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ORIGINAL ARTICLE

Histone deacetylase inhibitor apicidin downregulates DNA methyltransferase 1 expression and induces repressive histone modifications via recruitment of corepressor complex to promoter region in human cervix cancer cells

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Dysregulation of DNA methyltransferase (DNMT)1 expression is associated with cellular transformation, and inhibition of DNMT1 exerts antitumorigenic effects. Here, we report that DNMT1 abnormally expressed in HeLa cells is downregulated by a histone deacetylase (HDAC) inhibitor apicidin, which is correlated with induction of repressive histone modifications on the promoter site. Apicidin selectively represses the expression of DNMT1 among DNMTs in HeLa cells, independent of cell cycle arrest at G_0/G_1. Furthermore, apicidin causes a significant reduction in the recruitment of RNA polymerase II into the promoter. Chromatin immunoprecipitation analysis shows that even though apicidin causes global hyperacetylation of histone H3 and H4, localized deacetylation of histone H3 and H4 occurs at the E2F binding site, which is accompanied by the recruitment of pRB and the replacement of P/CAF with HDAC1 into the sites. In addition, K4-trimethylated H3 on nucleosomes associated with the transcriptional start site is depleted following apicidin treatment, whereas repressive markers, K9- and K27-trimethylation of H3 are enriched on the site. The downregulation of DNMT1 expression seems to require *de novo* protein synthesis, because the apicidin effect is antagonized by cycloheximide treatment. Moreover, knock down of DNMT1 with siRNA induces the apoptosis of HeLa cells, indicating that downregulation of DNMT1 might be a good strategy for therapeutics of human cervix cancer. Collectively, our findings will provide a mechanistic rationale for the use of HDAC inhibitors in cancer therapeutics.
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Introduction

It is well known that histone deacetylase (HDAC) inhibitors alter expression of many tumor-associated proteins and exhibit clinical activity against a variety of human malignancies; this antitumor activity may be characterized as either inducing cytostasis (cell cycle arrest), differentiation or apoptosis. It has also been generally accepted that more actively transcribed chromatin regions are associated with histone hyperacetylation and recruitment of histone acetyltransferases (HATs), and histone deacetylation associated with recruitment of HDACs often restores these genomically active regions to a more repressed and condensed chromatin state (Beaulieu *et al*., 2002). Thus, an attractive paradigm for the antitumor action of HDAC inhibitors has been the induction of histone acetylation producing transcriptional activation of critical genes needed for tumor growth arrest (Cress and Seto, 2000). Aside from the upregulation of epigenetically silenced tumor suppressor proteins or induction of caspases and other proapoptotic proteins by HDAC inhibitors (Juan *et al*., 2000; Kwon *et al*., 2002; Fuino *et al*., 2003; Mitsiades *et al*., 2004), emerging data are showing HDAC inhibitor-induced repression of critical transforming growth factor mechanisms, such as those involving oncogenic tyrosine kinases like bcr/abl, ErbB2 and DNA methyltransferase (DNMT) 3B (Fuino *et al*., 2003; Nimmanapalli *et al*., 2003). Indeed, several studies using DNA microarray analysis have now revealed that fewer than 10% of expressed genes in a given malignant cell population are affected by an antitumor dose of an HDAC inhibitor, with a near equal number of transcriptionally active genes being repressed as those

being stimulated (Drummond *et al*., 2005). However, the HDAC-dependent mechanisms accounting for the observed and rather selective modulation of gene expression, as well as specific patterns of antitumor activity, remain poorly understood.

Epigenetic modifications such as DNA methylation, histone acetylation and histone methylation regulate chromatin structure and function. Generally, DNA methylation on cytosine in CpG dinucleotides in the regulatory regions of genes is associated with gene silencing either by directly inhibiting the interaction of transcription factors with their regulatory sequences (Watt and Molloy, 1988) or by attracting methylated DNA-binding proteins such as MeCP2, which in turn recruits HDACs and histone methyltransferases, resulting in an inactive chromatin structure (Boyes and Bird, 1991; Nan *et al*., 1998). DNA methylation in mammalian cells is regulated by a family of highly related DNMT, DNMT1, DNMT3A and DNMT3B, which catalyse the transfer of methyl groups from S-adenosyl-L-methionine to the 5′ position of cytosine bases in the dinucleotide sequence CpG (Bestor *et al*., 1988; Okano *et al*., 1998). *In vitro*, DNMT1 prefers hemimethylated DNA over non-methylated DNA as substrate and is therefore responsible for propagation and maintenance of established methylation patterns during embryonal development and cell division. DNMT1 expression is properly cell cycle-regulated in normal cells to establish their function (Leonhardt *et al*., 1992). DNMT1 is recruited to replication foci through protein interactions involving the DNMT1-associated protein-binding region (Rountree *et al*., 2000), the proliferating cell nuclear antigen-binding region (Chuang *et al*., 1997) and the replication foci targeting sequence. On the other hand, *de novo* methylation of DNA is believed to be performed by DNMT3A and DNMT3B (Okano *et al*., 1998). However, these classifications are oversimplified as DNMT1 is also known to possess *de novo* methylation activity (Hermann *et al*., 2004) and is the most abundant methyltransferase in somatic cells (Robertson *et al*., 1999).

