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Androgen suppresses PML protein expression in prostate cancer CWR22R cells☆

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Abstract

The ability of PML to modulate key suppressive pathways in tumor cells suggests that PML may act as a tumor suppressor. The detailed mechanism of how PML functions in prostate cancer progression, however, remains unknown. Here we demonstrate that in the presence of androgen, PML protein expression can be suppressed in CWR22R prostate cancer cells. Further studies reveal that PML can selectively suppress AR transactivation and PML protein expression positively correlates with increased p21 protein level and enhances p53 transcription ability in CWR22R cells. We also found that PML strongly inhibits CWR22R cell colony formation, while PML siRNA enhances AR activity and CWR22R cell colony formation. Together our results suggest that PML may suppress prostate cancer cell growth by inhibiting AR transactivation and/or enhancing p53 activity.

Keywords: PML; Androgen receptor; CWR22R

The promyelocytic leukemia protein, PML, is a ubiquitously expressed nuclear phosphoprotein that belongs to the TRIM protein superfamily, containing a characteristic TRI partite motif, which has a C3HC4 zinc RING finger, two alternate cysteine–histidine rich zinc binding domains, the B1 and B2 boxes, and an α-helical coiled-coil dimerization domain [1]. Alternative splicing of the carboxy-terminal region results in generation of over 15 different isoforms for which specific functions have only recently begun to be identified [2,3]. Understanding the PML biological role has become an area of intense research because of its involvement in the pathogenesis of acute promyelocytic leukemia (APL), a distinct subtype of myeloid leukemia. PML antagonizes the initiation, promotion, and progression of tumors of various histological origins, acting in vivo as a cell-growth suppressor and tumor suppressor [4]. PML is essential for multiple stress/DNA damage-activated apoptotic pathways [5]. It directly interacts with DNA binding domain of p53, colocalizes with p53 in the PML-nuclear body (NB), and acts as a p53 transcriptional coactivator [5]. PML also mediates IFN-regulated cellular functions, including growth and tumor-suppressive activities, and induces a block in the G1 phase of the cell cycle [6–8]. PML physically interacts with the tumor suppressor retinoblastoma protein (Rb), which is also found in the PML-NB and is implicated in the control of cell cycle entry and cellular senescence [9]. The ability of PML to modulate key suppressive pathways suggests that PML may participate in the human tumorigenesis other than APL. However, how PML functions in prostate cancer progression remains largely unknown.

Prostate cancer (PC) is the second leading cause of cancer death among men in most western countries. PC

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growth depends on the ratio of cells proliferating to those dying. Androgens are the main regulator of this ratio by both stimulating proliferation and inhibiting apoptosis [10]. Androgen ablation causes regression of androgen-dependent tumors and is the mainstay of therapy for progressive prostate cancer. Androgen exerts its role through the androgen receptor (AR), which is a member of the nuclear receptor superfamily that cooperates with coregulators in order to exert its biological function [11–14]. Upon binding to testosterone/dihydrotestosterone (T/DHT), the liganded AR can bind to the androgen-response-element (ARE) on the 5′ promoter of the target gene, which results in the modulation of cell growth [15,16].

Here we show the changes in PML protein expression and cell growth following treatment with or without androgen in prostate cancer CWR22R cells.

Materials and methods

Materials. The PG13-luc was kindly provided by Dr. R.B. Ray [17]. DHT was from Sigma. The anti-AR polyclonal antibody, NH27, was produced as previously described [18]. The anti-Flag M2 monoclonal antibody was from Sigma, and anti-PML monoclonal antibody, AP-conjugated secondary anti-mouse, and anti-rabbit antibodies were from Santa Cruz. pSG5-PML3 was a gift of Pier Paolo Pandolfi [19]. pCMV2-Flag-PML3 and pCDNA3-PML3 were generated by inserting the *Eco*RI fragments released from pSG5-PML3 into pCMV2-Flag (Sigma) and pCDNA3 (Invitrogen) vectors which have been digested by *Eco*RI. The orientation was confirmed by sequencing assay.

Cell culture and transfections. The human prostate cancer CWR22R cells were maintained in RPMI-1640 with 10% FCS. Transfections were performed using SuperFect (Qiagen). In brief, cells were plated in 10% charcoal-dextran treated FBS (CD-FBS)-containing medium in 24-well plates at 8×10^4 cells/well. One day after plating, the cells were transfected according to standard procedures.

