Synergistic effect of p53 on TSA-induced stanniocalcin 1 expression in human nasopharyngeal carcinoma cells, CNE2

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Abstract

Human stanniocalcin 1 (STC1) has recently been identified as a putative protein factor involved in cellular apoptosis. The use of histone deacetylase inhibitor (i.e. trichostatin A (TSA)) and doxorubicin (Dox) is one of the common treatment methods to induce apoptosis in human cancer cells. A study on TSA and Dox-mediated apoptosis may shed light on the regulation and function of STC1 in cancer treatment. In this study, TSA and Dox cotreatment in human nasopharyngeal carcinoma cells (CNE2) elicited synergistic effects on *STC1* gene expression and cellular apoptosis. An activation of p53 (TP53) transcriptional activity in Dox- or Dox + TSA-treated cells was revealed by the increased expression levels of p53 mRNA/protein as well as p53-driven luciferase activities. To elucidate the possible involvement of p53 in *STC1* gene transcription, a vector expressing wild-type or dominant negative (DN) p53 was transiently transfected into the cells. Both *STC1* promoter luciferase constructs and chromatin immunoprecipitation assays did not support the direct role of p53 in *STC1* gene transactivation. However, the synergistic effects of p53 on the induction of NF- κ B phosphorylation and the recruitment of acetylated histone H3 in *STC1* promoter were observed in TSA-cotreated cells. The overexpression of exogenous STC1 sensitized apoptosis in Dox-treated cells. Taken together, this study provides data to show the cross talk of NF- κ B, p53, and histone protein in the regulation of *STC1* expression and function.

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Introduction

Stanniocalcin-1 (STC1) is known to be a hypocalcemic hormone in bony fish (Gerritsen & Wagner 2005). It is secreted from the endocrine gland, corpuscle of Stannius and acts on the target tissues to regulate whole-body Ca^{2+} and P_i homeostasis (Wagner *et al.* 1993, 1998, Lu *et al.* 1994). The mammalian homologue of STC1 was identified almost 15 years ago; its function, however, is largely not known (Chang *et al.* 1995, 1996, Olsen *et al.* 1996). Different from piscine, mammalian STC1 is expressed in a wide variety of tissues, acting on its target cells via autocrine/paracrine pathways (Varghese *et al.* 1998, De Niu *et al.* 2000). Therefore, the site of synthesis and release as well as the mode of action of mammalian STC1 may be altered during evolution (Yeung *et al.* 2012). This alternation might lead to the change in the physiological functions of STC1 from the regulation of whole-body $\text{Ca}^{2+}/\text{P}_i$ homeostasis in fish to signaling at cellular and tissue levels in mammals. This assumption is sensible as in mammals serum $\text{Ca}^{2+}/\text{P}_i$ homeostasis is known to be regulated by parathyroid hormone, calcitonin, and 1,25-dihydroxycholecalciferol (Khanal & Nemere 2008, Suzuki *et al.* 2008). Moreover, there is no solid evidence to illustrate that mammalian STC1 plays an important

role in this aspect. With increasing evidence to demonstrate the local actions of STC1, the focus of STC1 research has been moved from serum $\text{Ca}^{2+}/\text{P}_i$ homeostasis to cellular inflammation and carcinogenesis (Wagner *et al.* 1997, Madsen *et al.* 1998, Chang *et al.* 2003, Yoshiko & Aubin 2004, Ellard *et al.* 2007, Re & Cook 2010, Sheikh-Hamad 2010).

Cancer has been considered as a chronic inflammatory disease (Lopez-Novoa & Nieto 2009, Gupta *et al.* 2010, Pani *et al.* 2010). Cancer progression is basically the consequence of interplay among genetic, epigenetic, immunological, and microenvironmental factors (Allen & Louise Jones 2011, Schreiber *et al.* 2011, Shibata 2011). Human *STC1* gene is mapped at the metastatic susceptibility locus, 8p, which is known to be related to tumor progression and metastases (Chughtai *et al.* 1999, Macartney-Coxson *et al.* 2008). The identification of the long stretches of CAG repeats in the 5'-untranslated region infers the propensity of genetic instability and transcriptional silencing of the gene (Chang *et al.* 1998, Parniewski & Staczek 2002). Our previous study has demonstrated the epigenetic regulation of *STC1* gene expression in apoptotic cancer cells treated with histone deacetylase (HDAC) inhibitors (Law *et al.* 2008). The possible roles of STC1 in apoptosis have been reported by different

