Trichostatin A induces mesenchymal-like morphological change and gene expression but inhibits migration and colony formation in human cancer cells

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Abstract. Histone deacetylases (HDACs) are enzymes that catalyze the removal of acetyl from lysine residues in histones and other proteins, which results in gene transcriptional repression and subsequent changes in signaling events. HDACs inhibitors (HDACIs) have been used to reverse the aberrant epigenetic changes associated with cancer. However, the effects of HDACIs on epithelial-mesenchymal transition (EMT) in human cancer cells remain unclear. EMT is a fundamental process governing morphogenesis in multicellular organisms and promotes cancer invasion and metastasis. In this study, human cancer cells were treated with the HDACI trichostatin A (TSA). TSA was found to induce mesenchymal-like morphological changes in BGC-823 human gastric cancer and MCF-7 breast cancer cells, and increase the expression levels of the mesenchymal markers Vimentin and Twist. However, the expression levels of the epithelial cell marker E-cadherin were also increased in response to TSA treatment, while cell migration was reduced by TSA. Furthermore, TSA decreased cancer cell colony formation in BGC-823 and MCF-7 cells, and led to the deregulation of β-catenin, a critical signaling molecule involved in EMT. In conclusion, the results suggested that TSA exhibits dual functions in EMT induction and inhibition in human cancer cells, but the detailed mechanisms require further investigation.

Introduction

Cancer is one of the most common causes of fatality and up to 90% of cancer-associated mortality results from metastasis; however, the underlying mechanisms of metastasis remain poorly understood (1). The initial steps of local invasion include the activation of signaling pathways that control cytoskeletal dynamics in tumor cells, and the turnover of cell matrix and cell-cell junctions, followed by tumor cell migration into the adjacent tissue (2).

Epithelial-to-mesenchymal transition (EMT) is a key step during embryonic morphogenesis, but is also involved in the progression of primary tumors toward metastasis. The EMT phenotype is distinguished by the loss of cell-to-cell adhesion together with the disassembly of tight, adherens and gap junctions, and phenotypic changes in the cells from an epithelial to a motile, fibroblast-like morphology (3). Invasion and metastasis, mediated by EMT, are largely determined by the loss of E-cadherin functionality, as E-cadherin is critical for the maintenance of adherent junctions between neighboring cells and it preserves physical integrity in epithelial cells (4). In addition, during tumorigenesis, certain epithelial cells develop the ability of self-renewal, a trait associated with cancer stem cells, and undergo EMT to facilitate cancer metastasis and recurrence (5).

Histone acetylation and deacetylation are important in the modulation of chromatin topology and the regulation of gene transcription. Histone deacetylase inhibitors (HDACIs) are novel anticancer drugs, which induce histone (hyper-) acetylation and counteract aberrant gene repression. HDACI treatment may also result in the inhibition of tumor cell proliferation, in culture and *in vivo*, by inducing cell cycle arrest, differentiation and/or apoptosis. HDACIs have been shown to induce non-histone protein acetylation, which alters signaling networks relevant for tumorigenesis (6). Several HDACIs have been used in phase I and II clinical trials for the treatment of a number of hematological malignancies and solid tumors (7).

Previous studies have suggested that HDACIs act as EMT suppressors in normal epithelial and cancer cells (8,9). However, HDACIs have also been reported to induce significant morphological changes, resembling EMT in prostate cancer cells, in addition to the ability to upregulate the expression of mesenchymal cell markers, such as Vimentin, Snail, Slug and Twist (10). These findings provoke controversy as to whether HDACIs are EMT suppressors or inducers, and the implications of this for cancer therapy. To investigate these

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Abbreviations: TSA, trichostatin A; EMT, epithelial-mesenchymal transition; HDAC, histone deacetylase; HDACI, HDAC inhibitor; HAT, histone acetyltransferase

Key words: TSA, EMT, migration, cancer cells

questions, a panel of human cancer cells was treated with TSA and its effect on cell morphology, molecular expression and migration ability was evaluated.

