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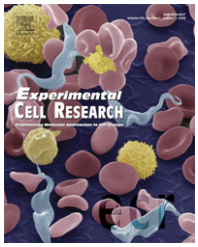

Research Article

# Histone deacetylase inhibitor-induced cellular apoptosis involves stanniocalcin-1 activation

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ABSTRACT

Our previous studies have demonstrated the involvement of HIF-1 and p53 in the regulation of stanniocalcin-1 (STC1) gene transcription in human cancer cells. In this study, we reported that the treatment of human colon adenoma HT29 cells with a histone deacetylase (HDAC) inhibitor (i.e. trichostatin A, TSA) induced both cellular apoptosis and STC1 expression. The activation of STC1 expression was also observed in other TSA-treated human cancer cells (i.e. SKOV3, CaCo-2, Jurkat and CNE-2 cells). STC1 mRNA was rapidly induced within 4 h in TSA-treated HT29 cells, and was found to be transcriptionally regulated and was independent of new protein synthesis as revealed by ActD and CHX treatment respectively. The induction was correlated with increased cellular levels of acetyl histone H3 and H4 and acetyl NFκB. Chromatin immunoprecipitation (ChIP) assay showed the increased binding of acetyl histone H3 and H4 to STC1 promoter in the TSA-treated cells. A cotreatment of HT29 cells with a NFκB inhibitor (parthenolide) significantly inhibited the TSA-induced cellular levels of acetyl NFκB p65 and abolished the stimulation of STC1 gene expression. ChIP assay also demonstrated that TSA treatment increased while TSA/parthenolide cotreatment decreased NFκB p65 binding to STC1 gene promoter. In the STC1-luciferase promoter construct (1 kb) study, the data implied that the promoter can be activated by TSA treatment. Interestingly, the promoter region contains 2 putative NFκB binding sites. Consistent with the STC1 mRNA expression data, TSA/parthenolide cotreatment also significantly inhibited the TSA-induced STC1 promoter-driven luciferase activity. Importantly, TSA-induced apoptotic process was found to be significantly reduced by the silencing of STC1 expression. This is the first study to show that histone hyper-acetylation and the recruitment of activated NFκB stimulated STC1 gene expression. In addition, our results support the notion that STC1 is a pro-apoptotic factor.



## Introduction

The putative tumor suppressor gene, stanniocalcin-1 (STC1) is differentially expressed in tumors as compared to surrounding normal tissues [1]. This putative role is supported by a high rate of loss of heterozygosity at the chromosomal loci at 8p11.2–p12 in many cancer cases [2,3], whereas STC1 is localized at 8p11.2–p21 [4]. In addition, the presence of CAG repeats in the non-coding regions of STC1 may relate to genetic instability and transcriptional silencing [5]. Despite its putative tumor suppressor function, little is known about the roles of STC1 in human malignancies. Recent evidence has highlighted that STC1 expression is markedly increased in hypoxia, a common phenomenon that occurs in regions of most solid human tumors in the latter stages of carcinogenesis [6]. In addition, STC1 has been proposed to be a putative pro-apoptotic factor in the regulation of programmed cell death [7] and was found to be regulated by p53 [8]. Current evidence implies the possible role of STC1 in cell death and survival mechanism.

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It is well-known that cell death activation is critical in cancer therapy. Histone deacetylase (HDAC) inhibitors are a new class of drugs for cancer treatment [9]. They are known to promote histone and non-histone protein acetylation, which is an essential post-translational process in regulating chromatin topology, gene transcription and intracellular signaling [10]. Treatment of tumor cells with HDAC inhibitors has been shown to induce transcriptional activation of genes that are essential for the inhibition of cell growth, metastasis, and angiogenesis but induce apoptosis [9]. It has been reported that less than 10% of expressed gene can be modulated in cells treated with HDAC inhibitors [9]. Despite the putative pro-apoptotic function of STC1 is proposed [11], the role of HDAC inhibitor on STC1 expression in human cancer cells is not known. Therefore, it would be of great interest to elucidate the molecular mechanism governing by HDAC inhibitors on the regulation of STC1 gene expression.

In this study, we aimed to evaluate and decipher the effect of HDAC inhibitors, (i.e. trichostatin A (TSA), apicidin and sodium butyrate) on STC1 expression in different human cancer cells. Using the HT29 cell model, we reported the first evidence that the hyper-acetylation of the STC1 promoter region and the recruitment of chromatin associated non-histone protein, NFκB, were involved in the activation of STC1 gene expression. The silencing of STC1 gene expression significantly reduced the population of TSA-induced apoptotic cells, implying the possible role of STC1 as a pro-apoptotic factor.

## Materials and methods

### *Human cancer cells lines*

Four human cancer cell lines obtained from ATCC, including tumors of ovary (SKOV3), colon (HT29, CaCo-2), lymphoblastic leukemia (Jurkat), and the CNE-2 nasopharyngeal carcinoma cells were used. The cells were grown and maintained in their respective growth media supplemented with 10% FBS. The cells were treated with 500 nM of TSA (Calbiochem) for 24 h. Total RNA was then extracted with TRIZOL (Invitrogen) and was subjected to cDNA synthesis and real-time PCR assay.