MTT assay. Viable cells were plated at 1×10^5/well in 24-well plates and allowed to adhere in 10% FBS RPMI 1640 for 48 h. Viability of cells was determined by visual inspection under a microscope. The media were then aspirated, cells were rinsed once with serum-free RPMI 1640, and then media were replaced with either 10% CD FBS supplemented with DHT or ethanol vehicle control. The cell numbers were assayed via MTT according to the manufacturer's protocol (Sigma).

Luciferase reporter assays. The luciferase reporter assays have been described previously [20]. Briefly, the cells were transfected with plasmids in the 10% CD-FBS media for 16 h and then treated with ethanol or 10 nM DHT for 24 h. After the cells were washed with PBS and harvested, cell lysates were prepared and used for luciferase assay according to the manufacturer's instructions (Promega). The results were obtained from at least three sets of transfection and presented as means ± SD.

Western-blot analysis. The cells were rinsed with ice-cold PBS and lysed on ice in RIPA buffer containing 1× PBS, 1% Igepal CA-630 (Sigma Chemicals), 0.5% sodium deoxycholate, and 0.1% SDS and supplemented with 10 μl/ml protease inhibitor PMSF (10 mg/ml). Cell lysates were clarified by centrifugation (14,000 rpm 15 min at 4 °C), total protein concentration was adjusted with the lysis buffer, and samples were boiled in an equal volume of 2× SDS-sample buffer. For Western blotting, protein extracts were separated on 10% SDS–polyacrylamide gels, transferred to Immobilon paper (Millipore), and probed with a mouse monoclonal antibody against PML and goat polyclonal antibody against β-actin (Santa Cruz Biotechnology). p27kip (K25020), and p21Cip1 (C24420) antibodies were purchased from Transduction Laboratories. The blots were developed using horseradish peroxidase-coupled goat anti-mouse or donkey anti-rabbit antibodies (Santa Cruz Biotechnology) and the chemoluminescence (ECL) substrate (Amersham–Pharmacia Biotech) according to the manufacturer's instructions.

Colony formation assay. CWR22R cells were transfected with 10 μg pCDNA3-PML3, 10 μg pMSCV/U6, or equimolar quantities of the empty vector. At 48 h after transfection, each plate was trypsinized and replated at a dilution of 1:100. After an additional 24 h, 250 μg/ml active concentration of G418 (for pCDNA3 and pCDNA3-PML3) and 1 μg/ml final concentration of puromycin (for pMSCV/U6 and pMSCV/U6-PML3-siRNA) were added to the medium. After 15 days of selection, G418 and puromycin-resistant colonies were colored with crystal violet and counted.

Construction of PML siRNA expression plasmid. PML siRNA was constructed using a DNA-based vector pMSCV/U6, which was a gift from Dr. P. Silver (Harvard Medical School, Boston, MA) that contains puromycin resistance marker. The oligo GTGCTTCGAGG CACACCAGTTCAAGAGA CTGGGGTGCCTCGAAGCACTTT TTT was subcloned into the *Apa*I–*Eco*RI site of pMSCV/U6 vector to generate pMSCV/U6-PML-siRNA.

Real-time PCR. Analysis Total RNAs from 5×10^6 CWR22R cells, in which were transfected with 10 μg pMSCV/U6-PML-siRNA and empty vector, were isolated 24 h after transfection by using TRIZOL following the manufacturer's protocol. Briefly, RNA was extracted with chloroform, precipitated by isopropanol, and resuspended in RNase-free water and then denatured at 65 °C for 5 min in a total volume of 12 μl containing 5 μg RNA, 0.5 μg random hexamers (Invitrogen), and 1 μl of 10 mM dNTP. Denatured RNA was then reverse-transcribed in a final volume of 20 μl containing 1× RT buffer, 10 U RNasin RNase inhibitor (Invitrogen), 10 mM dithiothreitol, and 50 U Superscript II reverse transcriptase (Invitrogen) at 42 °C for 50 min. Reverse transcriptase was inactivated by heating at 70 °C for 15 min. cDNA was subjected to real-time PCR using the SYBR Green PCR Reagents kit (Bio-Rad). The thermal cycling conditions comprised an initial denaturation step at 95 °C for 3 min and 40 cycles at 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s. Experiments were performed in triplicate for each data point. PCR primers were as follows: PML forward: 5′ CCG CAA GAC CAA CAA CAT C 3′ and PML Reverse: 5′ ACT GTG GCT GCT GTC AAG 3′.

