/threonine protein kinase and/or phosphatase (6). This proline/serine-rich region is deleted in the PML/RARα. Similar events have been seen in other members of that family in the context of oncogenic fusion (7).

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[1] *Abbreviations used in this paper:* APL, acute promyelocytic leukemia; BCS, bovine calf serum; G418^R, G418 resistant; HXM, hypoxanthine, xanthine, and mycophenolic acid; Neo^R, neomycin resistance gene; PKC, protein kinase c; PLC, phospholipase C; RA, retinoic acid; RARα, retinoic acid receptor α; RXR, retinoid X receptor; TBS, Tris-buffered saline.

The PML-RARα fusion protein is able to form a homodimer through the coiled-coil regions of the PML protein. This homodimer was shown to have distinct DNA binding properties when compared with the RAR/RXR heterodimer (19). Physiologically, heterodimerization between RAR and RXR is required for efficient binding of the PML-RARα fusion protein to target sequences (7, 19, 20). In APL, PML-RARα can heterodimerize with PML or RXR. Thus the fusion protein can potentially be a dominant negative inhibitor of PML and RXR (7, 19). It is noteworthy that sequestration of RXR by heterodimerization with PML-RARα may inhibit the heterodimerization of RXR with some hormone receptors like RARs, thyroid hormone receptor, and vitamin D_3 receptor (7, 9, 19). In turn, the normal biological function of these receptors will be impeded and hamper the ability of the cell to respond to differentiation induction by its ligands (e.g., vitamin D, thyroid hormone) and therefore may result in a differentiation arrest.

A multistep process of oncogenesis is required for most other tumors, but in APL the t(15;17) translocation is usually the only consistent cytogenetic abnormality found (2), and no other molecular events, for example, mutations in the RAS and p53 genes, have been noted (21). Consequently, we decided to address whether the loss of PML function in APL, in addition to sequestration of RXR and alteration of RARα, may contribute to leukemogenesis. In our previous study, we showed that PML is a growth suppressor and able to suppress foci formation induced by the activated neu oncogene (22). Thus, we chose the mutation-activated neu-transformed NIH/3T3 line, B104-1-1, as a model to study the effects of PML on oncogenesis. In this study, we found that constitutive expression of PML could reverse the phenotype of B104-1-1 cells, suppress clonogenicity in a soft agar assay, and suppress tumorigenicity in nude mice. The possible mechanism of PML effect on growth and the role of PML in APL pathogenesis were also discussed.

Materials and Methods

Cell Lines. The B104-1-1 cell line was obtained from M.-C. Hung (The University of Texas M.D. Anderson Cancer Center). As previously described, each B104-1-1 cell is an NIH/3T3 cell derivative containing ~10 copies of the mutation-activated genomic neu oncogene (23). B104-1-1 cells, NIH/3T3 cells, and their derivatives were maintained at 37°C in DME with 10% (vol/vol) bovine calf serum (BCS) (GIBCO BRL, Gaithersburg, MD). The packaging cell lines, GP + E-86 and PA317 (24, 25) and their derivatives were maintained in HXM complete medium (containing 15 μg/ml of hypoxanthine, 250 μg/ml of xanthine, and 25 μg/ml of mycophenolic acid) and HAT complete medium (containing 1.36 mg/ml of hypoxanthine, 17.6 μg/ml of aminopterin, and 378 μg/ml of thymidine). Each medium was supplemented with penicillin (5 U/ml) and streptomycin (5 U/ml).

Plasmids, Transfections, and Infection. The PML retroviral construct pLPMLSN was constructed by inserting the full-length cDNA of PML (22) into the unique BamHI restriction site of the retroviral vector, pLXSN, a gift from Dr. A. D. Miller (Fred Hutchinson Cancer Research Center, Seattle, WA) (26). pLXSN or pLPMLSN were transfected into the ecotropic cell line GP+ E-86 or the amphotropic PA317 packaging cell lines by calcium phosphate coprecipitation (27, 28). After 24 h of coprecipitation, the

transfected packaging cells were split and grown in either HXM or HAT selective medium with 800 μg/ml of active G418. G418-resistant (G418ᴿ) colonies were picked and expanded in selective medium and assayed for PML expression. The expression of transfected PML cDNA was determined by RNA slot blot analysis using the PML cDNA as described in our previous report (3). Expression of the PML protein was determined by Western blotting as described below.

