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Role of hTERT in apoptosis of cervical cancer induced by histone deacetylase inhibitor ☆,☆☆

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Abstract

Human telomerase reverse transcriptase (hTERT) is the catalytic subunit of telomerase holoenzyme as well as the rate-limiting component of the telomerase enzyme complex. However, the role of the hTERT in apoptosis induced by histone deacetylase inhibitor has only been marginally addressed. For the first time, our study demonstrated that trichostatin A (TSA) briefly activated the proliferation of cervical cancer cell lines, HeLa and SiHa, within 12 h, but then inhibited cell growth after that time point. In response to TSA, hTERT expression, telomerase activity, and telomere length also underwent similar changes during the same time frame. Furthermore, the data in our study showed that cells transfected with dominant negative hTERT were more likely to undergo apoptosis induced by TSA than cells transfected with wild-type hTERT. The cyclin/cdk inhibitor $p21^{waf1}$ was down-regulated by hTERT without changing the expression of p53. Results from this study suggest that the hTERT might be a primary target of TSA and the anti-apoptosis effect of hTERT might be carried out through a $p21^{waf1}$-dependent and p53-independent pathway.

Keywords: Histone deacetylase inhibitor; Telomerase; Apoptosis; Human telomerase reverse transcriptase; Cervical cancer; $p21^{waf1}$; p53

The human telomerase reverse transcriptase (hTERT) is the catalytic subunit of telomerase holoenzyme as well as the rate-limiting component of the telomerase enzyme complex. By reverse transcribing the telomerase RNA component (hTR) to the telomeric DNA sequence, hTERT synthesizes and elongates the terminal telomeric DNA, thereby counteracting the cellular senescent program orchestrated by telomere shortening. Thus, hTERT activation is essential for cell immortalization, malignant transformation [1–5], and cancer cell survival [6,7]. The evidence that telomerase activity present in most human tumors has made telomerase not only an important target for the diagnosis of malignancy, but also a potential candidate for the development of novel therapeutic agents.

Histone proteins package and organize DNA into nucleosomes, the repeating units of chromatin. The histone–DNA interaction determines the accessibility of transcription factors with their regulatory DNA elements. Histone acetylation is one of the important post-translational modifications, which may lead to the alteration of chromatin structure and therefore regulate

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☆☆ *Abbreviations:* TSA, trichostatin A; hTERT, human telomerase reverse transcriptase; HDAC, histone deacetylase; HPV, human papillomavirus; WT, wild type; DN, dominant negative.
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gene expression. This process is mediated by the counteracting activities of histone acetyltransferase (HAT) and histone deacetylase (HDAC). Disruption of HAT or HDAC activity has been shown in several cancers [8]. HDAC inhibitors, such as trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), and sodium butyrate (NaB), can inhibit cancer cell growth in vitro [9,10] and in vivo [11,12], reverse oncogene-transformed cell morphology [13,14], and enhance cell differentiation [15]. TSA was originally characterized as an anti-fungal antibiotic and later identified as a specific HDAC inhibitor [16]. It is a stronger and a more specific HDAC inhibitor, which can induce apoptosis in highly proliferative cells such as lymphocytes and various carcinoma cells at low concentrations [17–21]. Cancer cells are much more sensitive to the treatment of HDAC inhibitors than normal cells. The HDAC inhibitors are proving to be an exciting anti-cancer therapeutic approach and several of them are in clinical trials. However, the mechanism of HDAC inhibitor-induced apoptosis has only been marginally addressed. An understanding of events in the TSA-induced apoptosis could be valuable for improving the efficacy of cancer therapy. We previously demonstrated that HDAC inhibitor sodium butyrate could induce apoptosis in HeLa cells without changing the expression level of bcl-2 and bax [22]. Activators/inhibitors of hTERT expression have been identified at the levels of transcriptional control and epigenetic modification. TSA has been reported to activate telomerase in a few telomerase negative cell types. The present study was designed to evaluate the change of hTERT expression, telomerase activity, and telomere length of cervical cancer cells in apoptosis process induced by TSA and to explore the mechanism of anti-apoptosis effect of hTERT in this process. Here, we reported that hTERT could play protective role in apoptosis induced by TSA and the effect of anti-apoptosis of hTERT was through a $p21^{waf1}$-dependent and p53-independent pathway.

Materials and methods

Cell culture and reagents. Human cervical cancer lines, HeLa and SiHa cells, were obtained from the American Type Culture Collection (ATCC) and maintained in phenol-free Eagle's minimal essential medium supplemented with 10% fetal calf serum (Hyclone). Cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere. Trichostatin A (TSA) was purchased from Sigma.

Cell proliferation assay. Cells were plated in 96-well plates. Various concentrations of TSA were added into the wells and cell growth was monitored. Each treatment group had triple replicates. Cells were fixed with trichloroacetic acid (final concentration 10%) at 4 °C for 1 h at the end of the experiment and stained with 0.4% sulforhodamine B (SRB; Sigma). The bound dye was eluted with 100 μl of Tris–HCl (pH 10.5) and the absorbance was monitored at 570 nm. One plate was fixed with trichloroacetic acid 6 h after plating the cells. The absorbance obtained with this plate was taken as 0% growth. The absorbance obtained with wells containing untreated cells was taken as 100% growth. An increase and decrease of A_{570} values in the experimental wells relative to the initial value indicated cell growth and death, respectively. When plotted as a percentage of untreated control growth and death, values appear on the positive and negative scales of the *Y*-axis.

Stable transfection. The wild-type and dominant negative hTERT cDNAs were cloned into the vector pBabe-puro, which was kindly provided by Professor Weinberg (Massachusetts Institute of Technology, USA). Substituting the aspartic acid and alanine residues at positions 710 and 711 with valine and isoleucine residues created DN-hTERT, and the wild-type and resulting mutant was sequenced completely. For transfection, cells at a density of 10^6 cells/100 mm diameter plate were transfected with plasmid pBabe-puro, pBabe-puro-WT-hTERT, and pBabe-puro-DN-hTERT by electroporation according to the manufacturer's instruction (BTX, USA). Cells were selected continuously in puromycin (4 μg/ml) and the medium was changed every 3–4 days for 14 days. Monoclones were isolated by ring cloning and expanded. The expression of telomerase in individually isolated clones was measured using a PCR-based telomeric repeat amplification protocol (TRAP)-enzyme-linked immunosorbent assay (ELISA) kit (Roche). Once a stable cell line from each clone had been established, the clonal lines were then maintained in a medium with 2 μg/ml puromycin and the telomerase expression was examined periodically.