Although genetic alterations such as deletion, amplification and translocation have been well demonstrated to play a critical role in cancer initiation and progression, epigenetic modifications of DNA are also associated with these processes through the regulation of gene transcription (Eden and Cedar, 1994; Pradhan *et al*., 1999). Aberrant CpG methylation has been observed in several tumors (Baylin and Herman, 2000). Some CpG islands are hypermethylated in tumor cells (Baylin *et al*., 1998; Yan *et al*., 2003), suggesting that a fundamental disruption of methylation control has occurred. This aberrant hypermethylation can act in a manner analogous to deletion or mutation of growth inhibitory genes, thereby producing a selective growth advantage (Wu *et al*., 1993; Jones and Baylin, 2002; Gupta *et al*., 2003). DNMT1 has been shown to act as an oncogene in cultured fibroblasts (Wu *et al*., 1993). Furthermore, NIH 3T3 cells transformed by DNMT1 show enhanced DNA methylation; these cells form tumors when transplanted into nude mice (Wu *et al*., 1993). Conditional expression of c-fos upregulates expression of Dnmt1, resulting in morphological transformation of rat fibroblasts (Bakin and Curran, 1999). Interestingly, a low level of DNMT1 overexpression leads to cell transformation, whereas a high level is toxic (Wu *et al*., 1993). In addition, subcutaneous injection of the DNMT1 inhibitor 5-aza-2′-deoxycytidine decreases polyp number in Min mice that are heterozygous for the multiple intestinal neoplasia mutation of the adenomatosis polyposis coli gene (Laird *et al*., 1995). The mechanisms through which DNMT1 causes cellular transformation and through which inhibition of DNMT1 reverses cellular transformation are unknown (Baylin *et al*., 2001). The most obvious mechanism is that aberrant expression of DNMT1 causes methylation and silencing of tumor suppressor genes (Beaulieu *et al*., 2002; Robert *et al*., 2003; McCabe *et al*., 2006a). This hypothesis is supported by numerous documentations of methylated tumor suppressor genes in tumors (Lopez-Serra *et al*., 2006; Marsit *et al*., 2006). In accordance with this hypothesis, knock down of DNMT1 by either antisense or siRNA results in demethylation and activation of tumor suppressor genes, such as p16 and p21 (Fournel *et al*., 1999; Milutinovic *et al*., 2000; Robert *et al*., 2003). These data suggest that increased DNMT levels and activity affect the methylation status of genes critical to tumor formation. Thus, DNMT1 expression must be tightly regulated during normal cell growth.

In this study, we investigate the selective modulation of DNMT1 gene expression in HeLa cells by an HDAC inhibitor, apicidin, which inhibits proliferation of various tumor cells with a broad spectrum of antiproliferative activity against various cancer cell lines (Han *et al*., 2000). In particular, we focus on the transcriptional regulation of DNMT1 by apicidin and demonstrate that apicidin downregulation is attributed to the inhibition of transcription initiation, independent of cell cycle arrest at G_0/G_1, and the effect is mediated through induction of repressive histone modifications in the promoter region via recruitment of the corepressor complex.

Results

DNMT1 expression in HeLa cells is dysregulated during cell cycle and apicidin decreases DNMT1 mRNA and protein levels in various cancer cell lines

DNMT1 expression has been reported to be regulated in cell cycle-dependent manner (Szyf *et al*., 1991; Leonhardt *et al*., 1992). To examine if DNMT1 is normally S phase regulated in the human cervix cancer cell line HeLa, we first analysed changes in DNMT1 expression during the cell cycle. HeLa cells synchronized by serum starvation were stimulated with serum; fractions of G_0/G_1, S and G_2/M phase were determined by propidium iodide staining of DNA content and flow cytometry. While HeLa cells progress through the cell cycle as evidenced by the distribution of cell population (Figure 1b), there is no change in DNMT1 protein level during cell cycle, indicating dysregulation of DNMT expression in HeLa cells (Figure 1a).

Gene silencing of DNMT, which can act as an oncogene, has been proposed to be a good strategy for cancer therapeutics (Lee *et al*., 2005; Gore *et al*., 2006). Therefore, we then examined the potential of HDAC inhibitor apicidin to suppress the expression of DNMT1, DNMT3A and DNMT3B in HeLa cells. Apicidin treatment markedly reduces the level of DNMT1 mRNA and protein in a dose-dependent manner (Figures 1c and 2a), while apicidin has little effect on expression of DNMT3A and DNMT3B (Figure 1c). In contrast, the expression of cyclin-dependent kinase inhibitor $p21^{WAF1/Cip1}$ increases following apicidin treatment (Figures 1c and 2a), consistent with a previous report (Han *et al*., 2000).

To examine whether the observed apicidin effect is specific to HeLa cells, we expanded the investigation to include changes of apicidin-mediated DNMT1 expression in other cancer cell lines. Following treatment with apicidin for 24 h, DNMT1 protein levels dramatically decrease in NCCIT, human embryonic carcinoma cells, MCF-7, human breast cancer cells and HCC1954, human ductal carcinoma cells (Figure 2b). These results indicate that apicidin downregulates DNMT1 expression in various types of cancer cells.

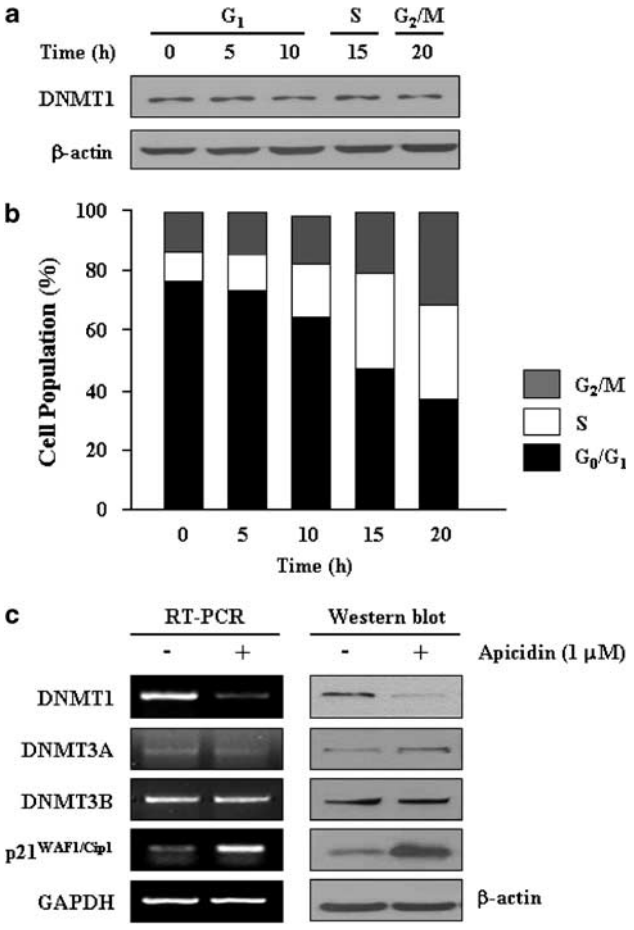

Figure 1 Dysregulation of DNA methyltransferase (DNMT)1 expression in HeLa cells is dysregulated during cell cycle and apicidin decreases DNMT1 mRNA and protein levels. (**a**) Quiescent HeLa cells were stimulated with 10% serum for the indicated time points. DNMT1 protein levels were determined by western blot analysis. (**b**) DNA content was determined by flow cytometry. (**c**) HeLa cells were treated with 1 μM apicidin for 24 h. The mRNA and protein levels were determined by reverse transcription (RT)–PCR and western blot analysis, respectively. Results shown are representative of three independent experiments.

