

USP22 transcriptional activity is negatively regulated by the histone deacetylase inhibitor trichostatin A

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Abstract. The ubiquitin-specific protease 22 (*USP22*) gene is overexpressed in the majority of types of cancer cells, and has been implicated in tumorigenesis. However, the mechanisms that regulate its expression remain unclear. The results of the present study demonstrated that the expression of *USP22* is negatively regulated by trichostatin A (TSA), a classical histone deacetylase inhibitor. Furthermore, TSA was revealed to interfere with the binding of RNA polymerase II to the *USP22* promoter, directly suppressing its transcription. In addition, the overexpression of *USP22* was observed to attenuate TSA-induced apoptosis in HeLa cells. To the best of our knowledge, these results provide the first insight into the regulation of the *USP22* gene by antitumor drugs and into the mechanisms underlying the anticancer activity of TSA.

Introduction

The human ubiquitin-specific protease 22 (*USP22*) gene is located on chromosome 17p11.2. Its product is a 525 amino acid protein with a molecular weight of ~60 kDa that functions as a deubiquitinating enzyme *in vivo* and *in vitro* (1). In humans, *USP22* is a subunit of the SAGA coactivator complex that is required for activator-driven transcription. Substrates of *USP22* include the histones H2A and H2B, the shelterin protein TRF1, the transcription factor p53, and FBP1 (2-5). In addition, *USP22* is overexpressed in numerous human tumors and is associated with treatment resistance, tumor aggressiveness and increased metastasis in cancer patients (6,7). However, little is known regarding the transcriptional regulation of *USP22*.

Acetylation is a posttranslational modification that regulates the physiological functions of proteins. Histone acetylases (HAT) and deacetylases (HDACs) regulate the dynamic equilibrium of acetylation (8). HDAC inhibitors have been identified as a novel class of potential drugs for use in cancer therapy, based on observations that the overexpression of HDAC was associated with tumorigenesis (9). The antitumor effects of HDAC inhibitors are due to the transcriptional alteration of specific cancer-related genes, including regulators of the cell cycle, apoptosis, differentiation and invasion (10-12). Therefore, it is hypothesized that HDAC-induced apoptosis may alter *USP22* expression.

The aim of the present study was to determine the effect of the HDAC inhibitor trichostatin A (TSA) on the expression levels of endogenous *USP22* and its promoter activity in human cancer cells. In addition, the mechanisms underlying the anticancer activity of TSA were investigated.

Materials and methods

Cell culture. Human HeLa cells (American Type Culture Collection, Manassas, VA, USA) were used in this study. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco-BRL, Carlsbad, CA, USA) at 37°C with 5% CO₂.

Total RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA extraction and qPCR were performed as described previously (13). Briefly, total RNA was isolated from cells using TRIzol Reagent (Invitrogen), and reverse-transcribed using M-MLV reverse transcriptase. Real-time PCR was performed using SYBR Green PCR Master Mix (Takara Bio, Dalian, China) on an ABI 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The mRNA levels of *USP22* and *GAPDH* were measured with the following specific primers: Forward: 5'-GTGCTTCTTCGGCTGTTTA-3' and reverse: 5'-CTCCTCCTTGCGATTATTT-3' for *USP22*; and forward: 5'-AGAAGGCTGGGGCTCATTTG-3' and reverse: 5'-AGGGCCATCCACAGTCTTC-3' for *GAPDH*. All qPCR results are presented as the mean of at least three independent experiments using duplicate qPCR analysis.

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Western blotting. Whole cell lysates were separated by SDS-PAGE using 10% polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA). After overnight blocking at 4°C in a buffer containing 5% (wt/vol) non-fat milk powder, the membrane was incubated in fresh buffer with the appropriate antibody for 1 h at room temperature. Monoclonal antibodies against USP22 and GAPDH were purchased from Santa Cruz Biotechnology, Inc. (sc-69082 and sc-20358; Dallas, TX, USA). The antigen-antibody complex was detected by incubating the membrane for 1 h at room temperature in buffer containing a 1:10,000 dilution of horseradish peroxidase-conjugated anti-goat IgG secondary antibody (Santa Cruz Biotechnology). Western blots were visualized using an enhanced chemiluminescence system (Pierce Biotechnology, Inc. Rockford, IL, USA).