Materials and methods

Cell culture and reagents. The BGC-823 human gastric cancer cell line was purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Science, Chinese Academy of Sciences (Shanghai, China). The MCF-7 human breast cancer and KYSE-510 human esophageal squamous cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained in RPMI 1640 medium containing penicillin (100 U/ml), streptomycin (100 mg/ml) and 10% fetal bovine serum at 37˚C in a humidified incubator supplemented with 5% CO_2 in air. Cell morphology was observed with an Olympus IX71 inverted microscope (Olympus Corporation, Tokyo, Japan). TSA was purchased from Beyotime Institute of Biotechnology (Haimen, China) and dissolved in dimethylsulfoxide (6.62 mm) for storage. The TSA was diluted to the appropriate concentrations with phosphate-buffered saline (PBS) prior to use. The primary antibodies used in this study were a rabbit monoclonal antibody to E-cadherin (Cell Signaling Technology, Inc., Danvers, MA, USA), a goat polyclonal antibody to β-catenin (Santa Cruz Biotechnology, Inc., Dalls, TX, USA) and a mouse monoclonal antibody to β-actin (Abmart, Shanghai, China). The secondary antibodies were HRP-conjugated anti-rabbit, mouse or goat secondary antibodies (Cell Signaling Technology, Inc.).

Immunocytochemistry assay. The cells were seeded on 96-well plates and allowed to adhere overnight. Following treatment with TSA for 24 h, the cells were washed with cold PBS and fixed in 4% paraformaldehyde for 15 min, followed by permeabilization in 0.1% Triton X-100 for 5 min. The cells were then blocked with 5% goat serum for 30 min, followed by incubation with primary antibody overnight at 4˚C. Following careful washing, the cells were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature, then stained with 3,3'-diaminobenzidine subsequent to additional thorough washing.

RNA isolation and quantitative polymerase chain reaction (qPCR). Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and quantified by spectrophotometric measurement (Molecular Devices, Sunnyvale, CA, USA). RNA (2 μg) was reverse-transcribed to cDNA using the RevertAid first strand cDNA synthesis kit (Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer's instructions. qPCR analysis was performed using SYBR Green-based detection on a StepOnePlus real-time PCR instrument (Applied Biosystems, Inc., Foster City, CA, USA). The relative mRNA expression levels of the genes of interest were normalized to those of GAPDH and calculated using the $2^{-\Delta\Delta Ct}$ method. The following primers were used: Sense: 5'-GAC CGC ACA CAG CAA GGC GAT-3' and antisense: 5'-GCT GTC CCG CCG ATT GAG GG-3' for Vimentin; sense: 5'-TCA GCA GGG CCG GAG ACC TA-3' and antisense: 5'-TCC ACG GGC CTG TCT CGC TT-3' for Twist; sense: 5'-CGC CCC CAT ACC AGA ACC TCG-3' and antisense: 5'-GTC CAG TTG GCA CTC GCC CC-3' for E-cadherin; sense: 5'-TGG TGC CCA GGG AGA ACC CC-3' and antisense: 5'-TGT CAC CTG GAG GCA GCC CA-3' for β-catenin; and sense: 5'-CTG GGC TAC ACT GAG CAC C-3' and antisense: 5'-AAG TGG TCG TTG AGG GCA ATG-3' for GAPDH.

Western blot analysis. To prepare the whole-cell extract, the cells were washed with cold PBS once and harvested by scraping in radioimmunoprecipitation assay lysis buffer. The protein content was determined by the Bradford assay (Beyotime Institute of Biotechnology, Shanghai, China). The extracted proteins were separated by SDS polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Beyotime Institute of Biotechnolgy, Haimen, China). The membranes were first blocked with 5% (w/v) non-fat dry milk in Tris-buffered saline with Tween-20 and then probed with the indicated primary antibodies with gentle agitation at 4˚C overnight. Subsequent to washing four times, the membranes were incubated with HRP-conjugated secondary antibodies 1 h. The signals were detected using an enhanced chemiluminescence detection kit (Thermo Fisher Scientific).