Detection of telomerase activity. The telomerase activity was measured using a PCR-TRAP ELISA kit (Roche, USA) according to the manufacturer's description with some modifications. For the TRAP reaction, 2 μg protein was added to 25 μl of reaction mixture with the appropriate amount of sterile water to create a final volume of 50 μl. Hybridization and the ELISA reaction were carried out following the manufacturer's instructions.

Flow cytometry. For flow cytometric analysis, floating and attached cells were collected. Cells were fixed in ice-cold 70% ethanol and stored at −20 °C. Samples were then washed once in PBS and resuspended in a solution of propidium iodide (5 mg/ml) and RNase A (0.5 mg/ml) in PBS. The single-cell suspensions were analyzed on a FACSCalibur (Becton–Dickinson, USA) using Cell Quest and Modfit data analysis software.

Annexin V assays. For quantitative analysis of the apoptosis, the living cells were trypsinized, washed once in ice-cold PBS, and incubated with an annexin V–FITC/propidium iodide (PI) kit (BD, USA) in binding buffer. The cells were then immediately analyzed with a FACScan machine (Becton–Dickinson, USA).

RNA extraction, reverse transcription, and competitive PCR. Total RNA was isolated from cultured cells using TRIZOL (Invitrogen, USA). Two micrograms of total RNA was reverse-transcribed with random hexamer and then the hTERT mRNA was separately amplified with gene-specific primers. The PCR primer sets were as follows: 5′-CGG AAG AGT GTC TGG AGC AA-3′ and 5′-GGA TGA AGC GGA GTC TGG A-3′. The reaction conditions were 31 cycles of 94 °C for 40 s, 60 °C for 40 s, and 72 °C for 60 s. The amplified products for hTERT mRNA were separated by 1.5% agarose gel electrophoresis and then visualized by SYBER Green staining (Molecular Probes, Eugene, OR).

Immunofluorescence staining. For indirect immunofluorescence staining, the cells on the coverslips were treated with the anti-hTERT antibody (oncogene), then washed twice, and incubated with the relative FITC-conjugated secondary antibodies (Santa Cruz) for 30 min at 37 °C. Cells were examined with a laser scan microscope (Leica TCS NT) equipped with a 488–534λ argon laser and a 633λ helium–neon laser.

Flow cytometric analysis of hTERT protein expression. HeLa and SiHa cells were maintained in 1.5 μM TSA for 12 and 24 h, and then gently recovered with 0.53 mM EDTA in PBS. The cells were then washed and resuspended in the same medium at 5.0×10^6 cells/ml with an antibody against hTERT(oncogene) and an isotype-matched control antibody at a concentration of 20 μg/ml for 90 min at 4 °C. Cells were washed twice and incubated with FITC-conjugated goat anti-mouse IgG antibody (Santa Cruz) at a dilution of 1:150 for 30 min at

4 °C, then washed and fixed at 4 °C in 2% paraformaldehyde. Cells were subjected to flow cytometry and the relative fluorescence intensity of the cells was compared with the fluorescence intensity of the same cells stained with the control antibody. Results were presented as the number of cells (10,000/analysis) versus the log of fluorescence intensity.

Measurement of telomere length using fluorescence in situ hybridization. Fluorescence in situ hybridization was carried out following the procedure described (Dako Cytomation, Denmark). The treated cells were analyzed with FACSCalibur (Becton–Dickinson, USA). 1×10^4 cells in each experiment were collected and analyzed using Cell Quest and Modfit data analysis software. The telomere fluorescence signal was defined as the mean fluorescence signal in cells after subtraction of the background fluorescence.

Western blot analysis for p53 and $p21^{waf1}$ protein expression. For Western blotting of p53 and $p21^{waf1}$ proteins, 50 mg of total cell lysate was separated by 12.5% SDS–PAGE and transferred to a nitrocellulose membrane. Membranes were blocked overnight with 5% dry skimmed milk in TBST. For detection of $p21^{waf1}$ and p53, polyclonal antibodies (Santa Cruz) were used. The protein–antibody complexes were detected by an HRP-conjugated secondary antibody (Santa Cruz) using the enhanced chemiluminescence system (Pierce, USA).

Statistical analysis. Standard error for all measured biological parameters is displayed in the appropriate figures. To determine the significant difference (95% probability) of parameters between sample groups, a paired (for the same sample populations with different treatments) or unpaired (for different sample populations) Student's *t* test was utilized.

Results

Effects of trichostatin A on cell proliferation and apoptosis in cervical cancer cells

RSB binding assay showed that HeLa and SiHa cells' treatment with TSA resulted in time-dependent induction of proliferation inhibition in 72 h. In particular, 1.0–2.0 μM TSA clearly inhibited cell growth after treatment only for 24 h (Fig. 1A). It was noticed that 0.1–1.0 μM TSA briefly activated cell growth within 12 h and then turned to suppress them marginally but did not induce cell death after 24 h. To determine the relationship of these anti-proliferation effects to the cell cycle or apoptosis, different time points of cell cycle and apoptosis rates were detected. In both cell lines, TSA at high concentrations (2.0 μM) induced a profound reduction of cells in the G_2/G_1 phase and an appearance of sub-G_1 populations after 48 h (Table 1).

