Biochemical and Biophysical Research Communications 391 (2010) 449–454

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

TSA-induced *DNMT1* down-regulation represses *hTERT* expression via recruiting CTCF into demethylated core promoter region of *hTERT* in HCT116

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ARTICLE INFO

Article history:
Received 9 November 2009
Available online 13 November 2009

Keywords:
DNA methylation
DNA methyltransferase 1
Epigenetic regulation
Human telomerase reverse transcriptase
Trichostatin A

ABSTRACT

Trichostatin A (TSA), an inhibitor of histone deacetylase, is a well-known antitumor agent that effectively and selectively induces tumor growth arrest and apoptosis. Recently, it was reported that *hTERT* is one of the primary targets for TSA-induced apoptosis in cancer cells but the mechanism of which has not yet been elucidated. In the present study, to better understand the epigenetic regulation mechanism responsible for the repression of *hTERT* by TSA, we examined expression of *hTERT* in the HCT116 colon cancer cell line after treatment with TSA and performed site-specific CpG methylation analysis of the *hTERT* promoter. We found that TSA-induced the demethylation of site-specific CpGs on the promoter of *hTERT*, which was caused by down-regulation of DNA methyltransferase 1 (*DNMT1*). Among the demethylated region, the 31st–33rd CpGs contained a binding site for CTCF, an inhibitor of *hTERT* transcription. ChIP analysis revealed that TSA-induced demethylation of the 31st–33rd CpGs promoted CTCF binding on *hTERT* promoter, leading to repression of *hTERT*. Taken together, down-regulation of *DNMT1* by TSA caused demethylation of a CTCF binding site on the *hTERT* promoter, the result of which was repression of *hTERT* via recruitment of CTCF to the promoter.

Introduction

Modulation of chromatin structure by histone acetylation/deacetylation is well known to be one of the major mechanisms involved in the regulation of gene expression [1]. Two opposing enzyme activities determine the acetylation state of histones: histone acetyltransferase (HATs) and histone deacetylase (HDACs), which acetylate and deacetylate the epsilon-amino groups of lysine residues located in the amino-terminal tails of the histones, respectively [2,3]. A number of structurally divergent classes of HDAC inhibitors have been identified [4]. They have been shown to induce cell-cycle arrest, terminal differentiation, and apoptosis in various cancer cell lines as well as inhibit tumor growth [5]. In particular, the reversible HDAC inhibitor trichostatin A (TSA) can effectively and selectively induce tumor growth arrest at very low concentrations [6,7]. Therefore, an understanding of the events in TSA-induced apoptosis may be valuable for improving the efficacy of cancer therapy.

Recently, it was reported that the human telomerase reverse transcriptase (*hTERT*) gene that encodes the catalytic subunit of telomerase holoenzyme may be a primary target of TSA for induction of apoptosis in various cancer cells [8–10]. *hTERT* is activated in a cancer cell-specific manner and is well-known as an important target for the diagnosis of malignancy and a potential candidate for the development of cancer therapy [11]. Numerous researchers have been interested in studying the role of *hTERT* in TSA-induced apoptosis [10,12,13]; however, these reports have only focused on the down-stream mechanisms of *hTERT* expression in TSA-induced apoptosis rather than how the expression of *hTERT* can be regulated by TSA. In addition, it has been reported that TSA acts differently in cancer cells compared with normal cells. Specifically, TSA induces transcriptional activation of *hTERT* expression in normal cells, but significantly represses the expression of *hTERT* in cancer cells [8,9]. In particular, inhibition of HDACs by TSA generally increases histone acetylation on the promoter of their target genes, which consequently results in gene activation. Nevertheless, histone acetylation induced by TSA in cancer cells does not induce increased *hTERT* expression, suggesting that there may be another epigenetic regulation mechanism controlling *hTERT* expression by TSA in a cancer cell-specific manner.