The B104-1-1 cells, as target cells, were transduced with pLXSN or pLPMLSN by the supernatant gene transfer technique, as previously described (29). In brief, B104-1-1 cells in exponential growth were plated with 10^6 cells in 100-mm culture dishes 1 d before recombinant virus infection. Each recipient culture dish was then fed with 10 ml of recombinant viral supernatant containing polybrene at a final concentration of 6 μg/ml. The recipient cells were then infected with viral particles in the supernatant for 48 h. The viral supernatant was prepared by incubating fresh medium for 1 d with the growth-accelerated viral producer. The viral supernatant was then harvested and centrifuged at 3,000 g for 5 min to remove cells and debris. The pLXSN- or pLPMLSN-containing virus-infected B104-1-1 cells were designated as B-LXSN and B-PMLSN, respectively. The transiently infected B-PMLSN cells were then split, selected, and expanded in DME complete medium containing 500 μg/ml of G418. A G418ᴿ single-cell colony was selected and used directly for other experiments. The titers of recombinant retrovirus were determined using the NIH/3T3 cells and were expressed as G418ᴿ NIH/3T3 CFU/ml (G418ᴿ CFU/ml) as previously described (30).

Antibody, Gel Electrophoresis, and Immunoblotting. The antipeptide antibody against PML, PML-C7, was raised in rabbits as described in our previous report (22). The neu oncogene product was detected by c-neu (Ab-3) mouse mAb (Oncogene Science, Inc., Manhasset, NY).

For expression analysis of PML or neu protein, cells were washed with cold PBS and scraped into 0.5 ml of RIPA buffer (10 mM Tris-HCL, pH 7.5, 120 mM NaCl, 1% NP-40, 1% deoxycholate, 0.1% SDS, 1 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin, and 50 mM sodium fluoride per 100-mm dish). After 15 min on ice, the lysed cells were spun at 5,000 g for 15 min at 4°C. Protein samples were mixed 1:1 with 2× SDS sample buffer (containing 0.1 M Tris-HCl, pH 6.8, 0.2 M dithiothreitol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) and then heated to 100°C for 5 min before a 7.5% SDS-PAGE. After electrophoresis, proteins were transferred onto nitrocellulose filter (Schleicher & Schuell, Inc., Keene, NH) in a buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol at 0.2 Å for 1 h at 4°C. Filters were then blocked by incubating overnight in Tris-buffered saline (TBS; 10 mM Tris, pH 8.0, 150 mM NaCl) containing 5% nonfat dried milk. The blots were then washed in TBS and incubated for 2 h with PML antibody or Ab-3. The primary antibody was removed and the blots were washed four times in TBS. To detect antibody reactions, the blots were incubated for 1.5 h with alkaline phosphatase–conjugated anti–rabbit or anti–mouse IgG (Bio-Rad Laboratories, Inc., Richmond, CA) diluted 1:2,000 in TBS, washed three times in TBS, and then placed in a buffer containing 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂, 330 μg of Nitroblue tetrazolium/ml, and 150 μg of 5-bromo-4-chloro-3-indolyl phosphate/m for 10–20 min. Enzymatic color development was stopped by rinsing the filters in deionized water.

Immunofluorescence staining of the PML protein was performed by using the affinity-purified antipeptide antibody as previously described (22).

[³H]Thymidine Incorporation. A total of 2.5 × 10⁴ cells were

plated in triplicate in 24-well plates and cultured in complete medium. The cells received a 1-h pulse of 1 μCi of [methyl-³H]thymidine and then were harvested 16, 40, and 64 h after labeling. Cells were washed in cold PBS and fixed with methanol. Unincorporated [methyl-³H]thymidine was removed by washing with cold 10% TCA. Total cells in each well were harvested after lysis with 0.3 N sodium hydroxide and 1% sodium lauryl sarcosinate. Radioactivity of the incorporated thymidine was then determined with a scintillation counter (model LS5000 TD; Beckman Instruments, Inc., Fullerton, CA).

Growth Rate Assay. Equal numbers of cells (2 × 10⁵) from individual clones were seeded onto 60-mm dishes. Cells were then cultured in 10% or 0.5% BCS and subsequently trypsinized, and viable cells were counted by the trypan blue dye exclusion method at daily intervals.

Soft Agar Colony-forming Assay. Soft agar colony-forming assays were done at least in triplicate in 0.367% agar. Equal numbers of cells (10³) from each of the indicated clones were seeded onto 60-mm culture dishes with or without 500 μg/ml of G418 (active concentration) (GIBCO BRL). After cooling to 4°C, cells were cultured at 37°C in 5% CO_2 for 14 d. The colonies were visible and counted after staining with 0.2% p-iodonitrotetrazolium violet (Sigma Chemical Co., St. Louis, MO).

Tumorigenicity Assay in Nude Mice. Tumorigenicity assays were performed in nude mice as previously described (31). In brief, 10⁵ cells from each clone were subcutaneously injected on day 0 into the flanks of athymic nude mice of the same age. Each clone was assayed at four injection sites. Each tumor was measured with a tumorimeter and its three-dimensional diameter determined. The experimental mice were sacrificed on day 17, after which the tumors were dissected and their apparent weights recorded.