Results

Effect of androgen on CWR22R cell proliferation

CWR22R cell line, as described previously, which can be weakly stimulated by DHT [21], was derived from a xenograft that was serially propagated in mice after castration-induced regression and relapse of the parental, androgen-dependent CWR22 xenograft. We first tested the DHT effect on the proliferation rate of CWR22R cells. As shown in Fig. 1, addition of 10^{-8} nM DHT can slightly enhance cell proliferation in CWR22R cells, which is consistent with a previous report [21].

Androgen decreases PML protein expression

Prostate cancer cells can survive in an environment with low-androgen levels in a number of ways. These include mutations of AR that alter ligand specificity leading to activation by progesterone, estrogen, glucocorticoids, or HER-2/*neu* [22,23]; ligand-independent

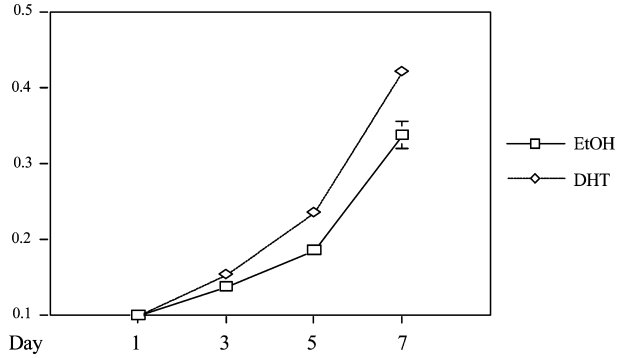

Fig. 1. Androgen effect on CWR22R cell proliferation rate. CWR22R cells were plated in 24-well dishes at a concentration of 1×10^5 cells/well. After 24 h growth in 10% FBS, media were changed to 10% CD-FBS and treated with ethanol (EtOH) as vehicle control and 10 nM DHT. Cell numbers were determined every 3 days by MTT. Results are representative of three independent experiments as means ± SD.

activation of AR by growth factors and cytokines, at least in vitro [22]; AR amplification, which is found in ~30% of cases [22]; and modulation due to interaction with AR coregulators [24–33]. Prostate cancer cells can also achieve the transition to androgen-independent growth by different multistep routes, including inhibition of apoptosis and bypassing or adapting the AR signaling pathway [22,24,34]. PML has been demonstrated as a tumor suppressor to control the induction of apoptosis, growth suppression, and cellular senescence upon oncogenic transformation [5]. Thus, we investigated the possible role of PML in CWR22R cell proliferation and transformation.

CWR22R cells were seeded into 60-mm dishes under the CD-medium culture conditions for 24 h and treated with or without DHT for another 24 h. Then the cells were harvested for the Western-blot analysis. Interestingly, we found that androgen treatment obviously suppressed the PML protein expression in CWR22R cells (Fig. 2).

PML selectively suppresses AR transactivation

The AR is expressed in normal prostate tissue and prostate cancers, and is a key transcription factor to control prostate cell growth. Activation of the AR in prostate cancer is being intensively investigated. However, how PML influences AR signaling is not clear. We applied the ARE reporter gene assay to see whether PML has an effect on AR activity. PML and four copies of a synthetic ARE-luciferase ((ARE)4-luc) reporter gene were co-transfected into CWR22R cells. As shown in Fig. 3A, 10 nM DHT induces AR transactivation up to 35-fold in CWR22R cells and the addition of PML can then repress AR transactivation in a dose-dependent manner, indicating that CWR22R cells also contain functional AR, which can be suppressed by transfection of PML. In order to further prove the repression effect of PML on AR, we used PML siRNA construct pMSCV/U6-PML-siRNA to do luciferase assay. pMSCV/U6-PML-siRNA was generated as described under Materials and methods. The decreased efficiency of PML by siRNA was determined by using real-time PCR analysis. As shown in Fig. 3B, endogenous PML mRNA expression level was reduced by around 55%. Due to the limitation of transfection efficiency, not all of the CWR22R cells can be transfected. Thus, a 55% reduction of PML mRNA level is thought to be significant. In Fig. 3C, PML siRNA obviously enhances AR activity, which further proves the repression effect of PML on AR transactivation.

