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Knocking Down PML Impairs p53 Signaling Transduction Pathway and Suppresses Irradiation Induced Apoptosis in Breast Carcinoma Cell MCF-7

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Abstract The promyelocytic leukemia (PML) can selectively and dynamically recruit a number of proteins including p53 to form a sub-nuclear multiprotein chamber named PML-NBs. In DNA damage response, p53 is recruited into PML-NBs and modified by phosphorylations and acetylations, which in turn potentiate its transcriptional and pro-apoptotic activities. In contrast, in carcinoma cells, the role of PML in the irradiation induced p53-mediated apoptosis is not precisely understood. In this study, we have used the breast carcinoma cell line, MCF-7, and stably suppressed the expression of PML. Inhibition of PML expression had no detectable effect on the expression of endogenous p53 at the mRNA level; however, a significant decrease of p53 protein was observed. There was also an increase in the p53–MDM2 complexes, which may facilitate p53 protein degradation by the ubiquitin-proteasome pathway, also in irradiation treated cells. The p53 transcriptional activity was attenuated both in unstressed and 10 Gy irradiation treated cells. Moreover, inhibition of PML expression in MCF-7 cells significantly reduced p53 downstream genes, cell cycle arrest gene $p21^{WAF/cip-1}$ and pro-apoptotic gene Bax expression, then irradiation-induced apoptosis. These results suggest that PML is a key regulator in the irradiation activated p53 apoptotic pathway in breast carcinoma cells. J. Cell. Biochem. 97: 561–571, 2006. © 2005 Wiley-Liss, Inc.

Key words: PML; RNAi; p53; MDM2; MCF-7; irradiation; apoptosis

Mutations in the *p53* gene have been reported in around 50% human tumors [Hickman et al., 2002]. p53 is known as a key element in control of human cell growth and differentiation and plays an important role in maintenance of genome integrity [Ko and Prives, 1996; Levine, 1997]. Most of its functions include transcriptional activation of genes involved in cell cycle, apoptosis, and DNA repair [Kho et al., 2004]. p53 induces the cells in which DNA damage cannot be repaired to undergo apoptosis, an important property for suppressing tumor genesis and tumor therapy. In unstressed cells, p53 is present in the latent state and maintained at low levels through mouse double minute 2 (MDM2) mediated ubiquitin-proteasome degradation pathway [Hofmann et al., 2002]. In DNA damage responses induced by genotoxic agents, such as IR, UV, and chemotherapeutic reagents, p53 is activated by post-translational modifications (phosphorylations, acetylations, and sumoylations), resulting its accumulation in nuclear and enhanced transcriptional activity. When the damage is severe, p53 binds to the promoters of pro-apoptosis genes in a sequence-specific manner, turns on the transcription of those genes, and eventually leads to cell apoptosis [Wahl and Carr, 2001; Hickman et al., 2002].

PML (promyelocytic leukemia protein) is a cell growth and tumor suppressor that harbours a RING-finger, two B-boxes and a predicted α helical Coiled-Coil domain, that together form a BRCC/TRIM motif. The BRCC motif is essential for PML to recruit other proteins to form PML-NBs. PML-NBs are a multi-protein, cell cycle regulated, matrix-associated sub-nuclear structure that appears as punctuate foci in the interphase nuclei [Jensen et al., 2001; Borden,

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2002]. Sizes of PML-NBs are various in different cell cycle and cell type. Usually, there are $5\sim30$ PML-NBs in one cell, from 0.3 to 1 μm in diameter. The structural integrity of those multi-protein complexes is important for normal cell growth and development. Disruption of PML-NBs formation leads to human diseases, like acute promyelocytic leukemia (APL) or spinnocerebellar ataxia type I (SCAI) [Zimber et al., 2004]. Studies on PML knockout mice have revealed the pro-apoptosis functions of PML and PML-NBs. PML and PML-NBs are well known to be very important regulators in TNFα, Fas, IFN, chemotherapeutic agents, especially irradiation induced cell apoptosis [Qugnon et al., 1998; Wang et al., 1998; Zhong et al., 1999; Bernardi and Pandolfi, 2003]. PML and PML-NBs can act as molecular hubs for the induction and/or reinforcement of apoptosis through a selective and dynamic regulation of pro-apoptosis transcriptional events in DNA damage response. PML-NBs provide a "platform" for other protein interaction and modification to occur.

Previous studies have showed that in DNA damage response p53 is recruited into PML-NBs, and PML, the most prominent component of PML-NBs, is a new partner of p53 [Fogal et al., 2000; Guo et al., 2000]. Once p53 was recruited into PML-NBs, it was modified by other enzymes, such as phosphorylation by home-domain interacting protein kinase-2 (HIPK2) or checkpiont kinase 2 (Chk2), acetylation by p300/CBP (CREB (cAMP-response-element-binding protein) binding protein). Those modifications are known to be key factors for p53 activating downstream pro-apoptosis genes and inducing cell apoptosis [Pearson et al., 2000; D'Orazi et al., 2001; Wang et al., 2001; Louria-Hayon et al., 2003]. So PML and PML-NBs are new partners of p53 in inducing apoptosis in DNA damage response [Ferbeyre et al., 2001; Bode and Dong, 2004].

