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p21^{WAF1} negatively regulates DNMT1 expression in mammalian cells

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ABSTRACT

The expression of DNMT1, the major maintenance DNA methyltransferases, is critical in coordinating DNMT1 activity with biological processes and therefore must be tightly regulated in the cell cycle. Here, we report p21^{WAF1} as a novel upstream regulator of DNMT1 expression. Ectopic expression of p21^{WAF1} or TSA-mediated p21^{WAF1} induction inhibits DNMT1 at the transcriptional level, and this observation consistently coincides with a reduction in p300. Furthermore, siRNA-mediated p300 knockdown significantly abolishes *DNMT1* mRNA levels, demonstrating the dependence of DNMT1 expression on p300. Consistent with this, p300 enhances transactivation of *DNMT1* promoter 340 bp upstream of the initiation start site harboring the E2F1 and Sp1/3 binding sites. Collectively, we identified p300 as a crucial transcription regulator for DNMT1. We proposed that the reduction in p300 following p21^{WAF1} up-regulation contributes to DNMT1 down-regulation. This novel p21^{WAF1}-p300-DNMT1 pathway may play a pivotal role to ensure regulated DNMT1 expression and DNA methylation in mammalian cell division.

Introduction

The transfer of a methyl group from S-adenosylmethionine to the C-5 position of cytosines residing in CpG dinucleotides is catalyzed by DNA (5-cytosine) methyltransferase. In mammals, DNA methyltransferase 1 (DNMT1) exhibits high preference for hemimethylated DNA and is the major DNA methyltransferase responsible for maintaining DNA methylation patterns in newly synthesized DNA [1]. DNMT1 is recruited to active DNA replication sites through its interaction with Proliferating Cell Nuclear Antigen (PCNA), a processivity factor for DNA Polymerases δ and β [2], which then facilitates the methylation of hemi-methylated DNA in S phase nuclei [3]. Interestingly, the cyclin-dependent kinase (Cdks)-inhibitor p21^{WAF1} is capable of competing with DNMT1 for binding to its co-factor PCNA, thereby accounting for its ability to inhibit DNA replication and DNA methylation events [4].

Although DNMT1 is constitutively expressed in mammalian cells, regulation of *DNMT1* gene expression is critical in coordinating DNMT1 activity with biological processes and therefore must be tightly regulated in the cell cycle. The discovery of new 5′ regions in murine and human *DNMT1* genes inevitably positioned a new transcription initiation site upstream of the previously identified promoter [5]. Putative binding sites for transcription factors such as TCF-1, AP-1 (Activator Protein 1), E2F1 and Sp1/Sp3 were identified upstream of this newly characterized start site [6]. These findings directed new interest in resolving how *DNMT1* gene is regulated. The *DNMT1* promoter is a TATA-less promoter and therefore its transcriptional activity is highly dependent on the binding of transcription factors to the basal promoter region. Transcription of murine *Dnmt1* promoter was shown to be independently controlled by Sp1 and Sp3 in a cell cycle dependent manner [7,8]. p300, a general transcriptional co-activator that exhibits histone acetyltransferase activity, associates with Sp1/3 to direct transcriptional co-activation of the *Dnmt1* promoter in S phase cells [7,8]. Furthermore, the E2F-binding site located within the transcription initiation region is critical for the regulation of *DNMT1* transcription in proliferating cells via the E2F1/pRB pathway [9].

Several groups had independently reported an inverse relationship between DNMT1 and p21^{WAF1} expression in mammalian cells [4,10–12], which strongly suggest that the two proteins may be linked in a regulatory pathway. Although DNMT1 has been shown to repress *p21^{WAF1}* transcription, the exact mechanism remains controversial. The involvement of p21^{WAF1} in transcriptional regulation [13–15] prompted us to question whether p21^{WAF1} negatively regulates DNMT1 expression in a feedback mec hanism. We are further encouraged by the findings that p21^{WAF1} can inhibit the activity of E2F1, a transcription factor for DNMT1, by binding to E2F1 subunits and by promoting the formation of the inactive E2F1/pRB complex [16].