Flow Cytometric Analysis. For analysis of DNA content by flow cytometry, cells were fixed for at least 24 h in 70% ethanol, washed twice with PBS, and resuspended in PBS containing 20 μg/ml of propidium iodide (Sigma Chemical Co.), 0.5% Tween 20, and 400 U/ml of pancreatic RNase (Worthington Biochemical Corp., Freehold, NJ). Cells were left in the dark overnight before analysis in a FACSCAN® flow cytometer equipped with a doublet discriminator and LYSYS II and CELLFIT software (Becton-Dickinson & Co., Mountain View, CA).

Results

Isolation of pLPMLSN Recombinant Virus Producer and Stable Transfectants of pLPMLSN in B104-1-1 Cells. The G418ᴿ pLPMLSN-containing recombinant virus producers were selected after transfecting the GP+E-86 ecotropic packaging cell line with the recombinant retroviral construct pLPMLSN. Expression of the PML cDNA in these stable transfectants was confirmed by Western blotting. We found that two of six isolated packaging cell lines, designated GPE-PMLD and GPE-PMLF, expressed the 90-kD PML protein. For the negative control, we transfected the GP-E-86 and the PA317 packaging cell lines with pLXSN. Two clones of pLXSN-containing recombinant virus producer were isolated. They were confirmed as clones by their ability to infect NIH/3T3 cells and to produce G418ᴿ clones. In addition, Southern blot analysis of the genomic DNA isolated from these clones showed a 2.1-kb fragment of the BamHI/ApaLI-digested neomycin resistance gene (Neoᴿ) sequence of pLXSN (our unpublished data). The virus-producing titers were determined by their ability to produce Neoᴿ-containing colonies after infection with NIH/3T3 cells. The viral titers of the culture supernatants of GPE-PMLD and GPE-PMLF were determined to be 1.2–3.0 × 10⁶ CFU/ml, and the titer of the pLXSN
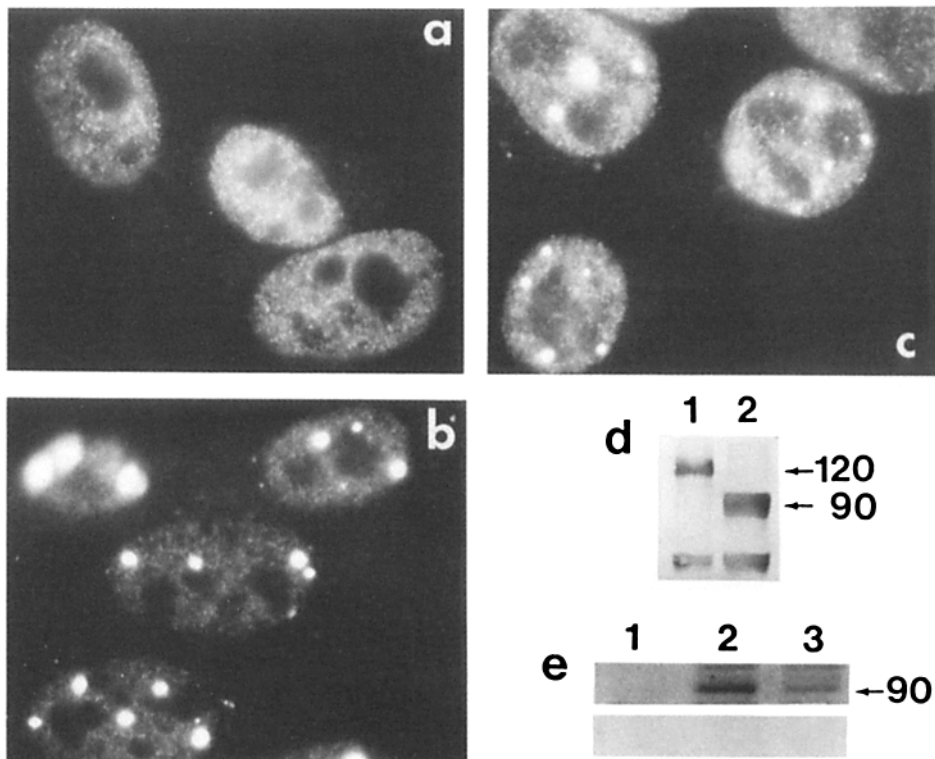

Figure 1. Expression of PML protein in PML-transfected B104-1-1 cells. Immunofluorescence staining of the PML protein in B-LXSN (mock-infected control) (*a*), PML1 (*b*), and PML2 (*c*) was performed by using the affinity-purified PML antipeptide antibody. Our PML antibody detected a single band of 120 (PML-RARα, lane *1*) and 90 kD (PML, lane *2*) proteins in NIH/3T3 cells transfected with the expression plasmids PML-RARα and PML (*d*). Expression of the 90-kD PML protein was detected in the stable transfectants PML1 (lane *2*) and PML2 (lane *3*) by Western blotting (*e*). Lane *1* represents the protein fraction isolated from the mock-infected control (B-LXSN).

recombinant virus producers (the control) was ~0.9–3.0 × 10⁶ CFU/ml.