Recent studies have found that PML can directly interact with MDM2, an important negative regulator of p53 stability [Kurki et al., 2003; Wei et al., 2003; Zhu et al., 2003]. Some researches on $PML^{-/-}$ fibroblast cells found that over-expression PML potentiate exogenous p53 stability [Bernardi et al., 2004; Bernassola et al., 2004], but sometimes over-expression has failed to stimulate the endogenous protein functions, so do c-Myc [Guo et al., 2000]! There are few studies on tumor cells, and the precise relationship between PML and p53 are poorly understood, especially in carcinoma cells and irradiation induced carcinoma cell apoptosis. In this study, we have used breast carcinoma cell line, MCF-7, as a cell model and stably reduced the expression level of PML by RNAi technique to study the effects on the p53 stability and transcriptional activity in unstressed and irradiation stressed situations. We found that RNAi-mediated reduction in the PML protein level decreased the p53 stability and transcriptional activity in both situations. Moreover, the cells subjected with PML RNAi treatment were resistant to irradiation-induced apoptosis.

MATERIALS AND METHODS

Cell Line, Culture, Transfection and Irradiation Treatment

Human breast carcinoma cell line MCF-7 was preserved by our lab, which expressed wild type p53 and PML proteins. It was cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS) and penicillin (100 unit/ml)/streptomycin (100 μg/ml). Cell culture was kept in a humidified incubator with 5% CO_2 at 37°C. All transfections were done with Lipofectamine regents (Invitrogen) according to the manufacture's protocols. Co^{60} was used as the source for irradiation treatment.

Plasmid and Antibodies

Our lab constructed the RNA interference plasmid pTET-U6, which can stably and effectively express stem-loop interference RNA in mammalian cells. This plasmid contained an U6 promoter, a RNA Pol III promoter that was used to synthesize siRNA in mammalian cells, and neomycin resistance was used for mammalian cell stable transfection. The p53 response element luciferase reporter plasmid pGL3-53RE-miniCMV was constructed as previously described (plasmid pRL-CMV from Promega) [Shan et al., 2004]. Red fluorescent protein-tagged p53 expression plasmid, pDsRed2-N1-p53 and control plasmid pDsRed2-N1 were kindly provided by Dr. LingQiang Zhang (Institute of Radiation Medicine, Academy of Military Medical Sciences).

The following antibodies were used: mouse monoclonal anti-p53 (DO-1, Santa Cruz), mouse polyclonal anti-PML (PG-M3, Santa Cruz), goat polyclonal anti-actin (Santa Cruz),

rabbit polyclonal anti-p21 (H-164, Santa Cruz); mouse polyclonal anti-Bax (B-9, Santa Cruz); mouse polyclonal anti-Bcl-2 (C-2, Santa Cruz); mouse monoclonal anti-MDM2 (SMP14, Zymed), FITC and TRITC labeled goat anti mouse IgG (Zhongshan, China).

RNA Interference and PML Knock-Down Cell Model Construction

Two complementary oligonucleotides were designed to target exon 2 of the *PML* gene (398–418) (Table I) [Bruno et al., 2003]. Those two complementary oligonucleotides were annealed gradually for 94° to 56° to form a double strands DNA fragment with a blunt 5' end, and a cohesive 3' end compatible to XbaI restriction site. pTET-U6 plasmid was digested by SalI and blunted by Mung Bean Nuclease (TAKARA), followed by digestion with XbaI. Then the annealed double strands DNA fragment was cloned into the treated pTET-U6 plasmid by T4 DNA ligase The resultant plasmid pTET-U6-PML was sequenced to confirm the correct insertion. The plasmid was transfected into MCF-7 cells by Lipofectamine (Promega), and the stably transfected cell line was screened with 600 μg/ml G418 for about 4 weeks, and cultured in DMEM supplemented with G418 (300 μg/ml).

p53 Half-Life Assay and MG132 Treatment

To assay p53 half-life, cycloheximide (100 μg/ml, dissolved in anhydrous alcohol, Fluka), a protein synthesis inhibitor, was added into cultured cells. At 1, 1.5, 2, 3 h after adding cycloheximide, cells were harvested and analyzed by Western-blot. For inhibition p53 degradation, MG132 (5 μM, dissolved in DMSO, Biochem), a protoseams inhibitor, was added into cultured cells.

Immunoprecipitation and Western-Blot

For Western blot analysis, cells were lysed in RIPA buffer(50 mM Tris-HCl, pH = 7.5; 150 mM NaCl; 1% NP40; 0.1% SDS; 0.5% Deoxycholic acid, sodium) containing protease-inhibitor PMSF (1 mM dissolved in anhydrous alcohol, Merck), Lysates were centrifuged to clear all cell debris. For co-immunoprecipitation, cells were lysed in immunopercipitation (IP) buffer (150 mM NaCl, 50 mM Tris-HCl at pH 7.5 and 1% NP40) supplemented with PMSF. The lysates were pre-cleared by incubated with protein G or protein A-agarose beads, then incubated with anti-p53 antibody (DO-1) and protein G or protein A-agarose beads overnight at 4°C. Immunoprecipitants were washed five times with ice-cold IP buffer and resolved by SDS–PAGE. Western blotting was performed according to standard procedures.