laboratories (Zhang *et al.* 2000, Wu *et al.* 2006, Lai *et al.* 2007, Block *et al.* 2009, Nguyen *et al.* 2009). The transcriptional relationship between p53 (TP53) and STC1 has also been suggested (Lai *et al.* 2007); however, the underlying regulatory mechanism is not clear. In this study, we were interested in this regulatory relationship, aiming to decipher the involvement of the transcriptional factor *p53* in the regulation of *STC1* expression. Using a pharmacological approach on *STC1* promoter, western blot analyses, and chromatin immunoprecipitation (ChIP) assay, our data revealed for the first time that p53-activated *STC1* gene expression was via the enhancement of trichostatin A (TSA)-stimulated histone acetylation and NF- κ B signaling in the human nasopharyngeal cancer cells, CNE2. Transient overexpression of exogenous STC1 augmented doxorubicin (Dox)-induced cellular apoptosis.

Materials and methods

Cell culture and treatments

The human nasopharyngeal carcinoma cell line CNE2 (a kind gift from Prof Mak, Hong Kong Baptist University, Hong Kong) was cultured in RPMI-1640 (Invitrogen, Gibco) supplemented with 10% fetal bovine serum (HyClone; Perbio, Cramlington, UK) and antibiotics (50 U/ml penicillin and 50 μ g/ml streptomycin; Invitrogen, Gibco) at 37 °C with 5% CO₂. Before treatment or transfection, cells were seeded at 70% confluence overnight and then treated with the NF- κ B inhibitor parthenolide (PTL; Calbiochem, La Jolla, CA, USA), the HDAC inhibitors, TSA (Calbiochem), valproic acid (VPA; Sigma), or apicidin (Calbiochem), and the chemotherapy drug Dox hydrochloride (Dox; Calbiochem) for an indicated time point.

Total RNA extraction and real-time PCR

Total RNAs were extracted using TRIZOL Reagent (Gibco/BRL) according to the manufacturer's instructions. The A₂₆₀:A₂₈₀ ratio was >1.8. cDNA was synthesized from 150 ng total cellular RNA using the High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA, USA). Real-time PCR was conducted using the StepOne real-time PCR detection system and POWER SYBR Green PCR Master Mix (Applied Biosystems). The amplification cycles were 95 °C for 3 min and 40 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min. Fluorescent signal was captured at 72 °C, and the occurrence of primer dimers and secondary products was inspected using melting curve analysis. The primers were as follows: human STC1 (CACACCCACGAGCTGACTTC–forward

and TCTCCCTGGTTATGCACTCTCA–reverse), p53 (CTCCTCAGCATCTTATCCGAGTG–forward and GTGGTACAGTCAGAGCCAACC–reverse), and actin (GACTACCTCATGAAGATCCTCACC–forward and TCTCCTTAATGTCACGCACGATT–reverse). Gene expression levels of *STC1* and *p53* were calculated by the ' $\Delta\Delta C_t$ method' in comparison to actin.

Western blot analysis

Protein lysates were harvested using a radioimmuno-precipitation assay buffer (50 mM Tris–HCL, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, and 0.1% SDS) following centrifugation at 10 000 *g* for 10 min. Protein concentration was measured using DC Protein Assay Kit II (Bio-Rad). Eighty micrograms of protein lysates were resolved in 10% SDS–PAGE and the protein bands were transferred onto PVDF membranes (Bio-Rad). The membrane was blocked in 5% nonfat milk/PBS for 1 h and then was probed with 1000 dilution of mouse antihuman p53 (Dako, Carpinteria, CA, USA) or rabbit antihuman acetylated p53 (lys382; Cell Signaling, Danvers, MA, USA), total NF- κ B RELA, phospho-NF- κ B RELA (Ser536) (93H1), acetyl-NF- κ B RELA (Lys310) (Cell Signaling), acetyl-histone H3 (Lys9/Lys14), histone H3 (Cell Signaling), and actin serum (Sigma). Using corresponding HRP-conjugated secondary antibodies (Bio-Rad Pacific Ltd.), specific bands were visualized by Western-Lightening Plus (PerkinElmer Life Sciences, Waltham, MA, USA).