Plasmid constructs. The USP22 promoters were constructed as described previously (13). To construct CMV-driven USP22 expression vectors, total RNA was isolated from human HeLa cells and reverse-transcribed using the reverse transcriptase M-MLV primed by an oligo (dT)15 primer. The primers used in the subsequent PCR amplification of USP22 cDNA were as follows: Forward: 5'-GAAGCTTATGAGCGACCAAGATCACTCCATG-3' and reverse: 5'-GGAATTCTCAGAAGCATTGCCACTGA-3'. The PCR products were analyzed on agarose gels and digested with *Hind*III and *Eco*RI and ligated into pCMV-His with T4 DNA ligase (Takara Bio). The fidelity of the constructs was confirmed by sequencing. The sequencing primer was TAATACGACTCACTATAGGG.

Luciferase assay. Cells were plated onto 24-well plates at a density of 5×10^4 cells per well and left overnight. This was followed by transfection with promoter reporter plasmid DNA using Lipofectamine 2000 reagent (Invitrogen Life Technologies). The media were changed 24 h later, for medium with or without TSA, and cell lysates were collected at the times indicated for analysis by luciferase assay using a Dual Luciferase Assay kit (Promega, Madison, WI, USA). All transfections were performed in triplicate.

Chromatin immunoprecipitation (ChIP). ChIP was performed as described previously (13). Briefly, cells were fixed by adding formaldehyde to a final concentration of 1% and incubated with moderate shaking for 30 min at room temperature. Thereafter, cells were washed twice with cold phosphate-buffered saline (PBS). The pellet was resuspended and lysed, and nuclei were isolated and sonicated until the chromatin had an average length of 500-1500 bp. Chromatin fragments were immunoprecipitated from cells using rabbit polyclonal antibody against RNA polymerase II (sc-899x; Santa Cruz Biotechnology) DNA samples from the immunoprecipitates and control inputs were analyzed using qPCR with the following primers: forward, 5'-GTCTACCCAGAGCCTAACGG-3', and reverse 5'-GCGGAGGCCGGACAAAGATGGG-3'.

Flow cytometry. Cells (1×10^5) were seeded onto 6-well plates, incubated overnight and transfected with USP22 or an empty vector. The media were changed 24 h later for media containing TSA, and cells were incubated for an additional 24

or 48 h. The cells were then harvested using trypsin, washed with 1X PBS, and resuspended in 50 μ l PBS. Cell death was assessed by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA) following staining with fluorescein isothiocyanate-Annexin V and potassium iodide.

Statistical analysis. Each experiment was performed at least three times independently. The SPSS 13.0 software program (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. The data between two groups was analyzed by a Student's t-test. The data between multiple groups were analyzed by one-way analysis of variance. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

TSA suppresses USP22 expression. To assess the effects of HDAC inhibitors on USP22 expression, HeLa cells were incubated with apoptosis-inducing concentrations of TSA (330 nM) (14) for different time periods, and USP22 mRNA levels were examined by RT-qPCR. As shown in Fig. 1A, the USP22 mRNA expression levels were reduced marginally following a 3 h incubation with TSA and were reduced significantly after 6-12 h, indicating that the suppression is a time-dependent. The effects of TSA on the expression levels of the USP22 protein were determined by western blotting. Consistent with the reduction in USP22 mRNA, TSA downregulated the expression levels of USP22 protein (Fig. 1B), with a significant reduction following 24 h of treatment. In addition, TSA downregulated the expression of USP22 in another cancer cell line, HepG2 (data not shown).

To exclude the possibility that TSA affected the stability of USP22 mRNA, actinomycin D was used to block mRNA transcription, and the levels of USP22 mRNA were assessed with RT-qPCR. When cells were maintained in normal culture conditions, the half-life of USP22 mRNA was ~6 h following the inhibition of transcription. When cells were cotreated with TSA and actinomycin D the half-life of USP22 mRNA was unchanged, indicating that TSA does not affect the stability of USP22 mRNA.