Immunofluorescence and Confocal Microscopy

Cells were seeded on cover-lips and cultured as described above, and immunostaining was carried out as follows at room temperature except indicated otherwise. After being washed with PBS, cells were fixed with 3% paraformaldehyde in PBS for 20 min, and incubated in PBS containing 0.1 M glycine–PBS for 5 min. Cells were then permeabilized with PBS containing 0.1% Triton X-100 for 5 min. Cells were incubated for 1 h at 37°C with primary antibody PG-M3 or SMP14 (1:50, diluted in PBS containing 5% bovine serum albumin (BSA)), followed by washing with PBS for three times. Cells were incubated with TRITC or FITC conjugated goat anti-mouse secondary antibody (1:200, diluted with 5% BSA–PBS) for 45 min, and then washed for three times with PBS. For nuclear staining, Hoechst 33258 (50 μg/ml, Fluka) were

TABLE I. Sequence of Primers Used in This Paper

Gene	Reaction	Primer (sense) (5′–3′)	Primer (anti-sense) (5′–3′)	Size and PCR condition
GAPDH	RT-PCR	CCATGGAGAAGG CTGGGG	CAAAGTTGTCATG GATGACC	195bp 94°C 30sec,58°C 30sec,72°C 30sec 30cycle
p53	RT-PCR	CGGGATCCATGGAG GAGCCGCAGTCAGA T	CCGCTCGAGTTTC TGGGAAGGGACAG AAGA	300bp 94°C 30sec,58°C 30sec,72°C 30sec 30cycle
PML	RT-PCR	GAGAATCGAAACTA AGCTG	CACTTGAGCTCAC TGTGGCT	710bp 94°C 30sec,58°C 30sec,72°C 45sec
PML RNAi	Anneal	AGTCGGCCGACTTCT GGTTTCAAGAGAAC CAGAAGTCGGCCGA CTCCTTTT	CTAGAAAAAGAGT CGGCCGACTTCTG GTTCTCTTGAAAC CAGAAGTCGGCCG ACT	35cycle 94°C 5min, 78°C 5min, 75°C 5min 71°C 10min, 68°C 10min, 65°C 10min 62°C 10min, 60°C 10min, 56°C 10min 50°C 10min

added, incubated for 10 min, and washed with PBS.

Total RNA Isolation and RT-PCR

Total RNA was isolated using TRLzol Regent (Invitrogen) according to the manufacturer's instructions and dissolved in DEPC treated water. 1 μg RNA was used for reverse transcription with M-MLV (Invitrogen) according to the manufacture's protocol. PCR was performed according to standard procedures. The primers used were given in Table I.

Dual-Luciferase Reporter Assay

For luciferase assay, cells were seeded on 6-well plates (1.5×10^5 cells/well) and transfected with 1 μg luciferase reporter plasmid and 0.005 μg pRL-CMV reference plasmid. At 24–36 h after transfection, cell extracts were analyzed with Daul-luciferase reporter assay system (Promega) following the manufacturer's instructions. Cells were irradiated with 10 Gy after 12 h post-transfection, and cells extract were analyzed $12 \sim 24$ h after irradiation.

Flow Cytometry

Cells were seeded in 24 cm^2 dishes and irradiated for 10 Gy after seeding for 24 h. At different time points, both floating and attached cells were harvested and washed with PBS, then re-suspended precipitation with 300 μl 5% FBS–PBS and 700 μl anhydrous alcohol, fixed it overnight at $-20°C$. After being fixed, cells were centrifugated and washed with PBS for three times. Precipitations were re-suspended with 500-μl propidium iodide (PI, 50 μg/ml) containing 50-μg/ml RNase A, incubated in 37°C water for 30 min, and analyzed by cytofluorimetry.

RESULT

pTET-U6-PML Efficiently and Stably Inhibited *PML* Expression in Breast Carcinoma Cell Line MCF-7

The plasmid pTET-U6-PML was constructed to transcribe small interference RNA (siRNA) that specifically target PML gene under the U6 promoter. It was introduced into human breast carcinoma cell line MCF-7 by transfection, and a stable cell line that can constitutively express PML siRNA was selected with G418 for about 1 month (see Materials and Methods). As shown in Figure 1, the expression of PML in these cells was significantly inhibited at the transcriptional (Fig. 1A) and translational (Fig. 1B) levels. There were not any detectable changes in PML expression in control stable cells transfected with the pTET-U6 empty plasmid. And there were no effects on the expression of unrelated genes (data not shown) and no significantly changes in inhibiting the expression of PML for at least 6 months culture (data not shown).

Lin et al. have recently reported that the cytoplasmic PML functions in TGF-β signaling pathway [Lin et al., 2004], however, the main function PML requires selective and dynamical recruitments of other proteins and formation of PML-NBs in the nuclear. In addition, the integrity of PML-NBs is necessary for PML function. The number and integrity of PML-NBs in the PML RNAi-treated cells were analyzed by immunofluorescence using anti-PML antibody. There were about 10 apparent PML-NBs in each nuclear in the stable cells transfected with empty plasmid and in the wild type MCF-7 cells (without transfection), however, no PML-NBs were detectable in *PML* RNAi treated cells (Fig. 1C). These results together evidently indicate that the expression of PML was significantly inhibited in the MCF-7 cells treated with siRNA.

PML RNAi Treatment Impairs p53 Stability by MDM2 Mediated Ubiquitin-Proteasomes Degradation Pathway

PML isoform IV (PML-IV) can recruit p53, via direct interaction, into PML-NBs, where p53 was phosphorylated at Ser 46 by HIPK2 although such modification does not affect p53 stability [Hofmann et al., 2002]. PML can also directly interact with MDM2, a key regulator of p53 stability, suggesting that PML may have effects on the p53 stability. The protein and mRNA levels of p53 in *PML* RNAi MCF-7 cells were examined by western blotting and RT-PCR. The results were showed in Figure 2. The endogenous p53 protein level in *PML* RNAi treatment cells was much lower than that in control cells (Fig. 2A). Protein levels were analyzed by AlphaImager system, results showed that the protein levels of PMlL and p53 in *PML* RNAi cells were only around 10% and 30% respectively compared to PML wild type cells (Fig. 2B), although there was no noticeable difference at the mRNA level (Fig. 2C). The half-life of p53 was significantly shortened in *PML* RNAi cells (Fig. 2D). These results suggested