Apicidin-induced downregulation of DNMT1 is independent of cell cycle arrest at G_0/G_1

Apicidin has been demonstrated to induce cell cycle arrest at G_0/G_1 in HeLa cells (Han *et al*., 2000). These findings led to the assumption that apicidin-induced downregulation of DNMT1 might be due to cell cycle arrest at G_0/G_1 phase. To examine the possibility, HeLa cells were arrested in G_0/G_1, and then changes in the levels of DNMT1 mRNA and protein were determined by reverse transcription (RT)–PCR and western blot analysis following treatment with apicidin or mevinolin, a pharmacological inhibitor of cell cycle at G_0/G_1 for 24 h (Rao *et al*., 1998). As expected, both apicidin and mevinolin treatments lead to the induction of $p21^{WAF1/Cip1}$ (Figure 3a) and the G_1 arrest, as evidenced by an increase in the number of cells at G_0/G_1 up to 87 and 85% from 67%, respectively (Figure 3b). Apicidin significantly reduces the levels of DNMT1 mRNA and protein, but mevinolin does not alter those expressions (Figure 3a). This finding indicates that apicidin downregulation of DNMT1 expression might be independent of apicidin-induced G_1 arrest or the induction of $p21^{WAF1/Cip1}$ expression.

Downregulation of DNMT1 expression by apicidin occurs at the transcriptional level

To address the possible mechanisms by which apicidin downregulates DNMT1 expression, we examined the effect of apicidin on the transcription of DNMT1 by determining the recruitment of RNA polymerase II (Pol II) into the transcription initiation site (B region) and coding region (C region) (Figure 4a; Bigey *et al*., 2000). Chromatin immunoprecipitation (ChIP) analysis using anti-Pol II antibody shows that apicidin significantly but not completely suppresses the transcriptional initiation, resulting in a decrease in the transcriptional elongation as evidenced by the 60 and 65% decrease in recruitment of Pol II into the transcription initiation site and coding region, respectively (Figure 4b). However, apicidin does not affect the recruitment of Pol II into the transcription initiation and coding region of glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Figure 4c). These results suggest that apicidin downregulates DNMT1 expression via inhibition of transcription, and that a mechanism other than transcriptional inhibition might also be partly associated with the downregulation of DNMT1 expression.

Apicidin induces repressive histone modifications and recruitment of pRB and HDAC1 to the E2F binding site

Histone modifications such as acetylation and methylation play an important role in regulation of gene expression through altering chromatin structure (Li *et al*., 2007). To examine whether downregulation of

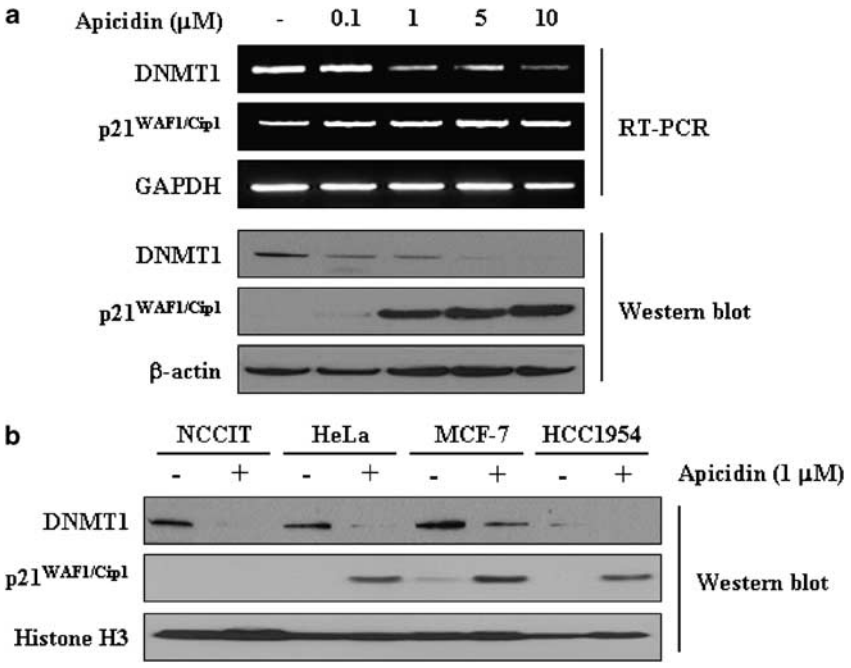

Figure 2 Apicidin downregulates the expression of dysregulation of DNA methyltransferase (DNMT)1 in various cancer cell lines. (**a**) HeLa cells were exposed to 0.1, 1, 5 and 10 μM apicidin for 24 h. The cells were subjected to reverse transcription (RT)–PCR and western blot analysis for DNMT1 and $p21^{WAF1/Cip1}$. (**b**) Various caner cell lines (NCCIT, HeLa, MCF-7 and HCC1954) were treated with apicidin (1 μM) for 24 h. Expression levels of DNMT1 and $p21^{WAF1/Cip1}$ were determined by western blot analysis. Results shown are representative of three independent experiments.

DNMT1 expression by apicidin is associated with alteration of histone modifications on the promoter region, we performed ChIP analysis. Apicidin treatment leads to the locally significant hypoacetylation of histone H3 and H4 on the transcriptional initiation site (B region) (Figure 5a), although the global acetylation of histone H3 and H4 increases as expected (Figure 5b).

Recent progress has highlighted the importance of three histone lysine methylations in epigenetics. Whereas methylation of histone 3 lysine 9 (H3-K9) and H3-K27 direct gene silencing, methylation of H3-K4 is associated with gene expression (Kouzarides, 2007). K4-trimethylated H3 on nucleosomes associated with the transcription initiation site is depleted following apicidin treatment, whereas repressive markers K9-trimethylation and K27-trimethylation of H3 are enriched on the site (Figure 5a). However, apicidin treatment dose not significantly alter the histone modifications upstream of the promoter region (A region).