### *Cell culture and treatments*

HT29, human colon adenocarcinoma cells were grown in McCoy5A supplemented with 10% FBS (HyClone®, Perbio) and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) (Invitrogen) with at a density of $5\times10^4$ cells per well in 12-well plates (Falcon). The cells were incubated in 5% $CO_2$ at 37 °C. After overnight incubation, the cells were exposed for 2, 4, 8 and 24 h to 500 nM of trichostatin A (TSA, Calbiochem). Total RNA was extracted, reverse-transcribed and STC1 mRNA levels were measured by real-time PCR. In the time course study, maximum induction of STC1 mRNA was detected at 8 h of post-treatment. To test the dose-dependent action of TSA, the cells were treated with 100, 200 and 500 nM of TSA for 8 h. Among those, the cells treated with 500 nM of TSA produced the highest response in STC1 mRNA induction. In addition to TSA, two other histone deacetylase inhibitors (apicidin (5 μM) and sodium butyrate (1 mM), Calbiochem) were used for the treatment. Cell lysates were also collected for Western blot.

HT-29 cells were seeded at a density of $5\times10^4$ cells/well in 12-well plates, grown overnight, then incubated with one of the following treatments: (a) 500 nM of TSA, (b) 1 μM actinomycin D (ActD, Calbiochem), (c) 2 μg/ml of cycloheximide (CHX, Calbiochem), (d) TSA + ActD, or (e) TSA + CHX. After incubation for the designed time, total RNA were collected for real-time PCR assay.

### *Annexin V assay*

HT29 cells were seeded at a density of $2\times10^5$ cells/well in 6-well plates (Falcon), grown overnight, and then treated with 500 nM TSA. After treatment for 24 h, adherent and floating cells were collected by trypsinization and centrifuged at 1000 ×*g* for 5 min. Cell pellets were resuspended and incubated in the complete medium for 20 min. After centrifugation, cell pellets were washed with PBS and resuspended in 100 μl of Annexin-V-FLUOS labeling solution (Roche Applied Science) as previously described [8]. The percentage of cells with annexin $V^+$/propidium iodide $(PI)^-$ was measured by flow cytometer (BD, FACSCalibur).

### *Effect of p53, Sp1, or NFκB inhibitors to STC1 mRNA expression*

HT-29 cells seeded at a density of $5\times10^4$ cells/well in 12-well plates were treated with one of the following treatments: (a) 500 nM TSA, (b) pifithrin-α (20 μM, an inhibitor for p53 signaling) (Sigma), (c) mithramycin (300 nM, an inhibitor for Sp1 binding) (Sigma), (d) parthenolide (10 μM, an inhibitor for NFκB) (Calbiochem), (e) TSA + pifithrin-α, (f) TSA + mithramycin or (g) TSA + parthenolide for 4 and 8 h. The cells were pre-incubated with the respective inhibitors for 30 min before the addition of TSA. Total RNA and protein lysates were collected for real-time PCR and Western blot analysis. Some of the treated cells were prepared for CHIP assay.

### *Chromatin immunoprecipitation*

ChIP assay was conducted using the ChIP assay kit according to the manufacturer's instruction (Upstate) and rabbit antibody against acetyl histone H3 (Lys9/Lys14), acetyl histone H4 (Lys8) or total NFκB p65 (Cell Signaling Technology®). The immunoprecipitated chromatin was analyzed in triplicate by PCR using the primers (TGGCTCACCAGACCAGTTGA-forward and CTTTCCCTCTCCTGGCTTGA-reverse) for human STC1 promoter.

### *RNA extraction, PCR product verification and real-time PCR*

Cells were dissolved in TRIZOL Reagent. Total RNA was extracted according to the manufacturer's instructions. The RNA $A_{260}/A_{280}$ ratios were between 1.6 and 1.8. The primers for human STC1 [CACACCCACGAGCTGACTTC—forward and TCTCCCTGGTTATGCACTCTCA—reverse], and actin [GACTACCTCATGAAGATCCTCACC—forward and TCTCCTTAATGTCACGCA CGATT—reverse] were used as described in our previous study [6,8]. Briefly, cDNA was synthesized from 1 μg of total cellular RNA using the iScript™ cDNA synthesis kit (Bio-Rad Pacific Ltd). Quantitated standards ($10^4$–$10^8$) and sample cDNAs were analyzed with the iCycler iQ real-time PCR detection system using iQ™ SYBR® Green Supermix (Bio-Rad Pacific Ltd). The copy number for each sample was calculated and all the data were normalized to actin. The PCR conditions were 95 °C for 3 min and 40 cycles of 95 °C for 30 s, 56 °C for 30 s and