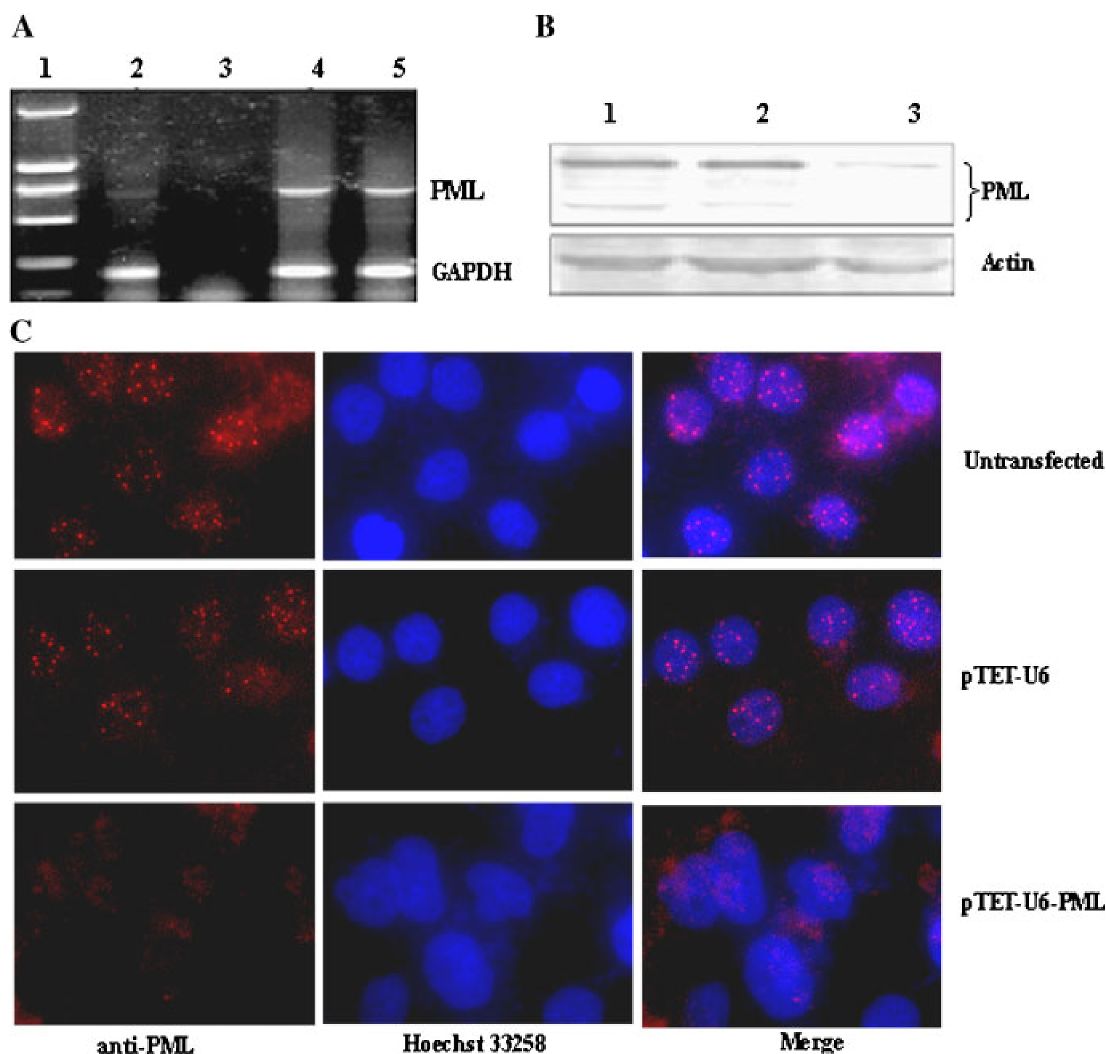

Fig. 1. *PML* RNAi cell model construction in breast carcinoma cell line MCF-7 (**A**) RT-PCR analysis of PML expression. **Lane 1**: DL2000 marker; **Lane 2**: transfected pTET-U6-PML; **Lane 3**: negative control (no cDNA added); **Lane 4**: transfected pTET-U6; **Lane 5**: untransfented. (**B**) PML protein levels were detected by western-blot assay. **Lane 1**: untransfected; **Lane 2**: transfencted pTET-U6; **Lane 3**: transfected pTET-U6-PML. (**C**) Stable lines and normal MCF-7 cell were immunoflurescenced with anti-PML antibody and TRITC labeled secondary antibody (left column), nuclei were stained with Hoechst 33258 (middle column). Merged pictures were present in the right column). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

that inhibition of PML expression resulted in no effects on the transcription of p53 but significantly reduced the protein level of p53 by decreasing its stability.