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0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.11.078

Recently, it has been reported that treatment with TSA is associated with a significant decrease in global DNA methylation [14], suggesting that DNA methylation of *hTERT* may be a new target of epigenetic regulation by TSA. The majority of DNA methylation results in the modification of cytosine at CpG sites, and this phenomenon is associated with repression of gene expression [15]. However, in the case of *hTERT*, increased DNA methylation of the *hTERT* promoter has been observed in *hTERT*-positive cancer cells, while lack of methylation has been found in normal *hTERT* negative cells [16]. This correlation is opposite of the general model of regulation by DNA methylation, in which the presence of methylated cytosine in a promoter typically inhibits gene transcription. Although DNA methylation of the *hTERT* promoter is an important factor for *hTERT* expression and *hTERT* is a strong candidate as a target gene of TSA, the correlation between TSA and DNA methylation of *hTERT* promoter has not yet been elucidated.

In the present study, we evaluated the changes in *hTERT* expression in the apoptosis process induced by TSA and explored DNA methylation of the *hTERT* promoter as one of the possible mechanisms of regulation of *hTERT* expression. Our results showed that TSA-induced demethylation of CpGs on the *hTERT* promoter via down-regulation of DNA methyltransferase 1 (*DNMT1*) after which the demethylation of CpGs promoted CTCF binding, an inhibitor of *hTERT* transcription, on *hTERT* promoter for the inhibition of *hTERT* expression. These findings suggest the possibility that demethylation of CpGs by TSA can epigenetically regulate the expression of tumor related genes and may provide important clues on how to approach cancer-specific therapies using TSA.

Materials and methods

Cell culture and drug treatments. The HCT116 human colon cancer cell line was obtained from the Korean Cell Line Bank (KCLB No. 10237) and cultured at 37 °C under a 5% CO_2 atmosphere in RPMI-1640 medium (WelGENE, Deagu, Korea) supplemented with 10% fetal bovine serum (WelGENE). Twenty-four hours after seeding, TSA was added to the culture medium to a final concentration of 1 μM. Afterwards cells were incubated with TSA for various time points as indicated in the figure legends.

Quantification of hTERT mRNA. Total RNA was extracted from cultured cells using the High Pure RNA isolation kit (Roche, Penzberg, Germany) according to the manufacturer's suggested protocol. Total RNA (2 μg) was reverse-transcribed with oligo (dT) primer according to the manufacturer's instructions using the Transcriptor first strand cDNA synthesis kit (Roche). The resulting product was then used for real-time PCR for quantification of *hTERT* mRNA. Real-time PCR was performed using LightCycler faststart DNA master SYBR Green I kit (Roche) with a LightCycler 2.0 (Roche). Reactions were performed in triplicate using 4 μl of cDNA per reaction and primers specific for *hTERT*, *DNMT1* and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). Primers for *hTERT* were used as mentioned by Zinn et al. [17]. The relative ratio of *hTERT* to *GAPDH* for each sample was then calculated.

Bisulfite modification and PCR-directed sequencing. To analyze the methylation status of the *hTERT* promoter, genomic DNA was extracted from the cultured cell lines using a Genomic DNA isolation kit (Promega, Madison, WI, USA) and then modified using a Genomic DNA mCpG modification kit (Chemicon, Temecula, CA, USA). Next, 2 μg of genomic DNA was used for bisulfite modification. Modified DNAs were then amplified by PCR using a 5′ AGTGGATT CGCGGGTATAGAC 3′ and 5′ GCCGCACGAACGTAACCAAC 3′ primer set. Amplification for PCR was performed using Go Taq (Promega) and the following PCR conditions: 40 cycles of 95 °C for 30 s, 55 °C for 45 s and 72 °C for 60 s. Following PCR amplification, purified PCR products were then cloned using a T&A cloning kit according to the manufacturer's instructions (Real Biotech Corp., Taipei, Taiwan). Plasmid DNA was isolated using a plasmid miniprep kit according to the manufacturer's instructions (Real Biotech Corp.). Plasmids DNA were sequenced using the M13 universal reverse primer (Macrogen, Seoul, Korea).

