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Trichostatin A, a histone deacetylase inhibitor, reverses epithelial–mesenchymal transition in colorectal cancer SW480 and prostate cancer PC3 cells

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ABSTRACT

Trichostatin A (TSA) is a kind of classical histone deacetylase (HDAC) inhibitor. In this study, we reported the reversal effects of TSA on EMT and investigated the possible involved molecular mechanisms in SW480 and PC3 cells. Firstly, we observed that TSA induced the reversal process of epithelial–mesenchymal transition (EMT) in SW480 and PC3 cells, resulting in attenuated cell invasion and migration abilities. TSA-induced EMT reversal was characterized by up-regulation of E-cadherin and down-regulation of Vimentin. Then, treatment with TSA also decreased the expression of transcription factor Slug. Furthermore, over-expression of Slug significantly caused down-regulation of E-cadherin and up-regulation of Vimentin. Meanwhile, TSA treatment in Slug-expressing cells could prevent these changes. These findings suggested that Slug played a crucial role in TSA-induced EMT reversal. Additionally, the study showed that TSA could induce the increase of HDAC1 and HDAC2 on the Slug gene promoter, which might be responsible for the suppression of Slug. Overall, TSA could reverse EMT in SW480 and PC3 cells and TSA-mediated down-regulation of Slug was involved in the reversal process.

1. Introduction

Histone deacetylase (HDAC) inhibitors are now considered to be promising anti-cancer agents and some of them are near clinical stage or already on the market. Their effects are correlated with the transcriptional regulation of specific cancer-related genes [1]. Trichostatin A (TSA) is the representative of four kinds of classical HDAC inhibitors, which inhibits HDACs in a noncompetitive and reversible way. TSA could inhibit proliferation and induce differentiation in a variety of cancer cells such as colorectal, prostate, neuroblastoma and skin cancer cells [2–5]. And its molecular mechanisms for inhibiting proliferation and inducing differentiation have been studied a lot and relatively well known. Also, some studies have observed TSA could suppress invasion and migration in prostate cancer cells [6]. But the underlying molecular mechanisms are rarely studied and still not clear.

Epithelial–mesenchymal transition (EMT) importantly contributes to invasion and migration of tumor cells [7,8]. The indeed clinical evidence reveal that regulators of EMT in tumor cells are closely associated with poor outcomes and tumor aggressiveness [9,10]. However, at the invasive front of many metastatic cancers, EMT occurs as reversible process that is possible to prevent [11]. EMT and its reversal process are two relative processes which could mutually transform. The cell polarity in these relative processes are decided by the expression levels of EMT related biomarkers such as E-cadherin, Vimentin, N-cadherin and Fibronectin [12]. Down-regulation of E-cadherin and up-regulation of Vimentin have been regarded as the significant symbol of EMT [12,13]. Thus, the expression levels of E-cadherin and Vimentin importantly reflect the mesenchymal and epithelial cellular states.

A group of transcription factors, including Slug, Snail, ZEB1 and Twist have been involved in regulation of EMT [14]. Slug is a zinc-finger transcription factor belonging to the Snail superfamily of transcription factors [15]. It binds to E-box elements at the promoter of the E-cadherin gene and consequently results in repressing endogenous E-cadherin expression. Activation of Slug is closely related to down-regulation of E-cadherin [16,17]. In addition, Slug

Abbreviations: TSA, trichostatin A; EMT, epithelial–mesenchymal transition; HDAC, histone deacetylase.

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Table 1
Primers used in this study.

Gene	Forward primer 5′–3′	Reverse primer 5′–3′	Application
E-cadherin	TACACTGCCCAGGAGCCAGA	TGGCACCAGTGTCCGGATTA	Real-time PCR
Vimentin	TGAGTACCGGAGACAGGTGCAG	TAGCAGCTTCAACGGCAAAGTTC	Real-time PCR
Slug	TTCGGACCCACACATTACCT	GCAGTGAGGGCAAGAAAAAG	Real-time PCR
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA	Real-time PCR
Slug^P	GTCCGTCTGCCGCACCTGAG	ACACGGCGGTCCCTACAGCA	CHIP assay

Slug^P: primers for Slug promoter.

could also increase Vimentin expression to induce EMT [18]. These findings highlight the importance of Slug in initiation of EMT. Thus, the suppression of Slug becomes significant to reverse EMT and overcome invasion and migration in tumor cells.

On the basis of the introduction above, we supposed EMT reversal were the crucial factors by which TSA exerted anti-invasion and anti-metastasis effects. Therefore, the TSA-mediated changes of E-cadherin, Vimentin, Slug and the possible involvement in regulation of EMT were investigated in this study.

