PML contributes to p53-independent p21 up-regulation in gamma-irradiation induced DNA damage responses

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The cyclin-dependent kinase inhibitor p21^{WAF1/Cip1} is a critical cell cycle regulator which translocates into the nucleus to participate in DNA repair during DNA damage responses. In the present study, we showed that the tumor suppressor, promyelocytic leukemia protein (PML) contributes to the up-regulation of p21 in a p53-independent pathway. Knock-down of PML in p53-null H1299 and HCT 116 (p53^{-/-}) tumor cells by specific siRNA resulted in down-regulation of p21 protein expression, inhibition of γ -irradiation-induced p21 up-regulation, and a decrease in p21 protein half-life. In PML knockdown H1299 cells, the down-regulation of p21 protein expression was reversed by MG132 treatment indicating that the proteasomal degradation of p21 protein was increased. Thus, PML positively regulates p21 expression by inhibiting proteasome-mediated proteolysis. Knock-down of PML decreased the repair of γ -irradiation-induced double strand breaks (DSBs) as indicated by the delayed disappearance of γ -H2AX foci and a decreased association between p21 and proliferating cell nuclear antigen (PCNA). Over-expression of p21 significantly restored the delayed DSB repair function. Taken together, these data provide evidence for a p53-independent functional relationship between PML and p21 in γ -irradiation-induced DNA damage responses, and identify PML as a positive post-translational regulator of p21 in p53-deficient tumor cells.

PML, p21, γ -irradiation, DNA damage, DSB repair

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Promyelocytic leukemia protein (PML) is an oncoprotein that is expressed primarily in myeloid, epithelial and endothelial cells. It was originally discovered due to its role as a potent tumor suppressor in the oncogenesis of acute promyelocytic leukemia (APL) [1]. More recent evidence shows that PML acts in other cancers [2], and that PML protein expression is reduced in multiple tumor cell lines [3,4]. In addition, reports indicate that PML is involved in many cellular processes including cell cycle progression, the DNA damage response, transcriptional regulation, viral infection, and apoptosis [5,6]. Taken together, these data indicate that PML is a key regulatory factor in many can-

cers, and a potential therapeutic target [7].

p53 is a tumor suppressor protein which regulates cell growth and cell death in response to stress, such as DNA damage. Previous studies identified PML as a transcriptional co-activator with p53 in a PML-dependent p53 pathway during the DNA damage response and apoptosis [8], and indicate that PML and p53 play essential roles during tumor suppression. However, nearly half of human tumors exhibit p53 mutation or deficiency, suggesting that the function and signaling pathways of PML in p53 deficient tumor cells may be different from in the wild-type. As PML is a potential therapeutic target in cancer treatment, we investigated the role of PML in p53 deficient tumor cells in an attempt to identify a basis for the development of novel tumor-therapy strategies.

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Among cell cycle regulatory proteins, the cyclin-dependent kinase inhibitor p21^{WAF1/Cip1} (hereafter referred to as p21) participates in multiple fundamental cellular processes, including the cell cycle progression, apoptosis, gene transcription, and DNA damage repair [9]. The functions of p21 in DNA repair and apoptosis remain controversial. It has been reported that p21 is the major transcriptional target of p53 after DNA damage [10], and that PML is indispensable for p53-dependent p21 induction [11]. In the PML regulated p53-p21 pathway, the activity of p53 is controlled by recruitment into PML-nuclear bodies (PML NBs) as well as stabilization through post-translational modifications, such as phosphorylation and acetylation [12]. However, the relationship between PML and p21 in p53-deficient tumor cells has not been investigated.

In this study, we knocked down PML expression in p53-null H1299 cells and HCT116 cells to identify the role of p53 in PML-induced p21 activation. Our results demonstrate that PML contributes to p53-independent p21 up-regulation, and that this PML-p21 pathway plays an important role in double strand break (DSB) DNA repair in γ -irradiation-induced DNA damage responses. As p53 is not involved in this process, this PML-p21 pathway may be a potential target for p53-deficient tumor therapy.

