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CCAAT Box Is Required for the Induction of Human Thrombospondin-1 Gene by Trichostatin A

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Abstract Histone deacetylase (HDAC) inhibitors have been reported to inhibit angiogenesis as well as tumor growth. Thrombospondin-1 (TSP1) has been recognized as a potent inhibitor of angiogenesis. Such an action of TSP1 may account for the effect of HDAC inhibitors. In the present study, we investigated the molecular mechanism by which trichostatin A, a HDAC inhibitor, induces the expression of TSP1 gene. Trichostatin A increased both mRNA and protein levels of TSP1 in HeLa cells. Promoter and actinomycin D chase assays showed that trichostatin A-induced TSP1 expression was regulated at the transcriptional level without changing mRNA stability. CCAAT box on the TSP1 promoter was found to primarily mediate the trichostatin A response by deletion and mutation analyses of the TSP1 promoter. Electrophoretic mobility shift assay indicated that CCAAT-binding factor (CBF) was specifically bound to the CCAAT box of TSP1 promoter. Moreover, chromatin immunoprecipitation assay showed that trichostatin A increased the binding of acetylated form of histone H3 to the CCAAT box region of TSP1 promoter. Taken together, these results strongly suggest that trichostatin A activates the transcription of TSP1 gene through the binding of transcription factor CBF to CCAAT box and the enhanced histone acetylation. Thus, the present study provides the clue that the inhibition of angiogenesis by trichostatin A is accomplished through the upregulation of TSP1, the anti-angiogenic factor. J. Cell. Biochem. 104: 1192–1203, 2008.

Key words: trichostatin A; thrombospondin-1; CCAAT box

Thrombospondin-1 (TSP1) is an extracellular matrix glycoprotein which is synthesized in various cells including platelets, endothelial cells, fibroblasts, and smooth muscle cells [Lawler, 1986; Bornstein, 1992]. Accumulating evidence suggests that TSP1 is a naturally occurring inhibitor of angiogenesis, the process of new capillary blood vessel formation. TSP1 inhibits endothelial cell adhesion, growth and motility in response to angiogenic stimuli [Panetti et al., 1997] and also has ability to induce apoptosis of endothelial cells [Guo et al., 1997]. On the contrary, downregulation of TSP1 expression in endothelial cells enhances in vitro angiogenesis [DiPietro et al., 1994].

TSP-1 gene expression appears to be regulated primarily at the transcriptional level through multiple binding sites within 5′ flanking region of TSP-1 gene in response to a variety of extracellular stimuli [Framson and Bornstein, 1993; Salnikow et al., 1997; Adams and Lawler, 2004]. Some studies provided evidence that a post-transcriptional control such as mRNA stability was also involved in the regulation of the gene [Okamoto et al., 2002; Kang et al., 2006]. In addition, epigenetic mechanism was shown to involve the silencing of TSP-1 expression through hypermethylation of CpG islands around transcription start site [Li et al., 1999; Yang et al., 2003].

Histone deacetylase (HDAC) inhibitors have been shown to inhibit proliferation and to

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induce apoptosis of various cancer cell types both in vitro and in vivo [Richon et al., 2000; Carey and La Thangue, 2006]. In addition, HDAC inhibitors have been reported to inhibit angiogenesis [Kim et al., 2001; Qian et al., 2006]. Among a small number of genes with an altered expression by various HDAC inhibitors were tumor suppressor and angiogenesis-related genes such as cell cycle inhibitor $p21^{WAF1/Cip1}$, tumor suppressor p53, hypoxia-inducible factor-1α (HIF-1α), and vascular endothelial growth factor (VEGF) [Suzuki et al., 2002; Yamashita et al., 2003; Chiba et al., 2004]. However, the underlying mechanisms whereby HDAC inhibitors induce these genes have not been fully understood, although transcriptional activation of $p21^{WAF1/Cip1}$ by trichostatin A was mediated through interaction of Sp1 binding site with transcription factors Sp1/Sp3 on the promoter [Sowa et al., 1997, 1999; Han et al., 2001].

In this study, we report that trichostatin A, a specific HDAC inhibitor, can induce TSP1 expression at the transcription level in HeLa cells. And the increased activity of the human TSP1 promoter attributes to the inverted CCAAT box located at −65 relative to transcription start site, on which CBF, a transcription factor, is specifically bound to the *cis*-element.

MATERIALS AND METHODS

Chemicals

Trichostatin A, [[(R-(E, E)]-7-[4-(Dimethylamino)phenyl]-*N*-hydroxy-4, 6-dimethyl-7-oxo-2, 4-heptadienamide)], and MS-275 were purchased from Sigma–Aldrich (St. Louis, MO).

Cell Culture

Human cervical carcinoma cell line HeLa was obtained from American Type Cell Culture (Rockville, MD). Cells were grown in DMEM medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified incubator with 5% CO_2.

Western Blot Analysis

The method for Western blot analysis was described previously. The antibody used in this study was monoclonal anti-mouse TSP1 (Neomarker, Fremont, CA) [Kang et al., 2006].

Northern Blot Analysis

Total RNA isolation and the method for Northern blot analysis were described previously [Kang et al., 2006]. The probes used in this study were dig-dUTP-labeled cDNAs. In all experiments GAPDH or 18S rRNA was used for normalization.

mRNA Stability Assay

HeLa cells were treated with trichostatin A (50 ng/ml) for 6 h and then exposed to actinomycin D (5 μg/ml) to block RNA synthesis. Total RNA was prepared at various time points and Northern blot analysis was performed.

Construction of the Serial-Deletion TSP1 Promoters

A 735 bp fragment spanning from −523 to +213 of the human TSP1 promoter was amplified by PCR using a plasmid (kindly gifted from Dr. Takeshi Uchiumi of Kyushu University, Japan) containing the sequence from −767 to +750 of human TSP1 promoter as a template. The PCR primers used were as follows: sense primer with *Kpn*I site at 5′ end (underlined), 5′-TCAGGTACCGGTGGAGGAGAGTCAGCGAGG-3′, anti-sense primer with *Xho*I at 5′ end (underlined), 5′-TCACTCGAGCAGCGCTCCAGGTGG ATGTC-3′. The PCR conditions were 30 s at 95°C, 30 s at 60°C, and 1 min at 72°C for 35 cycles, with a final extension for 10 min at 72°C. The amplified PCR product was digested with *Kpn*I and *Xho*I and subcloned into pGL3 vector (Promega, Madison, WI), designated as pTSP-523. The nucleotide sequence of the construct was confirmed by automated DNA sequencing. To prepare 5′ serially deleted TSP1 promoter constructs, PCR was performed using pTSP-523 construct as a template. The sense primers with *Kpn*I sites at their 5′ ends (underlined) were as follows: pTSP-212: sense primer (5′-TCAGGTACCCGACTTTTCTGAGAAGTTCTAGTGC -3′); pTSP-161, sense primer (5′-TCAGGTACCCACTTTCTAGCTGGAAAGTTGC-3′); pTSP-118, sense primer (5′-TCAGGTACCAGAGAGGAGCCCAGACTG-3′); pTSP-57, sense primer (5′-TCAGGTACC GAGGAATCCCCAGGAATGC-3′). The anti-sense primer was the same as that used for the pTSP-523.