Further experiments were performed to study the mechanisms of the decrease of p53 stability in *PML* RNAi cells, MG132, a proteasomes inhibitor, was added to *PML* RNAi and wild type MCF-7 cells respectively. As shown in Figure 3A, the protein level of p53 was significantly higher in *PML* RNAi cells treated with MG132 than those cells without MG132 treatment, and was comparable to that in wild type MCF-7 cells. Furthermore, compared with the wild type MCF-7 cells, the amount of p53–MDM2 complexes increased in *PML* RNAi cells. After 10 Gy irradiation treatment, p53–MDM2 complexes decreased obviously in both *PML* wild type and RNAi cells, but there were still more p53–MDM2 complexes in *PML* RNAi cells compared to *PML* wild type cells (Fig. 3B). To strengthen p53–MDM2 interaction, red fluorescent protein tagged p53 expression plasmid and control plasmid were transfected into PML wild type MCF-7 cells, then those cells were stained with anti-MDM2 antibody and FITC (green fluorescence) labeled secondary antibody. Results showed that red fluorescent

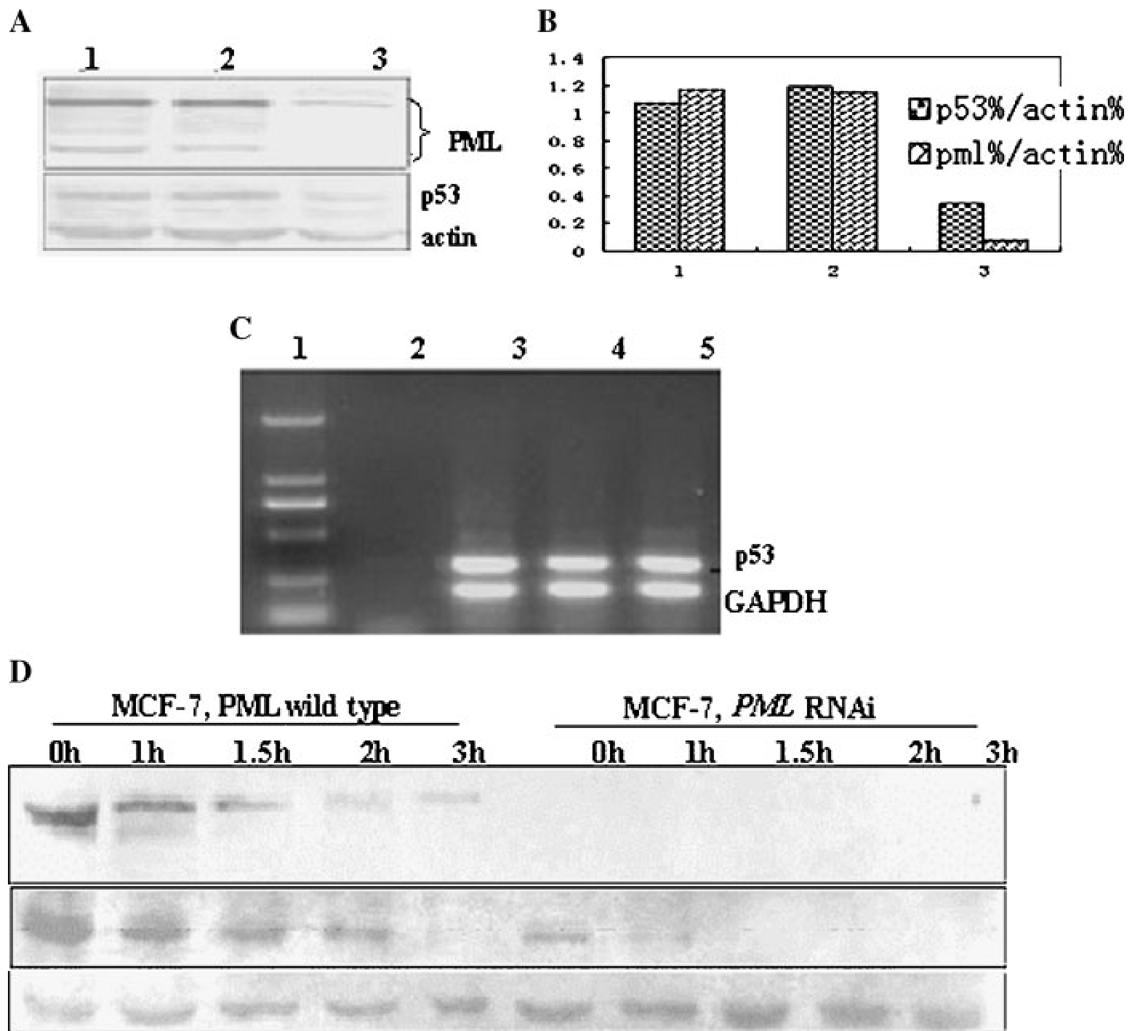

Fig. 2. *PML* RNAi led to a decrease in the p53 protein level in MCF-7 cells. (**A**) Western-blot was performed in *PML* wild type and *PML* RNAi cell with anti-p53 and anti-PML antibodies. **Lane 1**: untransfected; **Lane 2**: transfected pTET-U6; **Lane 3**: transfected pTET-U6-PML. (**B**) Analysis of the relative ratio of PML and p53 to actin by AlphaImager gel image system (Alpha innotech Co.) **Lane 1**: untransfected; **Lane 2**: transfected pTET-U6; **Lane 3**: transfected pTET-U6-PML. (**C**) p53 mRNA level in *PML* wild type and *PML* RNAi cell were detected by RT-PCR. **Lane 1**: DL2000 Marker; **Lane 2**: negative control (no cDNA were added); **Lane 3**: transtected pTET-U6-PML; **Lane 4**: transfected pTET-U6; **Lane 5**: untranfected. **D**: Inhibited protein synthesis by cycloheximide(100 μM), PML and p53 half-life were detected in *PML* wild type and *PML* RNAi cell by Western-blot.

protein tagged p53 and MDM2 co-localized in nuclei (Fig. 3C). All these results suggested that loss of PML potentiated p53 protein degradation by ubiquitin-proteasomes pathway both in untreated or irradiation treated MCF-7 cell.