TSA suppresses USP22 promoter activity. Subsequently, luciferase assays were used to assess the effects of TSA on the activity of the USP22 promoter. The 2.8 kb wild-type USP22 promoter P-2828/+52 was transfected into HeLa cells and incubated for 24 h. Following incubation, cells were treated with TSA for different time periods. As shown in Fig. 2A, treatment with TSA reduced the promoter activity in a time-dependent manner, which is consistent with the reduction of the USP22 mRNA expression levels. Subsequently, a series of truncated promoter-reporter gene constructs, derived from p-2828/+52, were transfected into cells to characterize the regulatory sequence that responds to TSA. Notably, the promoter activity of the 5' deletion constructs p-2828/+52, p-1306/+52, p-210/+52, and p-7/+52 and the 3' deletion constructs p-210/+31, p-210/+11 were all reduced by treatment with TSA compared with that of the untreated cells (Fig. 2B). Finally, the activity of a promoter harboring a mutated Sp1 binding site (p-210/sp1mut) located between -7 and -13 was also reduced by 40% following treatment with TSA compared with that of the wild-type promoter p-210/+52 (Fig. 2C). This suggests that the Sp1 binding

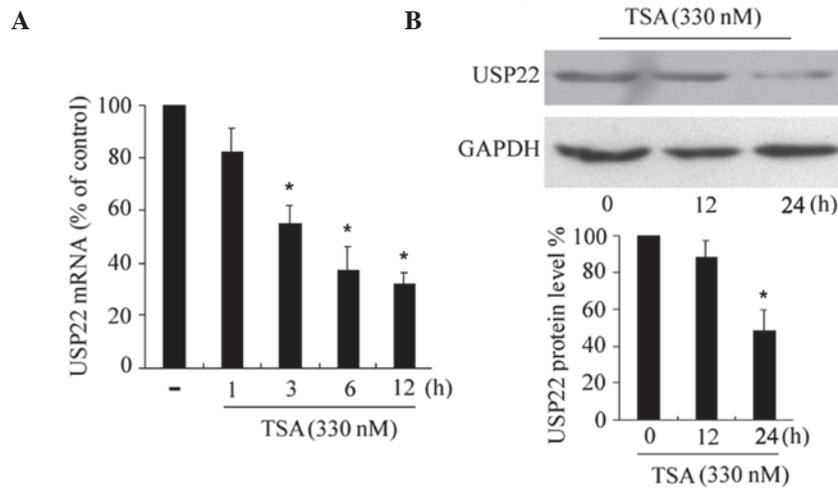


Figure 1. (A) Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of *USP22* mRNA expression in HeLa cells. Cells were treated with 330 nM trichostatin A (TSA) for 3, 6 or 12 h. Cells were harvested and their RNA was isolated, reverse transcribed into cDNA, and the expression levels of *USP22* mRNA were determined by RT-qPCR. Values are presented as the mean \pm standard error of the mean of three independent experiments. (B) Western blot analysis of *USP22* protein levels following treatment with 330 nM TSA for 12 or 24 h. TSA significantly decreased *USP22* protein level after 24 h incubation. * $P < 0.05$, one-way analysis of variance.