Site-Directed Mutagenesis

Four Sp1 sites and one CCAAT box in the TSP1 promoter were independently mutated as

previously described [Ko and Ma, 2005]. Briefly, the sequence from −523 to +213 of TSP1 promoter was amplified into two separate fragments using either a pair of a forward anchor primer and a reverse mutagenic primer or a pair of a reverse anchor primer and a forward mutagenic primer. The sequences of mutagenic primers used in this study and PCR condition for each primer are shown in Table I. Amplified fragments were digested with *Ear*I restriction enzyme, purified from 1% agarose using gel extraction kit, and ligated with T4 DNA ligase to generate 735 bp fragments containing the mutated Sp1 sites and CCAAT box. The ligated fragments were then purified and subcloned into pGL3 vector following double-digestion with *Kpn*I and *Xho*I. In addition, both CCAAT box and Sp1 site-mutated construct was prepared with QuikChange Site-Directed Mutagenesis Kit (Staratagene, La Jolla, CA) using CCAAT box-mutated construct as a template. The primer set was as follows: forward, 5′-TGC CCG GCC GCa tCC CAa cGG CCG GAG GAA TC-3′; reverse, 5′-GAT TCC TCC GGC Cgt TGG Gat GCG GCC GGG CA-3′. The mutation was verified by DNA sequence analysis.

Transient Transfection and Luciferase Assays

Either wild or mutant TSP1 promoter construct was transiently transfected into HeLa cells (0.5 μg/1×10^5 cells) using LipofectAMINE-PLUS reagent following the protocol provided by the supplier (Invitrogen, Carlsbad, CA). After transfection of 18 h, cells were treated with trichostatin A for 7 h and harvested for the determination of luciferase activity. The luciferase activity was normalized relative to the protein concentration determined by the Bradford method (Bio-Rad, Hercules, CA).

Preparation of Nuclear Extracts

HeLa cells (5×10^5 cells/ml) were washed twice with PBS and pelleted by centrifugation at 4°C. The pellet was resuspended in 500 μl of ice-cold hypotonic solution [5 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2% Nonidet P-40, 50 mM NaF, 1 mM Na_3VO_4, 5 mM dithiothreitol, 0.1 mg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin], incubated on ice for 10 min and repelleted by centrifugation at 4°C. The pellets were then incubated for 30 min at 4°C in 30 μl of ice-cold hypertonic solution [20 mM HEPES, pH 7.9, 0.5 M NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 5 mM dithiothreitol, 0.1 mg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml anti-pain, 1 μg/ml pepstatin]. After incubation, nuclear extract was collected by centrifugation. Protein concentrations were measured with the Bio-Rad Protein Assay.

Electrophoretic Mobility Shift Assay (EMSA)

Binding reactions for gel shift assays were performed in 20 μl of 10 mM HEPES, pH 8, 0.1 mM EDTA, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 4 mM spermidine, 2 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 10% glycerol, 1 μg of poly (dI-dC), ^{32}P-labeled oligonucleotide probe (30,000 cpm) and 10 μg of nuclear extract. The reaction was allowed to continue for 20 min at 4°C. The radiolabeled probe contains the sequence from −80 to −54 of the human TSP1

TABLE I. Nucleotides Sequences of the Primers Used

Mutants	Direction	Primers[a]	Anneling temperature (°C)
mSP1-1	Forward	5′-AACTCTTCT atG GGC ACC GAC TTT TCT GAG AAG TTC	60
	Reverse	5′-AACTCTTCT Cat CCA CGC AGC CTT GGC GCG CAC	62
mSP1-2	Forward	5′-AACTCTTCT atC CCC CTT CAC TTT CTA GCT GGA AAG	59
	Reverse	5′-AACTCTTCT Gat GGG GTC GGG GCT TGG GAG CAC	62
mSP1-3	Forward	5′-AACTCTTCT atG GAG AGA GGA GCC CAG ACT GG	60
	Reverse	5′-AACTCTTCT Cat CCC CCG CTG CCT GGC GCG CAA C	64
mSP1-4	Forward	5′-AACTCTTCT atC CCA TTG GCC GGA GGA ATC C	60
	Reverse	5′-AACTCTTCT Gat GCG GCC GGG CAG GAA GCG GGA G	64
mCCAAT	Forward	5′-AACTCTTCT acG GCC GGA GGA ATC CCC AGG	62
	Reverse	5′-AACTCTTCT Cgt TGG GCG GCG GCC GGG CAG	64
Anchor	Forward	5′-TCAGGTACC GGT GGA GGA GAG TCA GCG AGG	
	Reverse	5′-TCACTCGAG CAG CGC TCC AGG TGG ATG TC	

[a]For all primers, mutagenesis sites are indicated in lowercase and the recognition of restriction enzyme *Ear*I are underlined. *Kpn*I and *Xho*I recognition sites (underlined) on 5′ ends of anchor primer were used to facilitate subcloning of the mutant fragment into pGL3 vector. Standard PCR was performed with same conditions for all mutagenic primers; 94°C for 30 s except for annealing temperature, annealing temperatures indicated in Table I for 1 min, and 72°C for 1 min for 30 cycles.

promoter (5′-GCCCGGCCGCCGCCCATTGG-CCGGAGG-3′). In supershift studies, 2 μg of the appropriate antibody was preincubated with the crude nuclear extract for 10 min at 4°C before the addition of the radiolabeled probe. In competition experiments, the nuclear extract was incubated with a 100-fold molar excess of the appropriate unlabeled competitor oligonucleotides. Electrophoresis of the different samples was carried out on 6% nondenaturating polyacrylamide gels with 0.5× TBE [45 mM tris (hydroxymethyl) aminomethane, 45 mM boric acid, 1 mM EDTA, pH 8] and run at 15 V/cm for ~2 h. The gel was dried under vacuum and subjected to autoradiography. Oligonucleotides used in competition assays were as follows: CCAAT box mutant, 5′-GCCCGGCCGCCGCCC AacGGCCGGAGG-3′ and Sp1 mutant, 5′-GC-CCGGCCGCatCCCATTGGCCGGAGG-3′.

Chromatin Immunoprecipitation Assay (ChIP)

ChIP assay was performed with a commercial Kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's instructions [Kang et al., 2007]. The conditions for PCR were as follows: 30 s at 95°C, 30 s at 62°C, and 30 s at 72°C for 25 cycles, with a final extension for 10 min at 72°C. Primers for ChIP were as follows: forward, 5′-TCACTTTCTAGCTGGAA-AGTTGCG-3′; reverse, 5′-GACAGGAGCCCT-GAACTCG-3′.