Effects of TSA on hTERT expression, telomerase activity, and telomere length in cervical cancer cells

The limiting step in telomerase activation has previously conformed to be transcription of the catalytic subunit of telomerase, hTERT [23]. In order to examine the effects of TSA on hTERT gene transcription, RT-PCR was used to assess changes in hTERT expressions over the course of TSA-induced apoptosis in cervical cancer cells. As shown in Fig. 1B, hTERT mRNA expressions in two cell lines were activated as early as 6 h, up to its peak within 12 h after 1.5 μM TSA treatment, this was consistent with the cells' proliferation within 12 h. However, hTERT mRNA expressions were inhibited after 16 h of TSA treatment and this was especially evident at the 36 h time point. Immunofluorescence blot also revealed that hTERT protein expressions were briefly up-regulated and the strongest activation could be discerned at 12 h after 1.5 μM TSA treatment (Fig. 2A) in HeLa and SiHa cells. The quantitative analysis by FACS showed that hTERT protein expression increased 75.1% and 42.7% in HeLa and SiHa cells, respectively, within 12 h compared with untreated cells (Fig. 2B). These results showed that hTERT mRNA and protein expressions were both suppressed after 24 h with TSA treatment in cervical cancer cells.

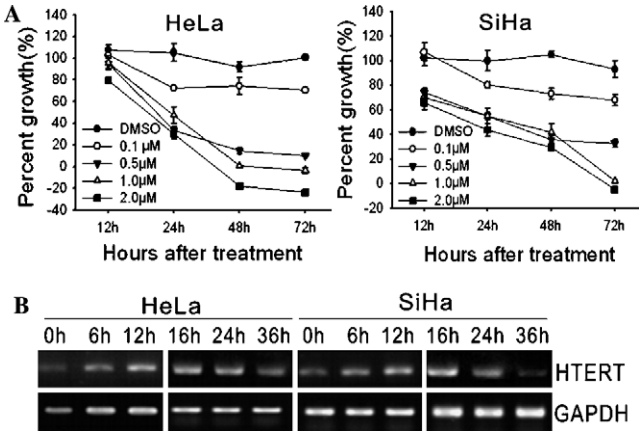

Fig. 1. The effects of TSA on cell growth and the mRNA expression of hTERT in HeLa and SiHa cells. (A) Cell proliferation was measured using RSB as described under Materials and methods. HeLa and SiHa cells were treated with 0.1, 0.5, 1.0, and 2.0 μM TSA or control DMSO for 12, 24, 48, and 72 h. Data are means ± SE. (B) The time-course of expression of hTERT mRNA by RT-PCR analysis.

Furthermore, to investigate the effects of TSA on telomerase activity and telomere length, HeLa and SiHa cells were treated with 1.5 μM TSA for 16 and

Table 1
Effects of TSA on cell cycle and apoptosis in HeLa and SiHa cells

	HeLa		SiHa	
	G_2/G_1	Sub-G_1 (%)	G_2/G_1	Sub-G_1 (%)
0 h	1.93 ± 0.01	0.12 ± 0.04	1.98 ± 0.04	0.11 ± 0.03
12 h	1.91 ± 0.03	0.05 ± 0.04	1.96 ± 0.02	0.70 ± 0.12
24 h	1.89 ± 0.07	1.21 ± 0.16	1.93 ± 0.04	2.34 ± 0.31
36 h	1.88 ± 0.03	8.29 ± 0.85	1.92 ± 0.07	2.47 ± 0.06
48 h	1.85 ± 0.06	10.05 ± 0.83	1.69 ± 0.09	15.97 ± 1.61
72 h	1.97 ± 0.12	32.39 ± 3.21	1.89 ± 0.13	34.76 ± 3.28

Cells were incubated with 2 μM TSA for 0, 12, 24, 36, 48, and 72 h; apoptosis rates were represented by Sub-G_1 proportion in DNA content analysis.

36 h. TRAP-ELISA and flow fluorescence in situ hybridization technique were employed to detect cell telomerase activity and to compare the telomere length separately. The data showed that the time course dependence of hTERT expression led to telomerase activity up to a peak at 16 h and its activity diminished after treatment for 36 h (Fig. 3A). Telomerase activity increased 11% in HeLa cells and 13% in SiHa cells, respectively, compared to untreated cells at 16 h. However, the telomerase activity at 36 h of these cells was slightly lower than untreated cells. In addition, similar results were obtained in telomere length assays, as shown in Fig. 3B. Quantitative analysis showed about 2-fold increase in telomeric fluorescence in 16 h when compared with the telomerase negative controls. The results verified that the expression of telomerase did extend the endogenous telomeres.

The effects of hTERT on proliferation and apoptosis in HeLa and SiHa cells

Our data suggest that TSA could inhibit telomerase activity and hTERT expression in cervical cancer cells, to determine whether hTERT plays a protective role in apoptosis induced by TSA, HeLa, and SiHa cells that were transfected with wild-type hTERT (WT-hTERT), dominant negative hTERT (DN-hTERT), and empty control vector (C-Vector). For each group, we choose