1 Materials and methods

1.1 Plasmid DNA and transfection

pCMV-Myc-tagged p21 expression vectors were generated as previously described [13]. Transfections in H1299 cells were carried out using FuGENE HD Transfection Reagent (Roche) according to the manufacturer's instructions.

1.2 Cell culture and γ -irradiation treatment

The p53-null non-small cell lung carcinoma cell line H1299, and the human colorectal cancer cell line HCT116 (p53^{-/-}) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone, UT), 2.4 g/L HEPES (Amresco), and 3.7 g/L sodium bicarbonate (Merck) in a 5% CO₂ humidified incubator at 37°C. For cell immunofluorescence studies, H1299 cells were sub-cultured on glass cover slips 1 d before use. γ -irradiation treatments (10 Gy) were performed with ⁶⁰Co. Before ionizing irradiation, culture media were removed and fresh media were added to the cells.

1.3 RNA interference

The target sequence of siRNA for PML was identified from published data as 5'-GAGUCGGCCGACUUCUGGU-3' (Shanghai GenePharma Co., Ltd.) [14]. A Nc siRNA duplex with the sequence 5'-UUCUCCGAACGUGUCACGUDtT-3' (Shanghai Gene-Pharma Co., Ltd.) was used as a negative

control. Proliferating H1299 cells were transfected with PML siRNA using the INTERFERinTM siRNA transfection reagent (PolyplustransfectionTM, France) according to the manufacturer's instructions. Cells were ready for gene expression analysis 48 h after transfection.

1.4 Flow cytometric analysis

For evaluation of DNA content by flow cytometry, cells were fixed for at least 24 h in 70% ethanol, washed twice with PBS, and re-suspended in PBS containing 50 mg/mL propidium iodide (Sigma) and 20 mg/mL DNase-free RNase A (Amresco). Cells were left in the dark at 37°C for 30 min and analyzed in a FACSCAN flow cytometer.

1.5 Western blot

For Western blot analysis, H1299 or HCT116 cells were lysed in ice-cold RIPA buffer containing a complete protease inhibitor cocktail (Roche), gently stirred for 1 h at 4°C, and centrifuged at 12000 r/min and 4°C for 20 min to remove insoluble material. After normalizing for protein concentration (BCATM Protein Assay Kit; PIERCE), 40 μ g of lysate were separated on 10% SDS-polyacrylamide gels, transferred to Hybond-ECL membranes (GE Healthcare), and visualized by incubation with various primary antibodies and peroxidase-conjugated secondary antibodies (Invitrogen). The following antibodies were used: rabbit polyclonal anti-PML antibody (H238, Santa Cruz), mouse monoclonal anti-p53 antibody (DO-1, Santa Cruz), mouse monoclonal anti-PCNA antibody (PC10, Santa Cruz), mouse monoclonal anti-p21 antibody (K0081-3, MBL), mouse monoclonal anti-Myc antibody (M047-3, MBL), and goat polyclonal anti- β -actin antibody (I149, Santa Cruz).

1.6 Immunofluorescence

H1299 cells were grown on glass cover slips and transfected. Cells were fixed in 4% paraformaldehyde/PBS (pH 7.5) for 15 min and permeabilization in PBS 0.5% Triton X-100 for 5 min at room temperature. Subsequently, cells were blocked in goat serum, incubated with mouse monoclonal anti- γ -H2AX (JBC1353003, Upstate), Alexa Fluor 488 goat anti-mouse IgG (H+L) (Molecular Probes, Invitrogen), and DAPI. Images were acquired using immunofluorescence microscopy (Nikon).