Statistical Analysis

Intensities of blots were scanned with a Fuji PhosphoImager (Fujifilm Medical Systems, Japan) and quantified using Scion Imaging software (Scion Corporation, Frederick, MD). Data are presented as mean values ± SD and P values were determined using unpaired Student's t-test. $P < 0.05$ was accepted as statistically significant.

RESULTS

Transcriptional Regulation Is Responsible for the Trichostatin A-Induced TSP1 Expression

To investigate whether trichostatin A could induce TSP1 expression, HeLa cells were exposed to trichostatin A at a concentration of 50 ng/ml (~0.15 μM). At this concentration, trichostatin A did not show cytotoxic effect on the cells.

Trichostatin A increased the level of TSP1 mRNA as early as at 3 h and the increase reached its maximum at 24 h, then thereafter the level decreased. However, MS-275, a class I HDAC inhibitor, maintained TSP 1 mRNA level until 48 h (Fig. 1A). The difference in the duration of TSP1 mRNA expression may be due to the biochemical structure difference of the drugs used. Though trichostatin A has a potent inhibitory action on HDAC, it has the limitation of low bioavailability and instability. Meanwhile, MS-275 has a pyridyl ring cap structure, which has better physiochemical actions than trichostatin A [Yoshida et al., 2001; Wittich et al., 2002]. TSP1 protein was also induced in trichostatin A-treated sample (Fig. 1B). Next, we determined whether transcriptional activation was involved in the increase in TSP1 mRNA level. Trichostatin A remarkably increased the activity of TSP1/luciferase reporter construct (pTSP-523) containing human TSP1 promoter sequence from −523 to +213 while it did not affect the activity of pGL3, a backbone vector (Fig. 1C). The possible involvement of post-transcriptional mechanism was tested by actinomycin D chase study to investigate the involvement of mRNA stability in the trichostatin A-induced TSP1 expression. As shown in Figure 1D, trichostatin A did not influence the decay rate of TSP1 mRNA. These results indicate that trichostatin A induces the expression of TSP1 mRNA transcriptionally rather than post-transcriptionally.

The Region Between −118 and −57 of the TSP1 Promoter Contains the Elements Responsive to Trichostatin A

To determine the *cis*-elements responsible for the trichostatin A-induced TSP1 expression, a set of 5′ serially-deleted TSP1 promoters was prepared (Fig. 2A) and each promoter was transiently transfected into HeLa cells. Following transfection, cells were treated with trichostatin A for 7 h and then luciferase activity was measured for the promoter activities. As shown in Figure 2B, deletion of sequences to −118 did not significantly influence the luciferase activity, still maintaining trichostatin A inducibility of about ninefold. However, further deletion of about 60 bases to −57 caused nearly complete loss of both basal and trichostatin A-induced promoter activities. These results indicate that the regulatory *cis*-elements required for the basal and trichostatin A-stimulated promoter