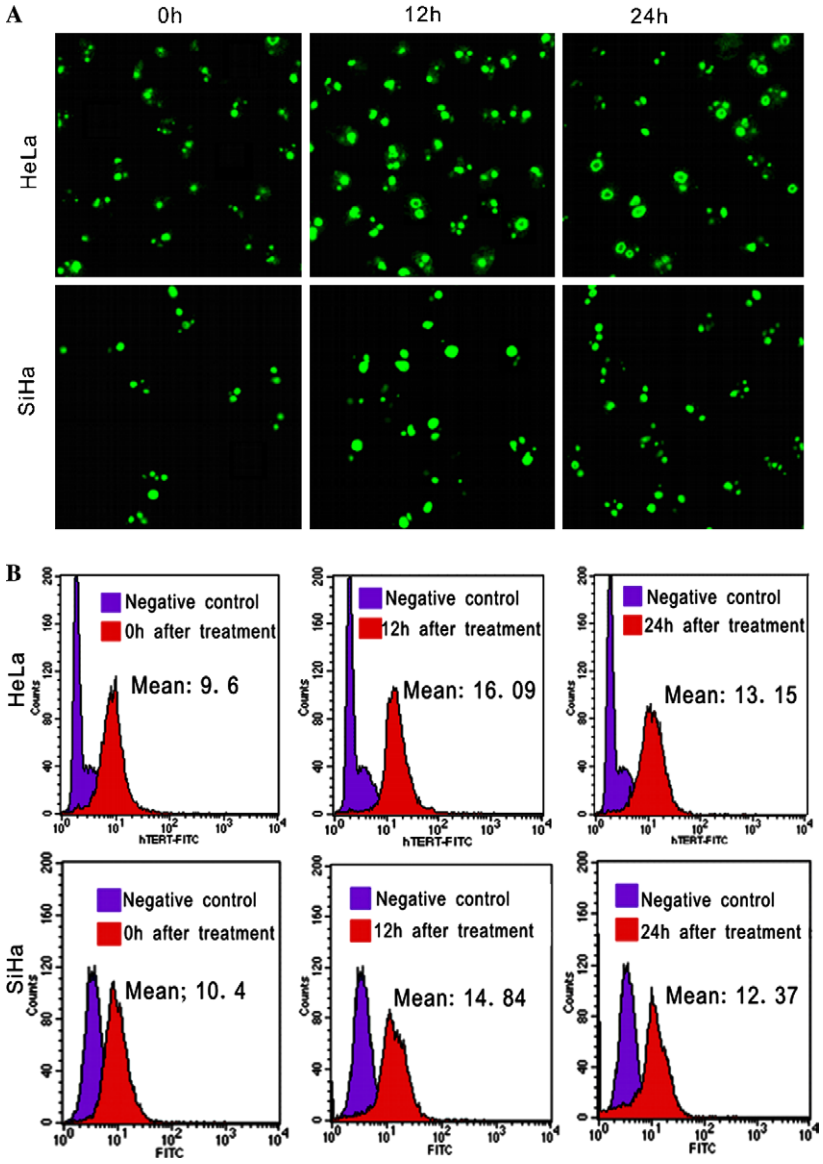

Fig. 2. The protein level of hTERT in HeLa and SiHa cells after TSA treatment. (A) The hTERT protein expression in TSA-treated HeLa and SiHa cells was detected by laser scan microscope. HeLa and SiHa cells were treated with 1.5 μM TSA for 0, 12, and 24 h. (B) hTERT protein was detected by cell immunofluorescence staining with or without anti-hTERT mAb in 12 and 24 h, the quantitative analysis was done by flow cytometry. The mean stands for relative fluorescence intensity.

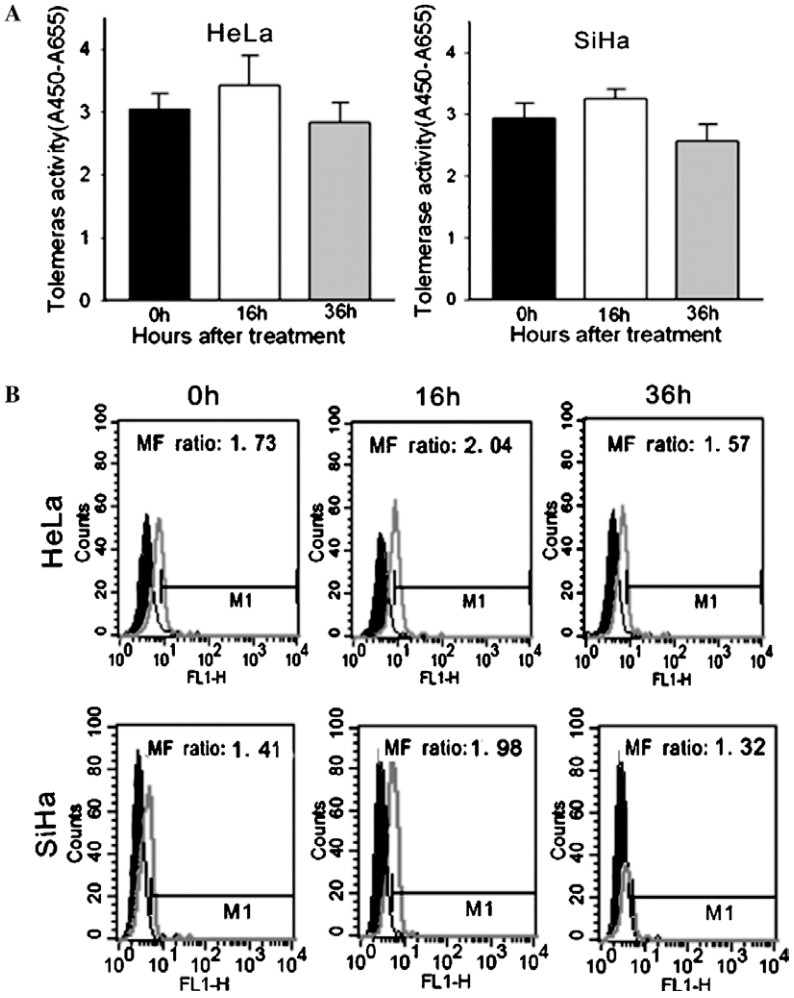

Fig. 3. The effects of TSA on the telomerase activity and telomere length in HeLa and SiHa cells. (A) The time-course analysis of telomerase activity in HeLa and SiHa cells by PCR-TRAP-ELISA. HeLa and SiHa cells were treated with 1.5 μM TSA for 0, 16, and 36 h. 10^5 cells were harvested for preparation of protein extract and 1 μg protein was subjected to TRAP assay. After hybridization and ELISA procedure, the absorbance of the samples at 450 nm (with a reference wavelength of approximately 655 nm) was measured. (B) The time-course analysis of telomere length in HeLa and SiHa cells by fluorescence in situ hybridization. 10^6 cells were harvested at 0, 16, and 36 h after treated with 1.5 μM TSA and analysis as described under Materials and methods. The black curve represents the mean fluorescence of background control and the white curve represents the mean fluorescence obtained from cells hybridized with telomere PNA probe. MF ratio stands for relative mean fluorescence ratio.

eight resultant stable clones to measure their telomerase activity. The telomerase activity was clearly induced in HeLa ($p = 0.004$) and SiHa ($p = 0.002$) cells transfected with WT-hETER. In contrast, an apparent decrease (HeLa, $p = 0.011$ and SiHa, $p = 0.015$) in telomerase activity was detected in the cells transfected with DN-hTERT when compared with C-Vector transfected cells (Fig. 4A).