To rule out the possibility that PML repressed AR transactivation is not due to the general transcriptional squenching, we investigated the PML effect on other nuclear receptor's activity in COS-1 cells. As shown in Fig. 4, the addition of PML also represses AR-induced MMTV-luc activity in a dose-dependent manner (Fig. 4A). In contrast, PML showed different degrees of enhancement effects on the glucocorticoid receptor (GR) (Fig. 4B) and estrogen receptor α (ERα)-mediated transactivation (Fig. 4C), indicating that PML selectively suppresses AR transactivation.

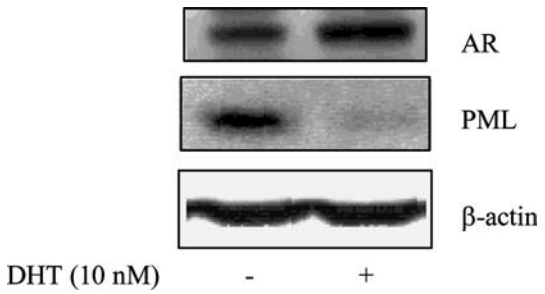

Fig. 2. Androgen suppresses PML protein expression. The CWR22R cells were cultured in 10% CD-FBS medium for 24 h and then treated with EtOH as vehicle control and 10 nM DHT. After 24 h, cells were harvested for Western-blot analysis with indicated antibodies.

PML enhanced p53 activity and p21 expression

The majority of p53 activity is dependent on its ability to act as a transcription factor. PML directly interacts with the DNA binding domain of p53, colocalizes with P53 in the PML-NB, and acts as a transcriptional coactivator [5]. Thus, it is conceivable that elevated PML protein level could enhance p53 transactivation in CWR22R cells. The *p21*$^{(WAF\text{-}1/CIP1)}$ (*p21*) gene, which has been identified as a key factor for the regulation of cell growth, is the target gene of p53. Enhanced p53 activity should also increase p21 expression. In order to prove this hypothesis, Western-blot assays were performed to analyze the p53 and p21 protein expression in CWR22R cells after treatment with or without DHT. As shown in Fig. 5A, androgen suppressed PML protein negatively correlated with increased p21 protein levels, but not $p27^{Kip1}$ protein levels,