In this study, we validated the relationship between DNMT1 and p21^{WAF1} in mammalian cell cultures, and investigated the underlying mechanisms involved. Our novel discovery that p21^{WAF1} negatively regulates DNMT1 expression by modulating p300 is of substantial importance in the regulation of DNMT1

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expression and DNA methylation in mammalian cell division, particularly during DNA damage when $p21^{WAF1}$ protein is induced.

Materials and methods

Chemical and antibodies. Trichostatin A (TSA) was purchased from Sigma–Aldrich. Proteasome inhibitor MG132 (Z-Leu-Leu-Leu-CHO) was purchased from BostonBiochem Inc. DNMT1 antibody was from Calbiochem. Anti-p300 (C20), anti-PCNA (PC10) and anti-cMyc (9e10) were purchased from Santa Cruz. Antibodies for β-actin (clone C4) and $p21^{WAF1/CIP1}$ (clone 70) were from Boehringer Mannheim and Transduction Laboratories, respectively.

Cell culture. MCF7 human breast carcinoma cells were purchased from ATCC and cultured in RPMI media supplemented with 10% FBS (Hyclone).

Western blot analysis. Cells were lysed in 5× volume of modified RIPA buffer (50 mM Tris, pH 8.0, 0.3 M NaCl, 10 mM EDTA, pH 8.0, 1% NP40, 50 mM NaF, 0.1% SDS, 0.5% sodium deoxycholate and 0.2 mM PMSF, sonicated at 50% power output). Equal amounts of proteins were subjected to electrophoresis along SDS–PAGE gels prepared using 30% acrylamide/bisacrylamide solution (Bio-Rad) and transferred to PVDF membranes (Bio-Rad). After blocking, the membranes were probed with 1:1000 primary antibodies in TBST (20 mM Tris–HCL, pH 8.0, 0.15 M NaCl and 0.05% Tween-20), washed thrice with TBST and then probed with 1:4000 anti-mouse IgG-HRPO DAKO prepared in 5% skimmed milk. Detections were performed with ECL™ reagents and visualized using hyperfilms (Amersham Biosciences). Protein signal was quantified using the QuantityOne465 Program (Bio-Rad).

mRNA analysis. Total RNA was extracted using the QIAshredder and RNeasy kit (QIAGEN) and reverse transcribed using the MuLV reverse transcriptase enzyme (NEB). RT-PCR was carried out using Taq DNA Polymerase in Storage Buffer B (Promega) (GAPDH, sense 5′-ACA TCG CTC AGA CAC CAT GG-3′ and antisense 5′-GTA GTT GAG GTC AAT GAA GGG-3′; DNMT1, sense 5′-GTG AAG GAG AAA TTG AAT CTC TT-3′ and antisense 5′-GCC TCT CCA TCG GAC TTG-3′; $p21^{WAF1}$, sense 5′-CCT GAC GGA TCC ATG TCA GAA CCG GCT-3′ and antisense 5′-CCT GAC GAA TTC TTA GGG CTT CCT CTT-3′), and the PCR samples were electrophoresed along 1.5% agarose gel (BRL).

DNA manipulations. Full-length human *DNMT1* sequence was released from the pM-DNMT1 construct (courtesy of Dr. L.S.H. Chuang, Institute of Molecular and Cell Biology, Singapore) and cloned into pXJ40-Flag (courtesy of Dr. H.K. Oh, Institute of Molecular and Cell Biology, Singapore) to yield the pXJ-Flag.DNMT1. The pXJ-$p21^{WAF1}$ plasmids were kindly provided by Dr L.S.H. Chuang. The human *DNMT1* proximal promoter region was amplified from MCF7 genomic DNA by PCR (sense 5′-TAT TGG TAC CCT TCT CGC TGC TTT ATC CC-3′ and antisense 5′-TAT TAA GCT TCT CGG AGG CTT CAG CAG A-3′) and cloned into pGL3-Basic vector (Promega).