1.7 Immunoprecipitation

Both NcsiRNA- and PMLsiRNA-transfected H1299 cells were lysed in M-PER[®] Mammalian Protein Extraction Reagent (Roche) supplemented with a complete protease inhibitor cocktail (Roche). The lysate was pre-cleared by incubation with mouse IgG and protein A/G PLUS-Agarose beads (sc-2003; Santa Cruz) for 4 h, and incubated with Agarose conjugated Anti-Myc-Tag (M047-8, MBL) at 4°C overnight. Immunoprecipitates were washed four times with

ice-cold NETN buffer (20 mmol/L Tris-HCl [pH 8.0], 1 mmol/L EDTA, 0.5% NP-40, 150 mmol/L NaCl, protease-inhibitor cocktail) and resolved by SDS-PAGE.

1.8 Statistical analysis

The differences between two groups were analyzed by ANOVA. $P < 0.05$ was considered statistically significant.

2 Results

2.1 PML is necessary for p21 expression before and after irradiation in p53 deficient tumor cells

To understand the role of endogenous PML in irradiation-induced cellular DNA damage responses, we knocked down PML expression in primary human lung fibroblasts (HEL) by RNA interference. Knockdown of PML in HEL cells (HEL-PMLsi) resulted in a decrease in cyclin-dependent kinase inhibitor p21 protein expression, 48 h after transfection. This was earlier than the decrease in p53 protein expression (96 h), suggesting that p21 may be regulated by PML in a p53-independent pathway (Figure S1). To verify this speculation, we knocked down PML expression in p53-null H1299 and HCT116 (p53^{-/-}) cells to determine whether p53 is necessary for PML-mediated p21 up-regulation. P53-null non-small cell lung carcinoma H1299 cells were transfected with PML specific siRNA (H1299-PMLsi) or negative control siRNA (H1299-control). PML and p21 protein expressions were detected in cell lysates by Western blot analyses. PML protein expression was inhibited and there was a significant reduction in p21 protein expression in H1299-PMLsi cells compared with the controls (Figure 1(a) and (b)). Similar results were identified in p53-null HCT116 (p53^{-/-}) cells (Figure 1(c)), indicating PML is physiologically involved in p21 maintenance in un-stressed p53 deficient cells. The MTT assay showed there were no significant differences in the proliferation of H1299-PMLsi and HCT116 (p53^{-/-})-PMLsi cells (Figure S2).

Western blot analyses showed PML and p21 protein expressions were increased in H1299-control cells 24 h after

10 Gy γ -irradiation. The irradiation-induced increase in p21 protein was inhibited by PML siRNA transfection (Figure 2). This suggests that PML plays an important role in induction of p21 in the γ -irradiation-induced DNA damage response.

2.2 PML contributes to up-regulation of p21 by inhibiting proteolysis in p53-null H1299 cells

p21 protein has a short half-life and is easily degraded by proteasome through ubiquitin-dependent and -independent pathways [15–17]. We tested whether PML knockdown influenced the stability of p21. We found that the half-life of p21 was significantly decreased in PML siRNA transfected H1299 cells (Figure 3(a)), and that this decrease was obviously reversed by MG132 (5 μ mol/mL, 6 h) (Figure 3(b)). To investigate whether the PML-regulated proteasomal degradation of p21 is influenced by the ubiquitin pathway, the ubiquitination of p21 was analyzed. There were no significant differences between H1299-PMLsi and H1299-control cells (Figure S3). This suggests that a ubiquitin-independent proteasome-degradation pathway contributes to the decreased expression of p21 protein in PML knockdown H1299 cells.

2.3 PML regulates cell cycle progression and DSB repair in p53-null H1299 cells

To investigate the roles of PML in DNA damage checkpoint function and DSB repair, we examined the effects of PML knockdown in H1299-PMLsi cell cycle progression by FACS analysis. An equal number of H1299-PMLsi and H1299-control cells (10^5) were seeded and grown for 48 h. Cells were irradiated and harvested at the indicated times, trypsinized, and stained with propidium iodide (Figure 4(a)). Our results showed, there were no significant differences in Nc siRNA transfected and untransfected control cell cycle profiles before and after irradiation; approximately 60% of H1299-PMLsi and 50% of H1299-control cells accumulated in G1 phase; the number of H1299-PMLsi