Our antibody fails to detect the murine PML protein in the parental B104-1-1 or B-LXSN cells; however, as expected, after retroviral transduction, a 90-kD PML protein was detected (Fig. 1). 10 single colonies infected with recombinant GPE-PMLD or GPE-PMLF virus were isolated by selection in 500 μg/ml of G418. Three of the stable transfectants expressing the 90-kD PML protein by Western blotting (PML1, PML2, and PML3) were selected for further studies. Expression of the PML protein in PML1 and PML2 was demonstrated by immunofluorescence staining and Western blotting (Fig. 1, b and c).

Effects of PML on the Morphology of B104-1-1 Cells. As shown in Fig. 2, a–c, in comparison with its parental NIH/3T3 fibroblast, B-104-1-1 cells showed the typical morphological characteristics of a transformed phenotype (32–34). They showed more anchorage-independent and less contact limitation, and they were able to overlap and pile up even when some cells were separated from each other. The negative control experiment was conducted by mock infection of B104-1-1 cells with pLXSN-containing virus. These cells demonstrated G418 resistance but showed no discernable change in their morphology (our unpublished data). On the

other hand, PML1 and other PML-stable transfectants (PML2 and PML3) showed a more differentiated morphology. These cells become more anchorage dependent and more contact limited. The cell volume became larger, and the cytoplasm/nucleus ratio significantly increased. The morphology of the PML-expressing B104-1-1 cells appears to be similar to that of the wild-type NIH/3T3 cells. This result indicates that the expression of PML in B104-1-1 cells suppressed the transformation phenotype. Under serum-deprived conditions, morphology of NIH/3T3, B104-1-1, and the PML1 cells appeared significantly different from each other (Fig. 2, d–f). A significant number of cytoplasmic vacuoles were found in the PML1 cells.

Effects of PML Expression on Clonogenicity in Soft-Agar Assay and Tumorigenicity in Nude Mice. According to the results of these studies, PML is able to suppress the transformed phenotype of B104-1-1 cells. To investigate whether PML also suppresses the clonogenicity of these cells, we first tested the effects of constitutive PML expression on the anchorage-independent growth of B104-1-1 cells on soft agar. As shown in Fig. 3, the negative (mock-infected) control, that is, B-LXSN–infected cells, showed no suppression of colony formation in soft agar assay. However, the number of colonies formed in the B-PMLSN–infected B104-1-1 cells was sig-
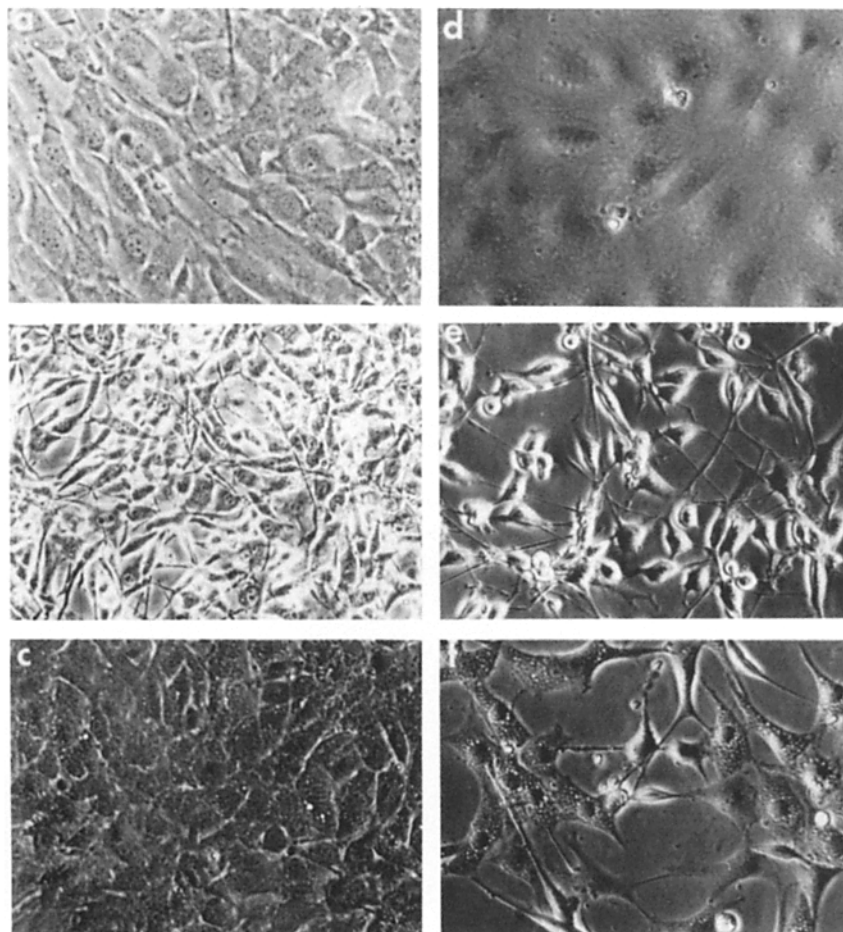