It is well known that in DNA damage responses, p53 is phosphorylated by a number of protein kinases and the phosphorylations led to an increase in the stability of p53. *PML* RNAi cells and wild type MCF-7 cell lines were treated with 10 Gy irradiations and harvested at different time points respectively. PML and p53 protein were analyzed by Western-blot assay. Both PML and p53 protein level increased after exposing to irradiation and the increasing tendency was coincident, but the levels of both proteins were significantly lower in *PML* RNAi cell than that of wild type cells (Fig. 4).

PML RNAi Impaired p53 Transcriptional Activity

To investigate the effect of *PML* expression level on the p53 transcriptional activity, luciferase reporter plasmid pGL3-53REs-miniCMV was transfected into *PML* RNAi cells and wild type MCF-7 cell line. This plasmid contained three-tandem p53RE and a miniCMV promoter just upstream of the luciferase gene cDNA. By

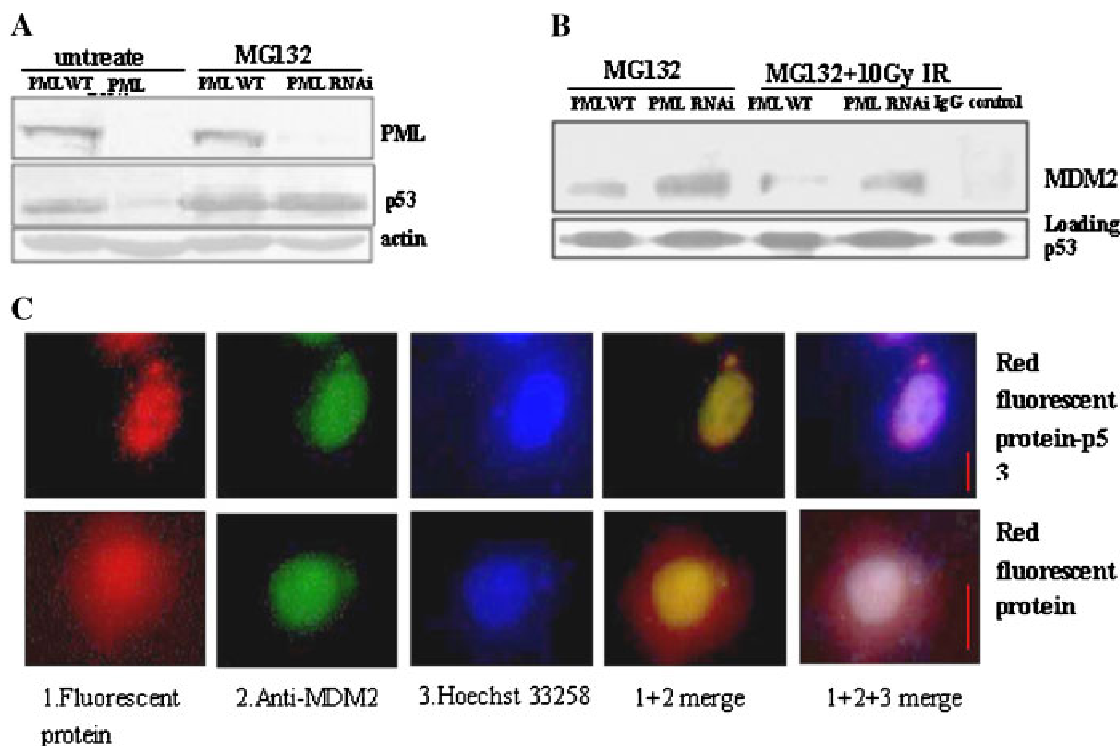

Fig. 3. *PML* RNAi reduced the p53 stability by MDM2 mediated ubiqitin-protosomes degradation pathway. **A**: Both *PML* RNAi and wild type MCF-7 were treated with 5 μM MG132 and cells were harvested at 6 hr after adding MG132, and were analyzed by Western-blot with anti-PML and anti-p53 antibodies. **B**: *PML* RNAi and wild type MCF-7 cell were treated with 10 Gy irradiation. After being treated, irradiated cells and non-treated cells were cultured with 5 μM MG132 for 6 h, all cells were harvested and co-immunoprecipitations were done with mouse anti-p53 antibody (mouse IgG as control) and precipitation was analyzed by Western-blot with anti-MDM2 antibody. **C**: pDsRed2-N1-p53 and control plasmid pDsRed2-N1 were transfected into wild type MCF-7 cell respectively (first column form left). Immunoflurescences were done with anti-MDM2 antibody and FITC labeled secondary antibody (second column from left). Nuclei were stained with Hoechst 33258 (third column from left). Merged pictures were present in the right two column. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

specifically recognizing and binding to p53RE, p53 can induce transcription of the downstream luciferase gene. The p53 transcriptional activity in *PML* RNAi cells decreased by over 50% compared to that of wild type MCF-7 cells. Exposure the cells to 10 Gy irradiation increased the p53 transcriptional activity by about 45% and 62% in *PML* RNAi cells and wild type MCF-7 cell line respectively. In that irradiation treated cells, the p53 transcriptional activity in *PML* RNAi cells was still more than 50% lower than that of wild type MCF-7 cells (Fig. 5A). To elucidate the effect of PML loss on p53 activating or repressing its downstream genes, cells were exposed to 10 Gy irradiation and the endogenous protein expression levels of p53 typical downstream genes, Bcl-2, Bax, and p21$^{WAF/cip-1}$ were detected. There was no obvious difference in anti-apoptotic protein Bcl-2, which was repressed by activated p53. But, the expression levels of pro-apoptotic protein Bax and cell-cycle arrest protein p21$^{WAF/cip-1}$ were significantly decreased in *PML* RNAi cells compared to *PML* wild type cells (Fig. 5B).

