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Promyelocytic leukemia protein-induced growth suppression and cell death in liver cancer cells

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The promyelocytic leukemia protein (PML), involved in the pathogenesis of acute promyelocytic leukemia, is a coactivator of p53 tumor suppressive functions. The ability of PML to inhibit growth and induce cell death in solid tumor cells, however, has not been determined. We therefore assayed the tumor suppressor activities of PML and compared them with those of p53 in four liver cancer cell lines. Following infection of cells with replication-deficient recombinant PML adenovirus, the exogenous PML localized in the nucleus and formed abnormally enlarged PML–nuclear bodies after 24 hours. *In vitro* growth curve analysis showed that the overexpressed PML initially induced a substantial G_1 cell cycle arrest and triggered massive cell death in all tested cell lines, irrespective of their p53 status. PML-induced cell death decreased by about 30% in the presence of a broad caspase inhibitor, zVAD. The cell death effect of PML was higher than that induced by p53 over a longer period of time. As with p53, overexpression of PML was closely related to upregulation of p21 and decrease of cyclin D1 expression. Unexpectedly, retinoic acid (RA) antagonized rather than enhanced PML-triggered cell death. RA enhanced the expression of adenovirus–cytomegalovirus-promoted PML at both transcription and protein levels within 12 hours after treatment; however, the PML protein was significantly degraded in the presence of RA at days 3–5 postinfection. PML degradation was also observed in SK-BR3 breast cancer cells treated with RA. Taken together, our findings strongly support the hypothesis that PML acts as a strong independent cell death inducer and that RA conversely abolishes the therapeutic effects of the PML proteins through proteasomal degradation of the protein.
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Understanding the function of the promyelocytic leukemia protein (PML) comes from characterizing the pathogenesis of acute promyelocytic leukemia (APL). In the vast majority of APL patients, the PML–retinoic acid (RA) receptor α (RARα) fusion protein renders hemopoietic progenitor cells resistant to apoptosis by deregulating the PML pathway and blocks the differentiation of myeloid cells induced by physiological concentrations of RA. PML is therefore regarded as a double-dominant-negative oncogene product, which interferes with both PML and RAR/RXR functions.[1–3]

Studies of APL tumorigenesis have shown that PML mediates tumor suppressive functions, including induction of apoptosis and growth arrest, at least in part through PML nuclear bodies (PML-NBs).[4] PML has also been reported to suppress the growth of breast and prostate cancer cells,[5,6] but it is not known whether this protein modulates key tumor suppressive pathways in the pathogenesis of various solid tumors.

PML has very similar characteristics to the well-characterized p53 tumor suppressor protein. For example, PML has been shown to act as a critical tumor suppressor in many cellular pathways, including those involved in apoptosis, cell proliferation and senescence.[4] PML−/− mice and cells are resistant to apoptosis induced by multiple stimuli,[7] and γ-irradiation of PML−/− splenocytes has been found to inhibit the activation of caspases 1 and 3,[8] suggesting that PML is essential for multiple apoptosis pathways and is a key protein in caspase-dependent apoptosis. Alternatively, PML overexpression has been reported to induce rapid cell death, but in the absence of typical features of apoptosis,[9] and this overexpressed PML may trigger a caspase-independent apoptosis pathway. Although these findings are contradictory, they are consistent in showing that PML plays a critical role in apoptosis.

PML was observed to interact directly with p53 and to colocalize with p53 in PML-NBs. In addition, p53−/− thymocytes are completely resistant to γ-irradiation-

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induced apoptosis, and the induction of p53 target genes, including BAX and p21, by γ-irradiation is impaired in PML−/− primary embryonic fibroblasts,[7] suggesting that PML can modulate p53 function as a transcriptional coactivator.[10] It was also reported, however, that PML can modulate p53-independent apoptotic pathways,[9] as well as stimulating the transcription of several apoptotic genes, including BAX, Fas L and p21. A recent study demonstrated that hCds/Chk2 activated by γ-irradiation phosphorylated PML and induced apoptosis in a p53-independent manner through the ATM-hCds/Chk2-PML pathway.[11]

RAs exert profound effects on cell proliferation, differentiation and apoptosis.[12,13] In APL patients, high doses of RA induce the expression of the RA-responsive genes, mediating myeloid cell differentiation.[14] PML is a critical component of the RA-dependent transactivation of p21, which regulates cell cycle progression and cell differentiation. In PML−/− cells, the ability of RA to suppress growth and induce differentiation are impaired.[15] RA signaling also regulates mammary epithelial cell growth and differentiation. Highly differentiated, estrogen receptor-positive mammary carcinoma cells express high levels of RARα and are therefore responsive to retinoid-dependent growth inhibition, G_1 cell cycle arrest and apoptotic cell death.[16,17]

The apoptosis-inducing activities of p53 and PML seem to be quite similar. In treating various cancers, reintroduction of wild-type p53 has been shown to induce apoptosis and tumor regression, thus confirming the therapeutic benefits of this protein.[18,19] Although PML is also essential for several apoptosis pathways and acts as a coactivator of p53 in PML-NBs, the ability of PML to independently suppress the growth of solid human tumors has not been determined. We therefore assayed whether the PML gene could act as an agent in treating liver cancer and compared the effects of this protein with those of p53. We report that overexpression of PML leads to significant G_1 cell cycle arrest, subsequently triggering cell death, in part through a caspase-dependent apoptosis pathway. Moreover, in comparing the cell killing effects of PML and p53, we show that PML expression resulted in a greater loss of cell viability over the long term, suggesting that this gene may be useful as a therapeutic agent. We also present that RA initially enhanced the PML expression through transcriptional activation of the adenovirus–cytomegalovirus (Ad-CMV) promoter during the first 12 hours, and subsequently it inhibited PML-induced apoptosis for the next 5 days due to the degradation of expressed PML proteins by the RA-activated proteasome degradation pathway.

Materials and methods

Cell culture and virus preparation

SK-Hep1 cells, derived from a human hepatic adenocarcinoma with nonfunctional p53 (ATCC HTB-52); Huh7 cells, derived from a hepatocellular carcinoma (HCC) expressing high levels of mutated p53 (point mutation at codon 220); and HepG2 cells, derived from a hepatoblastoma with wild-type p53 (ATCC CRL-11997) were maintained in DMEM supplemented with 10% FBS. Hep3B, derived from an HCC lacking the p53 gene (ATCC HB-8064) was grown in MEM with 10% FBS.