It has been demonstrated that a conserved E2F consensus-binding site is located in proximity to the transcription initiation points of murine and human DNMT1, and that the DNMT1 promoter is regulated by the pRB/E2F pathway (McCabe *et al*., 2005). Thus, we examined whether apicidin-induced downregulation of transcriptional initiation is mediated through alteration of this pathway. First, we tested the binding of E2F and pRB to the conserved E2F binding site *in vivo* in the absence of apicidin. ChIP analysis reveals that in the absence of apicidin, E2F is constitutively bound to the E2F binding site, while pRB is not able to bind to the site (Figure 5c). However, apicidin treatment causes the marked recruitment of pRB into the site. Furthermore, pRB recruitment is accompanied by an increase in the recruitment of HDAC1 into the site (Figure 5c). In contrast, P/CAF, an HAT, is dissociated from the site upon apicidin treatment (Figure 5c). Taken together, the results indicate that the transcriptional repression of DNMT1 by apicidin might be attributed to repressive histone modifications through the formation of corepressor complexes on the E2F binding site.

De novo *protein synthesis is required for the transcriptional repression of DNMT1 by apicidin*

We next investigated whether apicidin-induced downregulation of DNMT1 expression is mediated by *de novo* protein synthesis. As shown in Figure 6a, pretreatment of HeLa cells with the protein synthesis inhibitor cycloheximide (CHX) recovers the decrease of DNMT1 mRNA level induced by apicidin, indicating the requirement of protein synthesis for the transcriptional repression of DNMT1. Consistent with the results, CHX treatment abrogates the recruitment of pRB and HDAC1 to the E2F binding site by apicidin, leading to hyperacetylation of histone H3 (Figure 6b). Collectively, the results suggest that apicidin action on the transcription of DNMT1 requires *de novo* protein synthesis.

Knock down of DNMT1 induces expression of $p21^{WAF1/Cip1}$ *and apoptosis*

Recently, it has been shown that an inverse correlation exists between $p21^{WAF1/Cip1}$ and DNMT1 protein levels in SV40-transformed and -nontransformed cells (Chuang

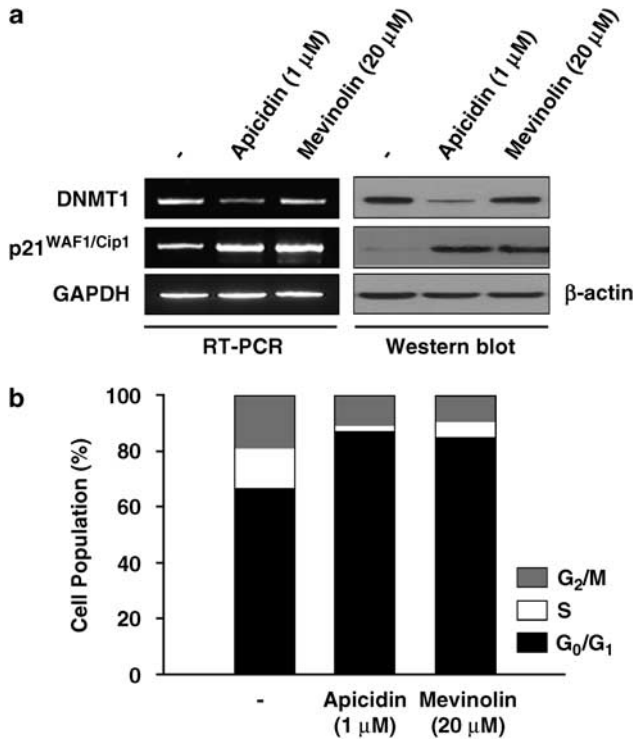

Figure 3 Apicidin-induced downregulation of dysregulation of DNA methyltransferase (DNMT)1 is independent of cell cycle arrest at G_0/G_1. (**a**) HeLa cells were incubated with apicidin (1 μM) or mevinolin (20 μM), a pharmacological inhibitor of G_1 arrest for 24 h. The mRNA and protein levels of DNMT1 and $p21^{WAF1/Cip1}$ were determined by reverse transcription (RT)–PCR and western blot, respectively. (**b**) DNA content was determined by flow cytometry. Results shown are representative of three independent experiments with similar results.

et al., 1997). To investigate whether alteration of DNMT1 protein levels affects the expression of the cell cycle inhibitor $p21^{WAF1/Cip1}$ in HeLa cells, we examined the effect of knock down of DNMT1 on expression of $p21^{WAF1/Cip1}$. Transfection of HeLa cells with siRNA against DNMT1 leads to significant reduction of mRNA and protein of DNMT1, while control siRNA dose not affect the levels of either DNMT1 or β-actin protein. Knock down of DNMT1 significantly but not fully induces the expression of $p21^{WAF1/Cip1}$, which can be further increased by apicidin treatment (Figure 7a). These results indicate that the upregulation of $p21^{WAF1/Cip1}$ by apicidin might be partially mediated through downregulation of DNMT1.

We then examined whether DNMT1 knockdown affects the biological process of HeLa cells. To do this, we determined the effect of DNMT1 knockdown on apoptosis of HeLa cells by analysing Annexin V staining using flow cytometry. As shown in Figure 7b, DNMT1 knockdown leads to significant increase in the number of Annexin V-positive cells (26.19%), compared to control siRNA (5.96%). To gain insights into the mechanism by which the inhibition of DNMT1 expression induces apoptosis, we investigated the effect of siRNA against DNMT1 on cleavages of caspase-3 and poly-(adenosine diphosphate-ribose) polymerase (PARP). DNMT1 knockdown induces the activation of capase-3, resulting in subsequent cleavage of PARP (Figure 7c). However, treatment of HeLa cells with apicidin does not cause cleavages of neither caspase-3 nor PARP (Figure 7c), consistent with our previous observation showing that apicidin does not induce apoptosis of HeLa cells (Kim *et al*., 2006). The results suggest that DNMT1 might be a good target for inducing apoptosis of HeLa cells.