Flow cytometry assay. For cell cycle analysis, cells were first harvested by trypsinization, then washed with cold DPBS, and fixed with 70% ethanol/DPBS at 4 °C for 1 h before harvesting. Cells were then permeabilized and labeled with 124 μg/ml propidium iodide (PI) using CycleTEST PLUS DNA reagents (BD Biosciences, USA). DNA contents were analyzed with a FACScan flow cytometer (BD Biosciences) using CellFit software (BD Biosciences).

Western blotting. For Western blotting, cells were suspended in Pro-prep solution (iNtRON Biotechnology, Seoul, Korea). Protein samples were run through sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Pall Corporation). Membranes were blocked with 5% BSA in tris-buffered saline (TBS; 25 mM Tris–HCl ph 8.0, 125 mM NaCl). The primary antibodies against the target proteins were Anti-p53 mouse polyclonal IgG (Santa Cruz, CA, USA), Anti-p21 rabbit polyclonal IgG (Santa Cruz), Anti-DNMT1 rabbit IgG (Bethyl, TX, USA), Anti-PARP goat polyclonal IgG (Santa Cruz) and Anti-β-actin mouse monoclonal IgG (Sigma, USA); all antibodies were diluted in TBS (1:500∼1:1000) and applied to membranes overnight at 4 °C. Secondary antibodies were diluted in TBS-T and applied to the membrane for 1 h at room temperature. At each step, membranes were washed 4 times with TBS-T (0.1% Tween 20 in TBS buffer) for 10 min. Signals were visualized using an Enhanced Chemiluminescence Western blotting detection kit (Santa Cruz).

Chromatin immunoprecipitation. For ChIP analysis, 1×10^6 cells were harvested by centrifugation for 3 min at 2000*g* and washed in 1× phosphate buffered saline (PBS) with a protease inhibitor cocktail (Roche). ChIP analysis was performed using a ChIP assay kit (Millipore, MA, USA) according to the manufacturer's instruction. Each antibody (5 μg) for SP1 (Millipore), c-MYC (Santa cruz) and CTCF (Bethyl) were added to the chromatin solution and incubated overnight at 4 °C with rotation. An experimental control without antibody was also performed to demonstrate specific binding to the antibody. Primers for *hTERT* were used as mentioned by Renaud et al. [18].