Transient overexpression of wild-type *p53* and *STC1*

pCMV-XL5 encoding wild-type *p53* (*p53* WT) and dominant-negative mutant *p53* (*p53* dominant negative (*p53* DN)) were purchased from BD Transduction Laboratories (Palo Alto, CA, USA), and pCMV-XL5 empty vector (pCMV) was obtained from Promega. For construction of *STC1* overexpression plasmid, a cDNA encoding full-length human *STC1* without a stop codon was ligated to an expression vector V5-tagged pLenti6.3/V5-DEST (Invitrogen). For transient transfection, 400 ng plasmid were transfected into CNE2 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Overexpression efficiency was confirmed at 24 h of posttransfection and over-expressed cells with minimum transfection efficiency of 70% were used for assays.

Annexin V assay

After TSA and/or Dox treatment, both adherent and floating cells were collected and centrifuged at 400 *g* for 3 min. Cell pellets were resuspended in the

Figure 1 Effects of TSA and/or Dox treatment on *STC1* mRNA expression and apoptosis in CNE2 cells. CNE2 cells were treated with TSA (200 ng/ml), Dox (500 nM), or TSA+Dox at the indicated time points. (A) Total RNAs were harvested and subjected to real-time RT-PCR. Gene expressions of *STC1* relative to actin at 8 h (left, $P < 0.001$) and 24 h (right, $P < 0.001$) are shown. (B) Treated cells were harvested at 24 h and stained with Annexin V and PI. Percentages of apoptotic cells (Annexin V-FITC⁺/PI⁺, the lower right quadrant in the plot) were counted using flow cytometric analysis ($P < 0.05$). Bars with the same letter are not significantly different according to the results of one-way ANOVA followed by Duncan's multiple range tests.

complete medium and then incubated in 5% CO₂ at 37°C for 10 min. After centrifugation, cell pellets were washed with PBS twice and resuspended in 500 µl of 1× Annexin V binding buffer with propidium iodide and Annexin V-FITC (BD Pharmingen, San Diego, CA, USA). The percentage of apoptotic cells was measured by flow cytometer (BD FACS Calibur).

Luciferase reporter assay

For construction of *STC1* promoter luciferase reporters, different regions of human *STC1* promoter (1k: -991/+225; 2k: -1842/+225; 3k: -2723/+225; and 4k: -3867/+225) were cloned into a pGL3 (SDHC) basic luciferase reporter (Promega). A pGL3 basic luciferase reporter encoding p53-responsive

promoter was purchased from Stratagene (La Jolla, CA, USA). For the assay, the reporter was cotransfected with an internal control, *pRL-SV40* plasmid (Promega) using Lipofectamine 2000 reagent (Invitrogen). The medium was replaced with complete medium after 6 h transfection and drugs were added at 24 h posttransfection. At the indicated time points, the cells were harvested and lysed in a $1 \times$ passive lysis buffer (Promega). The lysates were centrifuged at $10\,000\text{ g}$ for 2 min, and the supernatant was subjected for luciferase activity measurement using the Dual-Luciferase reporter assay system (Promega). Firefly and renilla luciferase activities were sequentially measured for each sample by VICTOR Multilabel Reader (PerkinElmer).

Chromatin immunoprecipitation

The ChIP assay was conducted using a ChIP assay kit (Upstate, Charlottesville, VA, USA) with slight modifications. Briefly, 1×10^7 cells were cross-linked with 1% formaldehyde (Sigma-Aldrich) and lysed in SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl, pH 8.0) followed by sonication to give average lengths of 200–1000 bp DNA. The sonicated chromatin was immunoprecipitated with 5 μg rabbit IgG control (Sigma-Aldrich) or rabbit antihuman acetylated histone H3 (K9/K14; Upstate). DNA–protein complexes were isolated with Protein A/G Plus Agarose beads (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) at room temperature for 2 h, washed, eluted in 1% SDS/100 mM NaHCO_3 , and cross-links reversed at 65°C overnight. DNA was purified using phenol:chloroform extraction, ethanol precipitation, and resuspended in 50 μl water. Then, 2 μl samples were subjected to real-time PCR, using the StepOne real-time PCR detection system and Power SYBR Green PCR Master Mix (Applied Biosystems). The amplification cycles were 95°C for 3 min and 40 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min. Fluorescent signal was captured at 72°C , and the occurrence of primer dimers and secondary products was inspected using melting curve analysis. The primers (CACCAGACCAGTTGAGG-GACCT-forward and TTTGCCAACTGGGGGCCCA-reverse) for human *STC1* promoter flanking -90 to $+11$ bp at the transcription initiation site were used. The degree of enrichment in the ChIP assay was normalized with their input control.