Figure 1 Knockdown of PML protein expression resulted in down-regulation of p21 protein expression in p53-null H1299 and HCT116 (p53^{-/-}) cells. (a) H1299 cells were transfected with 2.5–10 nmol/L PML siRNA (PMLsi) or negative control siRNA (NCsi). PML and p21 protein expressions were analyzed 48 h after transfection. (b) H1299 cells were transfected with 5 nmol/L PML siRNA (PMLsi) or negative control siRNA (NCsi). PML and p21 protein expressions were analyzed 24, 30, 36, and 48 h after transfection. (c) HCT116 (p53^{-/-}) cells were transfected with 5 nmol/L PML siRNA (PMLsi) or negative control siRNA (NCsi). PML and p21 protein expressions were analyzed 48 h after transfection.

Figure 2 γ -irradiation-induced up-regulation of p21 protein expression was inhibited by PML knockdown in H1299 cells. H1299 cells were transfected with 5 nmol/L PML siRNA (PMLsi) or negative control siRNA (NCsi) for 48 h, and subjected to γ -irradiation (10 Gy). Cells were harvested and analyzed by Western blot at the time points indicated.

cells accumulated in S phase was reduced by 10% compared with the control; there were no significant differences between the numbers of H1299-PMLsi and control cells accumulated in G2/M phase; the numbers of H1299-PMLsi and H1299-control cells in S phase began increasing 6 h after irradiation; and the population of H1299-PMLsi cells contained fewer S and G2/M, and more G1 cells 6–12 h following irradiation. Taken together, these data indicate that fewer H1299-PMLsi cells were arrested in S and G2/M phase, and that a deficiency in PML results in decreased S phase and G2/M-phase cell cycle checkpoint function. This suggests that PML plays an important role in the activation of S and G2/M DNA damage checkpoint function after irradiation (Figure 4(b)).

The function of PML in DSB repair in p53-null 1299 cells was investigated by analyzing the kinetics of phosphorylated histone H2AX (γ -H2AX) clearance after γ -irradiation. It has been shown that γ -H2AX acts as a marker of DSBs [18]. We found that γ -irradiation (10 Gy) induced the rapid appearance of γ -H2AX foci in PML siRNA transfected H1299, un-transfected control, and nonspecific siRNA transfected control cells (NCsi control). However, γ -irradiation-induced DSB repair was delayed in PML siRNA transfected H1299 cells, and there were significantly more γ -H2AX positive PML siRNA than Nc siRNA

cells 0.5 and 6 h after irradiation (Figure 4(c)).

2.4 p21 over-expression rectified the repair deficiency in PML knockdown H1299 cells

Evidence suggests that p21 is recruited to sites of DNA damage and involved in DNA repair [19–21]. To further investigate the role of the endogenous p53-independent PML-p21 pathway in maintaining genomic stability, we evaluated whether p21 functions downstream of PML in DSB repair in p53-null tumor cells. The kinetics of γ -H2AX clearance after radiation in p21 over-expressed H1299-PMLsi and control-vector transfected cells were analyzed. When PML expression was knocked down using siRNA, γ -H2AX foci were readily detected in cells transfected with control-vector 6 h after irradiation, whereas they were obscure in cells expressing ectopic p21 protein. This indicates that the decreased clearance of γ -H2AX foci in H1299-PMLsi cells was reversed by p21 over-expression (Figure 5(a)), and that a p53-independent PML-p21 pathway participates in DSB repair.

Previous reports show that p21 is recruited to UV irradiation-induced DNA damage sites where it interacts with proliferating cell nuclear antigen (PCNA), regulates the interaction of repair factors with PCNA, and protects PCNA from degradation [19]. Therefore, we investigated the effects of PML on the association between p21 and PCNA (Figure 5(b)). Our data showed that levels of p21-PCNA complex and DSB repair were lower in PML knockdown H1299 cells due to decreased p21 stability.