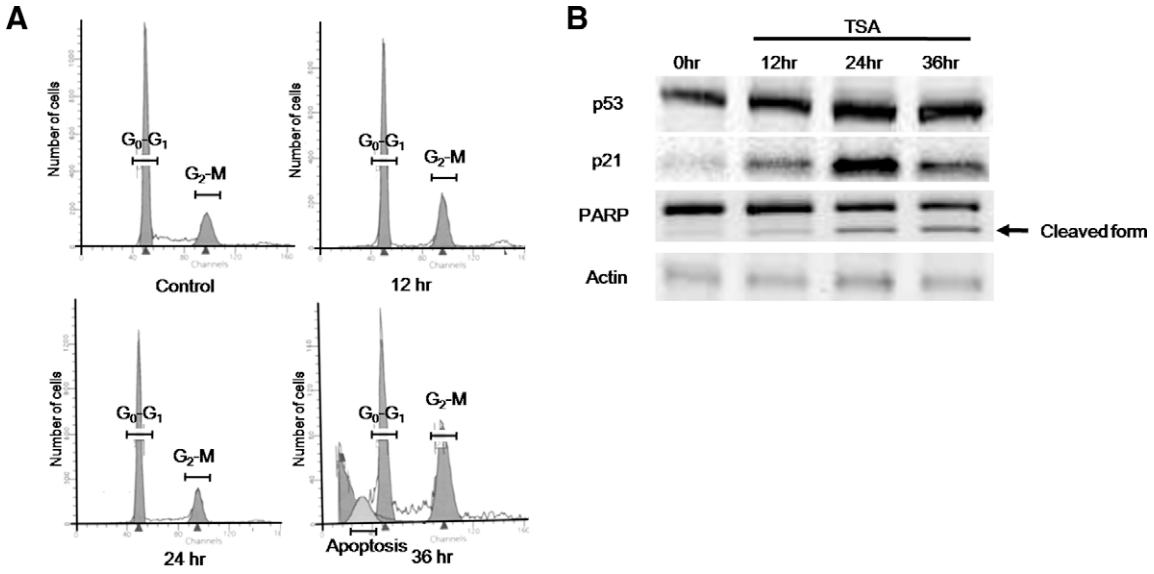
Results and discussion

Induction of cell-cycle arrest and apoptosis in colon cancer cell line by TSA

To analyze the induction of cell-cycle arrest and apoptosis by TSA in HCT116, we performed flow cytometric analysis using PI staining and Western blot analysis with p53, p21 and PARP antibodies. Flow cytometric analysis showed that 1 μM TSA-induced arrest of cell cycle at the G_2/M phase and resulted in the appearance of sub-G_1 populations after 48 h (Fig. 1A). In addition, the expression of p53 and p21 in cells increased in response to TSA treatment and the cleaved form of PARP was observed after a 24 h treatment with TSA (Fig. 1B). Consistent with other studies, our results indicate that TSA induces cell-cycle arrest and apoptosis in HCT116 cells [10].

Repression of hTERT expression by TSA via demethylation of site-specific CpGs on the hTERT promoter

To explore the expression pattern of *hTERT* by TSA treatment in HCT116 cells, we performed real-time PCR amplification of *hTERT* mRNA. Real-time PCR results showed that *hTERT* mRNA expression



	0 hr	12 hr	24 hr	36 hr
G_0-G_1 (%)	64.82	64.78	66.21	40.06
G_2-M (%)	13.67	19.51	30.56	40.26
Apoptosis (%)	0.41	3.72	4.41	17.78

Fig. 1. Induction of the apoptosis by TSA in HCT116 cells. (A) The distribution of cell cycle was assessed by flow cytometry after staining with propidium iodide (PI). Cells in the G_0–G_1, G_2–M and apoptosis phase of the cell cycle were plotted on one parameter histograms. Quantification of each phase is shown in respective boxes. (B) Cell lysates were prepared and subjected to SDS–PAGE and Western blot to analyze levels of p21, p53, PARP protein and actin.

in HCT116 cells was significantly decreased in a time dependent manner upon TSA stimulation (Fig 2A).

Since it has been reported that DNA methylation in the promoter region of *hTERT* affects its expression, we next investigated whether TSA could influence the methylation status of the *hTERT* promoter (region from −202 to +89). A total of 40 CpG sites were analyzed for site-specific methylation status (Fig. 2B). In mock treated HCT116 cells, the overall pattern of CpG methylation showed that the promoter region (1st–10th CpGs) as well as the translation start site (28th–34th CpGs) of *hTERT* was in a hypermethylated state while the CpGs at the transcription start site (13th–27th CpGs) tended to be hypomethylated (Fig. 2B).

In particular, the CpGs in their regions were containing binding sites of several transcription factors like as SP1, c-MYC and CTCF. Two SP1 binding sites (11th, 12th and 19th CpGs) in the transcription start site of *hTERT* were completely unmethylated, whereas a CTCF binding site (31st–33rd CpGs) in the translation start site was fully methylated in mock treated cells. Conversely, the methylated CpGs were dramatically demethylated in a time dependent manner by TSA treatment. That is, an irregular pattern of methylation at 6 h after TSA treatment was observed, and methylated CpGs were almost completely demethylated between 12 and 24 h, although the degree of methylation recovered slightly at 48 h. In particular, the methylation levels of the 31st–33rd CpGs of the CTCF binding site was decreased by up to 10% 24 h after TSA treatment. Further, the three CpGs in the SP1 binding site (11th, 12th and 19th) were not significantly changed and remained unmethylated between 12 and 48 h of TSA treatment despite a slightly increased methylation level (30–60%) with 6 h of TSA treatment.