2. Materials and methods

2.1. Chemicals and reagents

TSA was purchased from Sigma–Aldrich (Deisenhofen, Germany). Antibodies against E-cadherin, Vimentin, β-actin, GAPDH and the horseradish peroxidase (HRP)-conjugated secondary antibody were products of Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Antibodies against Slug, HDAC1, HDAC2, HDAC3 and HDAC4 were purchased from Cell Signaling Technology (Beverly, MA). PrimeScript RT reagent Kit and SYBR Premix Ex Taq™ were products of TaKaRa BIO Inc. (TBI, Japan). Alexa Fluor 488 conjugated secondary antibody, DAPI and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Cell culture and cytotoxicity test

SW480 and PC3 carcinoma cell lines were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Under a humidified 5% CO_2 atmosphere and at 37 °C incubator, cells were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (HyClone Laboratories, Logan, UT) and 1% penicillin/streptomycin (Invitrogen). Cytotoxicity of TSA on SW480 and PC3 cells was determined by the MTS assay (Promega, Madison, WI) according to the manufacturer's instruction.

2.3. Transwell invasion and migration assay

The polycarbonate filters (8 μm pore size, Corning) were coated with Matrigel Matrix (BD Biosciences), dried, and reconstituted at 37 °C with appropriate RPMI 1640 for invasion assay, and uncoated filters were used for migration assay. Then cells (1×10^5) in 300 μl medium containing 1% FBS were seeded to the upper chamber, while 600 μl medium was added to the lower chamber in RPMI 1640 containing 10% FBS. Cells were treated with or without TSA for 48 h and the drug concentration was the same in the lower and upper chambers. For invasion assay, the cells in the upper chamber were fixed in 4% paraformaldehyde for 20 min. After removing the matrigel with a cotton swab mechanically, the cells adhering to the under-side of the filter were stained with DAPI (10 μg/ml) and counted under upright fluorescent microscope (5 fields per chamber). For migration assay, the cells migrated and adhered onto the lower chamber were also fixed in 4% paraformaldehyde for 20 min, then stained with hematoxylin and counted under upright microscope (5 fields per chamber). Each invasion and migration assay was repeated in three independent experiments.

2.4. Quantitative real-time PCR

Cells plated on 6-well plates were treated with TSA for 48 h. Then, total mRNA of the cells was extracted and first strand cDNA synthesis was generated from 500 ng of total RNA. Quantification of target and reference (GAPDH) genes was carried out in triplicate on LightCyclerH 480 II (Roche, Applied Science). The relative amount of mRNA was normalized to the expression of GAPDH. Primer sequences are shown in the Table 1 [19].

2.5. Western blotting analysis

Protein lysates collected from cells were separated by SDS–PAGE and transferred onto PVDF membranes. The membranes were incubated with the primary antibodies (1:1000 dilution) overnight at 4 °C, and then incubated with the appropriate HRP-conjugated secondary antibodies (1:5000 dilution) for 2 h at room temperature. The detection of β-actin was used as a loading control [19].

2.6. Confocal microscopy

After 12 h of cultivation on chamber slides, cells were stimulated with TSA for 48 h. Cells were fixed in 4% paraformaldehyde for 30 min and blocked with goat serum for 30 min at 37 °C, and then incubated with anti-E-cadherin antibody at 1:100 for 1 h at 37 °C. Cells were washed with PBS and incubated with Alexa Fluor 488-conjugated secondary antibodies at 1:1000 for 45 min at 37 °C. After washed by PBS, the cells were stained with DAPI (10 μg/ml) for 15 min to visualize cell nuclei. Samples were examined with Confocal Laser Scanning Microscopy (Zeiss, Germany) to analyze expression of E-cadherin on cell membrane.

2.7. Gene over-expression

The cells were seeded on a 6-well plate (2×10^5 cells/well) and cultured for 12 h. Then they were transfected with 2 μg pcDNA-Slug or control vector pcDNA-3.1 mixed with lipofectamine 2000 reagent in serum-free medium according to the manufacturer's instructions. Medium was replaced by complete culture medium 6 h later, and then the cells were incubated at 37 °C in a CO_2 incubator.

