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Acetylated Sp1 inhibits PTEN expression through binding to PTEN core promoter and recruitment of HDAC1 and promotes cancer cell migration and invasion

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Specificity protein 1 (Sp1) is often overexpressed in cancer cells. Its binding sites are known to exist in the phosphatase and tension homolog deleted on chromosome 10 (PTEN) promoter. In this study, we hypothesized that Sp1 negatively regulates PTEN expression. We used several cell lines to determine the effects of Sp1. The results showed that Sp1 overexpression inhibited the expression and promoter activity of PTEN and correspondingly upregulated AKT phosphorylation, whereas Sp1 knockdown upregulated the expression and promoter ability of PTEN and downregulated AKT phosphorylation. Moreover, a series of deletion and site-directed mutations of the PTEN promoter indicated that Sp1 can inhibit PTEN promoter activity through a specific Sp1-binding site at the PTEN core promoter *in vivo*. Meanwhile, non-acetylated Sp1, with its loss of DNA binding activity, failed to inhibit the expression and promoter activity of PTEN. Histone deacetylase 1 was necessary for Sp1 to inhibit PTEN expression. The inverse expression of Sp1 and PTEN was found in tongue cancer cells and salivary adenoid cystic cancer (SACC)-LM cells (possessing higher potential for lung metastasis than SACC-83) as compared with that in adjacent normal tissue and SACC-83 cells, respectively. Sp1 knockdown decreased the migration and invasion of SACC-LM cells, whereas Sp1 overexpression increased the migration and invasion of SACC-83 cells. Overall, these results suggest that Sp1 is involved in the development and invasiveness of cancer through inhibition of PTEN.

Introduction

Specificity protein 1 (Sp1) is a ubiquitous transcription factor that recognizes GC-boxes with a consensus sequence of 5′-GGGCGG-3′ (1). Sp1 plays multiple roles in several cellular processes, including cell growth, differentiation and apoptosis (2). In general, Sp1 is an activator of housekeeping genes and other TATA-less genes (3). However, the overexpression of Sp1 in many types of tumors, such as in breast cancers (4), pancreatic tumors (5), thyroid tumors (6), gastric tumors (7), fibrosarcoma (8), liver cancers (9) and glioma (10), is a negative prognostic factor for survival. Sp1 overexpression is even suggested to be a new biomarker for a subset of aggressive pancreatic ductal adenocarcinoma (11). Sp1 is also believed to play a critical role in prostate cancers and could be a target in prostate cancer therapy (12).

Abbreviations: ANOVA, analysis of variance; ChIP, chromatin immunoprecipitation; FBS, fetal bovine serum; HDACs, histone deacetylases; MMP, matrix metalloproteinase; SACC, salivary adenoid cystic cancer; siRNA, small interfering RNA; Sp1, specificity protein 1.

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Sp1 is acetylated at a single site of lysine 703 (K703) (13). This Sp1 acetylation is involved in the regulation of gene expression (14–16). The interaction of Sp1 with histone deacetylases (HDACs) such as HDAC1 and histone acetyltransferases such as p300 either represses or activates gene expression (13,17–21).

Phosphatase and tension homolog deleted on chromosome ten (PTEN) is an important tumor-suppressor gene (22,23) and a dual-specificity phosphatase that removes phosphates from both proteins and lipids. PTEN dephosphorylates phosphatidylinositol (3,4,5)-trisphosphate, a product of phosphatidylinositol-3-kinase (PI3K), to antagonize the PI3K-AKT signaling pathway (24). It is often mutated or deleted in many types of tumors, such as glioblastomas, endometrial carcinomas, prostate cancers, breast cancers and so on (25). PTEN is downregulated in human lung and thyroid cancer cells (26) and is believed to contribute to the occurrence of invasive prostate cancer (27). The human PTEN promoter is GC rich and lacks a TATA box. It contains several transcriptional start sites with multiple putative binding sites for transcription factors, including several putative Sp1-binding sites (28). Transcription factors that bind to these PTEN promoter sites include CBF-1 (29), Snail1 (30), C-Jun (31), p53 (32), Egr-1 (33) and AP-2 (34), whereas those that regulate PTEN expression include NF-κB (26) and PPARγ (35). However, whether Sp1 could bind to the human PTEN promoter and regulate PTEN expression remains unknown. In this study, we hypothesized that Sp1 negatively regulates PTEN expression in tumors. Clinical and theoretical studies should be conducted to examine whether Sp1 could bind to the PTEN promoter and negatively regulate PTEN expression. The results of this study are important to understand the mechanisms underlying the role of Sp1 in tumorigenesis.