The recombinant replication-deficient adenovirus-PML (Ad-PML) was kindly provided by Dr Chang of the University of Texas, MD Anderson Cancer Center. To construct the recombinant adenovirus containing the human p53 gene (Ad-p53), p53 cDNA was isolated and cloned into pCA14 shuttle vector (Microbix, Onta., Canada) under the control of the CMV promoter. Ad-p53 was generated by homologous recombination after cotransformation with pTG-CMV, a cosmid cassette containing a full-length Ad5 genome with E1 and E3 deletions, a kind gift from Dr Verca (University of Fribourgh, Switzerland).[20] All viruses were propagated, purified, and titrated as described.[21] Transduction of the β-Gal gene by the recombinant adenoviruses was performed with a multiplicity of infection (MOI) of 20–200. After infection of Ad-β-Gal for 2 hours, cells were washed and incubated at 37°C in a 5% CO_2 incubator for 24 hours, after which the infected cells were fixed with 0.23% glutaraldehyde for 5 minutes and stained with X-Gal for 4 hours at 37°C. The mean percentage of blue cells was determined by counting three fields from three different experiments under light microscopy.

Analysis of cell growth

Cells were plated in triplicate at a density of 2×10^4 cells/well in 24-well tissue culture plates. After 24 hours, cells were infected with Ad-PML, Ad-p53 or Ad-β-Gal for 2 hours at 37°C, washed with culture medium and incubated at 37°C in 5% CO_2. Cell viability was determined by trypan blue exclusion. Live cells for each treatment were counted daily for 6 days.

Flow cytometry

Cells were seeded on 60-mm dishes and grown to 60–80% confluence, after which they were infected with adenoviral vectors. Cells were harvested 1, 3 and 5 days after infection, fixed in 70% ethanol and stored at 4°C. For determination of DNA content, harvested cells were resuspended in 50 μg/ml propidium iodide (PI), and assayed by flow cytometry on a FACSCalibur (Becton Dickinson), with the results analyzed with ModFit LT2.0 and Cellquest software.

Western blotting

Cells from 10-cm dishes, whether or not virally infected, were harvested and resuspended in extraction buffer containing 50 mM Tris-HCl (pH 7.5) 300 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, and proteinase inhibitor cocktail (1% Aprotinin, 1% Leupeptin, 2 mM PMSF). Cell extracts were centrifuged at 12,000 rpm for 5 minutes to remove cellular debris. Supernatants were electrophoresed through 10 or 12% SDS-PAGE gels, and the proteins were transferred onto nitrocellulose. Blots

were incubated with antibodies recognizing PML (Santa Cruz Biotechnology, Santa Cruz, CA), p21 (Upstate USA, Charlottesville, VA), Cyclin D1 (Cell Signaling, Beverly, MA), p53 (Upstate) and actin (Santa Cruz). Detection was accomplished using ECL reagent (Amersham, Piscataway, NJ) according to the manufacturer's protocol.

Treatment with RA and zVAD

SK-Hep1 cells were plated at a density of 5×10^4 in 60-mm plates, and the cells were infected for 2 hours with Ad-PML or Ad-p53 at MOI 50. The cells were washed, resuspended in fresh culture media and treated for 3 days with 5 μM all-*trans*-RA (ATRA, Sigma, St Louis, MO), reconstituted in DMSO, or with 0.1% DMSO, and, where indicated, with 50 μM zVAD-FMK (ICN, Bryan, OH) dissolved in DMSO for 3 or 5 days.

Immunofluorescence microscopy

Cells were seeded on 12-mm round coverslips in 24-well culture plates and infected with each virus. After the indicated days, cells were fixed with 4% paraformaldehyde, washed twice with PBS, blocked with 2% BSA in PBS for 30 minutes, and incubated with primary antibodies against p53 or PML for 1 hour at room temperature. The cells were washed with PBS, incubated with the appropriate secondary antibody (Zymed, South San Francisco, CA) for 30 minutes and with DAPI (0.5 μg/ml) for 5 minutes, and analyzed by fluorescence microscopy.

Real-time PCR

Primers for the PML gene were chosen with the assistance of the computer program Primer Express (Perkin-Elmer Applied Biosynthesis, Foster City, CA). For the RT-PCR, 5 μg of total RNA was retrotranscribed using the Superscript kit (Invitrogen, Carlsbad, CA). PCR was performed using the following oligonucleotides: forward primer 5′-ATG GAG CCT GCA CCC GCC CGA TCT C-3′, reverse primer 5′-GCA CTT GAG CTC ACT GTG GCT G-3′ and internal probe 5′-FAM-CGA GAG TCT GCA GCG GCG CCT GTC GGT GTA-TAMRA-3′. The GAPDH gene was used as an endogenous RNA control and each sample was normalized on the basis of its GAPDH content. The relative PML gene expression level was also normalized to a sample from uninfected SK-Hep1 cells. Final results, expressed as *n*-fold differences, were determined in exponent as follows:

$$n\mathrm{PML} = 2^{(\mathrm{GAPDH-PML})\mathrm{infected}} / 2^{(\mathrm{GAPDH-PML})\mathrm{uninfected}}$$

The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 minutes and 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Experiments were performed in duplicate for each data point.

Results

Overexpression of PML by infection with Ad-PML

To determine the transduction efficiency of recombinant adenoviruses in human liver cancer cell lines, we evaluated the percentages of β-Gal-positive cells at several MOI (Fig 1A). Based on these data, we used an MOI of 50 in SK-Hep1, Hep3B and HepG2 and an MOI of 200 in Huh7 cells for subsequent experiments. These MOIs resulted in efficiencies of 65–85%, while minimizing the cytotoxic effects of the recombinant adenovirus itself. By immunofluorescent staining, globular or enlarged ring-shaped PML-NBs were observed in Ad-PML-infected SK-Hep1 cells from day 1, whereas control cells infected with Ad-β-Gal showed no recognizable PML NBs (Fig 1B–a,c). Compared with uninfected cells, the levels of PML mRNA were 1.7×10^4 times higher in Ad-PML-infected cells at 12 hours postinfection with real-time RT-PCR assays as described in "Materials and methods" (data not shown), indicating that the basal level of PML is very low in the cell line. The staining pattern of newly formed PML-NBs was very similar in the four liver cell lines tested. p53 proteins were stained weakly in uninfected cells (Fig 1B–d) and significantly increased after Ad-p53 infection (Fig 1B–f).