Next, we characterized the proliferation properties of HeLa and SiHa cells expressing either WT-hTERT or DN-hTERT. Compared to the C-Vector carrying a vector that encoded only a drug resistance marker, the proliferation of both HeLa and SiHa cells transfected with DN-hTERT was inhibited after treatment with 1.5 μM TSA for 48 h, and the difference of growth rate in these two transfected cells was evident at 72 h time point. In contrast, the proliferation of WT-hTERT transfected HeLa and SiHa cells was slightly activated within 72 h (Fig. 4B).

It was reported that telomerase plays a central role in cellular resistance to apoptosis of cancer cell, further experiments showed whether telomerase activity inhibition would affect apoptosis induced by TSA. DN-hTERT, WT-hTERT, and C-Vector of HeLa and SiHa cells were treated with 1.5 μM TSA for 24 h. Annexin V assay, a quantitative analysis of apoptosis, was employed to detect apoptosis. It revealed that cells containing DN-hTERT displayed more sensitiveness to TSA. About 50% of HeLa and 56% SiHa DN-hTERT transfected cell lines were apoptotic. In contrast, only 13.5% apoptosis rate were detected in WT-hTERT transfected HeLa and SiHa cells. 19% of HeLa control

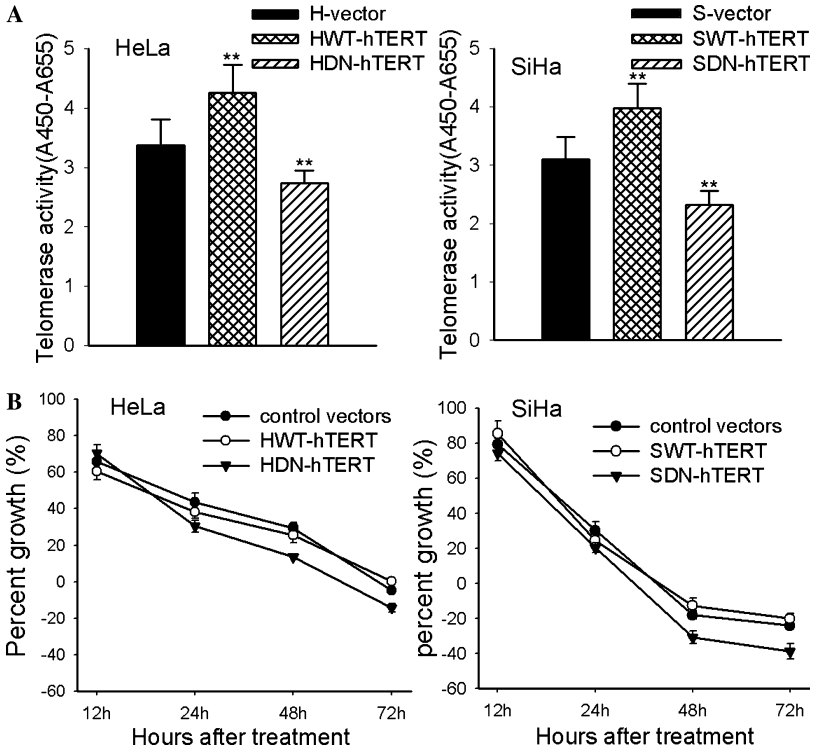

Fig. 4. Effect of hTERT on telomerase activity and proliferation in HeLa and SiHa cells. (A) After infection and cloning, telomerase activity was determined in C-Vector, WT-hTERT, and DN-hTERT cell lines of HeLa and SiHa cells by PCR-TRAP-ELISA. Values are shown as means ± SD of triplicates. There was a significant increase in WT-hTERT transfected cells (**$p < 0.05$) and decrease in DN-hTERT transfected cells (**$p < 0.05$), compared with vector transfected cells. (B) The C-Vector, WT-hTERT, and DN-hTERT cell lines of HeLa and SiHa cells were treated with 2 μM TSA for 0, 12, 24, 48, and 72 h. Cell growth was measured using RSB as described under Materials and methods. Data are means ± SE.

cells and 26% SiHa control cells underwent apoptosis with a background of 10% cell death in untreated cells (Table 2). These data provide convincing evidence for the apoptosis resistance of WT-hTERT of HeLa and SiHa cells.

Effect of TSA on p21^{waf1} and p53 expression

The cyclin/CDK inhibitor p21^{waf1} exerted an inhibitory effect on cyclin-dependent kinase and mediated cell growth arrest. During this process of HDAC inhibitor-induced apoptosis, the p21^{waf1} gene expression was often up-regulated [14]. We therefore determined if TSA treatment could cause an increasing expression of p21^{waf1}. Western blot analysis showed that in HeLa and SiHa cells, a sustained increase in expression of p21^{waf1} was observed after treatment with TSA for 12 h. As expression of p21^{waf1} could be regulated through both the p53-dependent and the p53-independent pathways [24,25], the cellular p53 level in these cells was detected. As shown in Fig. 5A, there was no change of p53 in treatment process. This suggested that the stimulation of p21^{waf1} expression was through a p53-independent pathway. After transfection, treatment with 1.5 μM TSA for 24 h, the DN-hTERT transfected cell lines had higher expression level of p21^{waf1} compared to WT-hTERT transfected cell lines (Fig. 5B). These data indicated that hTERT might inhibit the expression of p21^{waf1}. However, there were no changes of p53 in both HeLa and SiHa with different treatments. Taken together, it suggested that the hTERT plays a protective effect in TSA-induced apoptosis through a p21^{waf1}-dependent and a p53-independent pathway.

Table 2
Effects of hTERT on apoptosis in cervical cancer cells after treated with 1.5 μM TSA for 24 h

	HeLa		SiHa	
	0 h	24 h	0 h	24 h
C-vector (%)	7.1 ± 1.2	19.1 ± 4.2	12.8 ± 3.6	26.2 ± 2.7
WT-hTERT (%)	9.2 ± 1.8	13.8 ± 2.1*	6.9 ± 1.7	13.4 ± 2.5*
DN-hTERT (%)	10.6 ± 2.3	49.9 ± 6.3**	9.6 ± 4.8	55.9 ± 9.4**

Quantitative analysis of TSA-induced apoptosis. 10^6 cells were incubated in 1.5 μM TSA for 24 h and analyzed by annexin V assay.
* $p < 0.05$.
** $p < 0.01$.