In this study, we examined (i) whether Sp1 could inhibit the expression and promoter activity of PTEN through Sp1-binding sites on the PTEN promoter, (ii) whether Sp1 acetylation and HDAC1 recruitment are responsible for the Sp1 inhibition of PTEN and (iii) whether Sp1 is involved in the development and aggressiveness of cancers.

Materials and methods

Cell lines

HeLa cells derived from human cervical cancer cells, 293T cells from human embryonic kidney, U251 cells from human glioma and PC3 from human prostate cancer were incubated in Dulbecco's modified Eagle's medium (GIBCO) with 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. Human salivary adenoid cystic cancer (SACC)-derived SACC-83 cells and SACC-83 cell-derived SACC-LM with low and high lung metastatic potentials, respectively (36), were incubated in RPMI medium 1640 (GIBCO) with 10% FBS at 37°C with 5% CO_2. *Drosophila* Schneider 2 (S2) cells were cultured in Schneider's Drosophila Medium (Invitrogen) with 10% FBS at 28°C.

Clinical specimens

Clinical specimens of tongue squamous cell carcinoma and adjacent normal tissues were collected from 21 patients who underwent surgery in the Department of Oral and Maxillofacial Surgery, Peking University School of Stomatology. Lumps of tumors and adjacent normal tissues, which were at least 1.5 cm distal to the tumor margins, were confirmed by pathological examination. The experiment was approved by the Ethics Committee of Peking University School of Stomatology. Informed consents were obtained from all patients.

Plasmids and antibodies

Sp1 and Sp3 expression plasmids (pN3-Sp1/pN3-Sp3) were kindly provided by Guntram Suske at Philipps-University Marburg, Germany. Anti-Sp1 polyclonal antibody was purchased from Upstate. Anti-phospho-AKT (Thr 308) polyclonal, anti-AKT monoclonal, anti-PTEN polyclonal, anti-HDAC1 polyclonal and anti-Flag-Tag (DYKDDDDK) polyclonal antibodies were purchased from Cell Signaling. Anti-Sp3 monoclonal antibody was purchased from Epitomics. Anti-β-actin (I-19) polyclonal and anti-acetyl-lysine polyclonal antibodies were purchased from Santa Cruz Biotechnology.

Promoter/reporter constructs

The sequence of the human PTEN promoter was obtained from GenBank (AF067844.1). The putative full-length promoter (−2184 bp to +96 bp) of human PTEN was amplified from the genomic DNA of HeLa cells with a high-fidelity DNA polymerase (TOYOBO) using standard PCR techniques. The translational start site was defined as +1 because it has nine potential transcription start sites between −821 bp and −958 bp prior to the initiation codon ATG +1 (28). It was cloned into pCR2.1-TOPO cloning vector (Invitrogen) and recloned into luciferase reporter plasmid at NheI and HindIII sites using pGL3-Basic (Promega). Confirmation was conducted by DNA sequencing. Primers with restriction enzyme sites (underlined) for cloning the PTEN promoter were custom synthesized (Shanghai Sangon Biotech Co., Ltd, Shanghai, China): 5′-*GCTAGC*ACACGTGACCTCCTTCGGAAAGT-3′ (sense) and 5′-*AAGCTT*GCCGCAGAAATGGATACAGGTCAA-3′ (antisense). A series of deletion mutants of the PTEN promoter were constructed based on the full-length promoter-reporter construct. All constructs were confirmed by DNA sequencing.

Site-directed mutagenesis

Site-directed mutagenesis was performed via PCR using DpnI enzyme. PCR was performed with a high-fidelity DNA polymerase (TOYOBO) as described previously (21). All primers were custom synthesized (Shanghai Sangon Biotech Co., Ltd., Shanghai, China). The primers used for mutating Sp1-binding site B (-934/-929) on the PTEN promoter are as follows: 5′-GCGGCGGCGGAG*C*GA*G*A*TCCGC*GGCCGGC GGGCGGT-3′ (sense) and 5′-ACCGCCCGCCGGCC *GCGGATC*T*CG*CTCCGCCGCCGC-3′ (antisense). The primers used for mutating Sp1-binding site C (-918/-913) on the PTEN promoter are as follows: 5′-GATCCGCGGCC GGCGG*C*CGGTAAAGCTT GGCATTCC-3′ (sense) and 5′-GGAATGCCAAGC TTTACC*G*GCCGC CGGCCGCGGATC-3′ (antisense). The primers used for mutating the K703 of Sp1 are as follows: 5′-GCTTCATG AGGAGTGACCACCT GTCAA*C*ACATATCAAGAC CCACCAGAATAAG-3′ (sense) and 5′-CTTATTCTGG TGGGTCTTGATATGT*G*TTGACAGGTGGTCACTC CTCATGAAGC-3′ (antisense). The primers used for Flag-tag insertion in the Sp1 expression plasmid are as follows: 5′-ATCAGTGGCAA TGGCTTCTGA*GATTACAAGGATGACGACGATAAG*TGAGATCA GGCACCCGGGGCC-3′ (sense) and 5′-GGCCCCGGGTGCCTGATCTCA*C TTATCGTCGTCAT CCTTGTAATC*TCAGAA GCCATTG CCACTGAT-3′ (antisense). In the above primers, the *italics* refer to mutated nucleotides. All mutations were confirmed by DNA sequencing.