2.8. CHIP assay

SimpleChIPH Enzymatic Chromatin IP Kit (Magnetic Beads) and 6-Tube Magnetic Separation Rack purchased from Cell Signaling Technology were used for performing CHIP assay according to the manufacturer's instruction. Briefly, cells were treated with TSA or DMSO control for 48 h and 37% formaldehyde was

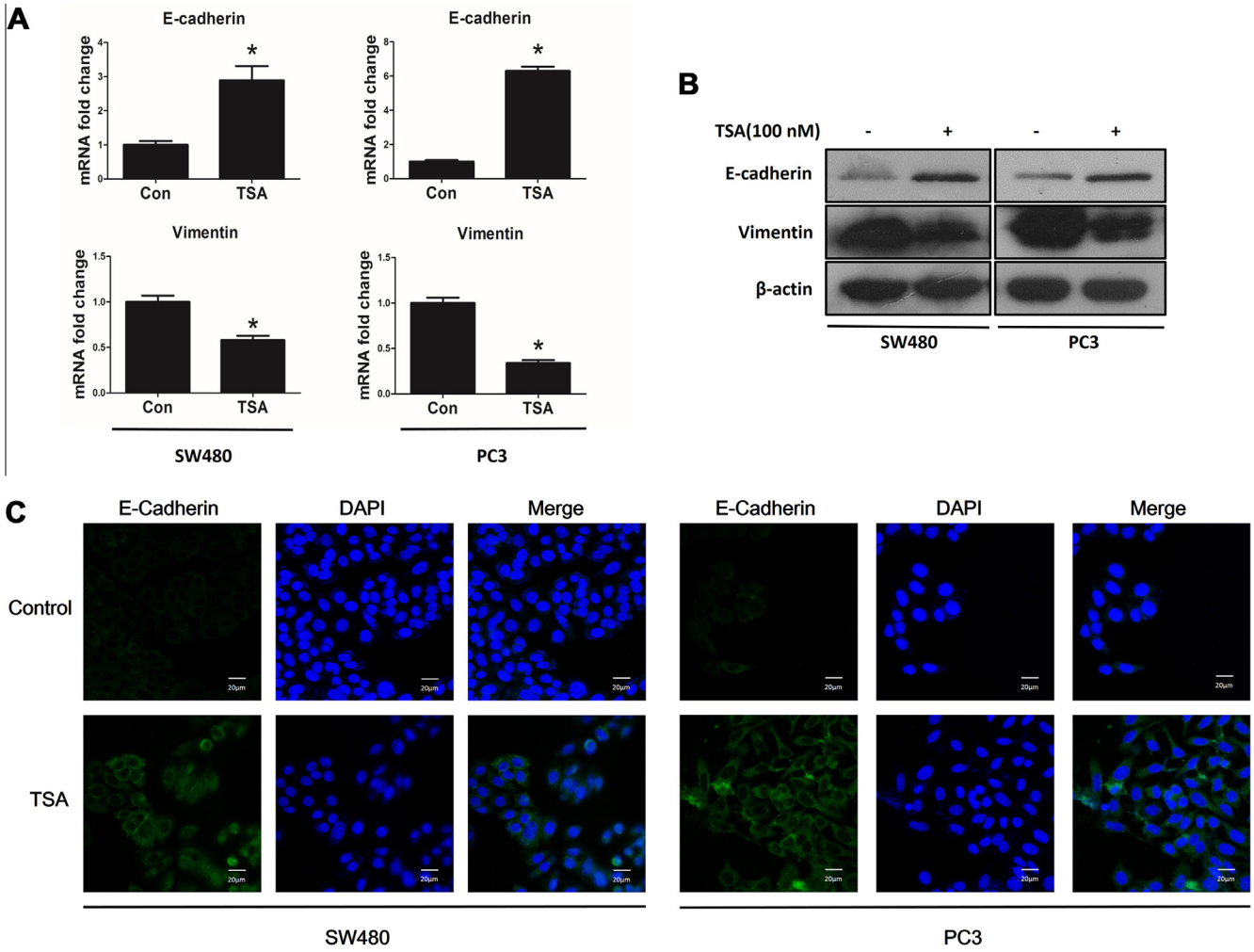

Fig. 1. Treatment with TSA reversed EMT in SW480 and PC3 cells. (A and B) SW480 and PC3 cells were treated with or without TSA (100 nM) for 48 h. The mRNA (A) and protein levels (B) of E-cadherin, Vimentin were analyzed by quantitative real-time PCR and Western blotting. $^{*}p < 0.05$ compared with control. (C) SW480 and PC3 cells were treated with or without TSA (100 nM) for 48 h. After fixation, the cellular location of E-cadherin (green) was examined by immunofluorescence staining and nuclei were stained with DAPI (blue). Bar, 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