CTCF is known to inhibit *hTERT* expression [18]. CTCF can associate with the first exon of *hTERT* when the binding site is unmethylated, whereas methylation of this site interferes with CTCF binding leading to abolished inhibitor activity [18,19]. Therefore, demethylation of CpGs on the CTCF binding site by TSA in our results may facilitate the binding of CTCF on the *hTERT* promoter to allow for CTCF-mediated repression of *hTERT* transcription. It may be able to explain the down-regulation of *hTERT* by TSA.

In the case of SP1, there is some discrepancy regarding whether SP1 acts as an activator or repressor of *hTERT* expression. As a transcriptional activator, SP1 has been shown to interact with the p300 coactivator possessing intrinsic HAT activity, leading to the activation of transcription [20]. As a transcriptional repressor, it was reported that SP1 recruits HDAC to the promoter, leading to the repression of transcription [21,22]. That is, the role of SP1 for regulation of *hTERT* was not certain yet. In addition, it has not been clearly determined whether DNA methylation affects the accessibility of SP1 binding to the *hTERT* promoter.

Alteration of SP1 and CTCF binding to the hTERT promoter by TSA treatment

To explore whether demethylation of CpGs could affect the ability CTCF and SP1 to bind to the *hTERT* promoter, we performed ChIP using CTCF and SP1 antibodies after TSA treatment. As shown in Fig. 2C, SP1 binding to the *hTERT* promoter was not changed 24 h after TSA treatment; however, CTCF binding was enhanced (Fig. 2C). These results coincided with the alteration of the CpG methylation status on the SP1 and CTCF binding site (Fig. 2B), suggesting that the demethylation of CTCF binding sites by TSA allowed CTCF binding to the *hTERT* promoter, which in turn led to repression of *hTERT* expression. We also observed that CpGs (11th, 12th and 19th) of SP1 binding sites were methylated in normal cell lines in which SP1 was not bound with the sites, indicating that SP1 binding may also be regulated by methylation status (data not shown). Thus, it seems that SP1 binding to unmethylated CpGs on the promoter of *hTERT* acts as an activator for the regulation of *hTERT* expression in cancer cells. Taken together, we believe that the binding alteration of the transcription factor CTCF, but not SP1, plays a very crucial role in the regulation of *hTERT* upon treatment with TSA.