Figure 2. Effect of PML on the morphology of the B104-1-1 cells. (a) Light microscopic photograph of NIH/3T3 cells showing the typical morphology of monolayer cells. (b) Light microscopic photographs of the B104-1-1 showing the transformed phenotype. (c) The three stable transfectants of B104-1-1 cells expressing the PML protein (PML1, PML2, and PML3) displayed morphology similar to that of the NIH/3T3 cells. Cells were cultured under 10% BCS (a–c) or 0.5% BCS (d–f).
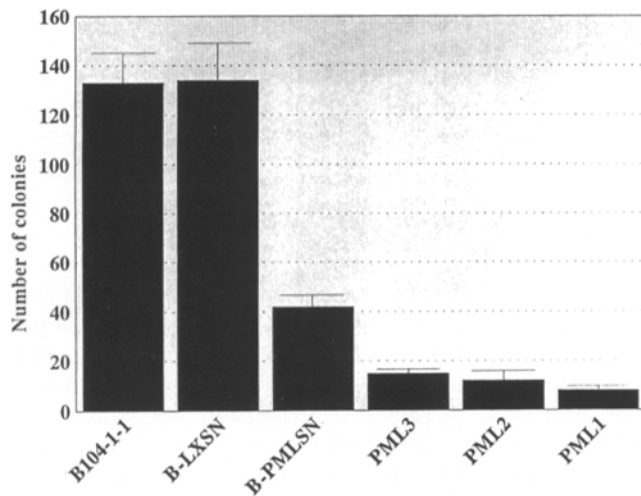
Figure 3. Suppression of clonogenicity of B104-1-1 cells by PML. Clonogenicity of B104-1-1, B-LXSN (mock-infected control), B-PMLSN (pLPMLSN containing virus-infected B104-1-1 cells), and the stable transfectants PML1, PML2, and PML3 were determined by soft agar assay as described in Materials and Methods. The number of colonies counted for each experiment represents the average counts of three plates. Bar heights represent mean ± SEM of three independent experiments.

Figure 4. The effect of PML on [³H]thymidine incorporation of B104-1-1 cells. Thymidine uptake in B104-1-1 (*B104*); B-LXSN (mock-infected control); B-PMLSN (transient PML transfectant), and PML1, PML2, and PML3 (stable PML transfectant) cells were performed as described in Materials and Methods. The results presented in each experiment represent the average of three plates. The bar height represents the mean ± SEM of two separate experiments. $P <$ *0.05, **0.02, ***0.01 vs control values. Statistical comparisons were performed by using the unpaired Student's t test; $P <0.05$ was considered statistically significant.

nificantly suppressed (up to 68%). Stable transfectants obtained from single colonies, PML1, PML2, and PML3, were able to suppress 89–94% of its CFU on soft agar.

The effects of PML on tumorigenicity of the B104-1-1 cells were assayed in athymic nude mice. As shown in Table 1, all nude mice injected with 10^5 B104-1-1 cells formed tumors as early as day 8. Similar results were observed in the group of mice injected with mock-infected B104-1-1 cells. No difference in the time to tumor appearance, average tumor size, or average tumor weight were found between the two groups of mice. However, the appearance of tumor was delayed until day 10 in nude mice that were injected with PML3 cells and until day 14 in those that were injected with PML2 cells;
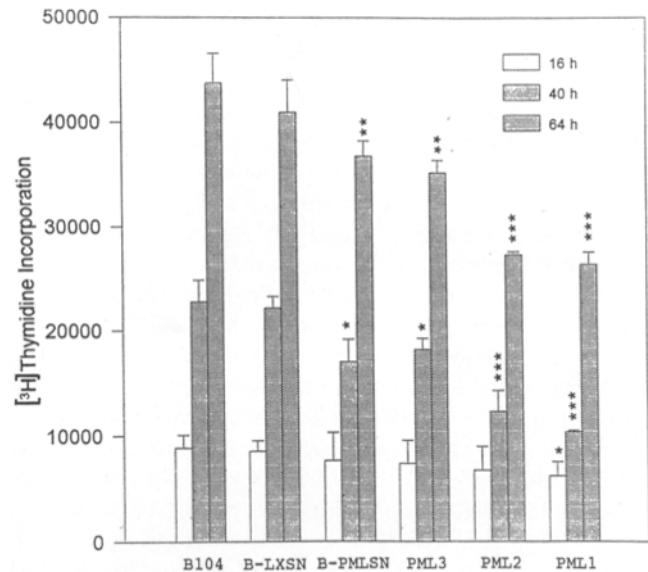
no tumor was observed on any site in the nude mice injected with PML1 cells. Tumor size and weight were significantly reduced in mice injected with PML2 and PML3 cells. These results demonstrate that PML is able to suppress the clonogenicity of B104-1-1 cells in soft agar assay and their tumorigenicity in nude mice.