Wound-healing assay. A total of $1x10^6$ cells were plated on 6-well plates. When the monolayer reached ~70% confluence, a scrape was inflicted using sterile pipette tips. The wound-healing rate was evaluated by capturing images of the cells after 24 h with an Olympus IX71 inverted microscope (Olympus Corporation).

Colony forming assay. The cells were seeded on 96-well plates at 10 cells per well and allowed to adhere overnight. Fresh medium either with or without 200 nm TSA was then added to each well. After three days, the numbers of colonies in each well with a diameter >100 μm were counted using an an Olympus IX71 inverted microscope (Olympus Corporation).

Statistical analysis. The data are presented as the mean ± standard error of the mean. Statistical analyses were performed using Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

TSA induces mesenchymal-like morphological changes in human cancer cells. TSA is known as a potent HDAC inhibitor, which binds to the zinc-containing catalytic domain of the class I and II HDACs, including HDACs 1 to 10 (11). To determine whether TSA induces EMT in cancer cells, BGC-823 human gastric cancer cells were treated with different concentrations of TSA. Untreated BGC-823 cells exhibited a cobblestone-like epithelial morphology. As shown in Fig. 1A, after 24 h treatment with 200 nm TSA, the cells exhibited a spindle-like shape similar to mesenchymal cell characteristics, but 50 nm TSA did not exert these effects (Fig. 1A). TSA also stimulated mesenchymal-like morphological changes in MCF-7 human breast cancer cells in a concentration-dependent