Discussion

DNMT1 is crucial for maintaining normal DNA methylation patterning during embryonal development and cell division (Li *et al*., 1992; Walsh and Bestor, 1999; Reik *et al*., 2001). Recently, several studies have demonstrated that dysregulation of DNMT1 expression plays a critical role in cellular transformation (McCabe *et al*., 2006a). Moreover, inhibition of DNMT1 in various cancer cells using antisense, RNA interference and pharmacological inhibitors induces reexpression of various methylation-silenced tumor suppressor genes, leading to a decrease in transformation and proliferation. In this study, we describe a novel molecular mechanism by which apicidin downregulates DNMT1 expression. Apicidin induces the dissociation of activator, P/CAF and the recruitment of corepressors, pRB and HDAC1, into the promoter region containing the E2F binding site, which is accompanied by repressive histone modifications on the site, including hypoacetylation of histone H3 and H4, hypomethylation of histone H3-K4 and hypermethylation of H3-K9 and H3-K27 (Figure 5). In parallel, the recruitment of Pol II into the promoter region and coding region is suppressed (Figure 4). These findings strongly suggest that repressive histone modifications associated with nucleosomes of the DNMT1 promoter by apicidin might be responsible for the repression of DNMT1 abnormally expressed in HeLa cells.

Numerous HDAC inhibitor studies have focused on mechanisms of gene activation and demonstrated that histone hyperacetylation induced by inhibition of HDAC activity is associated with gene transactivation (Marks *et al*., 2001). For example, suberoylanilide hydroxamic acid (SAHA) caused dissociation of HDAC1 from the p21 promoter without detectable change in the HDAC1 protein (Gui *et al*., 2004). The loss of HDAC1 on the promoter is correlated with the accumulation of acetylated H3 and H4, which may play a role in SAHA-induced increases in acetylation of the histones. In contrast, emerging evidence has shown that HDAC inhibitors can also induce localized histone hypoacetylation in the promoter region, leading to the repression of gene expression, although the molecular mechanism remains to be established (Ferguson *et al*., 2003). Recently, the HDAC inhibitor trichostatin A (TSA) has been shown to downregulate *de novo* DNMT3B expression by decreasing DNMT3B mRNA

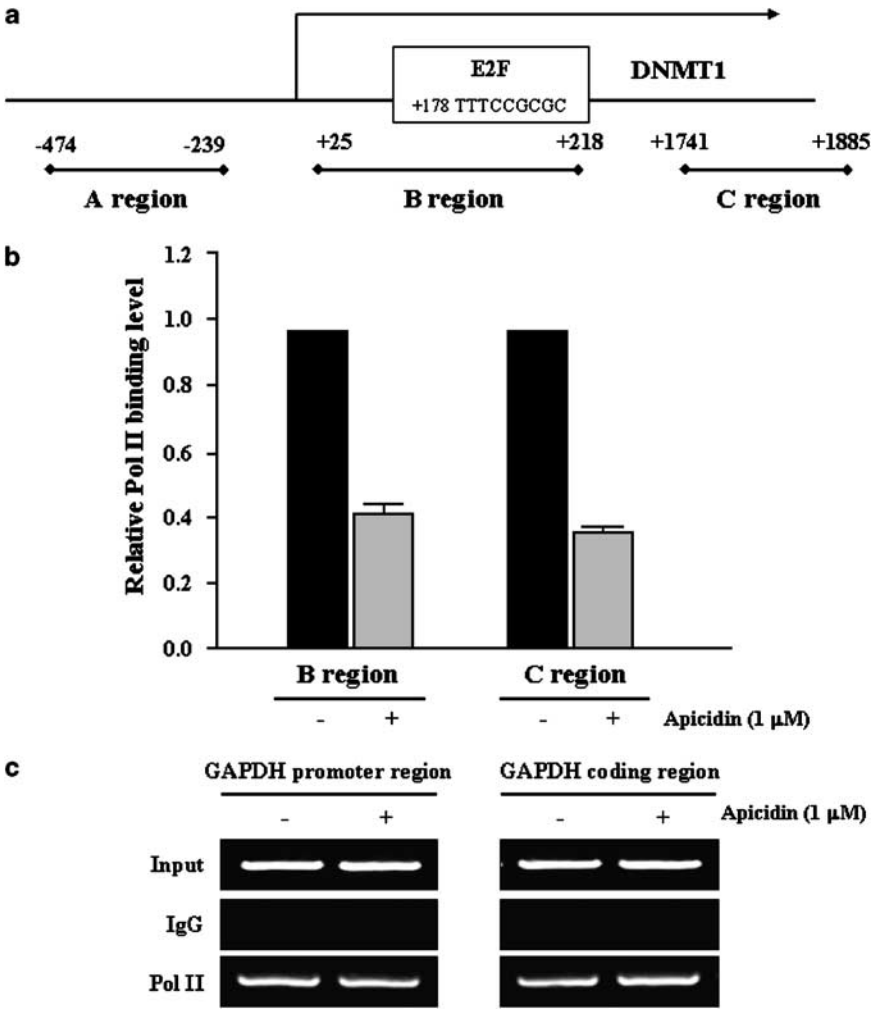

Figure 4 Decrease of dysregulation of DNA methyltransferase (DNMT)1 expression by apicidin occurs at the transcriptional level. (**a**) Schematic diagram of the DNMT1 promoter (A), transcription initiation (B) and coding regions (C). (**b** and **c**) Chromatin was immunoprecipitated with anti-RNA polymerase II 8WG16 (Pol II) antibodies following treatment of HeLa cells with 1 μM apicidin for 24 h. Pol II bindings to the transcription initiation and coding region of DNMT1and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were analysed by real-time PCR (**b**) or PCR (**c**) as described in the 'Materials and methods' section. Results shown are representative of three independent experiments with similar results.

stability (Xiong *et al*., 2005). A previous report also revealed that TSA treatment inhibits the mRNA synthesis of Her-2/neu as detected by a genomically integrated Her-2/neu promoter cell screen (Scott *et al*., 2002). While exposure to TSA had no effect on the open chromatin configuration of this gene, as monitored by DNase I hypersensitivity, TSA selectively destabilized and enhanced the decay of the mature Her-2/neu transcripts (Scott *et al*., 2002). In comparing the level of mRNA or protein of DNMT1 with loss of Pol II on the transcription initiation site, we show that while apicidin treatment almost completely depletes the mRNA and protein, about 60% of Pol II recruited on the promoter is dissociated, which is well correlated with previous observations showing that mutation in the E2F sites leads to a 65% loss of DNMT1 promoter activity (McCabe *et al*., 2005). The effect of apicidin on the dissociation of Pol II from the transcription initiation site does not completely account for changes in the levels of mRNA and protein. Therefore, we tested the possibility that apicidin partially affects the mRNA stability, and found that DNMT1 mRNA levels show minimal changes in HeLa cells in the presence of apicidin (data not shown).