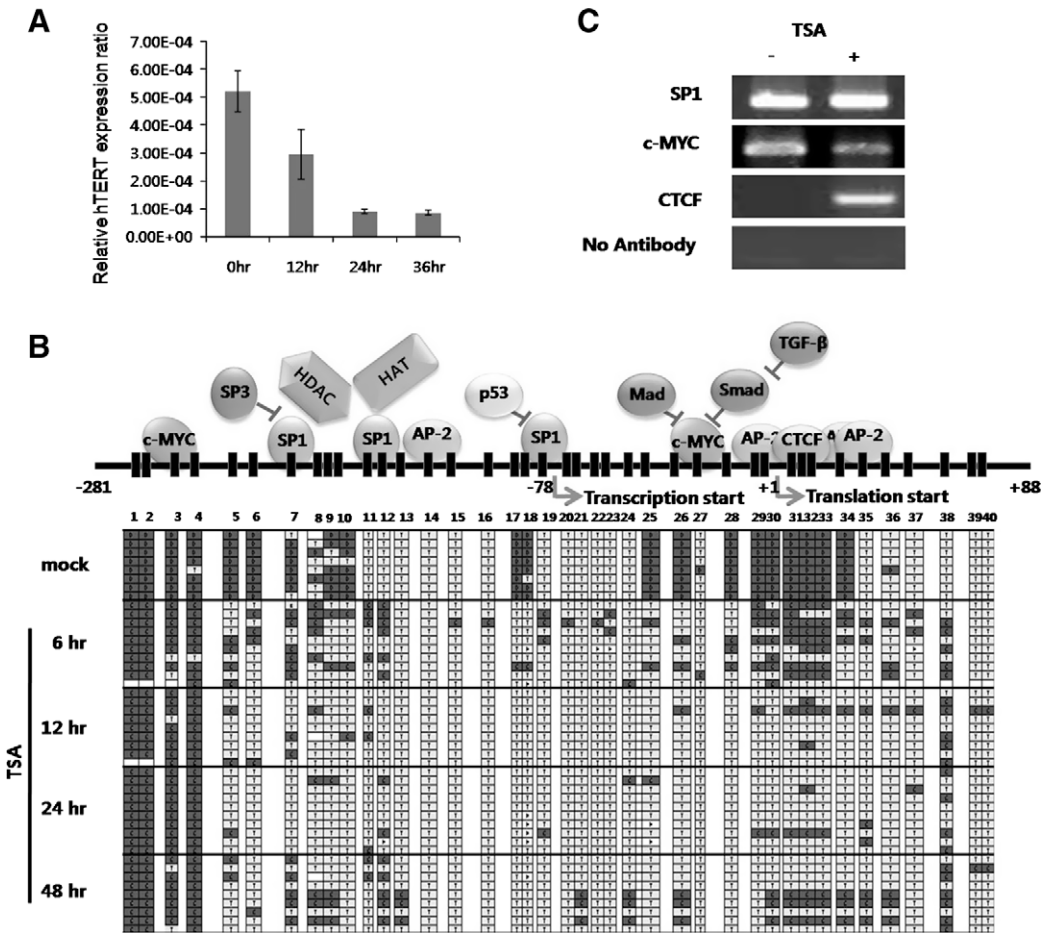


Fig. 2. Expression, DNA methylation and transcription factor binding to the *hTERT* promoter upon TSA treatment in HCT116 cells. (A) For quantitative analysis of *hTERT* expression in HCT116 cells after TSA treatment, samples were prepared at the indicated times and subjected to real-time RT-PCR. Data shown are the mean ± standard deviation for three independent experiments. (B) A total of 40 CpG sites on the promoter and 5′ proximal exon regions of *hTERT* in HCT 116 cells were analyzed by bisulfate modification sequencing. Each strait on the scale represents a CpG site in the analyzed region. Methylation at each CpG site is indicated as follows: Black box, methylated CpG; white box, unmethylated CpG. The schematic diagram on the scale represents a putative activator and repressor for *hTERT* expression. (C) Chromatin DNA from HCT116 cells was immunoprecipitated with antibodies against SP1, CTCF or c-MYC, or without antibody as a negative control. The resulting samples were amplified by PCR with the primer sets described in Materials and methods.

We also observed that several CpGs (17th, 18th, 25th, 26th and 28th) were strongly methylated in mock treated cells and were significantly demethylated by TSA treatment (Fig. 2B). Although the binding proteins for these CpGs have not yet been identified, it suggests the possibility that the methylation of these sites may affect the binding affinity of SP1 and c-MYC, because the 17th and 18th CpGs were located near the SP1 binding site and the 25th, 26th and 28th CpGs were located near the E box for c-MYC binding. Indeed, it was reported that methylation of adjacent CpG sites induces a significant decrease in SP1 binding and activity in the p21 promoter [23]. However, in our results, ChIP analysis using SP1 and c-MYC antibodies showed that binding of SP1 and c-MYC to the *hTERT* promoter was not altered after TSA treatment (Fig. 2C). The lack of a change in binding, despite a significant decrease in methylation status by TSA treatment, suggested that demethylation of the CpGs by TSA was not related to the binding affinity of SP1and c-MYC, and further, that the binding of SP1 and c-MYC was not critical for the repression of *hTERT*. Nevertheless, we cannot exclude the possibility that the alteration of the methylation status of these specific CpGs by TSA controls the binding of other unknown regulatory proteins to CpGs.

Decreasing hTERT methylation by TSA-mediated DNMT1 down-regulation

The foregoing results indicated that CpG methylation of the *hTERT* promoter is a critical event for TSA mediated down-regulation of *hTERT* in cancer cells. Although it has been reported that TSA induces DNA demethylation via a reversible crosstalk with histone acetylation, it is unclear which mechanisms are involved [14].