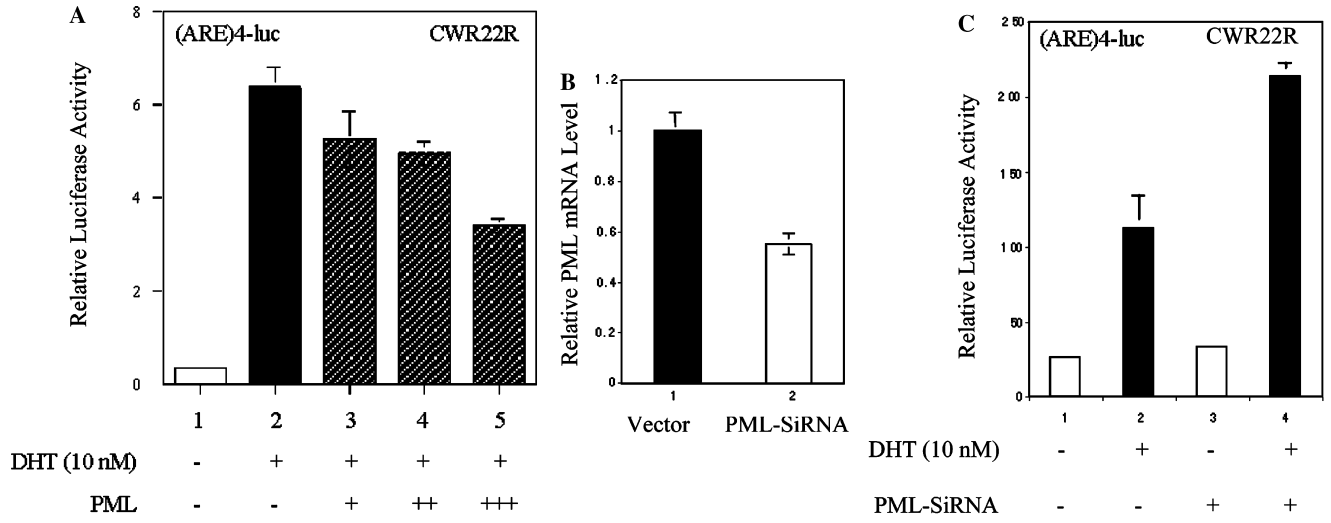

Fig. 3. PML suppresses AR transactivation. (A) CWR22R cells were transfected with 150 ng (ARE)4-luc, 2.5 ng pRL-SV40, and 50–300 ng PML (+, 50 ng; ++, 150 ng; and +++, 300 ng). The parent vector pCDNA-3 was used to balance the amounts of plasmid transfection. Transfected cells were treated for 24 h with 10^{-8} nM DHT or EtOH. Duplicate samples were analyzed for each single data point. (B) CWR22R cells were seeded into 100-mm dishes and transfected with 10 μg pMSCV/U6-PML-siRNA, or 10 μg of empty vector by electroporation. After transfection for 24 h, the cells were harvested and total RNA was isolated to perform real-time PCR as described in Materials and methods. (C) CWR22R cells were transfected with 150 ng (ARE)4-luc, 2.5 ng pRL-SV40, and 300 ng pMSCV/U6-PML-siRNA. The parent vector pMSCV/U6 was used to balance the amounts of plasmid transfection. Transfected cells were treated for 24 h with 10^{-8} nM DHT or EtOH. Duplicate samples were analyzed for each single data point.

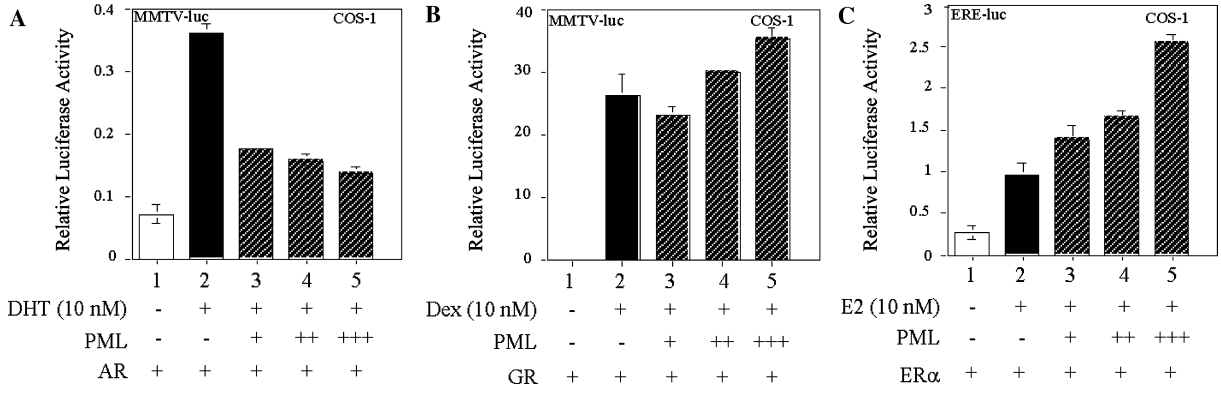

Fig. 4. PML enhances GR and ER-α transactivation. COS-1 cells were transfected with 150 ng MMTV-luc (A,B), or 150 ng ERE-luc (C), 2.5 ng pRL-SV40, 50–300 ng PML (+, 50 ng; ++, 150 ng; and +++, 300 ng), and 50 ng pSG5-AR (A), pSG5-GR (B), or pSG5-ER-α (C). The parent vector was used to balance the amounts of plasmid transfection. Transfected cells were treated for 24 h with 10^{-8} nM DHT or EtOH. Duplicate samples were analyzed for each single data point.

another key effector of cellular senescence. p53 protein level only marginally increased under the vehicle treatment. In order to demonstrate that PML is able to enhance p53 transactivation in CWR22R cells, we performed reporter gene assays in this cell line. PG13-luc reporter gene, the specific p53 target reporter gene [17], and different doses of PML were transfected into CWR22R cells. As shown in Fig. 5B, PML could enhance p53 transactivation in a dose-dependent manner. Moreover, over-expressed PML obviously increased p21 protein expression (Fig. 5C), suggesting that the growth retardation of CWR22R cells in the absence of androgen may be the consequence of enhanced p53 activity and increased p21 protein expression, both of which were regulated by PML.