Statistical analysis

Drug treatments were performed in triplicate at least. All data are represented as mean $\pm$ s.d. Statistical significance was assessed using Student's *t*-test or one-way ANOVA followed by Duncan's multiple range test. Groups were considered significantly different if $P < 0.05$.

Figure 2 Effects of TSA and/or Dox treatment on *p53* gene regulation. CNE2 cells were treated with TSA (200 ng/ml), Dox (500 nM), or TSA + Dox for 24 h. (A) Total RNAs were harvested and subjected to real-time RT-PCR. Gene expression of *p53* relative to actin was shown ($P < 0.01$). (B) Protein lysates were harvested. Western blot was performed using total *p53* antibody, whereas actin acted as a loading control. (C) Before treatment, CNE2 cells were transfected with *pGL3* plasmid containing human cloned *p53*-responsive promoter and a normalization control plasmid *pRL-SV40*. The cells were then exposed to TSA and/or Dox treatments for 24 h. *p53* luciferase reporter activities were measured using the dual-luciferase reporter assay system ($P < 0.01$). Bars with the same letter are not significantly different according to the results of one-way ANOVA followed by Duncan's multiple range tests.

Results

Effects of HDAC inhibitors and Dox cotreatment on the expression levels of *STC1*, p53, and apoptosis in CNE2 cells

In this study, we examined the effects of HDAC inhibitors (TSA, VPA, and apicidin) and/or Dox treatment on *STC1* mRNA expression in human nasopharyngeal carcinoma cells, CNE2. In 200 ng/ml TSA-treated CNE2 cells, *STC1* mRNA was significantly increased by 7.2- and 18.1-fold at both 8 and 24 h, respectively, compared with the vehicle control (Ctrl; Fig. 1A). In 500 nM Dox-treated cells, however, no noticeable effect on *STC1* mRNA expression was observed. Interestingly, cotreatment of TSA and Dox elicited a synergistic induction of *STC1* mRNA expression at both the time points (2.4-fold at 8 h and 24.4-fold at 24 h vs their TSA alone treatment). Like TSA treatment, the synergistic effect was also observed in the cotreatment of Dox with the other HDAC inhibitors, 500 μ M VPA (5.1-fold induction vs VPA alone, Supplementary Figure 1A, see section on supplementary data given at the end of this article), or 5 μ M apicidin (2.0-fold induction vs apicidin alone, Supplementary Figure 1B, see section on supplementary data given at the end of this article) at 24 h. Accompanied with *STC1* induction, cellular apoptosis was detected at 24 h treatment of either TSA (7.63% of apoptotic cells) or Dox (12.16% of apoptotic cells) and was further induced in TSA+Dox cotreatment (21.83% of apoptotic cells) compared with the control (3.79% of apoptotic cells; Fig. 1B). In the 24 h treatment, mRNA expression of p53 was suppressed by 6.6-fold in TSA treatment (vs Ctrl) but augmented by 3.7-fold in Dox treatment (vs Ctrl; Fig. 2A), which correlated with the total p53 protein expression level (Fig. 2B). Similar observations were observed in the treatment and cotreatment of Dox with VPA or apicidin (Supplementary Figure 2, see section on supplementary data given at the end of this article). Consistent with the expression levels, a 3.6-fold increase in p53-driven luciferase activities was measured at both Dox and TSA+Dox treatments (Fig. 2C).