Transient transfection

The cells were plated into six-well plates at 1×10^6 per well. After the cells reached 95% confluency, they were transfected with 2 µg plasmids or 100 nmol small interfering RNA (siRNA) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were harvested 48 h after transfection. Sp1 siRNA was purchased from Invitrogen with the following sequence: 5′-UGUAGAGUCUGCCAACUGACCUGUC-3′. Based on a previous study, HDAC1 siRNA was custom synthesized (RiboBio Co., Ltd, Guangzhou, China) with the following sequence: 5′-CAGCGACUGUUUGAGAACCTT-3′ (37).

Real-time quantitative PCR

Total RNA was extracted using TRIzol reagent (Invitrogen). Reverse transcription and real-time PCR were performed as described previously (38). The primers for human PTEN are as follows: 5′-GACCATAACCCACCACAGC-3′ (sense) and 5′-CCAGTTCGTCCCTTTCCAG-3′ (antisense). The primers for human β-actin are as follows: 5′-CGGGAAATCGTGCGTGAC-3′ (sense) and 5′-CAGGCAGCTCGTAGCTCTT-3′ (antisense). The primers for human p21 are as follows: 5′-TGGCACCTCACCTGCTCTG-3′ (sense) and 5′-CGGCGTTTGGAGTGGTAGAA-3′ (antisense). The primers for human c-myc are as follows: 5′-GAGTTTCATCTGCGACCCG-3′ (sense) and 5′-GCTGCCGCTGTCTTTGC-3′ (antisense). The primers for human bcl-2 are as follows: 5′-TGTGGCCTTCTTTGAGTTCG-3′ (sense) and 5′-CCCAGCCTCCGTTATCC-3′ (antisense). The primers for human Sp1 are as follows: 5′-CACCAGAATAAGAAGGGAGG-3′ (sense) and 5′-GGTGGTAATAAGGGCTGAA-3′ (antisense). The primers for human matrix metalloproteinase (MMP2) are as follows: 5′-CCGTCGCCCATCATCAAGTT-3′ (sense) and 5′-CTGTCTGGGGCAGTCCAAAG-3′ (antisense). All the primers were designed using the Primer Premier Version 5.0 software, except that the primers for human MMP2 were adopted from a previous study (39). The efficiency of all the primers was confirmed by sequencing their conventional PCR products. Real-time PCR was performed using a 7500 real-time PCR system of Applied Biosystems with FastStart Universal SYBR Green Master (Roche).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed using a ChIP assay kit (Upstate) as described in a previous study (21). Briefly, HeLa cells were cross-linked with 1% formaldehyde. The chromatin was sonicated into fragments ranging between 200 and 1000 bp and then was pulled down by anti-Sp1 antibody for PCR amplification. The primers for amplifying the fragments (−1138 to −606) containing Sp1-binding sites B and C (Figure 3) of the PTEN promoter are as follows: 5′-AGGCAGCTACACTGGGCAT-3′ (sense) and 5′-AGGAAGAGGCTGCACGGTTAGAAA-3′ (antisense). The PCR products were analyzed on 1.5% agarose gel and then photographed.

Luciferase assay

Luciferase assay was performed as described previously (21). Briefly, 1 µg PTEN reporter plasmid was transfected or cotransfected with 0.5 µg Sp1 expression plasmid with Lipofectamine 2000 into the HeLa or 293T cells in a 12-well plate. Transfection of the plasmids into S2 cells was performed using FuGene HD (Roche). The transfected cells were lysed in a cell lysis buffer 28 h after the transfection. Luciferase activity was measured with a FB12 luminometer (Berthold, Germany) using luciferin as the substrate according to the manufacturer's instructions (Promega).

Immunoprecipitation

Immunoprecipitation was performed as described previously (21). Briefly, whole-cell extracts (2.5 mg) were incubated in 500 µl extraction buffer with 4 µg anti-Sp1 antibody for 16 h at 4°C, added with 40 µl protein A/G-agarose beads (Santa Cruz Biotechnology), incubated again for 1 h at 4°C and then washed five times. The bound proteins were released by boiling in a loading buffer and then subjected to western blot analysis. Acetylated Sp1 was detected using acetyl-lysine antibody. The membranes were stripped for Sp1 detection.