PML inhibited the colony formation in CWR22 cells

To further demonstrate that PML is a growth suppressor, we employed a colony-formation assay. Briefly, CWR22R cells were transfected with pCDNA3-PML and empty vector of pCDNA3. Cells were selected with G418 for 14 days, after which resistant colonies were scored. Fig. 6A shows the mean values of three separate experiments performed with CWR22R cells (each experiment was performed in triplicate) where percentage of colonies formed in plates transfected with PML is compared to the value for the vector-transfected control. The results indicated that PML over-expression greatly reduced colony formation (average of 90%). More over, we used PML siRNA to prove the inhibition ability of

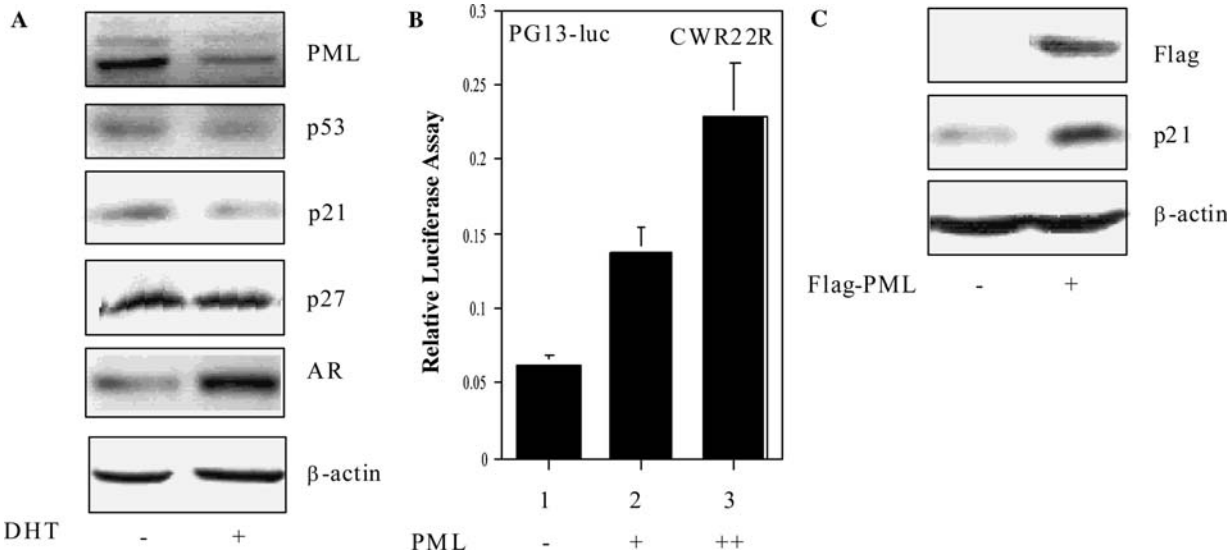

Fig. 5. PML enhances p53 activity and p21Cip1/Waf1 expression. (A) CWR22R cells were cultured in 10% CD-FBS-containing medium for 24 h and then treated with EtOH and 10 nM DHT. After 24 h, cells were harvested for Western-blot analysis with indicated antibodies. (B) CWR22R cells were transfected with 150 ng PG13-luc, 2.5 ng pRL-SV40, and 50–150 ng PLM (+, 50 ng; ++, 150 ng). The parent vector pCDNA-3 was used to balance the amounts of plasmid transfection. Transfected cells were cultured in normal growth medium. Duplicate samples were analyzed for each single data point. (C) CWR22R cells were transfected with pCMV-Flag-PML3 and pCMV-Flag, respectively. Cells were cultured in normal growth medium. After 24 h, cells were harvested for Western-blot assay with indicated antibodies.