A previous study has shown that the murine Dnmt1 gene contains two important *cis*-elements within the core promoter region (Kimura *et al*., 2003). One is an E2F binding site, and the other is a binding site for an unknown factor. Furthermore, transcription of DNMT1 is regulated in a complex fashion by E2F and other transcription factors through E2F-pRB-HDAC-dependent and -independent pathways. Recently, the transcriptional regulation of DNMT1 by the E2F-pRB pathway has been further confirmed by analysis of the protein expression and promoter activity of DNMT1 in $pRB^{+/+}$ and $pRB^{-/-}$ prostate epithelial cell lines rescued

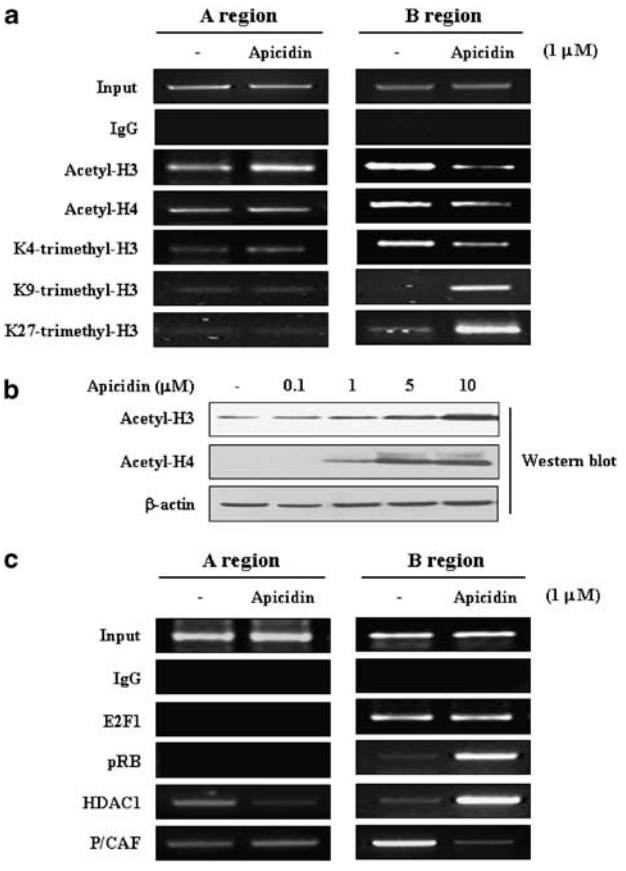

Figure 5 Apicidin induces repressive histone modifications through recruiting pRB and histone deacetylase (HDAC)1 to the E2F binding site. (**a**) HeLa cells were treated with apicidin (1 μM) for 24 h. Changes of epigenetic modifications on the promoter regions of DNA methyltransferase 1 in HeLa cells were determined by ChIP analysis using anti-acetylated or -methylated histone antibodies as described in the 'Materials and methods' section. (**b**) HeLa cells were treated with apicidin at various concentrations for 24 h, and the cell lysates were subjected to western blot analysis using anti-acetyl H3 and H4 antibodies. (**c**) Changes in the recruitment of E2F, pRB, HDAC1 and P/CAF to the promoter regions of DNMT1 were determined by ChIP analysis following treatment of HeLa cells with 1 μM apicidin for 24 h. Results shown are representative of three independent experiments with similar results.

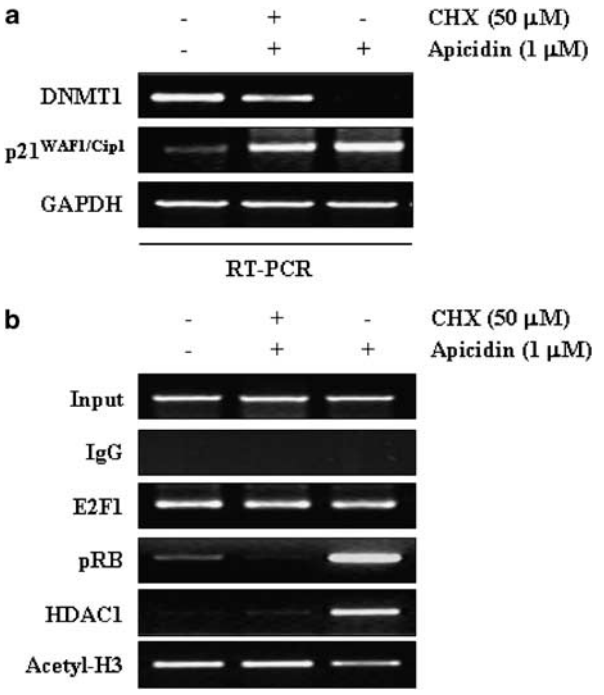

Figure 6 *De novo* protein synthesis is required for the transcriptional repression of dysregulation of DNA methyltransferase (DNMT)1 by apicidin. (**a**) HeLa cells were pretreated with cycloheximide (CHX, 50 μM) for 1 h, and treated with apicidin (1 μM) for an additional 24 h. Expression levels of DNMT1 and $p21^{WAF1/Cip1}$ were analysed by reverse transcription (RT)–PCR. (**b**) pRB and histone deacetylase (HDAC)1 recruitment to the E2F binding site was determined by ChIP analysis. Results shown are representative of three independent experiments with similar results.