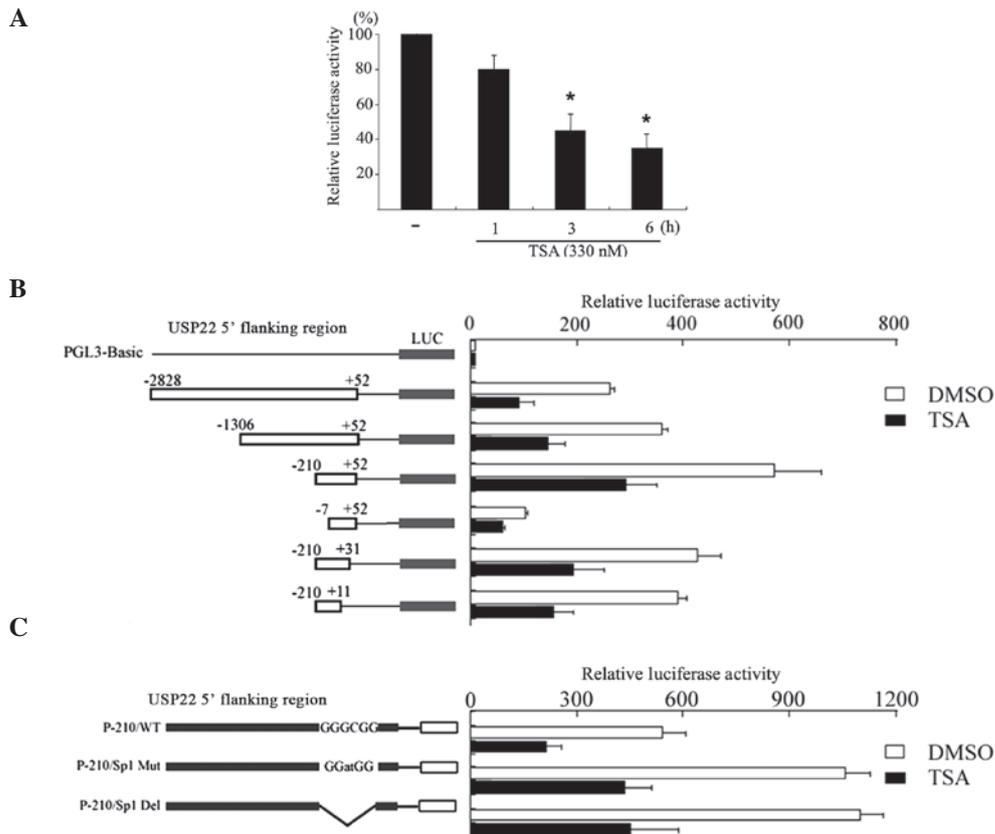


Figure 2. Effects of trichostatin A (TSA) on *USP22* promoter activity. (A) HeLa cells were transfected with the 2.8 kb *USP22* promoter, treated with 330 nM TSA for 1, 3 or 6 h and the luciferase activity was measured. TSA decreased *USP22* promoter activity in a time-dependent manner. (B) HeLa cells were transfected with different constructs of *USP22* promoters, treated with 330 nM TSA for 6 h and the luciferase activity was measured. TSA decreased *USP22* promoter activity in a time-dependent manner. (C) HeLa cells were transfected with p-210/sp1mut, treated with 330 nM TSA for 6 h and the luciferase activity was measured. Sp1 binding site is not involved in the TSA-mediated suppression of *USP22* promoter activity. * $P < 0.05$, one-way analysis of variance.

site is not involved in the TSA-mediated suppression of *USP22* promoter activity.

TSA-induced downregulation of USP22 involves the recruitment of RNA polymerase II. Recent studies have demonstrated

that HDAC activity is required for the recruitment of RNA polymerase II to several specific gene promoters (15). Since no regulatory elements were characterized that were responsive to TSA repression up or downstream of the *USP22* gene transcription start site, the effect of TSA on the recruitment

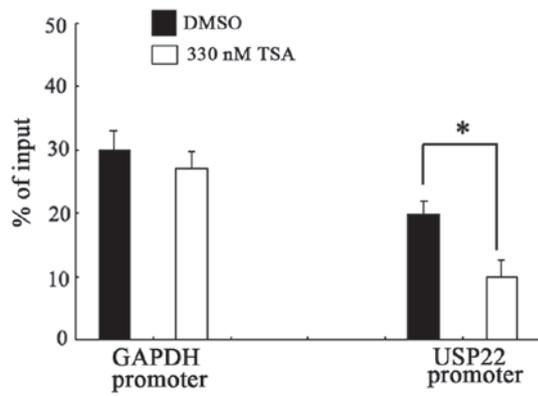


Figure 3. Trichostatin A (TSA) inhibits the recruitment of RNA polymerase II to the *USP22* promoter in HeLa cells. Cells were pretreated with 330 nM TSA for 6 h. Cross-linked DNA-protein complexes were then immunoprecipitated using anti-RNA polymerase II antibodies, and amplified by polymerase chain reaction using specific primers designed for the *USP22* promoter and *GAPDH* promoter regions, containing the transcription start site. The data are representative of three independent experiments. * $P < 0.05$.