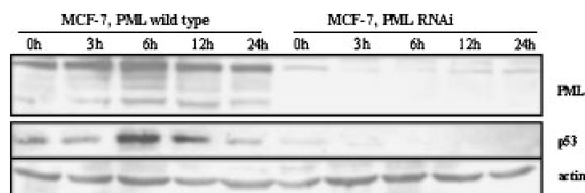

Fig. 4. Both *PML* RNAi and wild-type MCF-7 were treated with 10 Gy irradiation and harvested at 3, 6, 12, and 24 h after irradiation, then analyzed by Western-blot with anti-PML and p53 antibody.

PML Knockdown Suppressed Irradiation Induced MCF-7 Cell Apoptosis

Inhibition of PML expression led to a decrease in the stability and transcriptional activity of p53, suggesting that the cell cycle, cell growth rate, and DNA damage responses in *PML* RNAi

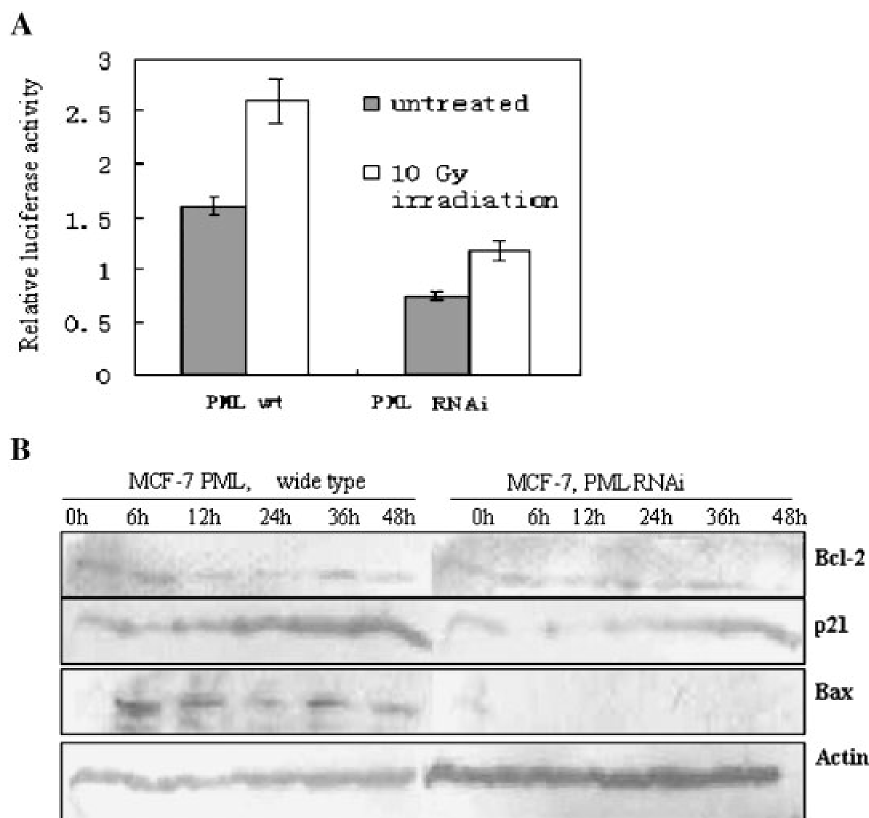

Fig. 5. *PML* RNAi impaired the p53 transcriptional activity both in treated and untreated MCF-7 cells. **A**: p GL3-53REs-miniCMV plasmid was transfected into *PML* RNAi and wild type MCF-7 cell. Those transfected cells were treated with 10 Gy irradiation at 12 h after transfected. All transfected cell were harvested at 24 h after transfection and assayed for relative luciferase activity. **B**: *PML* RNAi and wild type MCF-7 cells were treated with 10 Gy irradiation, cells were harvested at 6, 12, 24, 36, 48 h after irradiation and analyzed by western-blot with anti-p21, anti-Bcl-2, anti-Bax, and anti-actin antibodies respectively.

cells might be altered. However, there were not any significant differences in the cell cycle progression and the cell growth rate between *PML* RNAi and wild type MCF-7 cells using FCM and MTT assay (data not given). Both *PML* RNAi cells and wild type MCF-7 cells 24 h after exposing to 10 Gy irradiation showed typical cell cycle arrest at the G2/M phase and, 36 h later, the number of *PML* RNAi cells entering S phase was much higher than wild type MCF-7 cells. At the same time, the percentage of apoptosis cells increased gradually and reached a peak at 72 ~ 84 h, but the percentage of apoptosis cells in wild type MCF-7 cell line was 2.2 times high compared to that of PML RNAi cell. The results indicate that reduction of PML expression significantly suppresses apoptosis in MCF-7 cells.

DISCUSSION

RNA interference is a new powerful tool in gene function study. But using synthetic short 21 to 22 nt interfering RNAs(siRNA) suppression of gene expression is transient, which severely restricts its application [Elbashir et al., 2001; Brummelkamp et al., 2002; Krichevsky and Kosik, 2002; Sui et al., 2003; Hohjoh, 2004]. To overcome this limitation, we constructed a pTET-U6-PML plasmid which can express siRNA specifically target to all isoforms of *PML* gene in mammalian cells. And this plasmid contains neomycin resistance, so it can be used for stable transfection. Using this plasmid, we constructed a cell model which PML expression was stably inhibited successfully.