Discussion

TSA, a type of HDAC inhibitor, induced profound dose-dependent cytotoxicity in cancer cells. The present

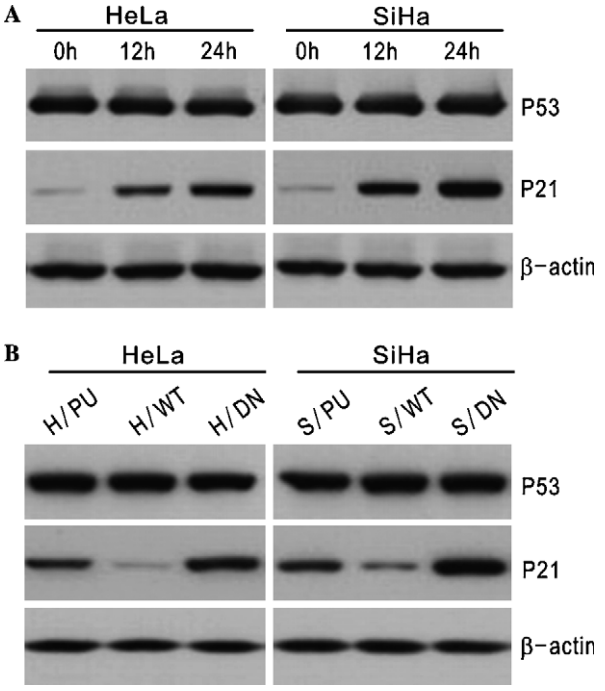

Fig. 5. Effects of TSA on $p21^{waf1}$ and p53 expression in cervical cancer cells. (A) TSA trigger $p21^{waf1}$ up-regulation in HeLa and SiHa cells. Cell lysates were prepared at indicated time points after TSA treatment. Western blot analysis of $p21^{waf1}$, p53, and β-actin was performed as described under Materials and methods. The β-actin was used as a control for loading. (B) After transfection, the expression of $p21^{waf1}$, p53 in C-Vector, WT-hTERT, and DN-hTERT cell lines of HeLa and SiHa cells was detected. H/PU, H/WT, and H/DN stand for HeLa cells transfected C-Vector, WT-hTERT, and DN-hTERT. S/PU, S/WT, and S/DN stand for SiHa cells transfected C-Vector, WT-hTERT, and DN-hTERT.

study found a novel phenomenon that TSA briefly activated the proliferation of cervical cancer cell lines, HeLa and SiHa, within 12 h, but then inhibited their growth after that time point. Consistent with cell proliferation, the hTERT expression was in a time-dependent manner during which it was briefly up-regulated to a peak at 12 h, but then suppressed after 24 h with a treatment of 1.5 μM TSA. This suggested that hTERT could be the primary target of TSA. During the first hours, treatment with TSA also induced a significant activation of hTERT expression in cervical cancer cells. This is different from previous data [26], which suggests that TSA treatment led to a significant induction of hTERT mRNA expression in normal cells, but not in cancer cells. Recent data suggested that telomerase plays a central role in cellular resistance to apoptosis of cancer cells [27], and inhibition of telomerase activity in tumor cell lines may induce apoptosis [28]. However, the effect of telomerase in HDAC inhibitor-induced apoptosis is controversial [29–32]. Here, we confirmed that TSA inhibited telomerase activity and telomere length as well as the hTERT expressions. These findings showed that telomerase might play an important protective role against apoptosis of cervical cancer cells induced by TSA.

Recent studies showed that cyclin-dependent kinases (Cdks) played an important role in the cell-cycle progression. The Cdk inhibitor proteins can negatively regulate cell-cycle progression by inhibiting Cdk activity through the physical association with their target cyclin–Cdk complexes [33–35]. Among them, $p21^{waf1}$ was cloned first and characterized as an inhibitor of cyclin E–Cdk2 complex kinase activity in p53-mediated cell-cycle arrest induced by DNA damage. The anti-proliferation effect of HDAC inhibitors in cancer cells is thought to be due to up-regulated expression of $p21^{waf1}$, but other studies did not observe $p21^{waf1}$ expression increase induced by HDAC inhibitor in lung cancer [30] and prostate cancer [29]. Our study showed that an induced expression of $p21^{waf1}$ was detected in human cervical cancer HeLa and SiHa cell lines after treatment with TSA for 12 h. However, before this time point, the expression of $p21^{waf1}$ was not changed (data not shown). The reason may be correlated with high hTERT expression during the first 12 h. Previous studies have shown that hTERT was up-regulated by c-myc overexpression [36,37] or down-regulated by $p21^{waf1}$ overexpression [38,39]. Therefore, an increased $p21^{waf1}$ expression induced by TSA in cancer cells supports the fact that HDAC inhibitor represses the growth of cervical cancer cells.

p53, a well-known tumor repressor, has been shown to activate $p21^{waf1}$ transcription and it was reported that expression of $p21^{waf1}$ can be regulated through both the p53-dependent and the p53-independent pathways [24,25], therefore we wondered if TSA could affect p53 expression and subsequently induce $p21^{waf1}$ expression. To address this question, we treated HeLa cells and SiHa cells with TSA and then detected the p53 protein level in these cells. It should be noted that HeLa and SiHa are human papillomaviruses (HPVs) positive cell lines, E6 oncoprotein HPVs encoded can recruit the cellular ubiquitin–protein ligase E6-AP to target p53 for ubiquitin–proteasome-mediated degradation. However, it has been reported that after DNA damage, p53 is not stabilized and activated in cells that ectopically express E6 and HPV-positive cancer cells [40]. Our data showed p53 could be detected but not changed with TSA treatment. This suggested that TSA-induced $p21^{waf1}$ expression was a p53-independent pathway.

The results in this study demonstrate the evidence that telomerase may have a protective effect in the process of TSA-induced apoptosis. However, the mechanism of this effect still remains unclear. It has been suggested that hTERT may promote survival by a mechanism other than telomere maintenance [28], and telomerase may inhibit an early event in the apoptotic cascade [27]. To further investigate the mechanism by which telomerase acts as a resistant factor in apoptosis

induced by TSA, hTERT was introduced into these cell lines. We found that cells transfected with DN-hTERT were rendered more sensitive to TSA treatment and that around half of these cells underwent apoptosis. Further study showed that the cells transfected with DN-hTERT had higher level expression of $p21^{waf1}$. Whereas, WT-hTERT transfected cells displayed a decreased expression level of $p21^{waf1}$. No difference of p53 expression was observed in these cell lines. These data indicated that the anti-apoptosis of hTERT might be through a $p21^{waf1}$-dependent and p53-independent cascade.