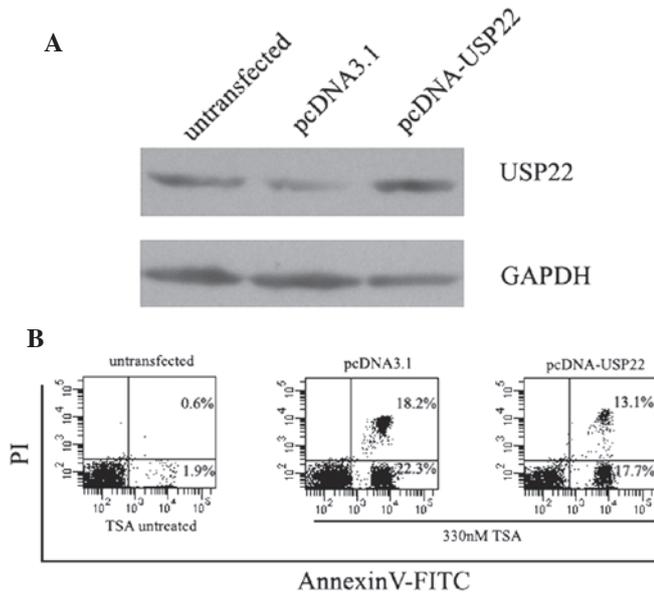


Figure 4. Untransfected HeLa cells and cells transiently transfected with an empty vector or a *USP22* expression plasmid were treated with 330 nM trichostatin A (TSA) for 24 h. Cells were then stained using propidium iodide and fluorescein isothiocyanate-Annexin V, and analyzed by flow cytometry using a BD FACScan flow cytometer.

of RNA polymerase II to the *USP22* promoter was investigated using a ChIP assay. As shown in Fig. 3, TSA treatment significantly blocked the binding of RNA polymerase II to the *USP22* promoter in HeLa cells. This inhibitory effect was not detected with the control promoter region of *GAPDH*, indicating that the inhibition was specific to the *USP22* promoter.

Overexpression of *USP22* attenuates TSA-induced apoptosis. The effect of *USP22* overexpression on TSA-induced apoptosis was investigated. A CMV-driven *USP22* expression vector was generated and used to transiently transfect HeLa cells. As shown in Fig. 4, a significant increase in the expres-

sion levels of *USP22* protein was observed in pcDNA-*USP22* transfected cells, whereas no change was detected in empty vector-transfected cells. As expected, the overexpression of *USP22* significantly decreased TSA-induced apoptosis in HeLa cells (Fig. 4).

Discussion

Recently, research interest in the expression of *USP22* has increased. In embryogenesis, *USP22* is expressed periodically and its depletion may lead to early embryonic mortality. Furthermore, the overexpression of *USP22* has been observed in the majority of types of human cancer cell and has been linked to cancer progression (2,6). Therefore, the reduction of *USP22* expression levels may provide a novel cancer therapy. However, the mechanisms that regulate *USP22* expression, particularly in human tumor cells remain to be elucidated. The results of the current study revealed for the first time that a HDAC inhibitor negatively regulates *USP22* expression.

TSA is a classic HDAC inhibitor that induces apoptosis by altering the expression levels of pro- and anti-apoptotic genes. For example, the expression levels of the cyclin-dependent kinase inhibitor p21 were upregulated during TSA-induced apoptosis (12), whereas the expression levels of survivin, an apoptosis inhibitor, were reduced (11). As *USP22* is an anti-apoptotic protein, it was hypothesized that *USP22* is downregulated by TSA, a hypothesis that was supported by the results of the qPCR and western blot analysis. However, the mechanisms that regulate TSA-induced gene silencing are complex, and not completely understood. Certain studies have indicated that TSA reduces gene expression at the transcriptional level (10), whereas others reported that TSA suppresses gene expression by affecting mRNA stability (16). An additional study revealed that TSA may lead to protein degradation (17). In the present study, TSA inhibited *USP22* expression at the transcription level, and did not affect the half-life of *USP22* mRNA. The TSA-mediated suppression of *USP22* was an early, time-dependent event. These results are supported by a previous study that determined that *USP22* transcription is regulated by extracellular signals (18).