Transient transfection. Unless otherwise stated, all transfections were carried out using SuperFect® transfection reagent (QIAGEN) at 37 °C for 5 h under the recommended protocol.

siRNA transfection. Cells were transfected with 100 nM of human siDNMT1, sip300 (Santa Cruz) or siLacZ (sense 5′-rCrGU rArCrG rCrGrG rArAU rArCU UrCrG rATT and antisense 5′-UrCrG rArArG UrAU UrCrC rGrCrG UrArC rGTT) (PrOligo) using Oligofectamine® transfection reagent (QIAGEN) prepared in OptiMEM medium (GIBCO-BRL) at 37 °C for 5 h. The transfected cells were washed twice with PBS, recovered in complete medium and harvested for further analysis.

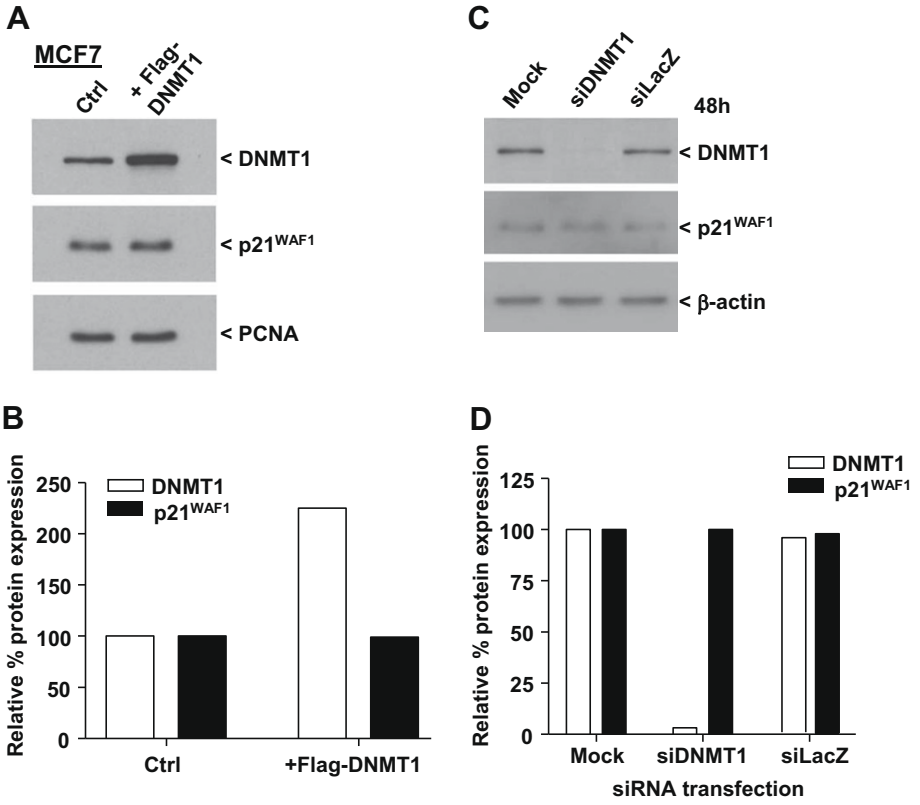

Fig. 1. Alteration in DNMT1 expression has no significant effect on $p21^{WAF1}$ protein levels in human breast carcinoma MCF7 cells. (A) Western blot analysis of DNMT1, $p21^{WAF1}$ and PCNA. Untransfected cells were used as a control (Ctrl; control). As MCF7 cells expressed negligible levels of $p21^{WAF1}$ under basal conditions, the Western blot for $p21^{WAF1}$ was over-exposed to enable visual detection. (B) Relative percentages of DNMT1 and $p21^{WAF1}$ in (A) normalized against PCNA. (C) Western blot analysis of DNMT1 and $p21^{WAF1}$ in cells transfected with the respective siRNA for 48 h. Empty transfection (Mock) was used as an internal control for the experiment. β-actin served as a loading control. (D) Relative percentages of DNMT1 and $p21^{WAF1}$ protein levels in (C) normalized against β-actin.