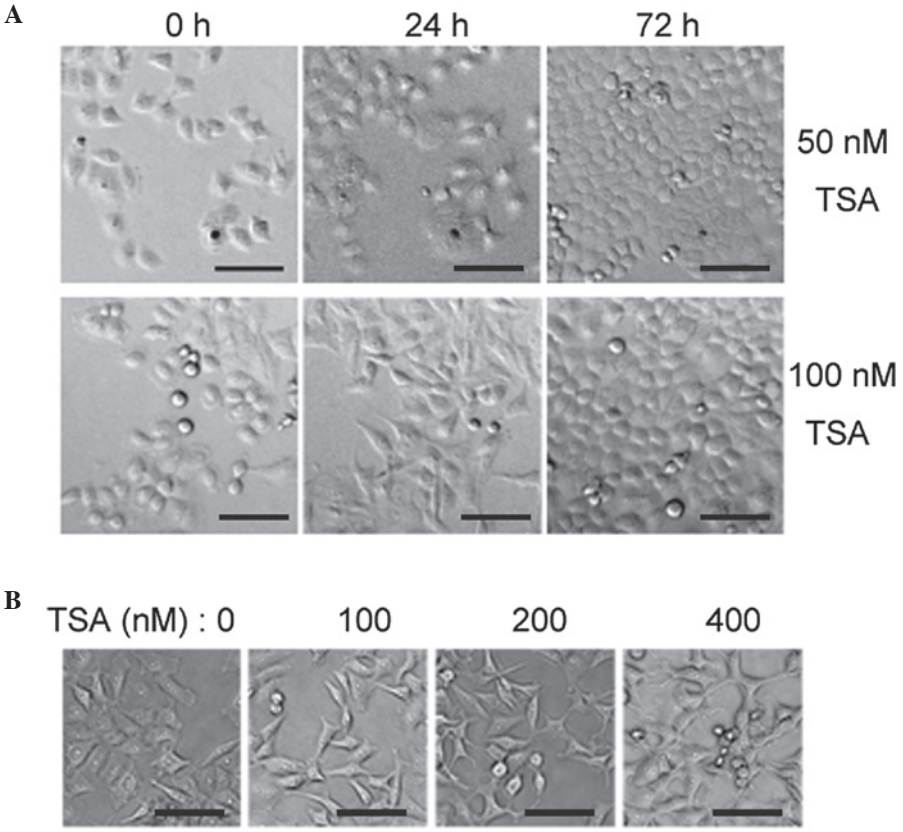

Figure 1. Trichostatin A (TSA) induced mesenchymal-like morphological changes in BGC-823 human gastric cancer cells and MCF-7 human breast cancer cells. (A) BGC-823 cells were treated with 50 or 200 nm TSA for the indicated time periods. (B) MCF-7 cells were treated with 0, 100, 200 and 400 nm TSA for 24 h. Bars indicate 50 μm.

manner (Fig. 1B). Following three days culture with 200 nM TSA, BGC-823 and MCF-7 cells all reached ~100% confluence and the mesenchymal cell morphology disappeared, indicating that 200 nM TSA induced no marked cytotoxicity in BGC-823 or MCF-7 cells (Fig. 1A).

TSA increases the expression levels of mesenchymal markers and E-cadherin. To confirm whether TSA functions as an inducer of EMT, the effect of TSA on the expression levels of mesenchymal and epithelial markers was determined by qPCR. As shown in Fig. 2A, 200 nm TSA treatment significantly upregulated Vimentin and Twist mRNA transcription in BGC-823 cells. In MCF-7 cells, the Vimentin mRNA expression levels were increased but the Twist expression levels were marginally reduced in response to TSA treatment (Fig. 2B). However, the E-cadherin transcription levels were significantly increased in the two cell lines following TSA treatment (Fig. 2A and B). Immunocytochemistry and western blotting assays further confirmed that TSA induced E-cadherin expression (Fig. 2C-E). The KYSE-510 human esophageal squamous cancer cells exhibited no marked morphological change in response to TSA treatment (Fig. 2F); however, E-cadherin expression levels were increased following TSA treatment, indicating that the upregulation of E-cadherin was independent of TSA-induced mesenchymal-like cytoskeleton remolding (Fig. 2G).

TSA treatment reduces cancer cell mobility. The cytoskeletal remodeling during EMT induces spindle-like cancer cell morphology, which facilitates cell mobility. A wound-healing assay was performed to examine the effect of TSA on cancer cell migratory ability. As shown in Fig. 3A and B, 200 nm TSA attenuated the wound-healing process in BGC-823 and MCF-7 cells.

TSA treatment reduces cancer cell colony formation. Cells that have undergone EMT have been demonstrated to be the source of cancer stem-like cells (5). High colony-forming efficiency is one of the hallmarks of cancer stem cells. It was determined whether TSA enhances the cancer cell colony-forming ability. Fig. 4 indicates that cancer cell colony formation was significantly inhibited in the TSA treatment groups compared with the control groups. Since BGC-823 cells are able to achieve confluency in 200 nm TSA (Fig. 1A), the reduced colony-forming ability in BGC-823 and MCF-7 was not due to the cytotoxic effect of TSA.

β-catenin is inhibited by TSA treatment. Diverse extra- and intracellular signals, including those of the Wnt/β-catenin signaling pathway, have been reported to induce and/or maintain EMT in various cell types (12). In the present study, β-catenin mRNA expression levels were found to be significantly upregulated in BGC-823 and MCF-7 cells following TSA treatment (Fig. 5A). However, β-catenin protein expression levels in BGC-823 cells were reduced following TSA treatment (Fig. 5B), suggesting that TSA is involved in either translational or post-translational regulation of β-catenin expression.