In conclusion, we demonstrated that telomerase might be a primary target of TSA and might play an important role in mediating TSA-induced apoptosis in cervical cancer cells. The hTERT activation might be the critical component in determining whether cells survive or undergo apoptosis and anti-apoptosis effect of hTERT might be through a $p21^{waf1}$-dependent and p53-independent pathway. Further research is needed to explore the mechanism that hTERT regulates $p21^{waf1}$ expression either in a direct way or indirectly by another cascade. Making this mechanism clear may help us to understand the role of hTERT and HDAC inhibitor in cell-cycle arrest and apoptosis induction. This information will be invaluable for improving the effectiveness of cancer therapy.

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References

[1] J.W. Shay, S. Bacchetti, A survey of telomerase activity in human cancer, Eur. J. Cancer 33 (1997) 787–791.
[2] M. Meyerson, C.M. Counter, E.N. Eaton, L.W. Ellisen, P. Steiner, S.D. Caddle, L. Ziaugra, R.L. Beijersbergen, M.J. Davidoff, Q. Liu, S. Bacchetti, D.A. Haber, R.A. Weinberg, hEST2, the putative human telomerase catalytic subunit gene is up-regulated in tumor cells and during immortalization, Cell 90 (1997) 785–795.
[3] A.G. Bodnar, M. Ouellette, M. Frolkis, S.E. Holt, C.P. Chiu, G.B. Morin, C.B. Harley, J.W. Shay, S. Lichtsteiner, W.E. Wright, Extension of life-span by introduction of telomerase into normal human cells, Science 279 (1998) 349–352.
[4] W.C. Hahn, C.M. Counter, A.S. Lundberg, R.L. Beijersbergen, M.W. Brooks, R.A. Weinberg, Creation of human tumour cells with defined genetic elements, Nature 400 (1999) 464–467.
[5] Y. Sonoda, T. Ozawa, Y. Hirose, K.D. Aldape, M. McMahon, M.S. Berger, R.O. Pieper, M.S. Berger, Formation of intracranial tumors by genetically modified human astrocytes defines four pathways critical in the development of human anaplastic astrocytoma, Cancer Res. 61 (2001) 4956–4960.
[6] W.C. Hahn, S.A. Stewart, M.W. Brooks, S.G. York, E. Eaton, A. Kurachi, R.L. Beijersbergen, J.H. Knoll, M. Meyerson, R.A. Weinberg, Inhibition of telomerase limits the growth of human cancer cells, Nat. Med. 5 (1999) 1164–1170.
[7] X. Zhang, V. Mar, W. Zhou, L. Harrington, M.O. Robinson, Telomere shortening and apoptosis in telomerase-inhibited human tumor cells, Genes Dev. 13 (1999) 2388–2399.
[8] P. Marks, R.A. Rifkind, V.M. Richon, R. Breslow, T. Miller, W.K. Kelly, Histone deacetylases and cancer: causes and therapies, Nat. Rev. Cancer 1 (2001) 194–202.
[9] M. Yoshida, M. Kijima, M. Akita, T. Beppu, Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A, J. Biol. Chem. 265 (1990) 17174–17179.
[10] W. Wharton, J. Savell, W.D. Cress, E. Seto, W.J. Pledger, Inhibition of mitogenesis in Balb/c-3T3 cells by trichostatin A. Multiple alterations in the induction and activation of cyclin–cyclin-dependent kinase complexes, J. Biol. Chem. 275 (2002) 33981–33987.
[11] M. Yoshida, Y. Hoshikawa, K. Koseki, K. Mori, T. Beppu, Structural specificity for biological activity of trichostatin A, a specific inhibitor of mammalian cell cycle with potent differentiation-inducing activity in Friend leukemia cells, J. Antibiot. (Tokyo) 43 (1990) 1101–1106.
[12] D.M. Vigushin, S. Ali, P.E. Pace, N. Mirsaidi, K. Ito, I. Adcock, R.C. Coombes, Trichostatin A is a histone deacetylase inhibitor with potent antitumor activity against breast cancer in vivo, Clin. Cancer Res. 7 (2001) 971–976.
[13] H.J. Kwon, T. Owa, C.A. Hassig, J. Shimada, S.L. Schreiber, Depudecin induces morphological reversion of transformed fibroblasts via the inhibition of histone deacetylase, Proc. Natl. Acad. Sci. USA 95 (1998) 3356–3361.
[14] P.A. Marks, V.M. Richon, R.A. Rifkind, Histone deacetylase inhibitors: inducers of differentiation or apoptosis of transformed cells, J. Natl. Cancer Inst. 92 (2000) 1210–1216.
[15] Q. Zhou, Z.K. Melkoumian, A. Lucktong, M. Moniwa, J.R. Davie, J.S. Strobl, Rapid induction of histone hyperacetylation and cellular differentiation in human breast tumor cell lines following degradation of histone deacetylase-1, J. Biol. Chem. 275 (2000) 35256–35263.
[16] N. Tsuji, M. Kobayashi, K. Nagashima, Y. Wakisaka, K. Koizumi, A new antifungal antibiotic, Trichostatin, J. Antibiot. 29 (1976) 1–6.
[17] J.A. McBain, A. Eastman, C.S. Nobel, G.C. Mueller, Apoptotic death in adenocarcinoma cell lines induced by butyrate and other histone deacetylase inhibitors, Biochem. Pharmacol. 53 (1997) 1357–1368.
[18] J.Y. Shin, H.S. Kim, K.S. Lee, J. Kim, J.B. Park, M.H. Won, Mutation and expression of the p27KIP1 and p57KIP2 genes in human gastric cancer, Exp. Mol. Med. 32 (2000) 79–83.
[19] K. Schmidt, R. Gust, M. Jung, Inhibitors of histone deacetylase suppress the growth of MCF-7 breast cancer cells, Arch. Pharm. 332 (1999) 353–357.
[20] V. Medina, B. Edmonds, G.P. Young, R. James, S. Appleton, P.D. Zalewsky, Induction of caspase-protease activity and apoptosis by Butyrate and Trichostatin A (inhibitors of histone deacetylase): dependence on protein synthesis and synergy with a mitochondrial/cytochrome c dependent pathway, Cancer Res. 57 (1997) 3697–3707.
[21] Y. Hoshikawa, H.J. Kwon, M. Yoshida, S. Horinnouchi, T. Beppu, Trichostatin A induces morphological changes and gelsolin expression by inhibiting histone deacetylases in human carcinoma cell lines, Exp. Cell Res. 214 (1994) 189–197.
[22] L. Xi, M.F. Wu, Y.D. Feng, W. Wang, Y.P. Lu, J.F. Zhou, D. Ma, Selective apoptosis in HeLa cells induced by sodium butyrate and its mechanism, ACTA Academiae Medicine Science 25 (2003) 401–405.
[23] Y.K. Kim, J.W. Han, Y.N. Woo, J.K. Chun, J.Y. Yoo, Expression of p21WAF1/Cip1 through Sp1 sites by histone deacetylase inhibitor apicidin requires PI 3-kinase–PKCe signaling pathway, Oncogene 22 (2003) 6023–6031.