The association between the cellular levels of p53 and the activation of *STC1* gene expression

To elucidate the possible involvement of p53 in *STC1* gene transcription, the pCMV vector expressing WT p53 was transiently transfected into CNE2 cells for 24 h followed by TSA treatment for 8 or 24 h. To ascertain the biological activity of p53 WT on CNE2 cells, cotransfection of p53 WT and p53 luciferase reporter was conducted and the respective luciferase activities were measured. As shown in Fig. 3A, the overexpression

of p53 WT dramatically stimulated the p53 reporter activities (108-fold induction vs pCMV). At the protein level, the overexpression of p53 WT increased the levels of p53 acetylation at Lys382 (Fig. 3B). Although p53 overexpression had no perceptible effect on *STC1* mRNA expression, it could enhance *STC1* mRNA expression in TSA cotreatment by 2.5-fold at 8 h (vs pCMV+TSA; Fig. 3C).

According to the core DNA-binding sequence (-CATG-) of p53, ten putative p53 binding sites (-2612/-2615, -2575/-2578, -2530/-2533, -1201/-1204, -1133/-1136, -892/-895, -851/-854, -505/-508, -497/-500, and -106/-109) were mapped on ~3 kb

Figure 3 Effects of p53 overexpression on *STC1* gene regulation. (A) CNE2 cells were cotransfected with pCMV encoding p53 WT and p53 luciferase reporter, and the luciferase activities were measured at 8 h. (B) The p53 WT-transfected cells were treated with 200 ng/ml TSA for 8 h, protein lysates were harvested, and protein expressions of acetylated p53 (lys382), total p53, and actin were determined using western blot. (C) Total RNAs after 8 h of TSA treatment in the transfected cells were harvested and *STC1* mRNA expression was examined by real-time PCR ($P < 0.05$). (D) After cotransfection of pCMV plasmids and *STC1* reporter encoding 1k *STC1* promoter regions for 24 h, the cells were treated with TSA and harvested at 8 and 24 h for the measurement of luciferase activities ($P < 0.001$). (E) After transfection of pCMV encoding p53 WT, p53 DN, or the empty vector, *STC1* mRNA expression was examined at 24 h. The protein expression levels of total p53 and actin are shown (right). (F) CNE2 cells were cotransfected with the p53 luciferase reporter and the pCMV encoding p53 WT, p53 DN or the empty vector alone, the respective luciferase activities were measured at 24 h. Bars with the same letter are not significantly different according to the results of one-way ANOVA followed by Duncan's multiple range tests.

STC1 promoter region. To test the effect of the exogenous *p53* WT overexpression on *STC1* promoter-driven activities, *STC1* luciferase reporters encoding different promoter regions (STC 1–4k) were constructed for the assays (Supplementary Figure 3A, see section on supplementary data given at the end of this article). Surprisingly, the overexpression of *p53* WT did not elicit noticeable effect on *STC1* promoter-driven luciferase activities among various *STC1* promoter constructs (Supplementary Figure 3B, see section on supplementary data given at the end of this article). Owing to the similar responses obtained from various *STC1* promoter regions, the construct encoding the proximal promoter region *STC1* 1k was used for the subsequent treatments. In *STC1* promoter analysis, the respective stimulatory or synergistic effects of TSA or TSA + *p53* WT treatments on *STC1* promoter-driven luciferase activity were observed at 8 and 24 h (1.2- and 1.3-fold induction in *p53* WT + TSA vs their pCMV + TSA at 8 and 24 h respectively) (Fig. 3D). To reveal whether the synergistic effects were due to the overexpression of the functional *p53*, the *p53* mutant DN was also used to test its effects on TSA-elicited *STC1* mRNA expression (Fig. 3E) and *STC1*-driven luciferase activity (Fig. 3F). Consistently, no noticeable effects were observed in TSA + *p53* DN-treated cells compared with TSA alone or TSA + empty vector treatments.

Our data suggested that *p53* could significantly induce TSA-mediated *STC1* gene expression; however, a ChIP assay using *p53* antibody did not show an enrichment of *STC1* promoter region (data not shown). The data suggested that *p53*-mediated activation of *STC1* gene expression might be attributed by other or indirect pathways. In the investigation of the signaling pathways involved in *p53*-mediated *STC1* expression, western blot analysis revealed significant increases in the levels of acetylated-NF- κ B and phospho-NF- κ B p65 (Fig. 4A). On addition of NF- κ B inhibitor PTL to TSA + Dox-cotreated cells, the level of *STC1* mRNA expression was significantly reduced (Fig. 4B). Moreover, TSA + Dox cotreatment significantly increased the levels of acetylated histone H3 compared with the TSA alone treatment (Fig. 4C). Acquiring ChIP assay using acetylated histone H3 antibody, *STC1* DNA was also markedly enriched after TSA + Dox cotreatment (Fig. 4D).