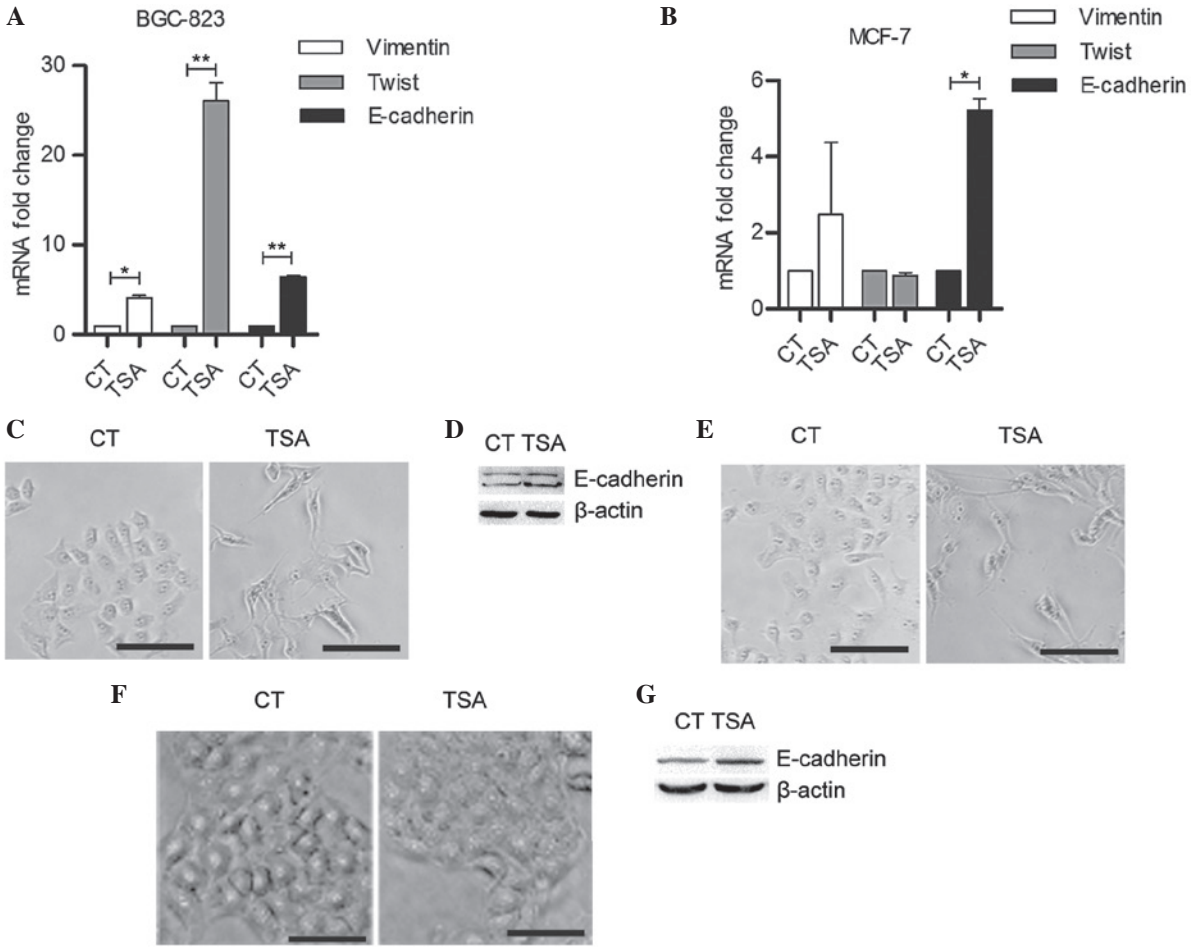

Figure 2. Trichostatin A (TSA) induced changes in the expression levels of mesenchymal and epithelial cell markers in cancer cells. (A) TSA induced an increased transcription of Vimentin, Twist and E-cadherin in BGC-823 human gastric cancer cells, assayed by reverse transcription polymerase chain reaction (RT-PCR). *P<0.01 and **P<0.001. (B) TSA induced increased transcription of Vimentin, Twist and E-cadherin in MCF-7 human breast cancer cells, assayed by RT-PCR. *P<0.01 and **P<0.001. (C-E) TSA enhanced the protein levels of E-cadherin in (C and D) BGC-823 cells and (E) MCF-7 cells. (F) KYSE-510 human esophageal squamous cancer cells exhibited no morphological changes following 200 nm TSA treatment. (G) TSA induced increased E-cadherin expression in KYSE-510 cells. Bars indicate 50 μm. CT, control group.