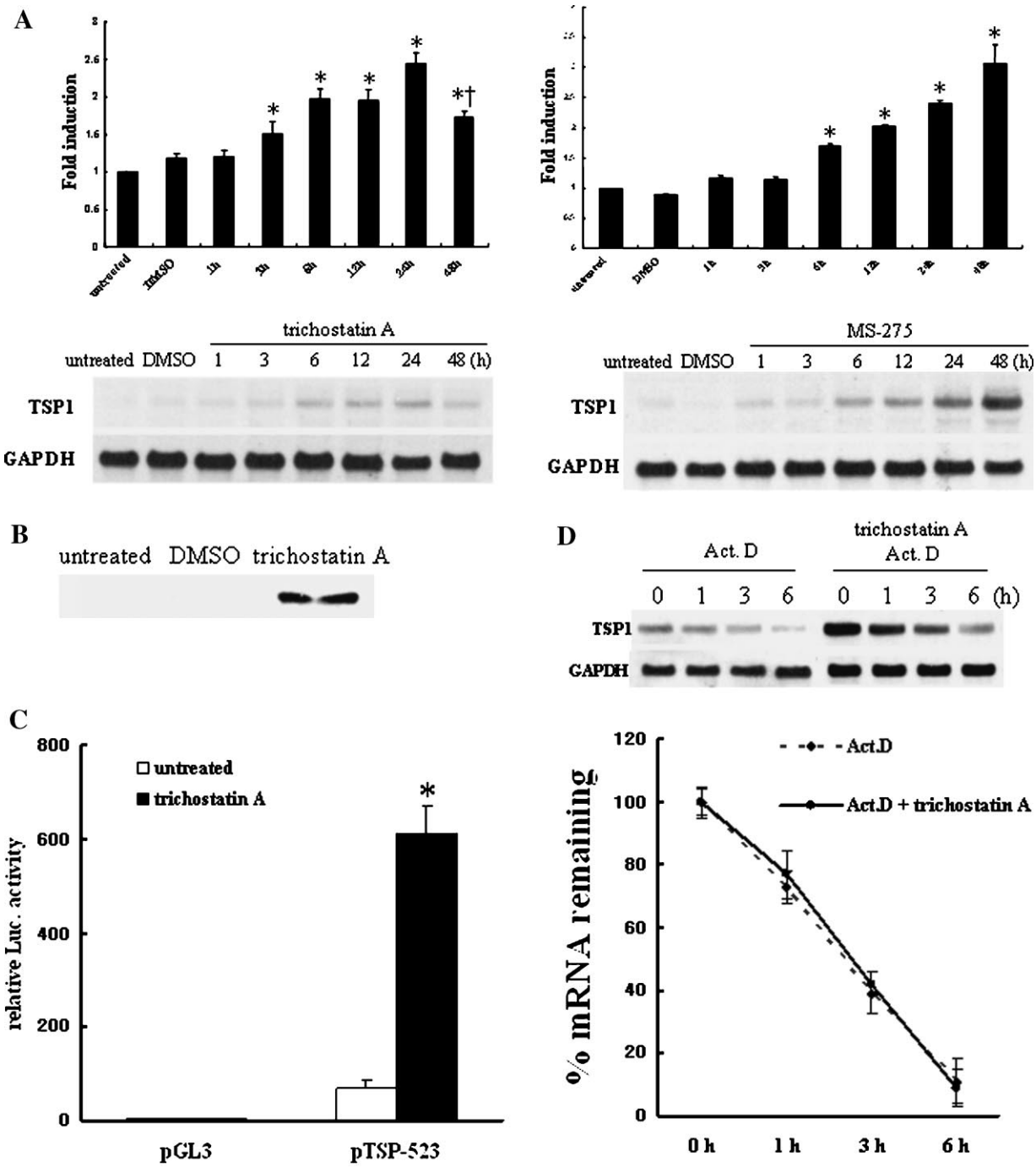

Fig. 1. Transcriptional activation of TSP1 gene by trichostatin A. **A**: Time course of TSP1 mRNA induction in HeLa cells. Cells were treated with trichostatin A (50 ng/ml) or MS-275 (1 μM) for the indicated time periods. Total cellular RNAs from untreated, vehicle (DMSO)- and trichostatin A- or MS-275-treated cells were analyzed by Northern blot using dig-dUTP-labeled cDNA probe specific for TSP1. Levels of GAPDH mRNA were used as a loading control. Data are expressed as the mean (±SD) density ratio of TSP1 to GAPDH from three independent experiments. $^{*}P < 0.05$ versus untreated control value; $^{\dagger}P < 0.05$ versus 24 h. **B**: Western blot analysis of TSP1 protein expression. TSP1 protein was only detected in the trichostatin A-treated sample. **C**: Activation of TSP1 promoter by trichostatin A. HeLa cells were transiently transfected with either vector alone (pGL3) or human TSP1 promoter/luciferase reporter construct (pTSP-523). Then, the cells were treated with trichostatin A (50 ng/ml) for 7 h. Luciferase activity was measured and normalized to protein concentration. The data represent the mean ± SD of five separate experiments performed in duplicate. $^{*}P < 0.05$ versus untreated value of pTSP-523. **D**: Effect of trichostatin A on the stability of TSP1 mRNA. Total RNA was isolated from HeLa cells treated with actinomycin D (Act.D) in the presence or absence of tichostatin A (50 ng/ml) and examined for TSP1 mRNA by Northern blot analysis. Lower graph, the bands of TSP1 mRNA was scanned and their intensity normalized to that of GAPDH mRNA were determined by NIH image software. Data were plotted on percentage of remaining mRNA relative to 0 h and represent the mean ± SD of three independent experiments.

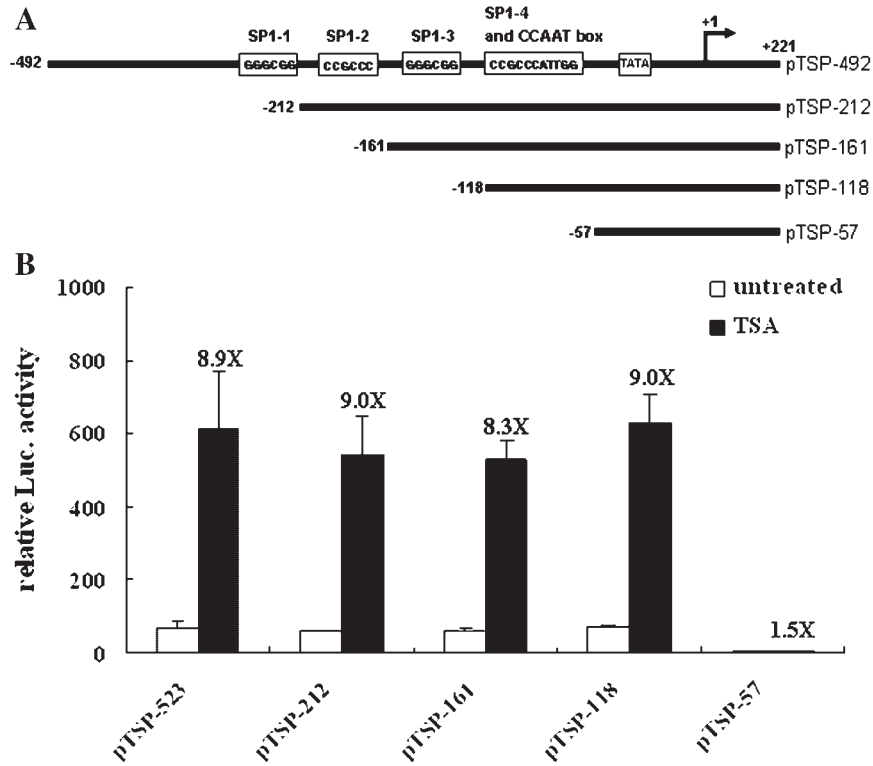

Fig. 2. Activities derived from the constructs containing serially 5′ deleted TSP1 promoter. **A**: Schematic representation of 5′ serially deleted TSP1 promoters. The nucleotides are numbered from the transcription start site that was indicated as arrow (+1). The positions and sequences for four Sp1 binding sites, CCAAT and TATA box were also indicated. **B**: The luciferase activities of the deleted promoter constructs. HeLa cells were transiently transfected with 0.5 μg of each construct. After transfection of 18 h, cells were not treated or treated with trichostatin A (TSA, 50 ng/ml) for 7 h and then harvested for luciferase assay. Luciferase activity was measured and normalized against protein concentration. The data represent the mean ± SD of four separate experiments performed in duplicate.

activities are located between −118 and −57 within TSP1 promoter.

TSP1 Promoter Requires Inverted CCAAT Box for Activation by Trichostatin A

Analysis of the sequence between −118 and −57 revealed the presence of two potential Sp1 binding sites and an inverted CCAAT box. These sites are important *cis*-elements for the activation of certain genes targeted by HDAC inhibitor like trichostatin A [Jin and Scotto, 1998; Sowa et al., 1999; Huang et al., 2000; Han et al., 2001]. To characterize *cis*-elements involved in trichostatin A response, the activities of TSP1/luciferase constructs in which each element (Sp1 and CCAAT box located at −71 and −65, respectively) was independently mutated in the context of the full-size promoter (pTSP-523), were determined. In addition, another three Sp1 binding sites (located at −221, −172, and −124) of the pTSP-523 located upstream of trichostatin A responsive region, were independently mutated to examine their relative contribution to the promoter activity. The wild and mutated sequences in the context of pTSP-523 construct were shown in Figure 3A. When four Sp1 binding sites were individually mutated (mSp1-1, -2, -3, and -4), the basal activity of each construct (open bar) was reduced to ∼50% compared with that of wild TSP-523. However, fold-induction (closed bar/open bar) of the mutated Sp1 constructs by trichostatin A was ∼10-fold and very similar to that of wild TSP-523 indicating partial involvement of the Sp1 binding sites in the basal promoter activity only. On the contrary, mutation of the inverted CCAAT box reduced markedly not only the basal activity but also the fold-induction by trichostatin A. Basal promoter activity of mutant CCAAT construct was only ∼18% of wild TSP-523. And the fold-induction of the mutant CCAAT construct by trichostatin A was decreased to 3.8-fold, although it is still statistically significant. Meanwhile, a further decrease in basal promoter activity was not