The pro-apoptotic role of *STC1* in Dox-induced apoptosis

We attempted to investigate whether *STC1* was able to modulate Dox-induced apoptosis. CNE2 cells were transiently transfected with V5-tagged *STC1* pLenti6.3/V5-DEST with the minimum transfection efficiency of 70%. By western blotting, V5-tagged *STC1* expression was remarkably induced in pLenti6.3-*STC1*-transfected

Figure 4 Effects of TSA + Dox cotreatment on NF- κ B signaling and acetylation of histone H3. (A) CNE2 cells were treated with TSA and/or Dox for 24 h and protein lysates were harvested for western blotting using antibodies for acetylated NF- κ B, phospho-NF- κ B p65, total NF- κ B, and actin. (B) CNE2 cells were pretreated with parthenolide (PTL) for 1 h before TSA + Dox cotreatment, then protein lysate and total RNA were prepared for western blotting and real-time PCR respectively ($P < 0.05$). (C) An increase in the protein expression levels of acetyl histone H3 was detected upon TSA and/or Dox treatments, whereas histone H3 was used as a loading control. (D) ChIP assay illustrated the significant increase in acetyl histone H3 (ACh3) binding on *STC1* promoter (–90 to +11 bp) in TSA and TSA + Dox-cotreated CNE2 cells ($P < 0.05$).

cells (vs plenti6.3) at 24 h posttransfection (Fig. 5A). With the overexpression of exogenous *STC1*, the percentage of apoptotic cells was noticeably enhanced in Dox-cotreated cells (1.4-fold vs plenti6.3+Dox; Fig. 5B).

Discussion

Considerable studies have demonstrated the involvement of *STC1* in human carcinogenesis. Current evidence illustrates the differential expression patterns of *STC1* in paired tumor and normal tissues (Liu *et al.* 2010, Tamura *et al.* 2011). A role of *STC1* in the tumor microenvironment has also been suggested (Yeung *et al.* 2012). This presumption is strengthened by the involvement of *STC1* in local inflammation (Sheikh-Hamad 2010) as well as the identification of *STC1* as a hypoxia and angiogenic responsive gene (Yeung *et al.* 2005, Law *et al.* 2010, Jauhainen *et al.* 2011, Roch & Sherwood 2011). To date, biological roles of *STC1* in carcinogenesis have been suggested, including cell motility, invasion, and apoptosis (Lai *et al.* 2007, Law *et al.* 2008, Liu *et al.* 2010). However, the mechanistic role of *STC1* in the cell death pathway is still not clear as both pro-apoptotic and antiapoptotic roles have been demonstrated. Moreover, the regulatory function of p53 in *STC1* gene transcription is still not clear. In this

study, we provided new evidence to relate the transcriptional role of p53 in *STC1* gene transactivation. A cross-talk mechanism among p53, NF- κ B, and histone H3 acetylation in the regulation of *STC1* gene expression was suggested. Furthermore, the overexpression of exogenous *STC1* was found to sensitize apoptosis induced by Dox treatment, supporting the pro-apoptotic role of the protein.

HDAC inhibitors, an important class of anticancer agents, have been developed to promote acetylation on chromatin-associated (histone) and non-chromatin-associated proteins (i.e. NF- κ B and p53). Generally, HDAC inhibitor treatment leads to multiple cellular consequences such as ROS generation, cell cycle arrest, apoptosis, and inhibition of angiogenesis (Bolden *et al.* 2006). Other studies have demonstrated that TSA could promote apoptosis induced by Dox treatment (Karagiannis *et al.* 2004, Rho *et al.* 2005) via the activation of p53-signaling pathway (Magnelli *et al.* 1995, Brantley-Finley *et al.* 2003, Tang *et al.* 2004, Wang *et al.* 2004). Human *STC1* has been suggested to be a putative p53 downstream target and is involved in apoptosis (Wu *et al.* 2006, Lai *et al.* 2007, Law *et al.* 2008, Nguyen *et al.* 2009). In this study, the data of western blotting, real-time PCR, and p53-luciferase reporter assays showed that p53 was activated in the Dox-treated cells. TSA and Dox cotreatment synergistically enhanced the induction of apoptosis and *STC1*