added (Final formaldehyde concentration was 1%) to crosslink proteins to DNA and mixed for 10 min at room temperature. Glycine was added into dishes for 5 min at room temperature. Following washing with ice-cold PBS, the nuclear extracts from the cells were prepared. To prepare cross-linked chromatin, the DNA suspension was digest to length of approximately 150–900 bp by Micrococcal Nuclease and this nuclease was inactivated by EDTA, and then sonicated with several pulses to break nuclear membrane. After purification, DNA fragment size was validated by electrophoresis and it turned out that DNA fragment size ranged from 150 bp length to 900 bp length approximately. For chromatin immunoprecipitation, 15 mg of chromatin DNA for each immunoprecipitation in CHIP buffer was incubated with the immune precipitating antibody: HDAC1, HDAC2, HDAC3, HDAC4, and the negative control (Normal Rabbit IgG) at 4 °C for overnight. After that, 30 ml of ChIP Grade Protein G Magnetic Beads were added for 2 h at 4 °C. By incubating with Proteinase K in 2 h at 65 °C, Chromatin was eluted from Antibody/Protein G Beads and cross-links was reversed. DNA was purified by Spin Columns. Quantification of DNA was carried out by PCR using specific primers (Table 1). The relative amount of promoter DNA was normalized to the input and IgG was the reference control and calculated as unit value of 1.0.

2.9. Statistical analysis

Values were expressed as Mean ± SD of three independent experiments unless otherwise specified. Data were analyzed by two-tailed unpaired Student's *t*-test between any two groups. One-way ANOVA analysis of variance was used to assess the difference of means among groups. These analyses were performed using GraphPad Prism Software Version 5.0 (GraphPad Software Inc., La Jolla, CA). A *p*-value of <0.05 was considered statistically significant.

3. Results

3.1. TSA reversed EMT in SW480 and PC3 cells

As mentioned in the introduction, expression levels of E-cadherin and Vimentin importantly reflect the mesenchymal and epithelial cellular states. Therefore, in SW480 and PC3 cells, we determined the expression of E-cadherin, Vimentin after treatment with TSA in both mRNA and protein levels. As shown in Fig. 1A and B, TSA led to increase of E-cadherin and decrease of Vimentin in mRNA (Fig. 1A) and protein levels (Fig. 1B). Since E-cadherin was a kind of membrane protein, the expression of E-cadherin was fur-