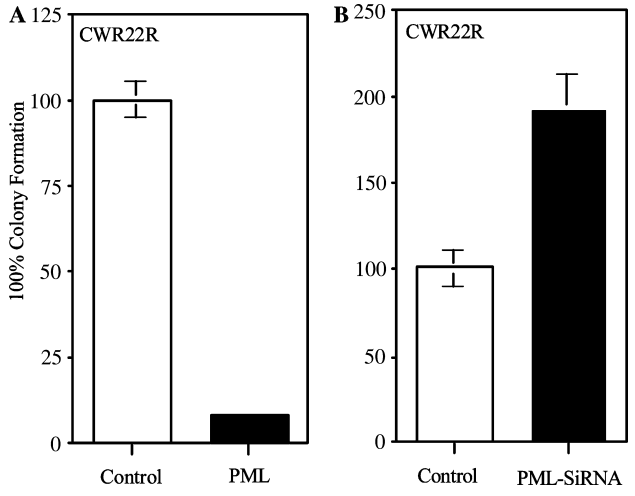

Fig. 6. PML inhibits CWR22R cell colony formation. CWR22R cells were transfected with pCDNA3-PML3 or empty vector (A), and pMSCV/U6-PML-siRNA or empty vector (B) as described in Materials and methods. The result represents the average of three separate experiments, each performed in triplicate.

PML on CWR22R cell colony formation. As shown in Fig. 6B, PML siRNA significantly increased the colony formation (average of 195%).

Discussion

The present study found androgen stimulates CWR22R cell growth, which is negatively correlated with PML protein level. Androgen treatment has little effect on PML mRNA expression (data not shown), which suggests that androgen may influence PML at the protein level. However, the mechanism of how androgen regulates PML protein expression and how PML is targeted for degradation remains unclear. PML is covalently modified by SUMO-1, a ubiquitin-like polypeptide. Three major sites of SUMO-1 modification have been identified in PML, K65 in the RING finger, K160 in the first B-box, and K490 in the nuclear localization signal [35,36]. SUMO-1 can be conjugated onto a variety of proteins including p53, IκB, Sp100, PML, and RanGAP. In the case of IκB, SUMO-1 appears to compete with ubiquitin for modification of the same target lysine, inhibiting proteasome-dependent IκB degradation [37]. Thus, it seems that SUMO-1 modification modulates the conformation of its target proteins rather than inducing their degradation. In contrast, Lallemand-Breitenbach et al. [38] found recently that sumoylation-promoted degradation and NB-associated proteolysis of PML upon As_2O_3 treatment. They demonstrated that As_2O_3 triggers the proteasome-dependent degradation of PML and that this process requires a specific sumoylation site in PML, K160. It will be interesting to see if androgen treatment in CWR22R cells might influence the PML-sumoylation status. Like ubiquitin, SUMO-1 covalently binds the lysine residues of target proteins in an ATP-dependent reaction requiring the E2-conjugating enzyme Ubc9. Ubc9 has been proven to be an AR interacting protein which can activate AR transactivation [39]. Although it not clear whether AR can also influence Ubc9 activity, future studies may go in this direction.

Although we found that PML is able to selectively repress AR transactivation in CWR22R cells, the mechanism of how PML plays its suppression role on AR activity is not clear. The cAMP enhancer binding protein (CREB)-binding protein (CBP) functions as a transcriptional coactivator for a variety of transcription factors, including nuclear receptors (NRs) [40], and the PML has been suggested to act as a CBP cofactor and contribute to the CBP-mediated transcriptional regulation [41]. Interestingly, previous studies revealed that PML associates with the N-terminal domain of CBP (amino acids 311–521) [41], while AR interacts with the region between residues 271 and 452 of CBP [42]. Thus, it may be possible that competing for the binding site on CBP by PML and AR, not other NRs, causes the decreased AR activity when PML is over-expressed, because it has been proven that other NRs interact with conserved motifs on CBP (amino acids 1–117) [40].

Here we found that androgen suppresses the PML protein level in CWR22R cells. We also demonstrated that PML suppresses AR transactivation, enhances p53 activity and p21 protein expression in CWR22R cells, and inhibits CWR22R cell colony formation. These findings may imply that the proliferation differences in CWR22R cells treated with or without androgen can be due to the PML protein expression induction. This may be clinically significant because successful induction of PML via chemicals or drugs in patients with advanced prostate cancer may result in the suppression of tumor growth. However, whether PML can influence the AR negative prostate cancer cells remains unclear. Further intensive study of potential side effects of PML may help us to better understand if PML can become a useful medicine in the treatment of prostate cancer.

Acknowledgments

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