In this study, we found that PML regulates p21 protein expression in p53-null tumor cells. We identified a novel p53-independent PML-p21 signaling pathway whereby PML-dependent p21 protein stabilization maintains genomic integrity by regulating DSB repair during γ -irradiation-induced DNA damage responses. Knockdown of PML in p53-null H1299 cells induced decreased p21 stability, delayed DSB repair, and impaired DNA damage checkpoint function. This mechanism may contribute to genomic instability in tumor cells.

Figure 3 The stability of p21 protein was decreased in PML knockdown H1299 cells. (a) H1299-PMLsi and H1299-control cells were treated with 20 μ g/mL CHX and harvested at the indicated times. PML and p21 protein expressions were analyzed. (b) H1299-PMLsi and H1299-control cells were treated with 5 μ mol/mL MG132, cells were harvested 6 h after MG132 treatment, PML and p21 protein expressions were analyzed.

Figure 4 PML knockdown inhibited DNA damage checkpoint function and delayed DSB repair in H1299 cells after γ -irradiation. (a) PML knockdown decreased S and G2/M checkpoint function after γ -irradiation. H1299-PMLsi and H1299-control cells were irradiated (10 Gy), harvested at the indicated times, and subjected to FACS analysis. (b) Histograms of cell cycle distribution in PML knockdown and control cells after irradiation (*, $P < 0.05$). (c) Knockdown of PML delayed DSB repair in H1299 cells. H1299-PMLsi and H1299-control cells were irradiated (10 Gy), and fixed for the γ -H2AX (green) immunofluorescence assay at 0.5 and 6 h after irradiation. Nuclear DNA was stained with DAPI (blue). Images were acquired using immunofluorescence microscopy (Nikon).

3 Discussion

This study showed that PML regulates p21 stability by a p53-independent signaling pathway during γ -irradiation-induced DNA damage responses. The function of p21 in DNA repair and apoptosis remains controversial [9]. Some evidence suggests that UV irradiation induces nuclear translocation of p21 and an interaction between p21 and chromatin-bound PCNA [20,21]. PCNA is a crucial protein involved in several aspects of DNA metabolism, including

DNA replication. The association between p21 and PCNA may cause competitive displacement of other PCNA interacting proteins resulting in enhanced DNA repair and cell survival. In contrast, other studies show that p21 is a selective negative regulator of PCNA-dependent DNA replication, nucleotide excision repair, and translesion synthesis [9,22]. We found decreased p21 stability and lower levels of the p21-PCNA complex in PML knockdown H1299 cells. These data indicate that PML regulates p21 stability and its association with interacting proteins (such as PCNA)

Figure 5 PML knockdown interrupts DSB repair in H1299 cells through impaired up-regulation of p21. (a) Over-expression of p21 reversed the DSB repair deficiency in PML knockdown H1299 cells. pCMV-Myc vector or pCMV-Myc-p21 transfected PML-knockdown H1299 cells were irradiated and fixed 6 h after irradiation. Antibodies against γ -H2AX (green) were used for immunofluorescence. Nuclear DNA was stained with DAPI. (b) The p21-PCNA complex was decreased in PML-KD H1299 cells. Myc-tagged p21 protein was expressed in H1299-PMLsi and control cells. Immunoprecipitation was performed using Agarose conjugated Anti-Myc-Tag, anti-p21 (K0081-3, MBL), and anti-PCNA (PC10, Santa Cruz) antibodies.

during DSB repair in p53-null tumor cells, and that p21 functions down-stream of PML in this pathway. In addition, they confirm the presence of a p53-independent pathway in the DNA damage response.