Luciferase reporter assay. Cells were transfected with 0.5 μg of pGL3-DNMT1Pro, 0.3 μg of pCMV-βGal and 0.5 μg of the respective plasmids using SuperFect® transfection reagent (QIAGEN) for 48 h. Cells were lyzed with 1× Passive Lysis Buffer (Promega) and protein concentration was quantified using the Bradford Assay. The β-galactosidase activity was quantified by incubating 50 μl lysate in 50 μl of 2× β-galactosidase buffer (150 mM Na_2HPO_4, 45 mM Na_2VO_4, 2 mM $MgCl_2$, 100 mM Mercaptoethanol and 1.33 mg/ml ONPG) at 37 °C for 1 h and measured at 420 nm using a microplate reader spectrophotometer (Bio-Rad). Luciferase activity was quantified using the Luciferase Assay System (Promega) and normalized against the β-galactosidase activities.

Results

Alteration in DNMT1 expression has no significant effect on p21^{WAF1}

We and others [4,10–12] have previously observed an inverse relationship between the expression levels of DNMT1 and p21^{WAF1} in mammalian cell cultures. We hypothesized that DNMT1 may act as an inhibitor of p21^{WAF1} so as to ensure negligible p21^{WAF1} expression during normal growth and cell division. To test this hypothesis, we studied the effect of DNMT1 over-expression on p21^{WAF1} in human breast carcinoma MCF7 cells. Although transfection of MCF7 cells with a mammalian vector expressing FLAG-tagged DNMT1 for 48 h efficiently over-expressed DNMT1 by >2-fold as compared to the control, both p21^{WAF1} and PCNA protein levels remained relatively unaffected (Fig. 1A and B). While this result shows no evidence that DNMT1 is an inhibitor of p21^{WAF1}, an alternative explanation may be that p21^{WAF1} is already at a basal level and the observed increase in DNMT1 was unable to deplete p21^{WAF1} any further. To examine this further, we knockdown DNMT1 in MCF7 cells by siRNA-mediated transfection. Unlike the Mock- and siLacZ-transfected cells, DNMT1 was undetectable in MCF7 cells transfected with 100 nM of siDNMT1 for 48 h (Fig. 1C and D). In contrast, p21^{WAF1} and β-actin protein levels remained relatively unchanged (Fig. 1C and D). Collectively, our results imply that DNMT1 potentially lies downstream of p21^{WAF1}.

Over-expression of p21^{WAF1} in MCF7 cells inhibits DNMT1

p21^{WAF1} may negatively regulate DNMT1 expression in mammalian cells. To explore this possibility, we transfected MCF7 cells with different concentrations of a mammalian construct expressing full-length p21^{WAF1} for 48 h. Whereas control and Mock-transfected MCF7 cells showed no change in the basal expression of p21^{WAF1}, transfection with pXJ-p21^{WAF1} yielded a dose-dependent increase in p21^{WAF1} (Fig. 2A and B). Interestingly, ectopic expression of p21^{WAF1} led to a gradual decrease in DNMT1 while β-actin remained relatively unchanged (Fig. 2A and C). Moreover, we also observed a significant reduction in the protein levels of transcription co-activator p300 in a similar temporal pattern as DNMT1 following the up-regulation of p21^{WAF1} (Fig. 2A and C). These results indicate that *in-vitro* over-expression of p21^{WAF1} inhibits DNMT1 and p300 in MCF7 cells.

Trichostatin A-mediated p21^{WAF1} induction inhibits DNMT1 at the transcriptional level

The histone deacetylase inhibitor TSA has been shown to induce p21^{WAF1} transcription in mammalian cells [17]. With regards to this, we sought to examine whether TSA-mediated p21^{WAF1} induction blocks DNMT1 expression. Treatment of MCF7 cells with increasing concentrations of TSA for 24 h induced p21^{WAF1} in a dose-dependent manner. On the contrary, DNMT1 were dramatically depleted corresponding to an increase in p21^{WAF1} protein, reaching an almost undetectable level at 300 nM TSA (Fig. 3A). Likewise, there was a significant reduction in p300 upon TSA treatment, while β-actin remained unaffected (Fig. 3A). The ability of p21^{WAF1} to inhibit transcription [13–15] prompted us to investigate whether the drastic reduction in DNMT1 occurs at the transcriptional level. As expected, *p21^{WAF1}* mRNA levels were induced in a dose-dependent manner in MCF7 cells following treatment with TSA for 24 h (Fig. 3B). In contrast, *DNMT1* mRNA levels were significantly depleted in a similar trend as its protein, while *GAPDH* remained unchanged (Fig. 3B). Furthermore, treatment with 200 nM TSA for various durations elicited a time-dependent induc-