The Effect of PML on Growth of neu-transformed Fibroblasts. [³H]Thymidine incorporation into DNA is generally well correlated with overall DNA synthesis, and it has

Table 1. *Suppression of Tumorigenicity of B104-1-1 Cells by PML*

Cell lines	Average tumor volume*					Average tumor weight*
	Day 8‡	Day 10	Day 12	Day 14	Day 16	Day 17
	mm^3					g
B104-1-1	49	175	459	1,479	2,160	2.57 ± 0.85
B-LXSN	53	171	477	1,562	3,061	2.57 ± 1.02
PML3	0	12	135	441	1,027	1.26 ± 0.90
PML2	0	0	0	45	112	0.28 ± 0.27
PML1	0	0	0	0	0	0.00 ± 0.00

Athymic nude mice of the same age were subcutaneously injected with 10^5 cells from each clone into the flanks. Tumor volume was determined with a tumorimeter. Tumor-bearing mice were killed on day 17, after which the tumors were removed and their apparent weights were recorded.
* An average of tumor volume and weight from four mice.
‡ Days after injection.

been used as a parameter to measure growth-affecting cells. B104-1-1, a neu-transformed NIH/3T3 fibroblast line, has been demonstrated to have an elevated growth rate (34). We thus determined the effect of PML expression on growth of the B104-1-1 cells by monitoring [³H]thymidine incorporation. The results presented in Fig. 4 indicate that no significant changes in thymidine incorporation were found in mock-infected cells. However, a significant reduction in thymidine incorporation was found in cells that were transfected with pLPMLSN and that expressed the PML protein (PML1 and PML2). Therefore, PML expression in the B104-1-1 cells could reverse, to a variable degree, the rate of DNA synthesis when cells were cultured under normal conditions. This observation of growth suppression by PML was further demonstrated by determining the growth curves of different cell types in the presence of various concentrations of BCS (10%, 0.5%). The results presented in Fig. 5 indicate that all cells expressing the PML protein showed a slower growth rate. The difference in growth rate between the mock-infected controls and the PML-expressing cells was more dramatic when the cells were cultured under low serum conditions. However, the effects of PML on the phase distribution of the cell cycle ap-

peared to be subtle and were insignificant either at normal or serum-deprived conditions (Table 2).

Effects of PML on the Expression of neu Oncogene. To investigate the mechanism of the PML suppression effect on B104-1-1 cells, we analyzed whether expression of PML protein in these cells affected the expression of neu. The neu gene encodes a 185-kD transmembrane protein of the tyrosine kinase receptor family (35–37). As shown in Fig. 6 a, B104-1-1 cells expressed high levels of the 185-kD NEU protein in both its phosphorylated (*upper band*) and unphosphorylated (*lower band*) forms. Expression of NEU in the GPE-PMLD– and GPE-PMLF–infected cells did not show any significant difference when compared with that of B104-1-1 and the mock-infected control. However, its expression was significantly suppressed in the stable PML transfectants, although a moderate level of NEU was still detectable in these cells (Fig. 6 b). Immunofluorescence staining of these cells indicated that almost 100% of the cells were PML⁺. This observation demonstrated that constitutive expression of PML in the neu-transformed NIH/3T3 cells significantly affects the expression of the NEU protein in vivo. Therefore, we conclude from these studies that PML did not significantly affect the expression of NEU in the transient assay, but did produce a dramatic reduction in NEU expression in the stable transfectants. Since there are 10 copies of activated neu oncogene transfected in the B104-1-1 cells, and high levels of the NEU protein is constitutively expressed, the moderate expression of NEU protein in the two stable transfectants suggested that constitutive expression of PML in these cells did not completely suppress the expression of activated NEU. Our results also suggest that growth suppression of the B104-1-1 cells by PML is a result of suppressing neu expression and the signals arising from the NEU protein.
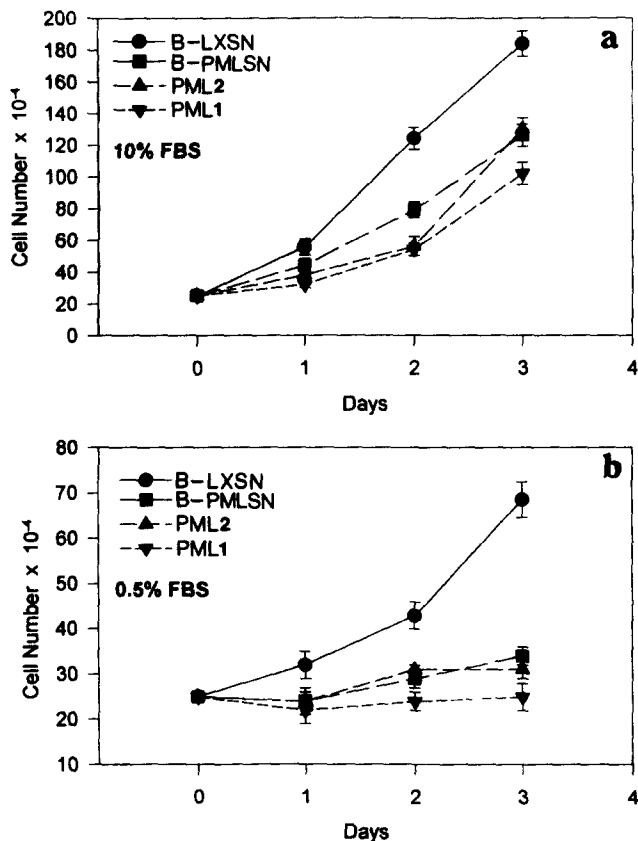