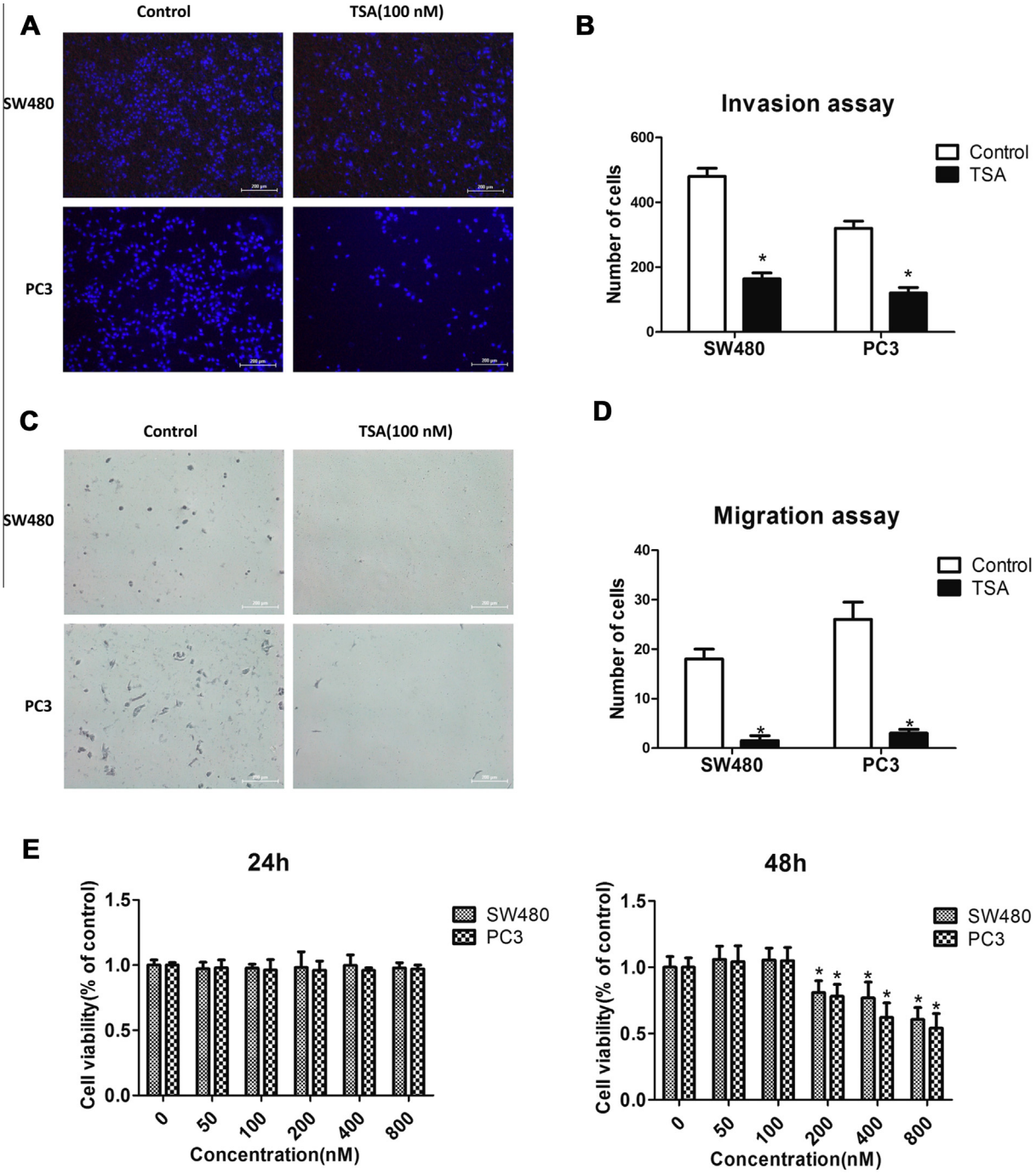

Fig. 2. TSA attenuated invasion and migration of SW480 and PC3 cells. (A) After treatment with or without TSA (100 nM) for 48 h, SW480 and PC3 cells which had spread through the matrix gel and into the under-side of the filter were fixed, stained with DAPI (10 μg/ml), and photographed. Bar, 200 μm. (B) The number of invasive cells. Data represented the average of three independent experiments. $^{*}p < 0.05$ compared with control. (C) SW480 and PC3 cells were allowed to migrate transwell chambers for 48 h in the presence or absence of TSA (100 nM). After 48 h, the migrated cells were fixed, stained, and photographed. Bar, 200 μm. (D) The number of migrated cells. Data represented the average of three independent experiments. $^{*}p < 0.05$ compared with control. (E) SW480 and PC3 cells were seeded at a density of 5×10^3/well in 96 well plates and treated with various concentrations of TSA for 24 h or 48 h. Cell viability was determined by MTS assay. $^{*}p < 0.05$ compared with control.

ther detected by confocal microscopy. Visually, the up-regulation of E-cadherin was observed on the cellular membrane by TSA treatment (Fig. 1C). These results suggested that TSA could reverse EMT in SW480 and PC3 cells.

3.2. TSA attenuated invasion and migration of SW480 and PC3 cells

Usually the attenuated invasive and migratory abilities of tumor cells were the functional reflection of the reversion process of EMT. Therefore, we detected the changes of invasion and migration abilities in SW480 and PC3 cells by transwell invasion and migration assay. The results revealed that after treatment with TSA, the invasive cells that had spread through the filter and adhered the underside (Fig. 2A) decreased significantly, as well as the migrated cells in the lower chamber (Fig. 2C). The numbers of invasive and migrated cells were counted in Fig. 2B and D. Meanwhile, we determined the anti-proliferative effects of TSA. As a result, treatment with TSA suppressed the growth of the SW480 and PC3 cells