Cell growth arrest by overexpression of PML

Infection with Ad-PML, and the resulting overexpression of PML, induced marked growth inhibition in the four liver cell lines (SK-Hep1, HepG2, Hep3B and Huh7) regardless of their p53 status (Fig 2). All controls, including mock-infected and Ad-β-Gal-infected cells, did not show significant growth inhibition, except for Hep3B cells infected with Ad-β-Gal, which showed partial growth suppression, probably due to the hypersensitivity of these cells to the adenoviral vector itself. The cell growth curves of Ad-PML-infected cells were very similar to those obtained after infection with Ad-p53. To further compare the inhibitory effects of PML on hepatic cancer cell growth, we used SK-Hep1 cells, which have a rearranged nonfunctional form of p53, rather than Huh7 and Hep3B cells, which were extremely sensitive to Ad-PML and Ad-p53, or HepG2 cells, which express wild-type p53.

Infection with either Ad-PML (73.32%) or Ad-p53 (76.12%) led to a significant increase in the G_1 population of SK-Hep1 cells compared with mock-infected (44.2%) or Ad-β-Gal-infected (49.40%) cells (Fig 3). PML overexpression was associated with a significant induction of p21 proteins and a decrease of cyclin D1, while there was no detectable alteration in the levels of endogenous p53 and hCds1/Chk2, a DNA damage checkpoint kinase that functions in γ-irradiation-induced apoptosis and in the stabilization of p53 (Fig 4).[22,23] Overexpression of p53 protein also induced expression of hCds1/Chk2 and p21 proteins initially; however, the latter declined to basal level after 3 days.

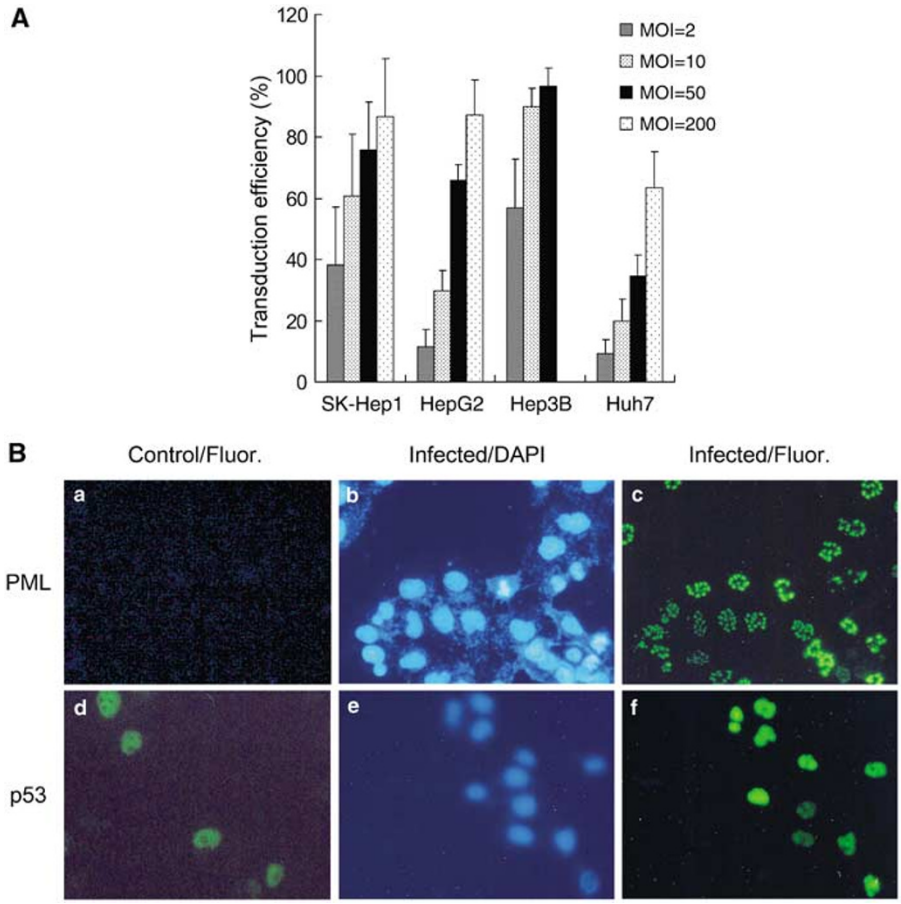

Figure 1 Determinations of transduction efficiencies and protein expression of four liver cancer cell lines. (**A**) The SK-Hep1, Hep3B, Hep2G and Huh7 liver cancer cell lines were infected with Ad-β-Gal adenoviral vector at different MOIs. After 48 hours, the infected cells were stained with X-Gal, and the mean percentage of blue cells was determined by counting three fields each from three different experiments. (**B**) Indirect immunofluorescence staining of SK-Hep1 cells for PML (**a** and **c**) and p53 (**d** and **f**) before (**a** and **d**) and after (**c** and **f**) Ad-PML and Ad-p53 infections at an MOI of 50: no PML NBs are visible before Ad-PML infection (**a**), while PML-NBs are enlarged and diffuse nucleoplasmic staining for PML is observed after Ad-PML infection (**c**). Endogenous p53 is stained very weakly in some cells (**d**) and the protein levels are significantly increased by Ad-p53 infection (**f**). Infected cells were counterstained with DAPI (**b** and **e**).

Cell death induction by overexpression of PML

When we infected SK-Hep1 cells with Ad-PML, we observed that the level of cell death after 3 days was slightly lower than that observed in SK-Hep1 cells infected with Ad-p53. At 5 days after viral infection, however, overexpression of PML resulted in massive cell death (Fig 5), with the degree of apoptosis (96.9%) being slightly higher than that observed after Ad-p53 infection (90.5%).