Figure 5. The effect of PML on growth rate of B104-1-1 cells. Stable PML transfectants of B104-1-1 (PML1 and PML2), B104-1-1 transiently infected with PML recombinant virus (B-PMLSN), and the mock-infected control (B-LXSN) were cultured in 10% (*a*) and 0.5% (*b*) BCS. The number of cells determined at each point represents the mean cell numbers of duplicated experiments.

Table 2. *Distribution of Cell Cycle Phases in B-LXSN and PML1 Cells in 10% and 0.5% BCS*

Cell type	Culture days	Percentage of BCS	Percentage of Cells		
			G1/G0	S	G2/M
B-LXSN	1	0.5	76.6	14.1	9.2
	1	10.0	65.5	20.9	13.5
	3	0.5	78.4	12.5	9.0
	3	10.0	64.3	21.1	14.5
PML1	1	0.5	72.9	17.4	9.6
	1	10.0	63.8	24.1	11.9
	3	0.5	80.2	11.8	8.0
	3	10.0	69.1	19.9	10.9

B-LXSN and PML1 cells (2 × 10⁵) were seeded onto 60-mm culture dishes in DME containing 10% or 0.5% BCS. Flow cytometry analysis of various phases of the cell cycle was performed as described in Materials and Methods.
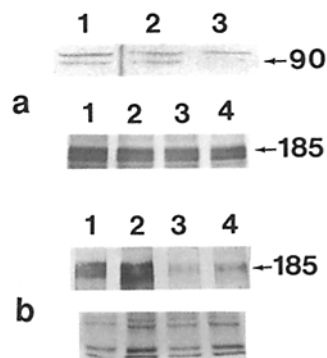
Figure 6. The effect of PML on the expression of NEU protein in stable transfectants and in transient infection. Expression of PML and NEU proteins in these cells was analyzed by Western blotting. For transient infection (*a*), total protein was isolated 72 h after viral infection. In the upper blots, lanes *1* and *2* represent proteins isolated from GPE-PMLD– and GPE-PMLF– infected cells; lane *3* represents protein isolated from the pLXSN mock-infected control. In the lower blots, lanes 1 and 2 represent proteins isolated from B104-1-1 and the mock-infected control, B-LXSN, respectively. Lanes *3* and *4* represent protein samples of GPE-PMLD and GPE-PMLF recombinant virus–infected cells. The upper and lower blots represent the expression of PML and NEU proteins, respectively. (*B*) Lanes *1* and *2* represent protein samples of B104-1-1 and the mock-infected control, respectively; lanes *3* and *4* represent protein samples of PML1 and PML2. The uppper blots show the expression of the NEU protein. The lower blots show the comparable quantity of protein samples of the upper blots stained with Coomassie blue. Expression of PML protein in PML1 and PML2 is shown in Fig. 1.