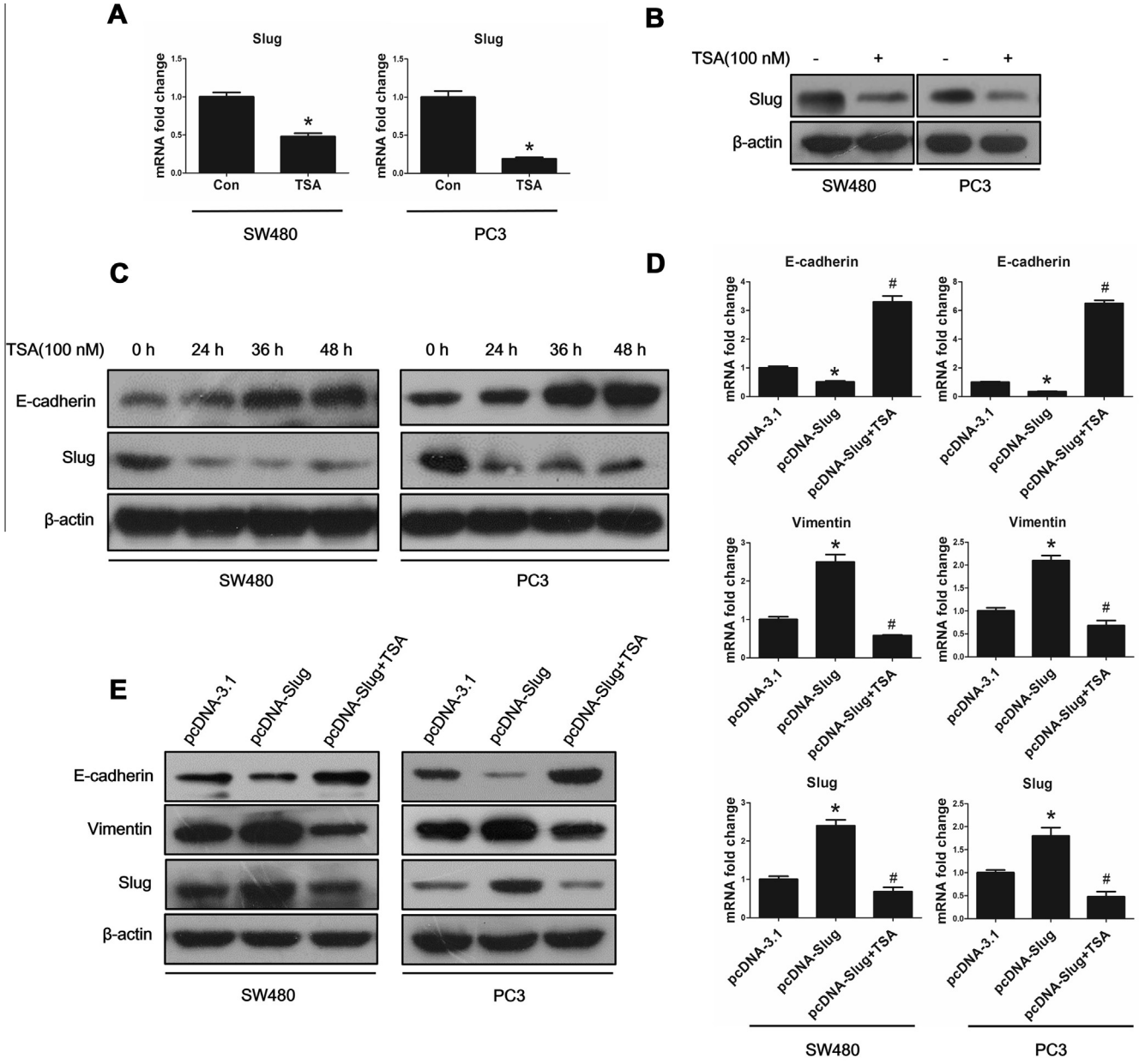

Fig. 3. Slug suppressed by TSA was involved in reversing EMT. (A and B) SW480 and PC3 cells were treated with or without TSA (100 nM) for 48 h. The mRNA (A) and protein levels (B) of Slug were analyzed by quantitative real-time PCR and Western blotting. $^{*}p < 0.05$ compared with control. (C) SW480 and PC3 cells were treated with or without TSA (100 nM) for 24–48 h. E-cadherin and Slug protein were detected by Western blotting. (D and E) pcDNA-3.1 and pcDNA-Slug were expressed in SW480, PC3 cells for 12 h, then cells with pcDNA-Slug were treated with or without TSA (100 nM) for additional 48 h. The mRNA (D) and protein levels (E) expression of E-cadherin, Vimentin, Slug were examined by quantitative real-time PCR and Western blotting. $^{*}p < 0.05$ compared with PcDNA-3.1, $^{\#}p < 0.05$ compared with pcDNA-Slug.

with similar efficacies and 100 nM TSA showed no inhibitory activity on cell proliferation within 48 h (Fig. 2E), suggesting that the decreased invasive and migrated cells by TSA were not due to its anti-proliferation effects. Collectively, these findings showed that TSA could attenuate the invasion and migration of SW480 and PC3 cells.