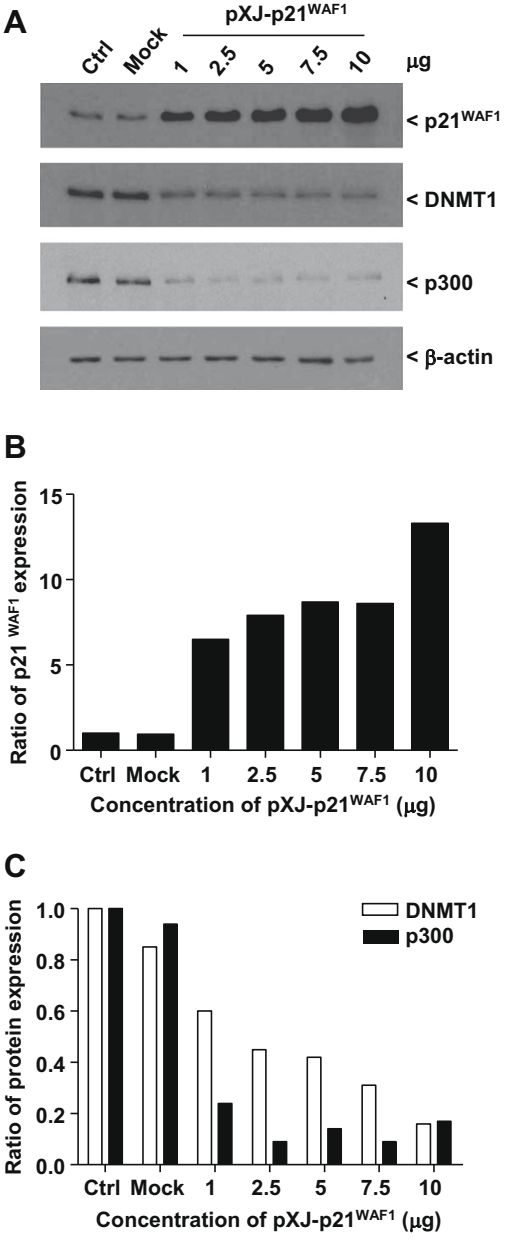

Fig. 2. Ectopic over-expression of p21^{WAF1} inhibits DNMT1. MCF7 cells were transfected with different concentrations of pXJ-p21^{WAF1} plasmids for 48 h. (A) Protein levels of p21^{WAF1}, DNMT1 and p300 were analyzed by Western blotting using specific antibodies. β-actin served as a loading control. (B) Relative ratio of p21^{WAF1} in (A) normalized against β-actin. (C) Relative ratio of DNMT1 and p300 in (A) normalized against β-actin. Similar data were obtained in two independent experiments.