Figure 5 The effects of *STC1* overexpression on Dox-induced apoptosis. (A) Protein lysates of *STC1* overexpressed cells were collected and probed with V5 antibody. (B) CNE2 cells were overexpressed with exogenous *STC1* followed by 24 h Dox treatment. Percentages of apoptotic cells (Annexin V-FITC⁺/PI⁻, the lower right quadrant in the plot) were counted using flow cytometric analysis (*P* < 0.05). Bars with the same letter are not significantly different according to the results of one-way ANOVA followed by Duncan's multiple range tests.

expression. To decipher the underlying mechanism of the synergistic effects, we attempted to overexpress the exogenous *p53* WT in TSA-treated cells. A similar observation of the synergistic effect on *STC1* expression was observed. Upon TSA treatment, the levels of *p53* acetylation (Lys382) were increased, which is crucial for its stabilization against ubiquitylation and its mediated function (Rodriguez *et al.* 2000, Ito *et al.* 2001, Luo *et al.* 2004). Indeed, in a breast cancer model, acetylated *p53* (Lys373/382) was recruited on gene promoter regions such as BANP after Dox treatment, leading to the activation of gene transcription (Singh *et al.* 2007). However, in a *STC1* promoter deletion assay, *p53* overexpression showed no noticeable effects on *STC1* promoter-driven luciferase activities. ChIP analysis also revealed no direct binding of *p53* on the *STC1* promoter (data on shown). The data suggest that *p53* was not involved in the direct transactivation of *STC1* gene expression. However, it has been reported that one of the key proteins that mediated *p53*-induced apoptosis is NF- κ B (Ryan *et al.* 2000), which is also known to regulate a *STC1* gene transcription (Law *et al.* 2008). In addition, *p53*-dependent histone acetylation has been reported (Kaesler & Iggo 2004). With the benefit of hindsight, our western blot data illustrated that Dox and TSA cotreatment significantly elevated the cellular levels of phospho-NF- κ B and acetylated histone H3. The demonstration of the inhibitory effects of NF- κ B inhibitor on *STC1* expression in TSA+Dox-cotreated cells denoted the dependence of NF- κ B-signaling on *STC1* gene expression. Moreover, the ChIP assay illustrated that the cotreatment caused a significant increase in acetylated histone H3 binding on *STC1* proximal promoter. The *p53*-mediated increase in the levels of phospho-NF- κ B was reported to be related to *p53*-stimulated pp90^{tsk} activity (Ryan *et al.* 2000), which could phosphorylate I κ B and lead to NF- κ B activation. The increase in histone acetylation may be associated with the progressive recruitment of chromatin-modifying enzymes on *p53*, promoting the activity of *p53*-dependent histone acetylation (Agalioti *et al.* 2002, Lee *et al.* 2002, Espinosa *et al.* 2003). Whatever the explanation, the TSA and Dox/*p53* WT cotreatment significantly induced *STC1* gene expression and cellular apoptosis. The pro-apoptotic role of *STC1* was supported by the *STC1* overexpression experiments, in which *STC1* sensitized the apoptotic process in Dox-treated cells. Although our data support the pro-apoptotic function of *STC1*, other studies have demonstrated the anti-apoptotic activity of the protein (Zhang *et al.* 2000, Block *et al.* 2009). This inconsistency may not be contradictory as the transcriptional factor, NF- κ B, is also known to be able to protect or contribute to apoptosis (Foo & Nolan 1999, Fan *et al.* 2008). Therefore, *STC1* may not be a key regulator for or against apoptosis; the particular role of *STC1* is

probably dependent on the extent of cellular stress imposed to the cells (Yeung *et al.* 2012).

Collectively, our study demonstrated that the cotreatment of TSA and Dox in CNE2 cells could remarkably induce *STC1* activation. Although the evidence of *p53*-mediated direct transactivation of *STC1* promoter regions was not identified, TSA+Dox-induced *STC1* was found to be mediated via *p53* to increase the levels of histone H3 acetylation and NF- κ B phosphorylation.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.101530/JME-11-0159>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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