To date several DNA methyltransferase (DNMTs) that catalyze the methylation of genomic DNA have been identified, including DNMT3A, DNMT3B and DNMT1 [24]. Of these, DNMT3A and DNMT3B exhibit *de novo* activity, in that they are involved in the biosynthesis of methyl cytosine in CpG, while DNMT1 preferentially attaches a methyl group on hemimethylated DNA during replication [25]. In addition, it has been reported that DNMT1 induces hypermethylation of tumor suppressor genes to epigenetically mediate their repression in the process of tumorigenesis in many cancers including colorectal cancer cell [26]. Together, these results suggest that DNMT1 may be involved not only in the methylation mechanism of the *hTERT* promoter in the process of carcinogenesis, but also TSA mediated demethylation of the *hTERT*

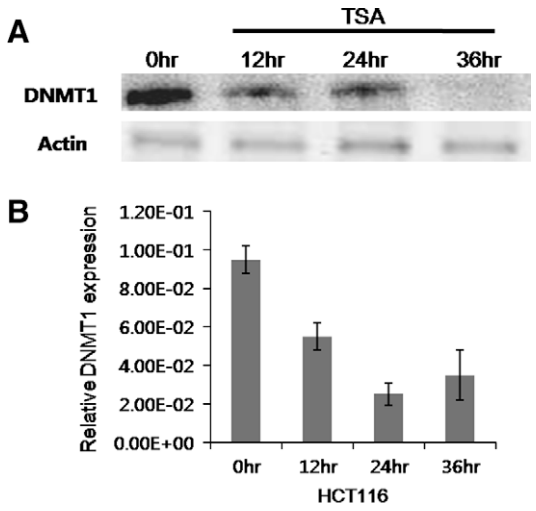


Fig. 3. Effect of TSA on DNMT1 expression in HCT116 cells. (A) Cell lysates were prepared at various time points as indicated and performed Western blotting to analyze *DNMT1* expression. Actin was used as an equal loading control. (B) Relative expression levels of *DNMT1* mRNA at various time points as indicated as determined by real-time PCR. The amount of *DNMT1* mRNA was normalized to an endogenous reference, *GAPDH*.

promoter. Western blot analysis with an anti-DNMT1 antibody showed that DNMT1 gradually disappeared after TSA treatment in HCT116 cells (Fig. 3A). To verify that the disappearance of DNMT1 by TSA was the result of transcriptional regulation of gene expression, we carried out real-time PCR to measure levels of the *DNMT1* transcript. The results showed that mRNA levels of *DNMT1* in HCT116 cells were also significantly decreased by TSA treatment (Fig. 3B), indicating that TSA down regulates *DNMT1* at the mRNA level, which may provide a molecular mechanism to explain how DNA demethylation occurs on the *hTERT* promoter following treatment with TSA. Recently, down-regulation of *DNMT1* by TSA was also reported in leukemia cells [27]. In addition, down-regulation of *DNMT1* and *hTERT* was observed in experiments using a siRNA against the *DNMT1* gene [27,28], indicating that *hTERT* expression can be controlled by *DNMT1*. These findings support a model by which down-regulation of *DNMT1* in HCT116 cells by TSA results in the promotion of demethylation of CpGs on the promoter of *hTERT*, which in turn may be a one of the major mechanisms by which TSA mediates repression of *hTERT* in a cancer cell-specific manner (Fig. 4). However, TSA-mediated repression of *hTERT* via down-regulation of *DNMT1* was not observed in all cancer cell lines. In MCF7, a breast cancer cell line, the expression of *DNMT1* was decreased by TSA treatment as well as in HCT116 (Data not shown). The decreasing of *DNMT1* induced demethylation of the fully methylated CpGs on the CTCF binding site and subsequently recruited CTCF into the promoter, resulting in significantly decreased expression of *hTERT* in these cell lines. On the contrary, HeLa, a cervical cancer cell line, and SK-OV3, an ovarian cancer cell line, were found to have partially demethylated or unmethylated CpGs, respectively, which correspond to CTCF binding site and showed no down-regulation of *DNMT1* after TSA treatment. Accordingly, there was no remarkable change by TSA in the down-stream responses such as methylation status of the *hTERT* promoter and *hTERT* expression. The expression of endogenous *hTERT* in HeLa and SK-OV3 was lower than that in HCT116 and MCF7 before TSA treatment. It seems that initial methylation status of CpGs on the CTCF binding sites in cancer cell lines examined predisposes to the differential cellular response after TSA treatment. Taken together, it is obvious that the binding of CTCF by demethylation of the 31st–33rd CpGs is closely linked to the control of *hTERT* expression by TSA in various cancer cells, although the serial phenomena such as the down-regulation of *DNMT1*, binding of CTCF to the demethylated CpGs on the *hTERT* promoter and inhibition of *hTERT* expression were not necessarily occurred in all cancer cells.