We also assayed whether cell death induced by PML overexpression occurs through a caspase-dependent mechanism. We found that the caspase inhibitor zVAD moderately decreased PML-induced cell death, as well as p53-dependent apoptosis, without significant differences in the rate (Fig 5a). Cell cycle analysis of the remaining cells, which underwent morphological changes but retained the ability to exclude trypan blue, revealed that PML expression dramatically induced cell cycle arrest at G_1 with a simultaneous decrease in the fraction of cells in S phase, and that caspase inhibitor blocked the prolonged G_1 arrest induced by PML (Fig 5b). To further exam the nature of cell death induced by PML, we analyzed morphological features after incubation with DNA-binding dye Hoechst 33342 (Fig 6A). The PML overexpressing cells showed significant cellular swelling and the irregular plasma membrane probably due to bleb rupture, which are characteristic features of a necrotic cell death process. The caspase-3 activation was not observed (data not shown), however, we found that the proapoptotic BAX protein increased more than two-fold in Ad-PML-infected cells (Fig 6B). Together, these findings support the notion that PML-induced cell death is the combined results of apoptotic and necrotic cell death, a phenomenon called necroapoptosis or aponecrosis.

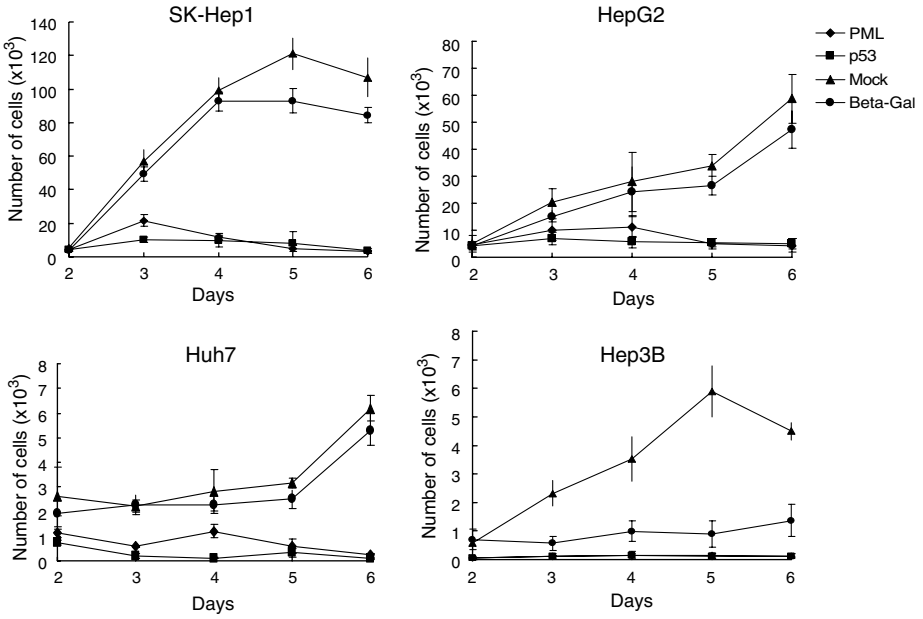

Figure 2 Cell growth curves of liver cancer cell lines infected with Ad-PML or Ad-p53. The growth of SK-Hep1, HepG2, Huh7 and Hep3B cells infected with Ad-PML was almost completely suppressed from day 1 to 6. All controls, including mock-infected (culture medium alone) and Ad-β-gal-infected cells, showed no significant growth inhibition, with the exception of Ad-β-Gal-infected Hep3B cells, the growth of which was partially suppressed. Each point represents the mean of triplicates. SD was always $<$12% of the mean. ◆, Ad-PML; ■, Ad-p53; ▲, Mock; ●, Ad-β-Gal.

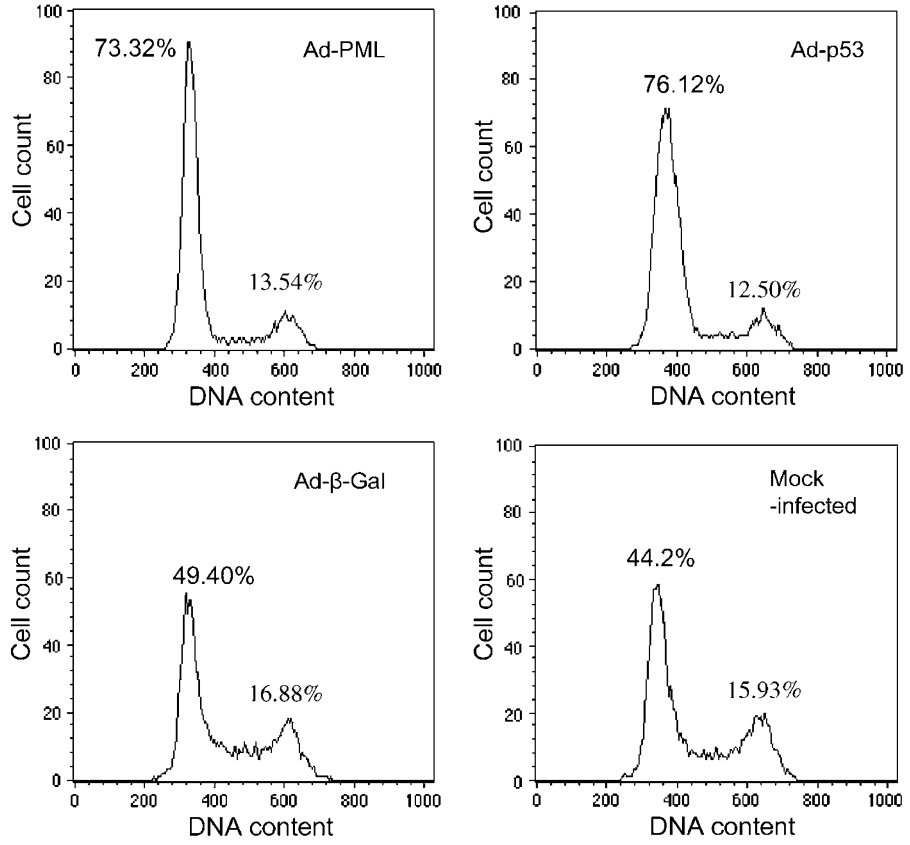

Figure 3 Cell cycle analysis after infection of SK-Hep1 cells with Ad-PML, Ad-p53 or Ad-β-Gal. The percentage of cells accumulated at the G_1/S and G_2/M phases of the cell cycle after 2 days of infection is indicated at the top of each peak. Significant proportions of both Ad-PML- and Ad-p53-infected SK-Hep1 cells were arrested in G_1 phase of cell cycle, compared with Ad-β-Gal- or mock-infected control cells.