The relationship of PML and p53 has been extensively studied recently. It has been showen that *PML* was a new partner of p53 in inducing cell apoptosis. PML-IV (PML isoform that binds to p53) can recruit p53 to the PML-NBs where it facilitates phosphorylation (Ser 46) and acetylation (Lys 382) of p53, mediated by HIPK2 and CBP respectively. But recent study found PML-IV could function as a p53

transcriptional co-activator, even after substitution of critical p53 lysine residues with arginines ($p53^{K382R}$) [Bernardi et al., 2004]. This suggests that PML may potentiate p53 activity by alternative mechanism. In this study, we revealed that PML protected p53 from MDM2 mediated degradation, potentiate p53 transcriptional activity and apoptosis induce in breast carcinoma MCF-7 cells.

Inhibition of the expression of *PML* led to a significant decrease in the p53 protein level. The decrease was due to the decrease of p53 protein stability instead of its transcription. It was reported that p53 turnover at the physiological level was mainly controlled by MDM2 [Jones et al., 1995]. We found that the p53–MDM2 complexes level significantly increased in *PML* RNAi MCF-7 cells, also in irradiation treated cells. MDM2 is an E3 ligase of p53, and the increase in the MDM2–p53 complexes may potentiate p53 ubiquitination and degradation by proteasome. Our results showed that PML can protect p53 from MDM2 mediated degradation at least in MCF-7 cells. There may be two pathways in PML regulating p53 stability. Firstly, PML can interfere interactions of p53 and MDM2 by interacting with MDM2 directly and sequestrating it in the nuclei [Bernardi et al., 2002]. Secondly, competition between ubiquitination and acetylation of overlapping lysine residues constitutes a novel mechanism by which the protein stability is regulated, and PML can exert effect on the p53 stability by regulating p53 acetylations [Ito et al., 2002; Li et al., 2002].

By using dual luciferase reporter system, we found that the decrease of the p53 protein level directly impaired p53 transcriptional activity both in unstressed or 10 Gy irradiated cells. When cells were exposed to 10 Gy irradiation, we found that the transcriptional activity of p53 to its downstream genes, $p21^{WAF/cip-1}$ and Bax decreased significantly. $p21^{WAF/cip-1}$ is a chief effector in p53 inducing cell G2/M arrest and Bax is an important pro-apoptotic protein in p53 inducing cell apoptosis. But anti-apoptotic protein Bcl-2 (Fig. 5B) and survivin (data not shown) expressions did not have apparent difference. Those two anti-apoptotic proteins expressions are usually repressed by activated p53. p53 was an important regulator in cell cycle, apoptosis and growth suppression. Its transcriptional activity decrease may exert effects on the cell growth rate or cell cycle. However, we failed to detect any significant differences in the growth rate and the cell cycle between *PML* RNAi cells and wild type MCF-7 cells (data not shown) when there were no pressures. It may be due to the fact that MCF-7 was a carcinoma cell line whose cell growth rate and cell cycle were abnormal compared to normal cells. But there were significant differences between those two cell lines when they were exposed to 10 Gy irradiation. More *PML* RNAi cells entered S phase compared to wild type MCF-7 cells. Inhibition of the expression of *PML* in MCF-7 cells also suppressed apoptosis induced by exposed to irradiation. Those results were accordance with G2/M arrest protein $p21^{WAF/cip-1}$ and pro-apoptotic protein Bax expression decrease. But more detailed work would be required to determine the role of PML and PML-p53 pathway in irradiation induced cell apoptosis (Fig. 6).

Our results revealed PML was an important regulator of p53 stability. In APL, one PML allele is involved in the chromosomal translocation and form PML-RAR α (retinoic acid receptor α) fusion protein that can disrupt PML-NBs formation. More importantly, Insinga et al.

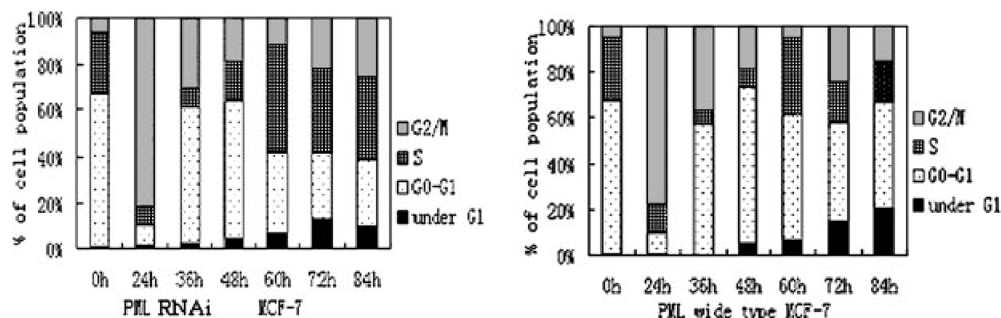

Fig. 6. *PML* RNAi suppressed irradiation induced cell apoptosis. PML RNAi and wild type MCF-7 cells were treated by 10 Gy irradiation, harvested on the different time points as indicated, fixed with ethanol and stained with propidium iodide. Stained cells were analyzed by Flow cytometry.

have found that PML-RARα destabilizes p53 by promoting its deacetylation and subsequent MDM2-dependent degradation [Insinga et al., 2004]. Their and this studies consistently suggest a role for p53 in the pathogenesis of APL. Recently, Tschan et al., found human p73, a member of p53 family, markedly increased when APL cell line NB4 were treated with retinoic acid [Tschan et al., 2000]. As retinoic acid treatment induces the degradation of the PML–RARα fusion protein and reorganization of the PML-NBs in NB4 cells, this may restore the ability of PML to stabilize and functionally activate p73 [Zhong et al., 2000]. Though previous and our results have showed a role for p53 in the APL, more work needs to be done to elucidate the precisely functions of p53 in the APL pathogenesis and treatment.

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