Conclusions

TSA was well known drug that it generally involved in up-regulation of gene expression through induction of histone acetylation on the promoter of target gene. However, in HCT116, TSA-induced the down-regulation of *hTERT* expression via demethylation of the site-specific CpGs on the *hTERT* promoter rather than induction of histone acetylation. Especially, the demethylation of the 31st–33rd CpGs by TSA resulted in the binding of CTCF on the *hTERT* promoter. The down-regulation of *DNMT1* for the demethylation of *hTERT* promoter was a primary event among the serial responses induced by TSA in HCT116. It is obvious that repression of *hTERT*

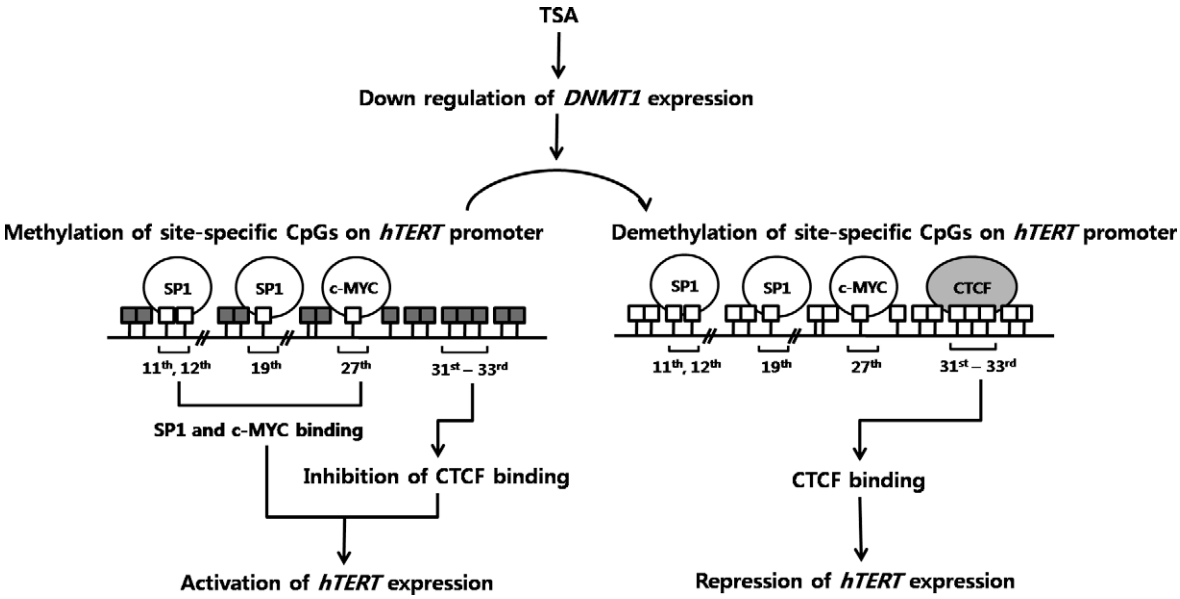


Fig. 4. Proposed model for TSA-induced repression of *hTERT* expression. Empty squares represent unmethylated CpG sites, and solid squares represent methylated CpG sites.

by TSA was caused by recruiting CTCF into the *hTERT* promoter demethylated by down-regulation of *DNMT1* in HCT116.

Acknowledgments

This research was supported by the Chung-Ang University Research Scholarship Grants in 2009.

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