by tissue recombination (McCabe *et al*., 2005). The human DNMT1 gene also exhibits putative E2F binding sites that align with those found in the murine, and the site is required for E2F-mediated transcriptional activation of DNMT1. The human DNMT1, like the murine Dnmt1 homologue, is positively and negatively regulated by E2F and pRB, respectively (McCabe *et al*., 2005). We provide the first indication that E2F and pRB is able to bind to DNMT1 promoter *in vivo*. Our results of the ChIP analyses clearly show that E2F but not pRB is constitutively bound to the DNMT1 promoter region containing the E2F binding site in HeLa cells that are abnormally expressing DNMT1, while pRB is recruited into the E2F binding site in the presence of apicidin. The transcriptional repression by pRB has been shown to be due to direct interaction with a chromatin-modifying enzyme HDAC (Brehm *et al*., 1998; Magnaghi-Jaulin *et al*., 1998). Consistent with the observation, apicidin-induced recruitment of pRB on the promoter region is accompanied by the replacement of P/CAF with HDAC1 on the E2F binding site. Among HATs and HDACs tested, only P/CAF and HDAC1 are involved in the process (data not shown). Apicidin treatment leads to local hypoacetylation of H3 and H4 associated with nucleosomes of the promoter region, even though the global acetylation of histones increases, indicating that pRB-dependent recruitment of HDAC1 on the promoter region might be responsible for the apicidin repression of DNMT1 expression through hypoacetylation of the histone H3 and H4. It has been hypothesized that histone modifications act alone, sequentially or in combination to moderate the code recognized by nonhistone protein complexes that regulate gene expression (Jenuwein and Allis, 2001; Zhang and Reinberg, 2001; Fischle *et al*., 2003). In addition to local hypoacetylation of histone H3 and H4, apicidin reduces the level of active marker, trimethyl-H3-K4 and increases that of repressive markers, trimethyl-H3-K9 and -K27 in the promoter region. This finding indicates that alteration of histone modifications induced by apicidin might be responsible for the downregulation of DNMT1, and that there is cross talk between histone

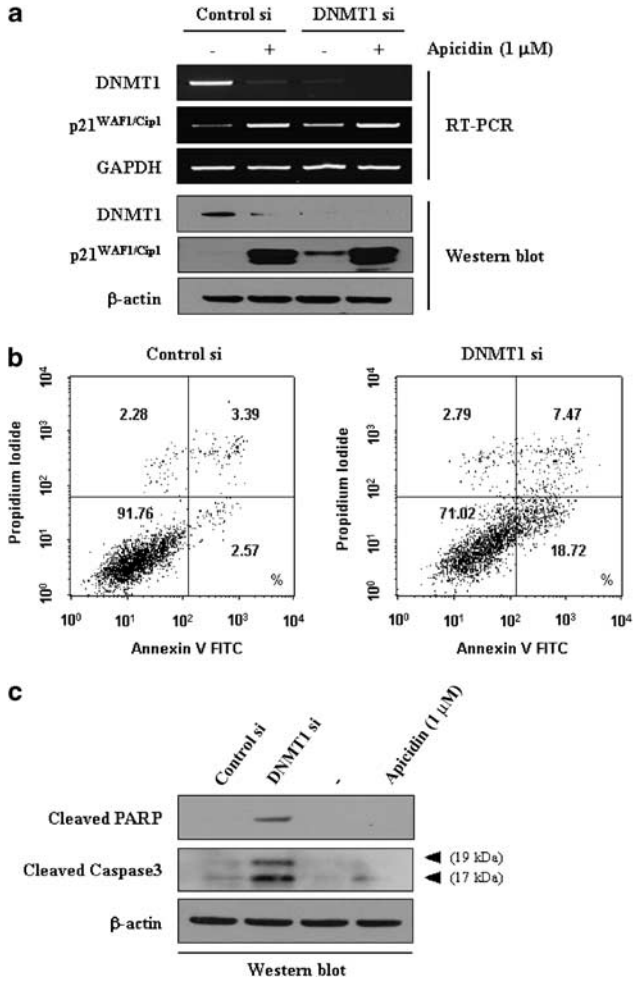

Figure 7 Knockdown of dysregulation of DNA methyltransferase (DNMT)1 induces the expression of $p21^{WAF1/Cip1}$ and apoptosis. (**a**) Transfection of HeLa cells with DNMT1 targeted and control siRNA was carried out using Lipofectamine 2000. At 48 h posttransfection, the cells were treated with apicidin (1 μM) or vehicle (DMSO) for an additional 24 h. The expression of target and internal genes was analysed by reverse transcription (RT)–PCR and western blot. (**b**) The extent of apoptosis of HeLa cells transfected with either targeted or control siRNA in the absence of apicidin was determined by flow cytometric analysis using Annexin-V-FITC apoptosis kit. (**c**) Cleavages of caspase-3 and poly-(adenosine diphosphate-ribose) polymerase (PARP) in HeLa cells either transfected with siRNA or treated with apicidin (1 μM) were analysed by western blot using antibodies against cleaved PARP and cleaved caspase3. Results shown are representative of three independent experiments.

acetylation and methylation in the E2F binding site, which might regulate the transcription of DNMT1. We also do not rule out the possibility that change in acetylation of E2F might be involved in the transcriptional repression of DNMT1 by apicidin, because E2F is thought to be regulated by reversible acetylation (Martinez-Balbas *et al*., 2000).

Apicidin-induced repression of DNMT1 mRNA expression seems to require *de novo* protein synthesis, because both apicidin-mediated declines in the mRNA levels of DNMT1 and the recruitment of pRB and HDAC1 into the E2F binding site in the DNMT1 promoter are reversed by cotreatment with CHX, a blocker of protein synthesis. Most cervical carcinoma cell lines, including HeLa cells are infected with human papilloma virus and express the E6 and E7 oncoproteins, which bind to and inhibit the p53 and pRB, respectively (Goodwin and DiMaio, 2000). Recently, DNMT1 has been demonstrated to be transcriptionally activated by the human polyomavirus Bradykinin B large T antigen and adenovirus E1a through pRb/E2F pathway (McCabe *et al*., 2006b). Thus, the apicidin effect could be related to interfering with the function of viral oncoproteins. The protein *de novo* synthesized by apicidin might disrupt the interaction pRB with E7, thereby dissociating pRB from pRB–E7 complex, which can be recruited along with HDAC1 on the E2F binding site. Alternatively, the protein might increase free pRB protein through inhibition of the expression of E7 protein. Although a specific protein required for the recruitment of pRB and HDAC1 on the E2F binding site is not elucidated in this study, the identification of the protein would be of great of interest.

Abnormal overexpression of DNMT1 in HeLa cells might contribute to the cellular transformation. Indeed, specific downregulation of DNMT1 with siRNA leads to apoptosis of HeLa cells. However, apicidin does not induce the apoptotic cell death of HeLa cells (Kim *et al*., 2006), although expression of DNMT1 in HeLa cells is downregulated by apicidin. These discrepancies might be due to the ability of cells to alter expression pattern of various genes involved in growth control or apoptosis through the modifications of chromatin structure by apicidin. As shown in our previous report (Kim *et al*., 2006), apicidin gains the apoptotic potential in HeLa cells, only when apicidin-induced activation of nuclear factor-κB, a survival factor, is downregulated, indicating that the apoptotic potential of apicidin in HeLa cells could be mediated by downregulation of DNMT1. Therefore, DNMT1 overexpressed in HeLa cells could be a target for therapeutics of human cervix cancer.