Discussion

The characteristic morphology of the activated *neu*-transformed NIH/3T3 cell line B104-1-1 makes it a good model for analyzing the functions of gene products that can affect the expression or the function of the activated *neu* oncogene. The B104-1-1 cell line in culture exhibited less anchorage dependence and less contact limitation by piling up and overlapping, especially during confluence. Our study demonstrated that the transformed phenotype could be reversed by infection with a retrovirally transduced *PML* gene. We have shown that PML is able to suppress anchorage-independent growth of B104-1-1 cells on soft agar. Furthermore, PML also suppressed activated-*neu*–induced transformation, which was demonstrated most stringently by the suppression of tumor formation in nude mice. These results indicated that PML can reverse *neu*-induced transformation. Since PML is a putative zinc finger transcription factor, this result implies that PML affects the activated *neu* oncogene either by inhibiting *neu* expression or by blocking the signals transduced by the NEU protein. Our results as shown in Fig. 6 suggest that constitutive expression of PML in B104-1-1 cells suppresses both the expression and biologic function of the *neu* gene. Suppression of NEU function in a *neu*-transformed cell line resulted in cell cycle arrest at the G_2/M phase (38, 39). However, PML-suppressed transformation of B104-1-1 cells did not significantly affect the cell cycle phases (Table 2), indicating that the effect of the molecular mechanism of PML suppression of *neu* is different from the effect of using specific monoclonal antibody against the NEU receptor (38, 39). In addition, morphology of PML1 is different from NIH/3T3 under serum-deprived conditions (Fig. 2).

The activated *neu* oncogene transfected in the B104-1-1 cell contains a single amino acid substitution in the transmembrane domain, and it has higher tyrosine kinase activity

than its normal counterpart (40–43). By Western blotting, we found that PML had only subtle effects on *neu* expression in the transient expression assay. However, the subtle change in *neu* expression cannot explain the significant reversal of the transformed phenotype and growth suppression that occurred in the PML-expressing B104-1-1 line. These results suggest that PML can reverse the *neu*-transformed phenotype of B104-1-1 cells and that this effect does not result from the suppression of *neu* expression, but mostly from the inhibition of signal transduction induced by activated *neu*.

Although the effects of tumorigenicity suppression by PML can be associated with that of growth retardation (31, 44, 45), the growth-suppressing effects of PML were demonstrated by decreased thymidine incorporation in PML-expressing B104-1-1 cells (B-PMLSN, PML1, PML2, and PML3). This was also reflected by the concordant suppression of cell growth in PML-expressing B104-1-1 lines shown in growth curves. The growth suppression effect of PML was more noticeable under conditions of serum deprivation. From our analysis of cell cycle distribution, it was clear that serum deprivation did arrest more fibroblasts in the G_0/G_1 phase, but that no significant difference resulted from the expression of PML. Thus, it is possible that the growth difference resulting from PML expression, especially in conditions of serum deprivation, came from a survival disadvantage, but not from cell cycle redistribution.

Our findings demonstrate that PML functions as a growth suppressor gene and, although it has yet to be demonstrated prospectively, the disruption of PML suppressor function after the t(15;17) will likely be an important contributor to the development of leukemic transformation.

In our previous study, we convincingly demonstrated that PML is a growth suppressor based on the following findings: (*a*) PML suppressed the anchorage-independent growth of APL-derived NB4 cells on soft agar and tumorigenicity in nude mice; (*b*) PML suppressed the oncogenic transformation of rat embryo fibroblast by cooperative oncogenes; and (*c*) PML suppressed transformation of NIH/3T3 cells by activated *neu* oncogene (22). We also showed by a cotransfection experiment that PML-RARα can suppress the transformation suppressor function of PML, possibly by a dominant negative-inhibitory effect. Our recent results demonstrated that PML is a phosphoprotein, and that at least one of the sites is phosphorylated by a tyrosine kinase. Our recent study also showed that PML is associated with the nuclear matrix (46). In addition to these results, we also found that PML is a promoter-specific transcription suppressor (22). Therefore, from these studies, we conclude that PML, the gene disrupted by the translocation breakpoint in APL, has many similar properties to tumor suppressors, for example, retinoblastoma gene product (Rb).

The mechanism of *neu* transformation may be coupled to phosphatidylinositol turnover through tyrosine phosphorylation of phospholipase C (PLC) (46, 47). It was also found in activated *neu*-transformed cells that PLCγ is constitutively phosphorylated on the tyrosine residues and forms a kinase-dependent complex with the mutation-activated *neu* receptor

(43). The coupling of activated *neu* to PLCγ produces inositol 1,4,5-triphosphate and diacylglycerol, which in turn induces Ca^{2+} and activates protein kinase C (PKC) (48). In view of the effects of PML on *neu*-induced transformation and survival promotion, it is possible that PML can attenuate or block the signal arising from activated *neu*. It was found that in lymphocytes, cell death (apoptosis) could be repressed by a PKC inhibitor, and that hormone-induced apoptosis could be sensitized by an inhibitor of tyrosine kinase (49).

In conclusion, the studies performed in this report further support the hypothesis that disruption of the PML gene by the t(15;17) translocation plays an important role in APL leukemogenesis.

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