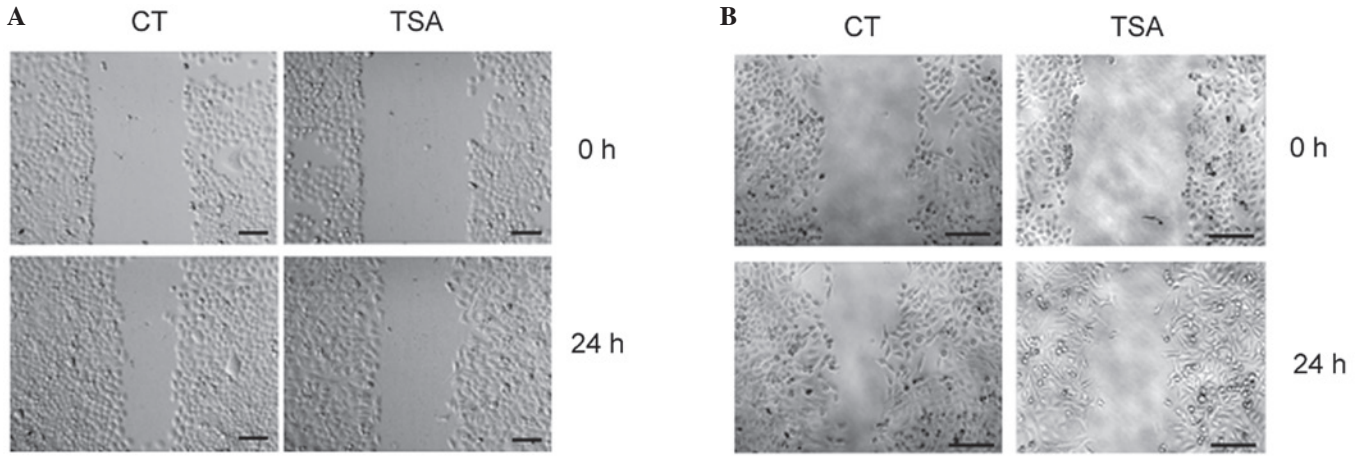

Figure 3. Trichostatin A (TSA) reduced cancer cell mobility. The wound-healing process was attenuated in (A) BGC-823 human gastric cells and (B) MCF-7 human breast cancer cells following TSA treatment. Bars indicate 100 μm. CT, control group.

Discussion

The status of histone acetylation is dependent on the balance between histone acetyltransferase (HAT) and HDAC activity. The opposing activity of HATs and HDACs tightly regulate gene expression through chromatin modification (13). HATs