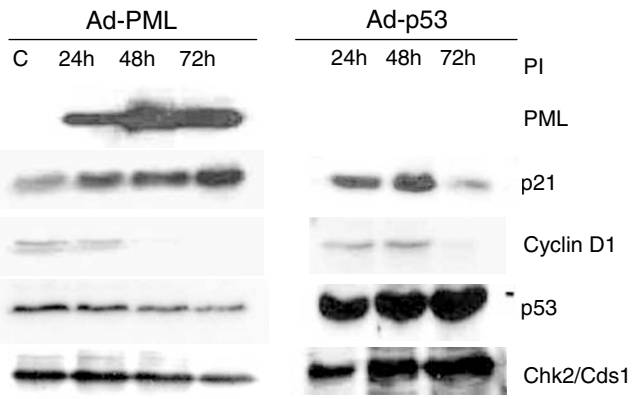

Figure 4 Expressions of cell cycle regulatory proteins after infection of SK-Hep1 cells with Ad-PML or Ad-p53. The level of p21 increased, while the amount of cyclin D1 decreased significantly after Ad-PML infection. There were no detectable alterations in the levels of endogenous p53 and hCds1/Chk2. Overexpression of p53-induced p21 expression, which declined to basal level after 3 days; however, it induced continuous overexpression of hCds1/Chk2.

Effects of RA on PML-induced cell death

RA is essential for differentiation of hemopoietic precursor cells and is a promising therapeutic agent for blood born and solid tumors. We therefore tested the combined effects of PML and RA on liver cancer cells. Surprisingly, we found that RA markedly inhibited cell death induced by Ad-PML infection of SK-Hep1 cells, with concomitant increases in the G_1 and G_2/M populations (Fig 7a). Ad-p53-infected SK-Hep1 cells showed similar responses to RA, indicating that RA does not act synergistically with the tumor suppressor genes PML and p53 in inducing cell death in SK-Hep1 cells.

To determine whether treatment with RA affects the expression of downstream targets associated with cell cycle arrest, we performed Western blot analyses of extracts of Ad-PML and Ad-p53-infected cells. Compared with untreated Ad-PML-infected SK-Hep1 cells, RA-treated cells expressed sharply decreased amounts of overexpressed PML and p53 (Fig 7b), with PML levels declining 15- to 20-fold after 5 days. RA, however, had no effect on expression of endogenous p53 in either infected or uninfected cells. RA also decreased expression of a CDK inhibitor, p21, to the basal level in infected cells, whereas uninfected SK-Hep1 cells, which underwent contact inhibition, showed induction of p21 expression.

DAPI staining revealed a few apoptotic bodies with disassembled fragments of nuclei in Ad-PML-infected cells at day 4 (Fig 7c), while some cells showed cytoplasmic swelling, a feature of necrotic cell death.[24,25] Cells infected with Ad-p53 underwent similar morphological changes. Interestingly, RA treatment significantly reduced these morphological changes in cells overexpressing PML or p53, with membrane blebbing and disassembly almost completely absent from infected, RA-treated cells compared with infected, RA-untreated cells. RA inhibition of cell death was more obvious in cells overexpressing p53 than in those overexpressing PML. Compared to p53-infected cells, we found that PML overexpression led to cell death continuously over a long period of time, suggesting that Ad-PML-infected cells may overcome the negative effects of RA, showing reduced, but still progressive, cell death. To further investigate the RA-induced degradation of PML and p53 protein, we infected Ad-PML virus and added RA in SKBR-3, a retinoid-sensitive breast cancer cell line.[16] Again, SKBR-3 cells exhibited complete degradation of PML proteins at 120 hours postinfection (Fig 8a). It has been known that the immediate early (IE) gene promoter of the CMV contains multiple RA-responsive elements, therefore, RA treatment enhances the expression of the transduced gene by an Ad–CMV-promoter vector.[26,27] Real-time PCR detection of PML transcripts presented that the expression of PML at the RNA level increased in a time-dependent manner at the early times of postinfection with RA treatment (Fig 8b). In parallel, SK-Hep1 cells exhibited an increase in PML proteins when treated with RA about 1.5-fold at 24 hours postinfection (Fig 8c). Together, these findings suggest that RA initially transactivates PML expressions from the CMV promoter-driven expression-cassette; however, after longer periods of incubation (3–5 days), RA treatment degrades the overexpressed proteins, presumably by RA-activated proteasome-mediated degradation.

Discussion

Gene therapy has recently emerged as a new and promising tool for cancer treatment.[28,29] Adenoviral delivery has confirmed the therapeutic effect of the p53 tumor suppressor gene in treating cancers.[30] It was recently reported that PML functions as a critical tumor suppressor gene in many cellular pathways, including those involved in apoptosis, cell proliferation and senescence.[3,31] Moreover, PML has been shown to inhibit colony formation of transformed cells and tumor growth in nude mice,[5] as well as the growth of cells derived from solid tumors, including those of breast and prostate.[5,6] We therefore investigated whether the PML gene may be useful in the treatment of HCC and cholangiocarcinoma of the liver. We overexpressed PML by using the recombinant adenoviral vector in various types of liver cancer cells (SK-Hep1, HepG2, Hep3B and Huh7), evaluated the antitumor activities of PML and compared its effects with those of overexpressed p53. Compared with control vector-infected cells, PML overexpression almost completely suppressed cell growth in all four liver cancer cell lines tested. In agreement with previous reports, p53 overexpression also resulted in strong growth arrest of these cell lines. We also found that the two genes were similar in inducing cell cycle arrest at the G_1 phase. However, they differed in that PML overexpression induced slow, but progressive growth arrest and cell death over a long period of time, whereas p53