3.3. Suppression of Slug by TSA was involved in reversing EMT

Slug has been regarded as a crucial transcription factor in EMT regulation. We examined the expression of Slug in SW480 and PC3 cells after treatment with TSA. As expected, TSA significantly suppressed Slug in mRNA and protein levels (Fig. 3A and B). Then we investigated TSA-induced suppressive effects of Slug in EMT regulation. After treatment with TSA for 24–48 h, the changes of E-cadherin and Slug protein were detected by Western blotting. As shown in Fig. 3C, the E-cadherin protein significantly increased after treatment with TSA for 36 h whereas the Slug protein obviously decreased after treatment with TSA for 24 h, which was earlier than the changes of E-cadherin protein. These observations implied that TSA-mediated down-regulation of Slug might act as a molecular incentive and organizer to the EMT reversal. To further validated, cells were transfected with pcDNA-Slug and the expression of E-cadherin, Vimentin and Slug were detected by q-PCR and Western blotting. In mRNA and protein levels, Slug, Vimentin were up-regulated and E-cadherin was down-regulated in SW480 and PC3 cells after being transfected with pcDNA-Slug (Fig. 3D and E). It suggested a successful over-expression of Slug could

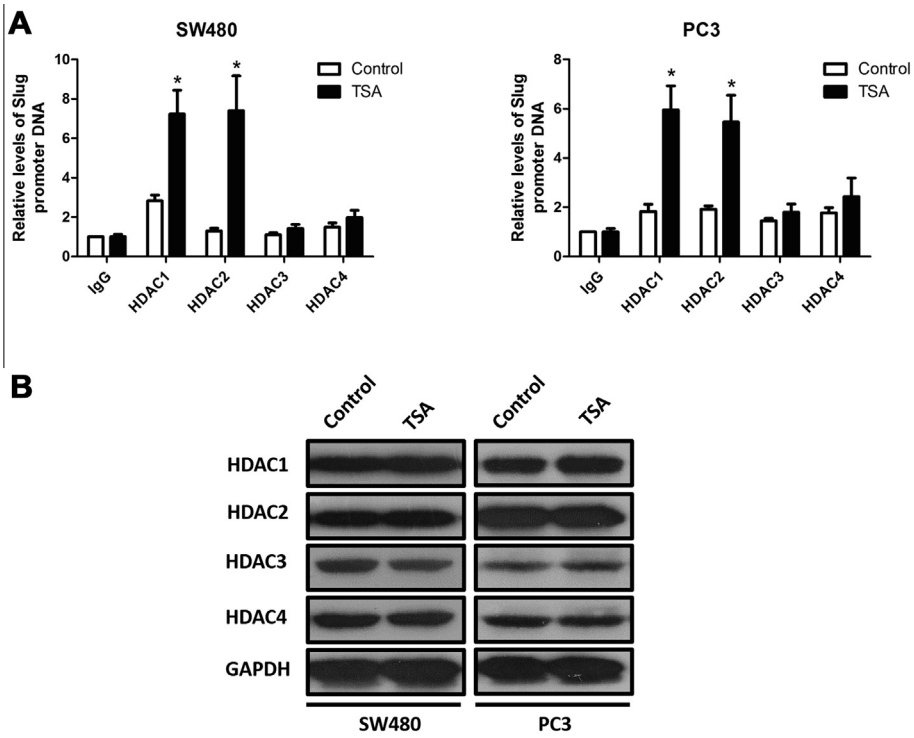

Fig. 4. TSA induced increase of HDAC1 and HDAC2 on Slug gene promoter. (A) SW480 and PC3 cells were treated with or without TSA (100 nM) for 48 h, CHIP assay was conducted to determine the amount of HDACs on the Slug gene promoter. *$p < 0.05$ compared with control. (B) SW480 and PC3 cells were treated with TSA (100 nM) for 48 h, and subsequently HDAC1, HDAC2, HDAC3, HDAC4 were detected by Western blotting.

induce down-regulation of E-cadherin and up-regulation of Vimentin, which implied a phenotypic transition of epithelial to mesenchymal. Meanwhile, TSA treatment on Slug-expressing cells could reverse these changes (Fig. 3D and E). These results revealed that TSA-induced suppression of Slug was involved in reversing EMT.