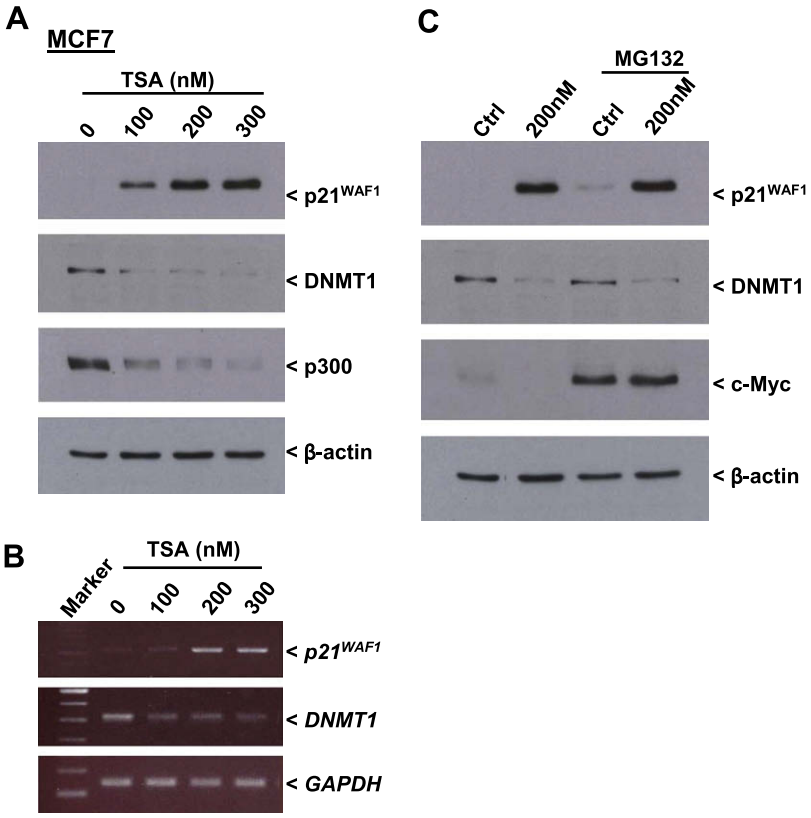

Fig. 3. TSA-mediated $p21^{WAF1}$ induction leads to down-regulation of DNMT1 at the transcriptional level. MCF7 cells were treated with different concentrations of TSA for 24 h and the protein and mRNA expression were analyzed. (A) Total lysates were extracted from the TSA-treated cells and Western blotting of $p21^{WAF1}$, DNMT1 and p300 were performed. β-actin was used as a loading control. (B) mRNA expression of *$p21^{WAF1}$*, *DNMT1* and *GAPDH* were determined by RT-PCR using specific primers. (C) Cells were pre-treated with 10 μM of proteasomal inhibitor MG132 for 6 h followed by treatment with 200 nM TSA for another 24 h. Ctrl; untreated cells. Protein levels of $p21^{WAF1}$, DNMT1 and c-Myc were determined by Western blot analysis. β-actin served as a loading control. Similar data were obtained in two independent experiments.

tion in $p21^{WAF1}$ and the corresponding down-regulation of DNMT1 (and p300) (Supplementary Fig. 1A. was also associated with a reduction in its mRNA levels (Supplementary Fig. 1B). This data further support the notion that $p21^{WAF1}$ is an inhibitor of DNMT1 expression.

To validate our observation, we examined whether blocking of the proteasomal degradation pathway can rescue DNMT1 depletion in MCF7 cells challenged with TSA. As shown in the Western blots, while pre-treatment with proteasomal inhibitor MG132 effectively blocks the physiological turn-over of c-Myc, which is a known target for proteasomal degradation pathway [18], it was unable to rescue the DNMT1 depletion mediated by TSA treatment (Fig. 3C). Taken together, our results demonstrate that $p21^{WAF1}$ inhibits DNMT1 at the transcriptional level.

Transcription co-activator p300 is necessary for DNMT1 expression

To determine if p300 plays a role in regulating DNMT1 expression and that the reduction in p300 protein following $p21^{WAF1}$ up-regulation contributes to the down-regulation of DNMT1, we went on to investigate the effects of siRNA-mediated p300 and DNMT1 knockdown in MCF7 cells. Both *p300* and *DNMT1* mRNA yields were effectively depleted in MCF7 cells transfected with siRNA targeting *p300* and *DNMT1*, respectively (Fig. 4A); and this was correlated with a reduction in the p300 and DNMT1 protein levels by ~80% and 100%, respectively, as compared to the controls (Fig. 4B and C). Strikingly, while the loss of DNMT1 did not affect p300 mRNA and protein yields, p300 knockdown led to a drastic reduction in DNMT1 mRNA and protein levels by >50%, which were comparable to the effects observed in the DNMT1 knockdown cells (Fig. 4A–C). Similar data was observed in human lung carcinoma H1299 cells (Supplementary Fig. 2A–C). These data suggest that the down-regulation of DNMT1 expression by p300 knockdown is not cell-type dependent. Furthermore, transactivation from the human *DNMT1* proximal promoter harboring the E2F1 and Sp1/3 binding sites (Supplementary Fig. 3. was significantly enhanced in the presence of p300 (Fig. 4D; Supplementary Fig. 2D). Overall, these data strongly suggest that p300 is necessary for DNMT1 expression, and that the reduction in p300 upon $p21^{WAF1}$ up-regulation contributes in part to the down-regulation of DNMT1.