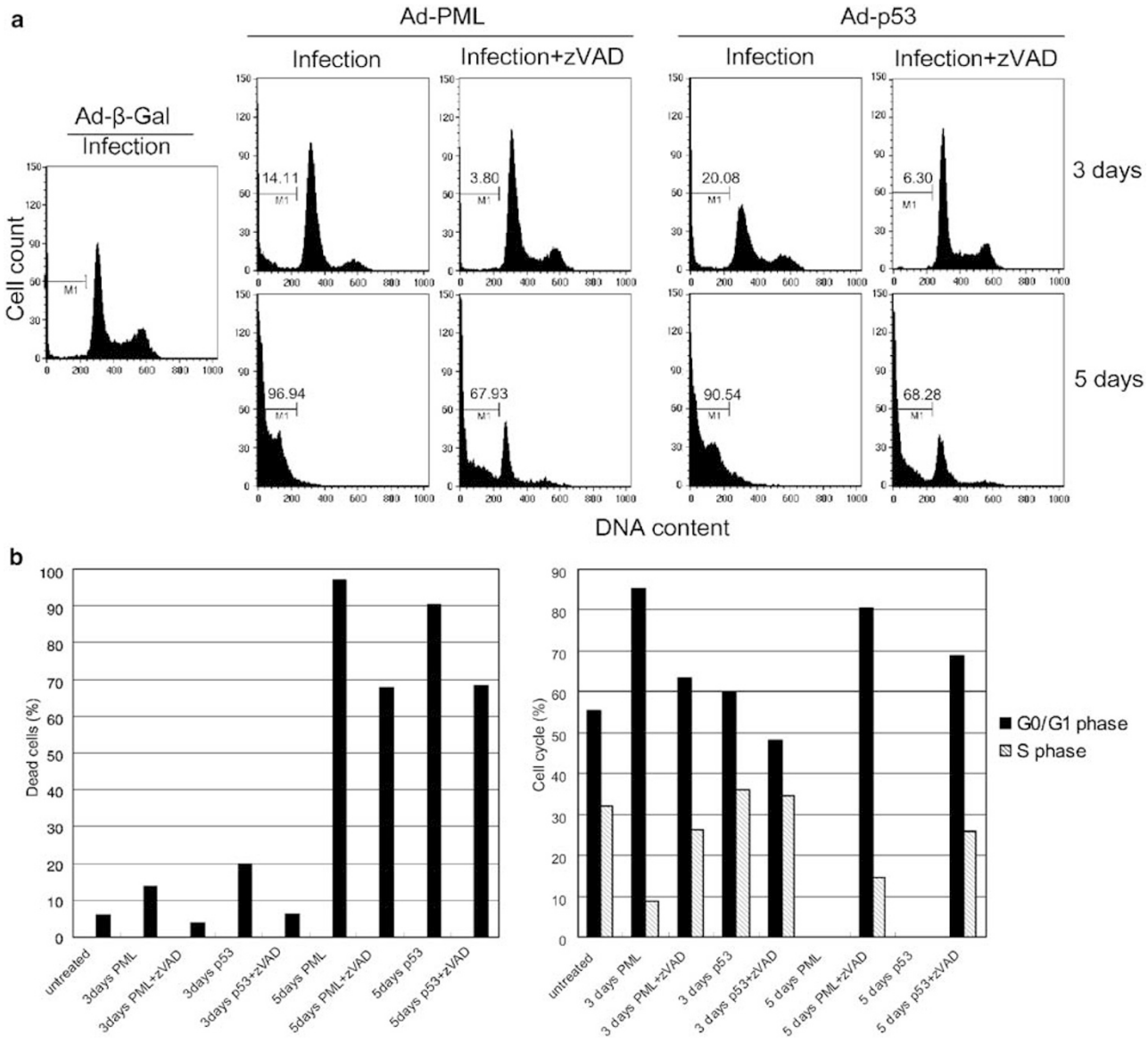

Figure 5 Effects of the caspase inhibitor zVAD on PML- and p53-induced cell death. (**a**) Flow cytometric analysis of apoptosis (M1) after treatment of Ad-PML- and Ad-p53-infected SK-Hep1 cells with zVAD. Cell death of Ad-PML-infected cells is moderately increased, being slightly lower at 3 days and slightly higher at 5 days, than the death rate of Ad-p53-infected cells. The caspase inhibitor zVAD decreased PML- and p53-induced cell death without significant alterations in the rate. (**b**) Quantitation of PML- and p53-induced cell death in (**a**) Results are derived from three separate experiments and represent means of triplicate determinations (right). Quantitative evaluation of the population of live cells in G_0/G_1 and S phases of the cell cycle, expressed as percentages. Means of three different experiments are shown (left). PML expression dramatically induced cell cycle arrest at G_1 phase, with simultaneous decrease in the percentage of cells at S phase, whereas the zVAD blocked the prolonged G_1 arrest induced by PML.

overexpression led to marked growth arrest within a relatively short period of time. These results indicate that PML is a potent gene for controlling cell proliferation and may be more useful than p53 as a long-term therapeutic agent in the treatment of liver cancer.

Although the molecular mechanisms underlying the tumor suppressive functions of PML need further elucidation, this protein has been shown to act as a transcriptional coactivator of p53 in PML-NBs.[7,10] The p53–DNA binding activity, as well as the induction of p53 target genes, such as p21 and Bax, was shown to be impaired in PML−/− thymocytes, suggesting a PML-dependent, p53-regulatory pathway[7,32,33] and the modulation of the tumor suppressor activity of PML through p53. In contrast, our results indicate that PML activates the cyclin-dependent kinase inhibitor p21, arrests cells at the G_1 phase and decreases cyclin D1, inhibiting the activity of CDK4-cyclin D and thus preventing