Collectively, the findings support the hypothesis that apicidin downregulates DNMT1 expression through inducing repressive histone modifications via the recruitment of pRB and HDAC1 into the E2F binding site, which require a newly synthesized protein. The findings provide a new insight into molecular mechanisms by which the HDAC inhibitor apicidin represses the expression of DNMT1 and suggest a mechanistic rationale for the use of HDAC inhibitors in cancer therapeutics.

Materials and methods

Cell culture

The HeLa cell line, derived from human cervix cancer, was cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT, USA) and 1% penicillin/streptomycin (Gibco).

Reagents and antibodies
Apicidin (cyclo(*N*-*O*-methyl-L-tryptophanyl-L-isoleucinyl-D-pipecolinyl-L-2-amino-8-oxodecanoyl)), a known antiprotozoal agent, was prepared from *Fusarium* sp. strain KCTC 16677 according to the method described previously (Park *et al*., 1999). Mevinolin (Sigma, St Louis, MO, USA) was used to synchronize the HeLa cells in the G_1 phase. CHX was obtained from Calbiochem (La Jolla, CA, USA). Commercial primary antibodies were anti-β-actin, anti-DNMT1, anti-DNMT3A, anti-DNMT3B, anti-trimethyl K4, K9 and K27 histone H3, and P/CAF (Abcam Inc., Cambridge, MA, USA); anti-$p21^{WAF1/Cip1}$ and anti-E2F (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-acetyl-histone H3 and H4 and anti-HDAC1 (Upstate Biotechnology, Lake Placid, NY, USA); anti-pRB (BD PharMingen, San Jose, CA, USA) and anti-Pol II 8WG16 (Covance Research Products Inc., Berkeley, CA, USA); and anti-cleaved Caspase3 and cleaved PARP (Cell Signaling Technology, Beverly, MA, USA).

RNA Extraction and reverse transcription-PCR
Total RNA was extracted using easy-Blue reagent (iNtRON Biotechnology, Sungnam, Gyeonggi, Korea). Integrity of RNA was checked by agarose gel electrophoresis and ethidium bromide staining. Using an RNA PCR kit (Promega, Madison, WI, USA), 1 μg of RNA was used as a template for each RT-mediated PCR. Primer sequences used for PCR were: DNMT1-5′, 5′-GATGAGTCCATCAAGGAAGA and DNMT1-3′, 5′-CTCCTTCAGTTTCTGTTTGG; GAPDH-5′, 5′-TGATGACATCAAGAAGGTGAAG and GAPDH-3′, 5′-TCCTTGGAGGCCATGTAGGCCAT; p21-5′, 5′-CAGAGGAGGCGCCATGTCAG and p21-3′, 5′-CCTGTGGGCGGATTAGGG.

Western blot analysis
Cell lysates were boiled in Laemmli sample buffer for 3 min, and then 30 μg of each protein was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes; the membranes were blocked for 30 min in Tris-buffered saline (TBS) containing 0.1% Tween 20 and 5% (w/v) dry skim milk powder and incubated overnight with primary antibodies. The membranes were then washed with TBS-0.1% Tween 20, incubated for 1 h with a secondary antibody and visualized with an enhanced chemiluminescence detection kit (Amersham Life Sciences, Arlington Heights, IL, USA).

Chromatin immunoprecipitation assay
Chromatin immunoprecipitation assays were performed using the Acetyl-Histone H3 Immunoprecipitation Assay Kit (Upstate Biotechnology, Charlottesville, VA, USA), according to the manufacturer's instructions. Chromatin from 1×10^6 HeLa cells sheared by a sonicator was precleared with salmon sperm DNA-saturated protein G sepharose and then precipitated by acetylated histone H3 antibody and others. After immunoprecipitation, recovered chromatin fragments were subjected to PCR or real-time PCR (PCR conditions: 30 cycles with denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 68 °C for 30 s; real-time PCR conditions: 40 cycles with denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s), using primer pairs specific for each region corresponding to the DNMT1 promoter and coding regions. Primer sequences were: A region-5′, 5′-CTGGTCTAAAACTCCTGGGCTCAAG and A region -3′, 5′-GTCCTCATCTGTAAAACGGGG; B region-5′, 5′-GGCCGGCTCCGTTCCATCCTTCTG and B region -3′, 5′-CGGCATCTCGGAGGCTTCAG; C region-5′ 5′-GAGTGTGGGAAATGTAAAGC and C region -3′, 5′-CTGGGATGTTATCATCGACT. Primer sequences of GAPDH were: promoter region -5′, 5′-ACAGTCCAGTCCTGGGAACC and promoter region -3′, 5′-CTTGAGGCCTGAGCTACGTG; coding region -5′, 5′-TGATGACATCAAGAAGGTGAAG and coding region -3′, 5′-TCCTTGGAGGCCATGTAGGCCAT.

Cell cycle assay
Cell cycle assays were performed using Cycletest Plus DNA Reagent Kit (BD Biosciences, San Diego, CA, USA), according to the manufacturer's instructions. The profiles of cells in the cell cycle were analysed with a FACScan flow cytometer (BD Biosciences).

Stealth RNAi
Stealth RNAi duplex (Invitrogen Corporation, Carlsbad, CA, USA) was designed to DNMT1. HeLa cells were transfected with 20 nM control siRNA or 20 nM DNMT1 stealth RNAi using Lipofectamine 2000 (Invitrogen Corporation).

Apoptosis analysis assays
After treatment of cells with DNMT1 siRNA and control siRNA, cells were harvested, and the extent of apoptosis was determined by flow cytometric analysis using Annexin-V-FITC apoptosis detection kit (BD Biosciences). Cleavages of caspase-3 and PARP were determined by immunoblot analysis using antibodies against cleaved caspase-3 and cleaved PARP.

Abbreviations

ChIP, chromatin immunoprecipitation; DNMT, DNA methyltransferase; HAT, histone acetyltransferase; HDAC, histone deacetylase; Pol II, RNA polymerase II.

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