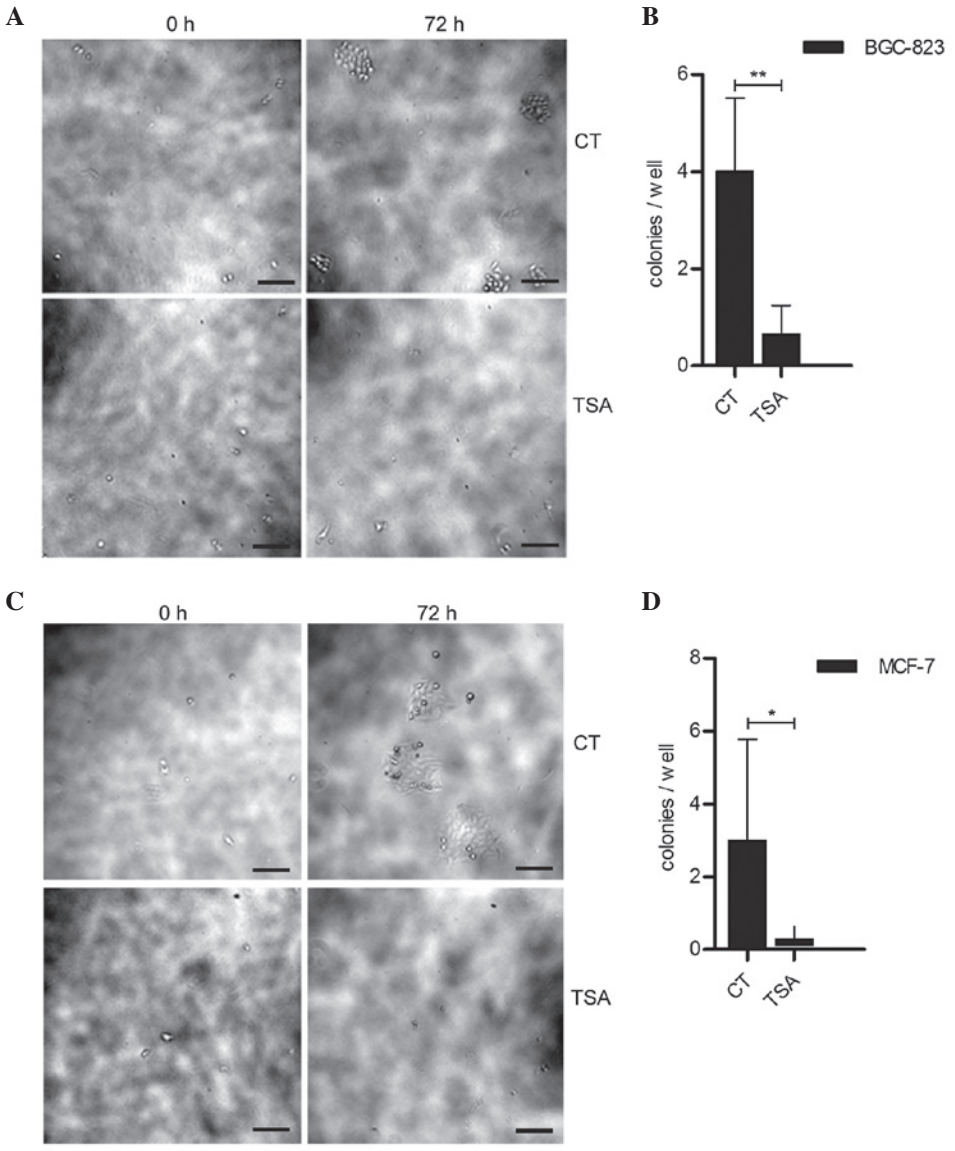

Figure 4. Trichostatin A (TSA) reduced cancer cell colony-forming ability. The number of colonies with diameters >100 μm was reduced in (A and B) BGC-823 human gastric cancer cells and in (C and D) MCF-7 human breast cancer cells following TSA treatment. Bars indicate 100 μm. CT, control group. *P<0.01 and **P<0.001.