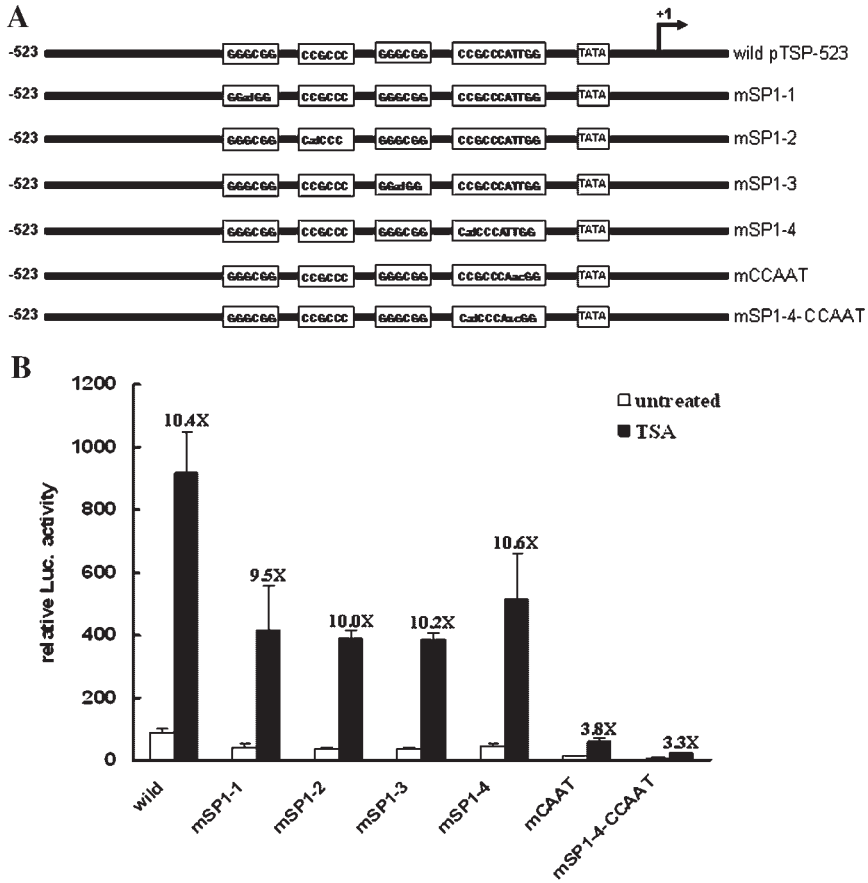

Fig. 3. Requirement of CCAAT box for trichostatin A induction of TSP1 promoter. **A**: Schematic representation of mutated TSP1 promoters. The sequences in the box for four Sp1 binding sites and one CCAAT box are identical to the wild-type pTSP-523 except for the mutations in lowercase letters. **B**: Mutation of the inverted CCAAT box (mCCAAT) remarkably decreases fold-induction of TSP1 promoter by trichostatin A. Wild-type construct pTSP-523 (WT), four Sp1 binding sites-mutated constructs (mSp1-1, mSp1-2, mSp1-3, mSp1-4), CCAAT box alone-mutated construct (mCCAAT), and both Sp1 and CCAAT box-mutated construct (mSP1-4-CCAAT) were transiently transfected into HeLa cells. After transfection of 18 h, cells were not treated or treated with trichostatin A (TSA, 50 ng/ml) for 7 h and then harvested for luciferase assay. Luciferase activity was measured and normalized against protein concentration. The data represent the mean ± SD of four separate experiments performed in duplicate.

observed in both CCAAT box and Sp1 site-mutated construct. These results suggest that the inverted CCAAT box is the main element required for the both basal and trichostatin A-induced activation of TSP1 promoter.

CBF Interacts With Trichostatin A-Responsive CCAAT Box in TSP1 Promoter

To determine the transcription factors bound to the inverted CCAAT box on the TSP1 promoter, EMSA was performed using nuclear extracts from untreated and trichostatin A-treated HeLa cells. Using wild oligonucleotide as a probe, two nuclear protein/DNA complexes were observed without any change in mobility and intensity of the complexes in response to trichostatin A (Fig. 4A,a,b). In a competition assay, formation of the complexes was competed away by adding a 100-fold excess of the wild or mutant Sp1 oligonucleotides (Fig. 4B, lanes 2,3), but not by oligonucleotide containing mutant inverted CCAAT box (Fig. 4B, lane 4), indicating that the formation of the complexes is CCAAT box-specific. The characterization of the nuclear factor binding to the inverted CCAAT box was determined by immune-supershift assay. Since CBF, which is comprised of three subunits, CBF-A, CBF-B, and CBF-C, is a well-known factor interacting with CCAAT box of various gene promoters, antibodies against CBF-A and CBF-B were used. Antibodies against Sp1, AP2, EGR1, and YY1 were also tested, because the sequence of the probe used in this study was high GC-rich (~85%) and

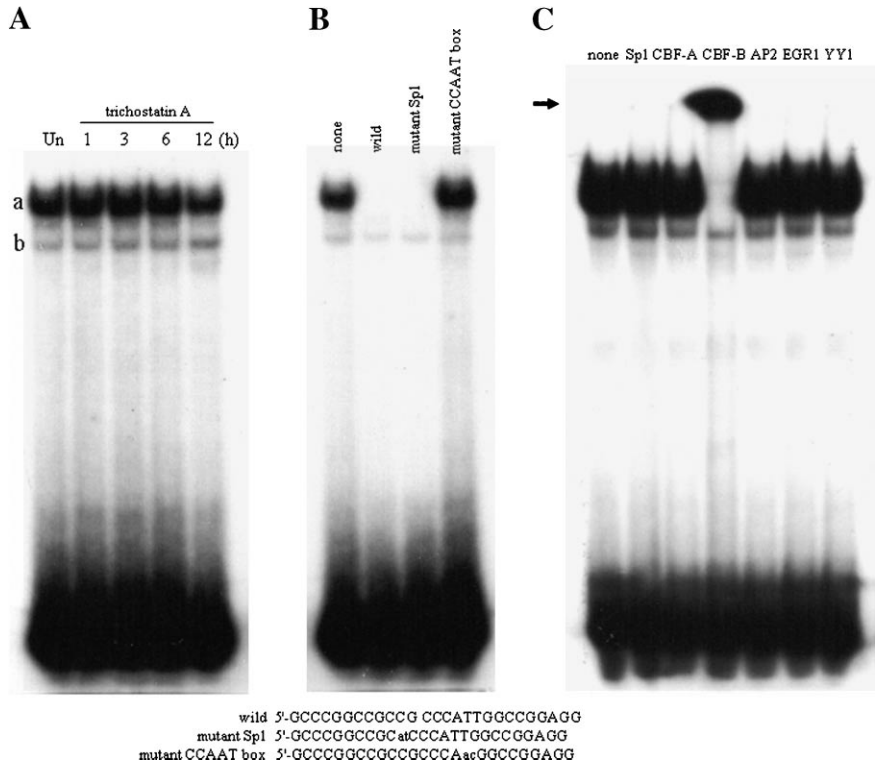

Fig. 4. The binding of CBF-B to the inverted CCAAT box of TSP1 promoter determined by EMSAs. **A**: HeLa cells were treated with trichostatin A (50 ng/ml) for the indicated time periods. Nuclear extracts (10 μg) were obtained and EMSAs were performed as described in Materials and Methods Section. Un: untreated cells. Two nuclear protein/DNA complexes were detected regardless of the treatment with trichostatin A (a,b). **B**: Competition assays for determination of the sequence specificities of CCAAT box. Nuclear extract treated with trichostatin A for 7 h was incubated with 100 × molar excess of unlabeled oligonucleotides containing wild or mutated sequences for Sp1-4 site and for CCAAT box. **C**: Immune supershift assay for CBF binding to the CCAAT box. The same nuclear extract used in the competition assay was preincubated with the indicated antibodies (1 μg) before the addition of a radiolabeled probe. Arrow points to specific complex that reacted with the respective antibody.