Discussion

In this study, we established $p21^{WAF1}$ as a negative regulator of DNMT1 expression in human cancer cells and unveiled a novel molecular basis for the inverse expression levels frequently observed between the two proteins in mammalian cells. Our findings challenged previous studies indicating that DNMT1 represses *$p21^{WAF1}$* transcription. Hypermethylation of *$p21^{WAF1}$* promoter is rare, and instead, several lines of evidence originally supported the association of DNMT1 with transcriptional repression of *$p21^{WAF1}$* mRNA expression via DNA methylation-independent mechanism [10,11,19]. Furthermore, siRNA-mediated disruption of DNMT1 expression in A549 lung carcinoma cells was shown to be accompanied by an increase in $p21^{WAF1}$ proteins [20]. However, contrary to these findings, we demonstrated that DNMT1 overexpression or knockdown did not alter $p21^{WAF1}$ expression in human breast cancer cells. Two consecutive studies showed that DNMT1 knockdown in A549 cells triggers a cascade of genotoxic

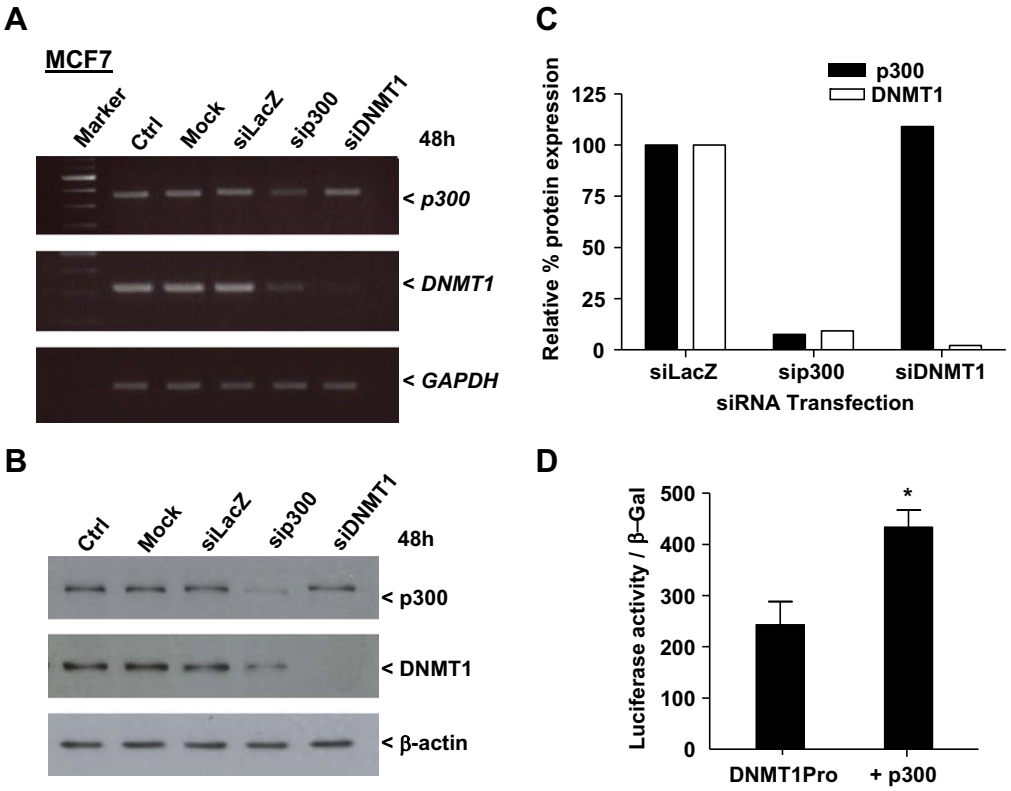

Fig. 4. Transcription co-activator p300 is necessary for DNMT1 expression. (A) MCF7 cells transfected with 100 nM siLacZ, sip300 or siDNMT1 for 48 h. Empty transfection (Mock) served as an internal control. Ctrl, untransfected cells. mRNA expression of *p300*, *DNMT1* and *GAPDH* were determined by RT-PCR using specific primers. (B) Protein levels of p300 and DNMT1 were analyzed by Western blot analysis. β-actin served as a loading control. (C) Relative percentages of DNMT1 and $p21^{WAF1}$ in (B) normalized against β-actin. (D) MCF7 cells were transfected with pGL3-DNMT1Pro construct (DNMT1-Pro) alone or with p300 plasmids (+p300). Plasmids expressing β-galactosidase (pCMV-βGal) were transfected into the cells as a measure of the transfection efficiency. The luciferase activity was measured using a luminometer and normalized against the β-galactosidase activity. Data obtained from duplicate sets of three independent experiments were plotted and presented as mean ± SEM. The comparison was performed with an unpaired Student's *t* test ($^{*}P < 0.05$).

stress checkpoint proteins via an ATR-dependent manner which induces an intra-S phase arrest to inhibit DNA replication [21,22]. Since the ATR pathway plays an important role in p53 activation and consequently $p21^{WAF1}$ induction, as demonstrated by the response of A549 cells to nucleoside analogue-induced DNA damage [23], the concomitant increase in $p21^{WAF1}$ observed by the previous groups might be an indirect consequence of the intra-S phase arrest, rather than the intrinsic effect of DNMT1 knockdown. Supporting our argument, we showed that siRNA-mediated DNMT1 knockdown efficiently depletes DNMT1 expression without significantly affecting the cell cycle profiles (data not shown). Collectively, our data provides no evidence that DNMT1 represses $p21^{WAF1}$ expression in human breast cancer cells.