[24] W.S. el-Deiry, T. Tokino, V.E. Velculescu, D.B. Levy, R. Parsons, J.M. Trent, D. Lin, W.E. Mercer, K.W. Kinzler, B. Vogelstein, WAF1 a potential mediator of p53 tumor suppression, Cell 75 (1993) 817–825.
[25] S.B. Parker, G. Eichele, P. Zhang, A. Rawls, A.T. Sands, A. Bradley, E.N. Olson, J.W. Harper, S.J. Elledge, p53-independent expression of p21Cip1 in muscle and other terminally differentiating cells, Science 267 (1995) 1024–1027.
[26] M. Takakura, S. Kyo, Y. Sowa, Z. Wang, N. Yatabe, Y. Maida, M. Tanaka, M. Inoue, Telomerase activation by histone deacetylase inhibitor in normal cells, Nucleic Acids Res. 29 (2001) 3006–3011.
[27] W. Fu, J.G. Begley, M.W. Killen, M.P. Mattson, Anti-apoptotic role of telomerase in pheochromocytoma cells, J. Biol. Chem. 274 (1999) 7264–7271.
[28] B. Herbert, A.E. Pitts, S.I. Baker, S.E. Hamilton, W.E. Wright, J.W. Shay, D.R. Corey, Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death, Proc. Natl. Acad. Sci. USA 96 (1999) 14276–14281.
[29] M. Suenaga, H. Soda, M. Oka, A. Yamaguchi, Histone deacetylase inhibitors suppress telomerase reverse transcriptase mRNA expression in prostate cancer cells, Int. J. Cancer 97 (2002) 621–625.
[30] J. Tsurutani, H. Soda, M. Oka, M. Suenaga, S. Doi, Y. Nakamura, K. Nakatomi, K. Shiozawa, Y.Y. Amada, S. Kamihira, S. Kohno, Antiproliferative effects of the histone deactylase inhibitor FR901228 on small-cell lung cancer lines and drug-resistant sublines, Int. J. Cancer 104 (2003) 238–242.
[31] M. Nakamura, H. Saito, H. Ebinuma, K. Wakabayashi, Y. Saito, T. Takagi, N. Nakamoto, H. Ishii, Reduction of telomerase activity in human liver cancer cells by a histone deacetylase inhibitor, J. Cell. Physiol. 187 (2001) 392–401.
[32] M. Hou, X. Wang, N. Popov, A. Zhang, X. Zhao, R. Zhou, A. Zetterberg, M. Bjorkholm, M. Henriksson, A. Gruber, D. Xu, The histone deacetylase inhibitor trichostatin A derepresses the telomerase reverse transcriptase (hTERT) gene in human cells, Exp. Cell Res. 274 (2002) 25–34.
[33] A. Noda, Y. Ning, S.F. Venable, O.M. Pereira, J.R. Smith, Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen, Exp. Cell Res. 211 (1994) 90–98.
[34] K. Polyak, J.Y. Kato, M.J. Solomon, C.J. Sherr, J. Massague, J.M. Roberts, A. Koff, p27Kip1, a cyclin–Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest, Genes Dev. 8 (1994) 9–22.
[35] O. Halevy, B.G. Novitch, D.B. Spicer, S.X. Skapek, J. Rhee, G.J. Hannon, D. Beach, A.B. Lassar, Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD, Science 267 (1995) 1018–1102.
[36] K.J. Wu, C. Grandori, M. Amacker, N. Simon-Vermot, A. Polack, J. Lingner, R. Dalla-Favera, Direct activation of TERT transcription by c-MYC, Nat. Genet. 21 (1999) 220–224.
[37] Y.-S. Cong, J. Wen, S. Bacchetti, The human telomerase catalytic subunit in hTERT: organization of the gene and characterization of the promoter, Hum. Mol. Genet. 8 (1999) 137–142.
[38] Z. Wang, S. Kyo, M. Takakura, M. Tanaka, N. Yatabe, Y. Maida, M. Fujiwara, J. Hayakawa, M. Ohmichi, K. Koike, M. Inoue, Progesterone regulates human telomerase reverse transcriptase gene expression via activation of mitogen-activated protein kinase signaling pathway, Cancer Res. 60 (2000) 5367–5381.
[39] Y.C. Henderson, R.L. Breau, T.J. Liu, G.L. Clayman, Telomerase activity in head and neck tumors after introduction of wild-type p53, p21, p16, and E2F-1 genes by means of recombinant adenovirus, Head Neck 22 (2000) 347–354.
[40] A. Hengstermann, L.K. Linares, A. Ciechanover, N.J. Whitaker, M. Scheffner, Complete switch from Mdm2 to human papillomavirus E6-mediated degradation of p53 in cervical cancer cells, Proc. Natl. Acad. Sci. USA 98 (2001) 1218–1223.