hence possibly contains putative Sp1 and EGR1 binding sites. As shown in Figure 4C, the nuclear extracts mixed with anti-CBF-B antibody only resulted in a complete supershift of the slowly migrating complex (arrow), suggesting specific interaction of probe with the subunit B of CBF. From these results, it is suggested that CBF is a nuclear factor involved in trichostatin A-induced TSP1 transcription.

Trichostatin A Increases Binding of Acetylated Form of Histone H3 to the CCAAT Box Region of TSP1 Promoter

ChIP assay was performed whether trichostatin A increases the binding of acetylated histone H3 to TSP1 promoter to activate the gene transcription. Immunoprecipitated and purified DNAs were amplified by standard PCR using a pair of primers flanking the CCAAT box region (−162 to +44) of the TSP1 promoter (Fig. 5A). As shown in Figure 5B, in vivo binding of acetylated form of histone H3 was clearly increased in response to trichostatin A, while the binding of histone H3 was not increased, indicating the recruitment of acetylated form of histone H3 to the chromatin around the trichostatin A-responsive region.

DISCUSSION

This study shows that trichostatin A upregulated human TSP1 gene at the transcriptional level. Using promoter analysis and EMSA, we demonstrate that trichostatin A stimulated the TSP1 promoter activity primarily through the inverted CCAAT box sequence located at −65 relative to the transcriptional start site, which subsequently lead to induction of the TSP1 mRNA in HeLa cells. Thus, TSP1 gene proved to be one of target genes regulated by HDAC inhibitors.

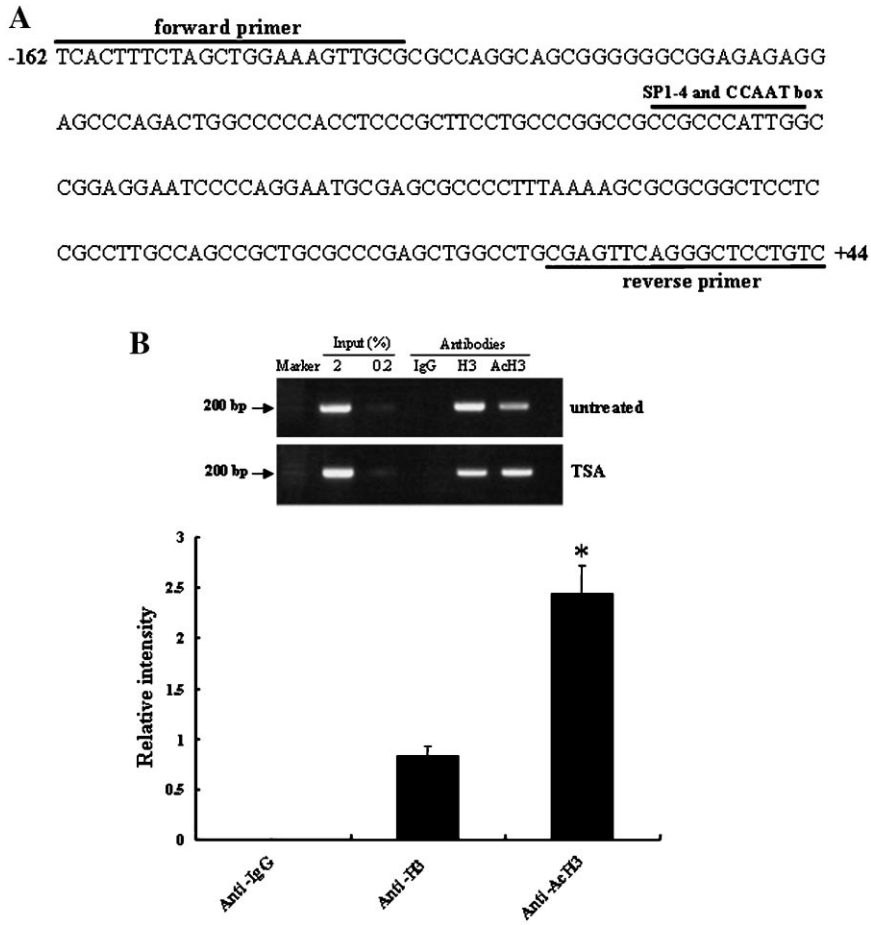

Fig. 5. Recruitment of acetylated histone H3 (AcH3) to the CCAAT box of the TSP1 promoter in vivo. **A**: The amplified region of TSP1 promoter and primers used in the chromatin immunoprecipitation assay. **B**: PCR was performed to amplify the region from −162 to +44. The levels of binding histone H3 (H3) and AcH3 to the TSP1 promoter region were expressed relative to the band intensity of the untreated control. Input samples are 2% and 0.2% of the total DNA, whereas the PCR products of the immunoprecipitations include 5% of the resuspended DNA. Data are expressed as mean ± SD of four separate experiments. $^*P < 0.05$ versus untreated control value.

Expression of TSP1 gene has been known to be regulated by three mechanisms: transcriptional regulation [Adams and Lawler, 2004], post-transcriptional regulation such as mRNA stability [Okamoto et al., 2002; Kang et al., 2006], and epigenetic control such as CpG methylation on the promoter [Li et al., 1999]. In this study, using TSP1 promoter analysis and actinomycin D chase assay, we demonstrated that the transcriptional activation was responsible for the induction of TSP1 gene by trichostatin A. This finding is well consistent with previous studies in that HDAC inhibitor including trichostatin A regulated target genes primarily at the transcriptional level [Sowa et al., 1997; Han et al., 2001; Camarero et al., 2003]. However, in the induction of $p21^{WAF1/Cip1}$ gene by HDAC, mRNA stability as well as transcriptional activation was also an important mechanism involved [Hirsch and Bonham, 2004].