3.4. TSA induced increase of HDAC1 and HDAC2 on Slug genes promoter

We had demonstrated that TSA significantly down-regulated the mRNA expression of Slug (Fig. 3A), which was more likely a transcriptional regulatory process. Since HDACs could create a non-permissive chromatin conformation that prevented the transcription of genes, the increase of HDACs on the gene promoter may be a kind of way to suppress gene transcription. We performed CHIP assay to detect the changes of HDACs on the promoter of Slug gene. As shown in Fig. 4A, after treatment with TSA for 48 h, the amount of HDAC1 and HDAC2, but not HDAC3 and HDAC4, associated with proximal promoters of Slug gene increased significantly in SW480 and PC3 cells compared to control cells. Meanwhile, in Fig. 4B, treatment with TSA for 48 h did not show any changes in the total amount of HDACs. These results suggested that TSA specifically induced the increase of HDAC1 and HDAC2 on promoters of Slug, which might be mechanistically responsible for suppression of Slug.

4. Discussion

As a classical HDAC inhibitor, TSA has been studied a lot to promote the development of HDAC inhibitors. Some studies reported that TSA could reverse EMT in non-tumor cells. In human renal tubular epithelial cells, TSA could suppress tubular EMT by inducing several inhibitory factors of TGF-β1 signals, such as Id2 and BMP-7 [20]. In hepatocytes, TSA abrogated TGF-β1-induced EMT and reversed fibrosis by epigenetic modulation of type I collagen [21]. Besides, TSA strongly prevented TGF-β2 induced morphological changes and the up-regulation of a-SMA, collagen type I, collagen type IV, Fibronectin, Snail and Slug in retinal pigment epithelium cells [22]. In this study, we observed that TSA could reverse EMT and consequently suppressed invasion and migration in SW480 and PC3 cells. And we also indicated TSA-mediated down-regulation of Slug closely involved in EMT reversal. These findings firstly extent TSA-induced reversion effects of EMT from non-tumor cells to tumor cells and enrich the mechanisms involved.

E-cadherin and Vimentin are significant EMT biomarkers and also perform their functions to suppress or promote invasion and migration in tumor cells. E-cadherin is an epithelial cell adhesion molecule connecting to cells' degree of epitheliality whereas Vimentin acts as a mesenchymal marker most commonly associated with facilitating EMT [12,13]. Therefore, expression of E-cadherin and Vimentin not only reflect the mesenchymal and epithelial cellular states but also influence the invasion and migration abilities of tumor cells. Our data suggested that TSA could induce the EMT reversal (Fig. 1) and the suppression effects of invasion and migration (Fig. 2) in tumor cells, which were the most direct effects against progression of malignant tumors. It would provide a theoretical basis for a reasonable guide of HDAC inhibitors in anti-tumor clinical use.

Transcriptional alterations are the main mechanisms responsible for EMT regulation in tumor cells. Transcription factors closely associated with EMT are classified into two groups according to their effects on E-cadherin. Slug, Snail, Zeb, E47 and KLF8 factors bind to and repress the activity of the E-cadherin promoter directly, whereas factors such as Twist, Goosecoid, E2.2 and FoxC2 repress E-cadherin transcription indirectly [14]. In this study, TSA-mediated suppression of Slug was closely involved in reversing

EMT in SW480 and PC3 cells (Fig. 3), but these results did not exclude the possibility that TSA could influence other regulation approaches to reverse EMT. Anyhow, considerable laboratory evidence indicated that Slug closely contribute to initial of EMT in colorectal, prostate, bladder, nasopharyngeal and lung cancer cells [23–27] and clinical evidence showed that Slug plays an important role in the invasion and metastasis and overexpression of Slug are associated with poor overall survival [28,29]. Given this, Slug might be an important target of HDAC inhibitors exerting anti-tumor effect in clinical cancer therapy.

By removing acetyl groups from histones, HDACs could create a non-permissive chromatin conformation that prevent the gene transcription. Further study found that the amount of HDAC1 and HDAC2 binding to Slug gene promoter was higher in TSA treated cells (Fig. 4), which might be responsible for TSA-induced suppression of Slug. However, HDAC inhibitors usually have direct effects on histone deacetylases and elevate the acetylation level of histones to regulate target gene expression. It is rarely reported that HDAC inhibitors could induce the increase of HDACs on the target gene promote, which might be an indirect and complex process, and more attentions should be paid on these similar effects.

In summary, we here demonstrated more molecular evidence of TSA for anti-cancer effects. The results showed that EMT was prevented by TSA and TSA-induced down-regulation of Slug was involved in reversing EMT in SW480 and PC3 cells. In addition, TSA induced increase of HDAC1 and HDAC2 binding to the promoter of Slug gene, which might contribute to the down-regulation of Slug. Though the precise mechanism remains undetermined, these findings will help to better understand the molecular mechanisms for anti-cancer potentials of HDAC inhibitors.

Acknowledgments

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