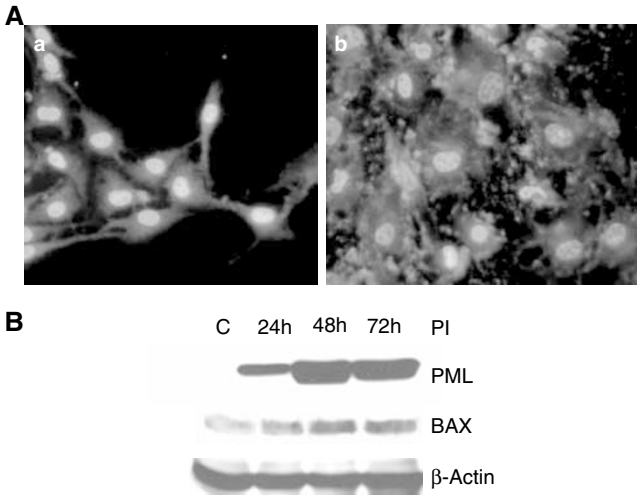

Figure 6 PML induces both apoptotic and necrotic pathways. (**A**) Nuclear changes of Ad-PML-infected cells were monitored with the DNA probe, Hoechst 33342 under LM at ×200 magnification. PML overexpressing cells showed morphological features of necrosis including enlarged nucleus, cellular swelling and surface blebbing, whereas other apoptotic changes such as chromatin condensation were not observed. (**B**) PML overexpression induced proapoptotic BAX protein in SK-Hep1 cells. BAX protein increased >two-fold in Ad-PML-infected cells at 48 hours postinfection as compared with that found in uninfected cells.

proliferating cells from progressing from G_1 to S phase. In addition, we found that the ability of PML to alter cell cycle regulatory proteins was not related to the status of p53, thus indicating that p53 is not critical for PML-induced cell cycle arrest in liver cancer cells.

There are two outstanding questions about the role of PML in apoptosis. The first is whether the ability of PML to induce apoptosis is dependent on p53 functions. Both PML−/− and p53−/− thymocytes have been shown to be resistant to γ-irradiation-induced apoptosis.[7] The tumor suppressive activities of p53 were found to be impaired in PML−/− primary cells, supporting the hypothesis that PML is required for proper functions of p53. In contrast, PML has been reported to modulate p53-independent proapoptotic pathways through interaction with DAXX, a positive mediator of FAS- and TGFβ-induced apoptosis.[34] We have shown here that infection of cells with Ad-PML resulted in massive cell killing, with the apoptotic activity of PML being more potent than that of p53 over a longer time. Although it is unclear whether the potent apoptotic effects of PML are due to its relative stability, in that it has a half-life of 5–6 hours, or to its relative activity, these effects suggest that PML may be of use in cancer gene therapy.

The second question is whether PML-induced apoptosis is caspase-dependent. Data obtained in transient expression studies showed that PML-induced cell death was enhanced, rather than blocked by the broad caspase inhibitor zVAD.[9] In contrast, caspase activation has been shown to be impaired in PML−/− cells in response to various stimuli such as TNF, ceramide and γ-irradiation.[8] When we tested the effects of zVAD in Ad-PML and Ad-p53-infected SK-Hep1 cells, we found that treatment with this inhibitor reduced cell death to a comparable extent in both, suggesting that PML and p53 participate in both caspase-dependent and -independent apoptosis programs. Flow cytometry analysis of preapoptotic cells showed that exposure to this caspase inhibitor can lead to a decrease of cells in G_1 phase, which subsequently results in modest cell death reduction. Since most dead cells by PML overexpression showed characteristic morphology of necrosis including cell swelling and plasma membrane protrusions, we believe that PML induces apoptotic and necrotic cell death at the same time in the cells.

RA is a potent therapeutic drug in the treatment of APL, and its effectiveness is now being evaluated in various solid tumors including neuroblastomas and breast cancers.[16,17,35] PML was shown to be required for RA to induce myeloid differentiation of precursor cells,[14,32,33] suggesting that RA could accelerate the tumor suppressive activity of PML. Surprisingly, we found that the cell death activity of PML was almost completely abrogated by RA in liver cancer cells. RA significantly delayed cell death and accumulated cells at the G_1/S transition, thus blocking PML-induced changes. Similar effects of RA were observed in Ad-p53-infected cells. Moreover, RA decreased the expression of PML, p53 and p21 in both infected cell lines. RA-induced PML decrease was consistently observed in SKBR-3 breast cancer cells, even though a real-time RT-PCR assay demonstrated that RA did not downregulate the PML transcripts, and rather upregulated PML, suggesting involvement of post-transcriptional mechanism in PML downregulation. RA triggers degradation of both PML/RARα and RARα through an as yet unidentified proteasome-dependent pathway.[36] The RA-induced degradation of PML/RARα, thus making an APL cell line dramatically sensitive to arsenic-triggered apoptosis, provides a basis by which RA could inversely control PML.[37] Proteasome or autocatalytic protease complex degrades most of the cryptozoic proteins and in particular of short-lived proteins critical for cell proliferation and cell cycle regulation including p53, the cycling-dependent kinase inhibitor $p27^{Kip1}$ as well as cytokines by ATP/ubiquitin-dependent proteolysis.[38] Interestingly, the cell death program can be activated by proteasomal inhibitors in HL60 cells.[38] On the basis of the above information and the results presented here, we speculated that RA treatment induces proteasomal degradation of PML after a long period incubation. Thus, combined therapy with PML and RA does not produce synergistic tumor cell killing and instead delays cell death by activating two conflict pathways of PML- promoted cell death and RA-induced proteasome-dependent degradation of PML.