Various HDAC inhibitors regulate a small list of genes through Sp1 site [Doetzlhofer et al., 1999; Huang et al., 2000; Takakura et al., 2001; Walker et al., 2001; Yang et al., 2001; Zhang and Dufau, 2002]. Among them are $p21^{WAF1/Cip1}$ gene, thymidine kinase gene, $G\alpha_{i2}$ gene, IGF binding protein-3 gene, telomerase gene, and human luteinizing hormone receptor (hLHR) gene. Two Sp1 binding sites on $p21^{WAF1/Cip1}$ gene promoter, located at −82 and −77 relative to the transcription start site, are critical elements in mediating the responses to HDAC inhibitors such as suberoylanilide hydroxamic acid and apicidin [Huang et al., 2000; Han et al., 2001]. In the case of the $p21^{WAF1/Cip1}$ and hLHR genes, transcription factor Sp1 and/or Sp3 has

been shown to interact with Sp1 binding site on the promoter to transcriptionally activate these genes [Sowa et al., 1999; Huang et al., 2000; Zhang et al., 2006].

Both human TSP1 and $p21^{WAF1/Cip1}$ promoters hold multiple Sp1 binding sites in the proximal region. Thus, it is possible that the Sp1 binding sites on the TSP1 promoter are involved in mediating the response by trichostatin A. In this study, however, serial deletion of Sp1-1, Sp1-2, and Sp1-3 sites did not affect the trichostatin A response. Moreover, the transiently transfected TSP1 promoter constructs containing mutant Sp1 binding sites including Sp1-4 site were still responsive to trichostatin A with a similar degree to wild TSP1 construct. These findings suggest that Sp1 binding sites are not involved in transcriptionally activating the TSP1 gene in response to trichostatin A.

In contrast, our deletion and mutational analysis identified the important role of inverted CCAAT box located at −65 on the TSP1 promoter in transcriptional activation of TSP1 promoter by trichostatin A. Others also reported the involvement of either CCAAT or inverted CCAAT box in transcriptional induction of certain genes mediating the response of HDAC inhibitors. The γ-globin gene expression is regulated through two CCAAT boxes and the distal CCAAT box is more critical [McCaffrey et al., 1997]. The multidrug resistant gene (MDR) and SH2 domain-containing protein tyrosine phosphatase SHP-1 gene are also modulated through the inverted CCAAT box [Jin and Scotto, 1998; Xu et al., 2001]. In the present study, despite the mutation of the CCAAT box, trichostatin A response was still remained by unknown mechanism although the response was markedly reduced. However, similar results were observed in other study in that the mutation of CCAAT box of γ-globin gene failed to abolish the trichostatin A response [Jin and Scotto, 1998]. Therefore, another *cis*-element(s) also appears to participate in the induction of the genes including TSP1 and γ-globin in concert with the CCAAT box. Actually, for the TSP1 promoter to be fully activated by serum, coordinated function of the inverted CCAAT box and upstream serum response element are required [Framson and Bornstein, 1993].

Transcription factor CBF (also designated NF-Y or CP1) is composed of three subunits, CBF-A, CBF-B, and CBF-C, all of which are necessary for DNA binding. CBF has been known to specifically bind to the CCAAT box and regulate transcription in a variety of genes [Mantovani, 1999]. In the present study, using a competition and an immune supershift assays, we clearly showed the binding of CBF to the CCAAT box in the TSP1 promoter. Furthermore, our ChIP assay demonstrated that trichostatin A increased the binding of acetylated form of histone H3 to the chromatin around the CCAAT box of TSP1 promoter. The condition of enhanced histone acetylation on H3 or/and H4 in gene promoters has been well known to activate gene transcription [Clayton et al., 2006]. Other researcher's study showed that inverted CCAAT box is essential for MDR1 gene activation, and trichostatin A activates this gene through recruitment of histone acetyltransferase (HAT) activity to the CCAAT box by CBF [Jin and Scotto, 1998]. The binding of CBF with CCAAT box would thereby result in histone acetylation, presumably leading to a local disruption of nucleosome structure to achieve the transcriptional activation of the gene. In the absence of trichostatin A, the HAT activity would be antagonized by a HDAC. Another study also showed that CBF was associated with p300, a transcriptional coactivator holding HAT activity, to upregulate the heat shock protein 70 gene transcriptionally [Landsberger and Wolffe, 1995]. Therefore, it is assumed that the HAT activity recruited by CBF is the mechanism mainly involved in trichostatin A-activated TSP1 gene.

In summary, we demonstrate here that the induction of TSP1 gene by trichostatin A is mediated through the inverted CCAAT box located at −65 relative to transcription start site on the promoter and the binding of transcription factor CBF is possibly involved.

REFERENCES

Adams JC, Lawler J. 2004. The thrombospondins. Int J Biochem Cell Biol 36:961–968.

Bornstein P. 1992. Thrombospondins: Structure and regulation of expression. FASEB J 6:3290–3299.

Camarero N, Nadal A, Barrero MJ, Haro D, Marrero PF. 2003. Histone deacetylase inhibitors stimulate mitochondrial HMG-CoA synthase gene expression via a promoter proximal Sp1 site. Nucleic Acids Res 31:1693–1703.

Carey N, La Thangue NB. 2006. Histone deacetylase inhibitors: Gathering pace. Curr Opin Pharmacol 6: 369–375.

Chiba T, Yokosuka O, Arai M, Tada M, Fukai K, Imazeki F, Kato M, Seki N, Saisho H. 2004. Identification of genes