Since TSA suppressed the expression levels of *USP22* mRNA, one of the aims of the current study was to characterize the specific elements in the *USP22* promoter that were targeted by TSA. Although several regulatory elements were found in the *USP22* promoter, no DNA element up- or downstream of the transcription start site was responsive to TSA, including the Sp1 binding site that was identified close to the *USP22* transcription start site. A previous study suggested that Sp1 is important in HDAC inhibitor-induced apoptosis (19). Sp1 is a transcription factor that is acetylated in the DNA binding domain by CBP/p300 and subsequently modulates transcriptional activity and influences protein-protein interactions (20). Therefore, it was originally hypothesized that TSA-stimulated suppression of *USP22* expression was mediated by this site. However, promoter deletion and mutagenesis analyses showed that the Sp1 binding site was not involved in TSA-induced *USP22* downregulation.

TSA responsive regions were not identified up- or downstream of the *USP22* transcription start site, indicating that the key region that mediates TSA-induced *USP22* suppres-

sion may be located close to the transcription initiation site. A previous study suggested that histone deacetylase activity is required to recruit RNA polymerase II to promoters, thus affecting gene transcription (15). Therefore, interfering with RNA polymerase recruitment is one mechanism by which HDAC inhibitors stimulate gene silencing (21). In the present study, ChIP analysis revealed that TSA blocked the recruitment of RNA polymerase II, indicating that TSA blocks the formation of the preinitiation complex at the *USP22* promoter. Nevertheless, the mechanism by which HDAC activity affected the binding of RNA polymerase II to the *USP22* promoter requires further investigation.

To confirm the anti-apoptotic role of TSA treatment, a *USP22* expression vector driven by a CMV promoter was produced. Since the activity of the CMV promoter increases marginally in response to TSA treatment (22), it was possible to obtain *USP22* over-expressing HeLa cells following treatment with TSA. The overexpression of *USP22* attenuates TSA-induced apoptosis, indicating that *USP22* has an important role in HDAC inhibitor-induced apoptotic signaling. Although the mechanism by which *USP22* attenuates TSA-induced apoptosis is not yet fully understood, the regulation of apoptosis-related genes by *USP22* is a possible route. Indeed, several key genes in TSA-induced apoptosis, such as the cyclin-dependent kinase inhibitor p21 WAF1, are also modulated by *USP22*. *p21* is a downstream target gene of *USP22*, and downregulation of *USP22* promoted p21 expression. By contrast, p21 is upregulated during TSA-induced apoptosis. Therefore, the suppression of *USP22* expression by TSA may regulate p21 expression. In addition, p53 may also participate in the TSA-*USP22* pathway as it has been linked to TSA-induced apoptosis. Whilst *USP22*-mediated stabilization of SIRT1 has been shown to lead to a reduction in the levels of p53 acetylation and the suppression of p53-mediated apoptosis (4), contradictory studies have reported that the activity and stability of SIRT1 are not affected by *USP22* (23). These contrasting results could be due to cell-specific effects. A greater understanding of the mechanisms by which *USP22* regulates gene transcription may identify additional molecules that are involved in TSA-induced apoptosis.

In conclusion, the present study revealed that the expression of *USP22* is negatively regulated by TSA. Furthermore, it was determined that this negative regulation occurred via interference of the binding between RNA polymerase II and the *USP22* promoter. In addition, the exogenous overexpression of *USP22* attenuated TSA-induced apoptosis. These results indicate that blocking *USP22* expression at the transcriptional level by TSA may provide a novel strategy for cancer therapy.

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