Given that $p21^{WAF1}$ is capable of regulating transcription [13–15], we raised the possibility that $p21^{WAF1}$ may also be linked to DNMT1 in a regulatory pathway. Here, we showed that ectopic over-expression of $p21^{WAF1}$ or TSA-mediated $p21^{WAF1}$ induction in human breast carcinoma cells considerably diminishes DNMT1 at the protein and mRNA levels. In line with this, it was recently reported that treatment with histone deacetylase inhibitor apicidin selectively represses DNMT1 expression in human cervical cancer cells [24]. Although the significance of this relationship is not fully understood, our data provides a platform demonstrating $p21^{WAF1}$ as an upstream regulator of DNMT1 expression potentially by negatively regulating *DNMT1* transcription. Our observation is not unusual, since $p21^{WAF1}$ has also been documented to repress several genes involved in cell cycle progression such as *c-Myc* and *Cdc25A* [13].

Strikingly, in our attempt to explore the underlying mechanism involved, transcriptional co-activator p300 surfaced as a potential candidate for further studies. Despite the fact that p300 is critical for eukaryotic gene expression [25] the functional importance of p300 on DNMT1 expression in human cancer cells remained obscure. Here, we showed that p300 knockdown leads to drastic reduction in *DNMT1* mRNA levels, demonstrating for the first time that p300 plays a crucial role in regulating human DNMT1 expression. The dependency of *DNMT1* transcription on p300 was further illuminated by the finding that p300 greatly enhanced transactivation of *DNMT1* promoter potentially by associating with the Sp1/3 and E2F1 binding sites located on the proximal promoter region. Based on these observations, we proposed that the reduction in p300 proteins following $p21^{WAF1}$ up-regulation contributes in part to the down-regulation of DNMT1 expression. However, thorough investigations will be needed to elucidate the exact mechanism on how $p21^{WAF1}$ modulates p300 protein expression in this context.

In conclusion, we presented a novel mechanism linking DNMT1 and $p21^{WAF1}$ in a regulatory pathway in human cancer cells. Furthermore, we identified p300 as a crucial regulator of DNMT1 expression. Our work further demonstrated p300 as a potential target for $p21^{WAF1}$ under physiological conditions, which may be necessary to ensure properly regulated DNMT1 expression and DNA methylation especially during cell cycle progression. The details of this novel $p21^{WAF1}$-p300-DNMT1 pathway may provide the molecular basis for understanding the coordination of DNMT1 expression with other critical biological processes such as those that occur during the DNA damage response.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.03.001.

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