Through the direct comparison of PML and p53 as cell death executioners, we have shown that the cytopathic effects of PML are comparable to those of p53 over a short period of time, while being more potent over a long period of time. We also found that PML and p53 modulate the expression of cell cycle regulatory proteins

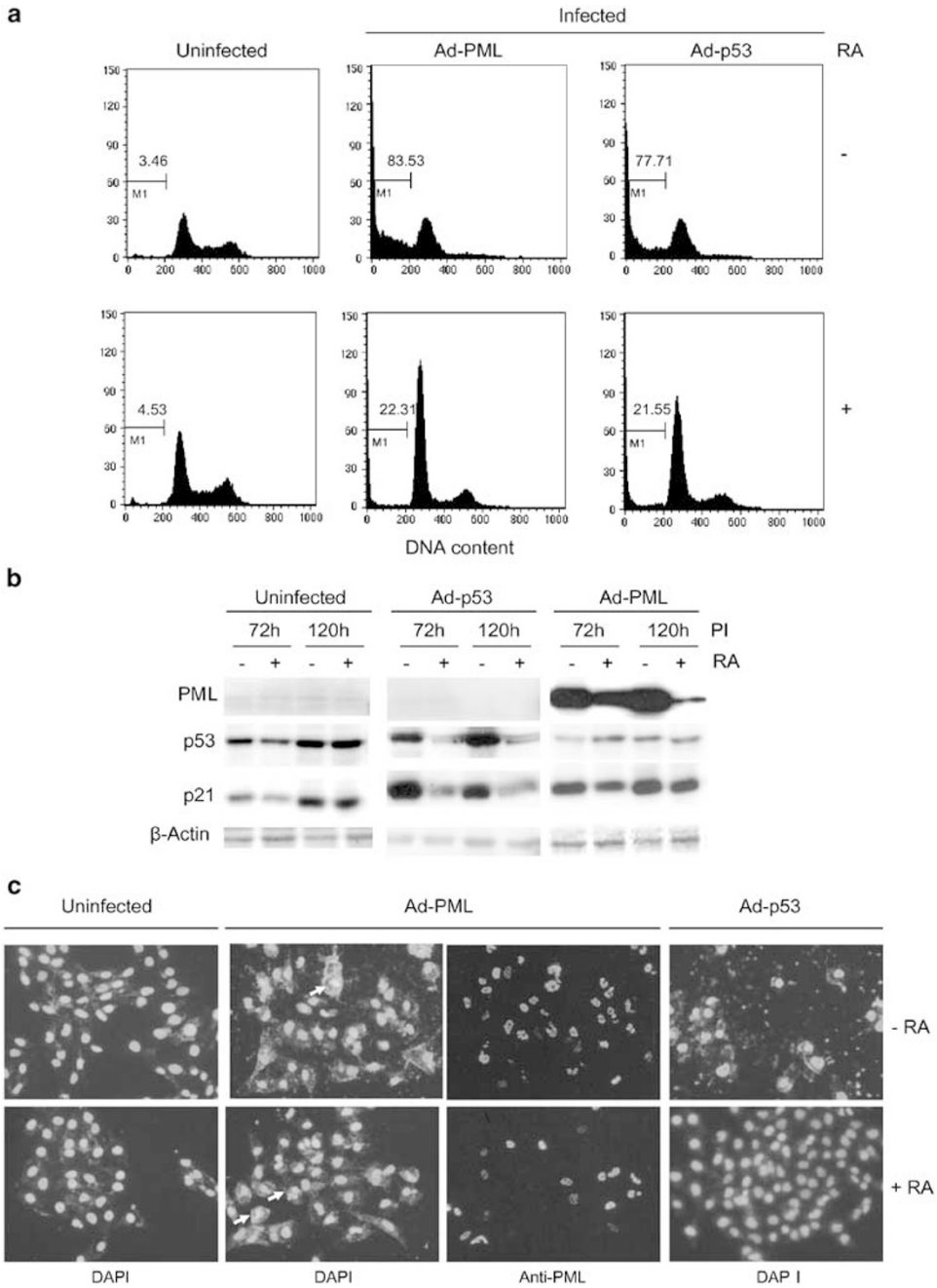

Figure 7 Effects of RA on PML-induced cell death. (**a**) Flow cytometric analysis of apoptosis after treatment with RA. Low fluorescent region (M1) shows apoptotic cells, and the relative percentages of dead cells are presented. RA markedly inhibited apoptosis of Ad-PML-infected SK-Hep1 cells, with concomitant increases in the populations at G_1 and G_2/M. (**b**) Western blot analysis of expression of PML, p53 and p21 in RA-treated, Ad-PML- or Ad-p53-infected cells. RA treatment decreased p21 expression in Ad-PML- and Ad-p53-infected cells, as well as the expression of PML and p53. (**c**) Morphological analysis of the Ad-PML- and Ad-p53-infected SK-Hep1 cells after RA treatment. Speckled PML-NBs were visualized by indirect immunofluorescent staining. Nuclear fragmentation of apoptotic cells is indicated by white arrows.

to a similar extent, suggesting that these two proteins execute cell death through very similar pathways. In conclusion, our results strongly support the potential usefulness of PML in cancer gene therapy, including the treatment of various types of liver cancers, and suggest that the PML gene can be effective in tumor cells resistant to p53-mediated apoptosis, such as advanced liver cancers and acute myeloid leukemias in adults.

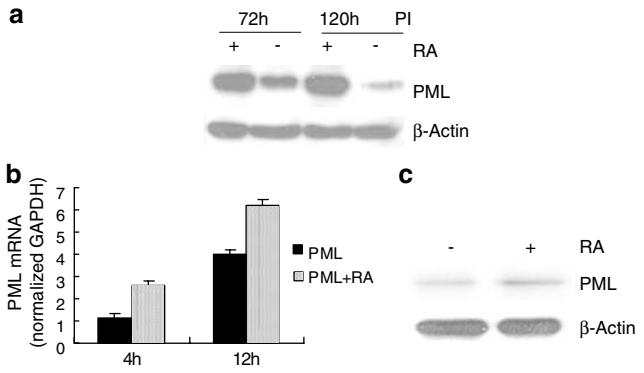

Figure 8 RA modulates PML expression at the RNA and protein levels. (**a**) The amounts of PML proteins decreased with RA treatment in Ad-PML-infected SKBR-3 cells, RA-responsive breast cancer cells at 3 and 5 days of infection. (**b**) RA enhances the expression of PML mRNA transcripts in Ad-PML-infected, RA-treated cells. Semiquantitative real-time PCR detection of PML mRNA using the total RNA prepaed from uninfected, Ad-PML infected at 4 or 12 hours postinfections was performed and normalized to GAPDH. (**c**) Western blotting of PML in the whole-cell extracts of Ad-PML-infected cells at 24 hours postinfection. The PML proteins were not detected at 12 hours postinfection of Ad-PML regardless of RA treatment (data not shown), however, the upregulation of PML protein in RA-treated cells was clearly visible at 24 hours postinfection of Ad-PML.

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