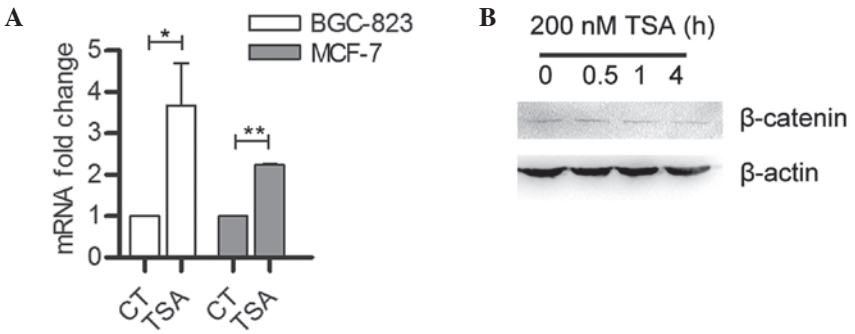

Figure 5. Effects of Trichostatin A (TSA) on β-catenin mRNA and protein expression levels. TSA (A) increased β-catenin mRNA expression levels in BGC-823 human gastric cancer and MCF-7 human breast cancer cells, (*P<0.01 and **P<0.001), but (B) reduced β-catenin protein expression levels in the BGC-823 cells. CT, control group.

transfer acetyl groups to N-terminal lysine residues in histones, which induces increases in local chromatin and elevates accessibility of regulatory proteins to DNA, whereas HDACs catalyze the removal of acetyl groups, resulting in chromatin condensation and transcriptional repression. The identification of increased HDAC expression levels

and activity in cancer tissues has resulted in the rational design of HDACIs as potential therapeutic agents for cancer therapy (11,14).

Studies have demonstrated that HDACIs increase E-cadherin expression levels and reverse EMT in normal human cells and human cancer cells (8,9). By contrast, other studies have shown that HDACIs are cytoskeleton remodelers, and induce mesenchymal markers and molecules mediating drug resistance, cell mobility and self-renewal, supporting the hypothesis that HDACIs act as EMT inducers in cancer cells (10). In the present study, TSA was found to induce epithelial-mesenchymal morphological transition in BGC-823 and MCF-7 cells in a dose-dependent manner (Fig. 1). TSA treatment enhanced the transcription levels of the mesenchymal markers Vimentin and Twist, but also induced the expression of E-cadherin in BGC-823, MCF-7 and KYSE-510 cells (Fig. 2). Loss of the epithelial homotypic adhesion molecule E-cadherin is a hallmark of EMT (4). E-cadherin is required for the formation of stable adherens junctions, and reduced expression levels of E-cadherin have been reported in various cancer cells, being associated with tumor progression and metastasis (15). In the present study, in accordance with the increased expression levels of E-cadherin, TSA treatment was found to reduce cell migration in the wound-healing assay (Fig. 3). Furthermore, the administration of TSA significantly reduced colony formation in BGC-823 and MCF-7 cells (Fig. 4). These results contradict those of previous studies that suggest that TSA is an inducer of EMT in human cancer cells (10).

Certain non-histone proteins are also targets of HDAC-catalyzed acetylation with varying functional effects. For example, the gene regulatory activity of transcription factors, such as p53 and nuclear factor-κB, is modulated through direct acetylation and deacetylation by HATs and HDACs (16-17). The Wnt/β-catenin signaling pathway is critical for spontaneous or induced EMT in cancer cells (12). In an unstimulated cell, β-catenin is phosphorylated and subsequently degraded in the adenomatous polyposis coli/Axis inhibitor/glycogen synthase kinase 3 complex. In the presence of stimuli, such as induction by Wnt ligand, β-catenin phosphorylation and degradation is inhibited. The accumulating β-catenin translocates to the nucleus, where it interacts with lymphoid enhancer/T cell transcription factors and regulates the expression of target genes, including c-myc and cyclin D1. The activity of β-catenin is tightly regulated by various modifications, including phosphorylation, acetylation and ubiquitination (18,19). In the present study, although TSA enhanced the transcription level of β-catenin in BGC-823 and MCF-7 cells, the protein levels were reduced following TSA treatment (Fig. 5), which was consistent with previous studies reporting that HDAC6 is required for β-catenin activation in colon cancer cells (20,21). Whether TSA-mediated EMT induction and inhibition, elevation of β-catenin mRNA levels and inhibition of β-catenin protein levels are associated with other signaling pathways, such as the decorin-β-catenin-E-cadherin signaling pathway (22), remains unclear and is currently under investigation.

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