up-regulated by histone deacetylase inhibition with cDNA microarray and exploration of epigenetic alterations on hepatoma cells. J Hepatol 41:436–445.
Clayton AL, Hazzalin CA, Mahadevan LC. 2006. Enhanced histone acetylation and transcription: A dynamic perspective. Mol Cell 23:289–296.
DiPietro LA, Nebgen DR, Polverini PJ. 1994. Downregulation of endothelial cell thrombospondin 1 enhances in vitro angiogenesis. J Vasc Res 31:178–185.
Doetzlhofer A, Rotheneder H, Lagger G, Koranda M, Kurtev V, Brosch G, Wintersberger E, Seiser C. 1999. Histone deacetylase 1 can repress transcription by binding to Sp1. Mol Cell Biol 19:5504–5511.
Framson P, Bornstein P. 1993. A serum response element and a binding site for NF-Y mediate the serum response of the human thrombospondin 1 gene. J Biol Chem 268: 4989–4996.
Guo N, Krutzsch HC, Inman JK, Roberts DD. 1997. Thrombospondin 1 and type I repeat peptides of thrombospondin 1 specifically induce apoptosis of endothelial cells. Cancer Res 57:1735–1742.
Han JW, Ahn SH, Kim YK, Bae GU, Yoon JW, Hong S, Lee HY, Lee YW, Lee HW. 2001. Activation of p21(WAF1/Cip1) transcription through Sp1 sites by histone deacetylase inhibitor apicidin: Involvement of protein kinase C. J Biol Chem 276:42084–42090.
Hirsch CL, Bonham K. 2004. Histone deacetylase inhibitors regulate p21WAF1 gene expression at the post-transcriptional level in HepG2 cells. FEBS Lett 570:37–40.
Huang L, Sowa Y, Sakai T, Pardee AB. 2000. Activation of the p21WAF1/CIP1 promoter independent of p53 by the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) through the Sp1 sites. Oncogene 19: 5712–5719.
Jin S, Scotto KW. 1998. Transcriptional regulation of the MDR1 gene by histone acetyltransferase and deacetylase is mediated by NF-Y. Mol Cell Biol 18:4377–4384.
Kang JH, Kim SA, Hong KJ. 2006. Induction of TSP1 gene expression by heat shock is mediated via an increase in mRNA stability. FEBS Lett 580:510–516.
Kang JH, Kim MJ, Jang HI, Koh KH, Yum KS, Rhie DJ, Yoon SH, Hahn SJ, Kim MS, Jo YH. 2007. Proximal cyclic cAMP response element is essential for exendin-4 induction of rat EGR-1 gene. Am J Physiol 292:E215–E222.
Kim MS, Kwon HJ, Lee YM, Baek JH, Jang JE, Lee SW, Moon EJ, Kim HS, Lee SK, Chung HY, Kim CW, Kim KW. 2001. Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes. Nat Med 7:437–443.
Ko JK, Ma J. 2005. A rapid and efficient PCR-based mutagenesis method applicable to cell physiology study. Am J Physiol Cell Physiol 288:C1273–C1278.
Landsberger N, Wolffe AP. 1995. Role of chromatin and Xenopus laevis heat shock transcription factor in regulation of transcription from the X. laevis hsp70 promoter in vivo. Mol Cell Biol 15:6013–6024.
Lawler J. 1986. The structural and functional properties of thrombospondin. Blood 67:1197–1209.
Li Q, Ahuja N, Burger PC, Issa JP. 1999. Methylation and silencing of the Thrombospondin-1 promoter in human cancer. Oncogene 18:3284–3289.
Mantovani R. 1999. The molecular biology of the CCAAT-binding factor NF-Y. Gene 239:15–27.
McCaffrey PG, Newsome DA, Fibach E, Yoshida M, Su MS. 1997. Induction of γ-globin by histone deacetylase inhibitors. Blood 90:2075–2083.
Okamoto M, Ono M, Uchiumi T, Ueno H, Kohno K, Sugimachi K, Kuwano M. 2002. Up-regulation of thrombospondin-1 gene by epidermal growth factor and transforming growth factor beta in human cancer cells–transcriptional activation and messenger RNA stabilization. Biochim Biophys Acta 1574:24–34.
Panetti TS, Chen H, Misenheimer TM, Getzler SB, Mosher DF. 1997. Endothelial cell mitogenesis induced by LPA: Inhibition by thrombospondin-1 and thrombospondin-2. J Lab Clin Med 129:208–216.
Qian DZ, Kato Y, Shabbeer S, Wei Y, Verheul HM, Salumbides B, Sanni T, Atadja P, Pili R. 2006. Targeting tumor angiogenesis with histone deacetylase inhibitors: The hydroxamic acid derivative L BH589. Clin Cancer Res 12:634–642.
Richon VM, Sandhoff TW, Rifkind RA, Marks PA. 2000. Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation. Proc Natl Acad Sci USA 97:10014–10019.
Salnikow K, Wang S, Costa M. 1997. Induction of activating transcription factor 1 by nickel and its role as a negative regulator of thrombospondin I gene expression. Cancer Res 57:5060–5066.
Sowa Y, Orita T, Minamikawa S, Nakano K, Mizuno T, Nomura H, Sakai T. 1997. Histone deacetylase inhibitor activates the WAF1/Cip1 gene promoter through the Sp1 sites. Biochem Biophys Res Commun 241:142–150.
Sowa Y, Orita T, Minamikawa-Hiranabe S, Mizuno T, Nomura H, Sakai T. 1999. Sp3, but not Sp1, mediates the transcriptional activation of the p21/WAF1/Cip1 gene promoter by histone deacetylase inhibitor. Cancer Res 59:4266–4270.
Suzuki H, Gabrielson E, Chen W, Anbazhagan R, van Engeland M, Weijenberg MP, Herman JG, Baylin SB. 2002. A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. Nat Genet 31:141–149.
Takakura M, Kyo S, Sowa Y, Wang Z, Yatabe N, Maida Y, Tanaka M, Inoue M. 2001. Telomerase activation by histone deacetylase inhibitor in normal cells. Nucleic Acids Res 29:3006–3011.
Walker GE, Wilson EM, Powell D, Oh Y. 2001. Butyrate, a histone deacetylase inhibitor, activates the human IGF binding protein-3 promoter in breast cancer cells: Molecular mechanism involves an Sp1/Sp3 multiprotein complex. Endocrinology 142:3817–3827.
Wittich S, Scherf H, Xie C, Brosch G, Loidl P, Gerhäuser C, Jung M. 2002. Structure-activity relationships on phenylalanine-containing inhibitors of histone deacetylase: In vitro enzyme inhibition, induction of differentiation, and inhibition of proliferation in Friend leukemic cells. J Med Chem 45:3296–3309.
Xu Y, Banville D, Zhao HF, Zhao X, Shen SH. 2001. Transcriptional activity of the SHP-1 gene in MCF7 cells is differentially regulated by binding of NF-Y factor to two distinct CCAAT-elements. Gene 269:141–153.
Yamashita Y, Shimada M, Harimoto N, Rikimaru T, Shirabe K, Tanaka S, Sugimachi K. 2003. Histone deacetylase inhibitor trichostatin A induces cell-cycle arrest/apoptosis and hepatocyte differentiation in human hepatoma cells. Int J Cancer 103:572–576.

Yang J, Kawai Y, Hanson RW, Arinze IJ. 2001. Sodium butyrate induces transcription from the G alpha(i2) gene promoter through multiple Sp1 sites in the promoter and by activating the MEK-ERK signal transduction pathway. J Biol Chem 276:25742–25752.

Yang QW, Liu S, Tian Y, Salwen HR, Chlenski A, Weinstein J, Cohn SL. 2003. Methylation-associated silencing of the thrombospondin-1 gene in human neuroblastoma. Cancer Res 63:6299–6310.

Yoshida M, Furumai R, Nishiyama M, Komatsu Y, Nishino N, Horinouchi S. 2001. Histone deacetylase as a new target for cancer chemotherapy. Cancer Chemother Pharmacol 1:S20–S26.

Zhang Y, Dufau ML. 2002. Silencing of transcription of the human luteinizing hormone receptor gene by histone deacetylase-mSin3A complex. J Biol Chem 277:33431–33438.

Zhang Y, Liao M, Dufau ML. 2006. Phosphatidylinositol 3-kinase/protein kinase Czeta-induced phosphorylation of Sp1 and p107 repressor release have a critical role in histone deacetylase inhibitor-mediated derepression [corrected] of transcription of the luteinizing hormone receptor gene. Mol Cell Biol 26:6748–6761.