PML plays an essential role in maintaining genomic integrity [7]. A decreased expression of PML results in loss of cell cycle control, prevention of apoptosis, and the promotion of oncogenesis. Previous reports show that PML-induces p53 up-regulation of p21 transcription during the DNA damage response [23,24]. In this study, we identified a PML-induced p53-independent post-translational pathway of p21 up-regulation. We propose that this p53-independent PML-p21 pathway plays an extensive role in maintaining genome stability by activating DNA damage checkpoint function and promoting DNA repair. PML acts as hyper-stable scaffold component within PML NBs which play important roles in DNA damage responses. After irradiation, PML NBs dynamically recruit or release a striking variety of important proteins, many of which are involved in DNA repair. As p21 was reported to localize into PML NBs after Kaposi's sarcoma-associated herpes virus infection, we investigated whether PML could directly interact with p21. At least seven PML isoforms have been identified [25]. PML IV is the most studied PML isoform known to induce p53 and apoptosis; therefore, we used over-expressed PML IV to identify p21 and PML interactions. Unfortunately, we could not identify a direct interaction between PML IV and p21 in H1299 cells (data not shown), suggesting that other molecules may be involved in this pathway. We propose that further studies are needed to evaluate which PML isoforms interact with and regulate p21. Furthermore, as PML NBs are not only docking sites

for transiting proteins, but also locations for post-translational modification of nuclear proteins, the mechanisms of PML-induced post-translational regulation of p21, including phosphorylation and acetylation, must also be investigated.

p21 is a stress response protein induced by various stimuli, including siRNA transfection. In this study, PML-siRNA transfection decreased the expression of p21 protein, but the levels of p21 protein were much higher in the negative control siRNA-transfected cells than in untransfected cells, although the PML protein contents were comparable. While these data indicate that other factors besides PML influence the p21 protein levels in p53-null H1299 cells, they confirm that PML is necessary for p53-independent maintenance of p21 protein levels.

It is essential to characterize the roles of PML-dependent p53-independent pathways during tumor suppression as nearly half of human tumors exhibit p53 mutations or deficiency. We propose that these studies may identify PML as a potential pharmacological target and contribute to the development of novel therapeutic strategies for the treatment of cancer.

4 Conclusions

(i) γ -irradiation induced PML and p21 protein expressions in p53-null H1299 cells.

(ii) PML increased p21 stability and facilitated p21-PCNA complex formation in p53-null H1299 cells.

(iii) PML-p21 pathway contributes to DSB repair after γ -irradiation.

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Supporting Information

Figure S1 PML-KD induced p21 down-regulation prior to p53 reduction in HEL cells. HEL cells were transfected with 10 nmol/L PML siRNA (PMLsi) or Negative control siRNA (NCsi). PML and p21 expression were analyzed at 24, 30, 36, and 48 h after transfection.

Figure S2 PML knockdown did not significantly affect p53-null H1299 and HCT116 (p53^{-/-}) cells proliferation. Proliferation of PML knock down H1299 cells (A) and HCT116 (p53^{-/-}) cells (B) were detected by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. Cells were seeded in 96-well plates at the same concentration (2×10^3 /well for H1299, 4×10^3 /well for HCT116). After incubation in complete medium containing 10% fetal bovine serum overnight, cells were transfected by using 5 nmol/L PML-siRNA or NC-siRNA. Transfected cells were then proliferated for 72 h. MTT (5 mg/ml) was added to the culture at 20 μ l per well, followed by incubation at 37°C for 4 h. Medium was aspirated, and cells were lysed with 150 μ l dimethyl sulfoxide. Absorbance of each well was recorded in a microplate reader at 492 nm. Data are presented as means $\pm$ SEM. Three wells per treatment were used.

Figure S3 Ubiquitinylation of p21 was not changed in H1299-PMLsi cells. Myc-p21 and HA-Ubiquitin were over-expressed in H1299-PMLsi or H1299-control cells. Immunoprecipitation were conducted by Agarose conjugated Anti-Myc-Tag antibodies, and analyzed by anti-p21 antibody (K0081-3, MBL).

Figure S4 The expression of p21 and PML in pCMV-Myc-p21 or pCMV-myc transfected PML-knockdown H1299 cells and control cells. pCMV-myc vector or pCMV-Myc-p21 were transfected into H1299 cells 24 h after PMLsiRNA transfection, PML and p21 expression were analyzed by Western Blot with anti-PML(H238,Sc) and anti-p21(K0081-3, MBL) antibodies.

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