Western blot

Western blot was performed as described previously (21). Briefly, proteins were separated using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to a polyvinylidene fluoride membrane. The membrane was blocked using non-fat milk for 1 h at room temperature (RT). After incubation with primary antibody in TBS-T for 2 h at RT or 16 h at 4°C, the membrane was washed extensively with TBS-T and then incubated with secondary horseradish peroxidase–conjugated antibody for 1 h at RT. After extensive washes with TBS-T, the membrane was visualized with enhanced chemiluminescence plus reagents (Thermo).

Transwell migration and invasion assays

Cell migration and invasion assays were performed in transwell chambers (Corning Costar) with a polycarbonate membrane as described previously (36). Transwell cell migration assays were conducted as follows. Briefly, the cells were transfected with or without Sp1 plasmid or Sp1 siRNA for 36 h. Subsequently, the cells were harvested, seeded at 1×10^5 cells/well in serum-free medium in the upper chambers, and then incubated for 12 h. The lower chamber contained the culture medium with 10% FBS. Cells on the top surface of the membrane were wiped off. The membrane was then fixed and stained with crystal violet. Cells on the bottom surface of the membrane were examined under a light microscope at ×200 magnification. Cells from 6 to 10 random fields across three replicate wells were counted and averaged by the number of fields. The same procedure was performed for transwell invasion assay, except that the upper chambers were coated with 20 µg extracellular matrix gel (Sigma-Aldrich).

DNA affinity purification assay

DNA affinity purification assay (DAPA) was performed as described previously (21). Briefly, 5′-end-biotinylated oligonucleotides were custom synthesized with the sequence corresponding to the PTEN promoter −926/−912 region containing C site (5′-biotin/AGGCCGGCGGGCGGT-3′). The mutant probe contains the same mutation of C site as that in the site-directed mutagenesis (5′-biotin/AGGCCGGCGG*C*CGGT-3′, the *italics* refers to the mutated nucleotide). The biotinylated sense strand was annealed with its non-labeled antisense strand and then incubated with 293T nuclear extracts. The DNA protein complex was precipitated using streptavidin–agarose beads (Sigma-Aldrich). The bound proteins were released by boiling in sodium dodecyl sulfate loading buffer and then subjected to western blot analysis.

Statistical analysis

Statistical analysis was performed using SPSS 11.5 for Windows. All data were presented as mean ± standard deviation (SD). Differences between multiple groups were analyzed by two-way analysis of variance (ANOVA). $P < 0.05$ was considered to indicate statistical significance.

Results

Sp1 overexpression inhibited PTEN expression and upregulated AKT phosphorylation

To explore whether Sp1 could negatively regulate PTEN expression, we first tested whether Sp1 overexpression could inhibit PTEN

expression. As shown in Figure 1, Sp1 overexpression by transfection of Sp1 expression plasmids for 48 h significantly decreased the messenger RNA (mRNA) and protein expressions of PTEN compared with the control group and upregulated AKT phosphorylation (Thr 308). Sp1 overexpression in HeLa cells upregulated the mRNA expressions of the oncogene c-myc and bcl-2 by more than 2-fold but showed no effect on the mRNA expression of the tumor-repressor gene p21/Waf1 compared with the control group.

Sp3 has a similar structure and shares the same consensus of binding site with Sp1 (40,41); moreover, it can repress Sp1-mediated transcriptional activation (42). Thus, we examined whether Sp3 could also affect PTEN expression or interfere with the Sp1-mediated inhibition of PTEN expression. As shown in Figure 1C, Sp3 overexpression in HeLa cells did not affect the mRNA expressions of PTEN, c-myc and bcl-2 but strongly induced that of p21 by more than 6-fold. After cotransfection of Sp1 with Sp3, Sp3 did not interfere with the Sp1-mediated inhibition of PTEN expression but blocked the Sp1-mediated induction of bcl-2 and c-myc. Conversely, Sp1 partially blocked the Sp3-mediated induction of p21. The protein levels of Sp1 and Sp3 were comparably overexpressed 48 h after transfection (Figure 1D).

Sp1 knockdown upregulated mRNA and protein expressions of PTEN and downregulated AKT phosphorylation

To confirm further the Sp1-mediated inhibition of PTEN, we used a Sp1-specific siRNA to knockdown Sp1. As shown in Figure 2A and 2B, Sp1 siRNA decreased the mRNA and protein expressions of Sp1, upregulated the mRNA expression of PTEN and downregulated the mRNA expression of MMP2 (Sp1-dependent expression). Meanwhile, Sp1 knockdown significantly upregulated PTEN protein expression and downregulated AKT phosphorylation in several cell lines. However, neither the overexpression nor the knockdown of Sp1

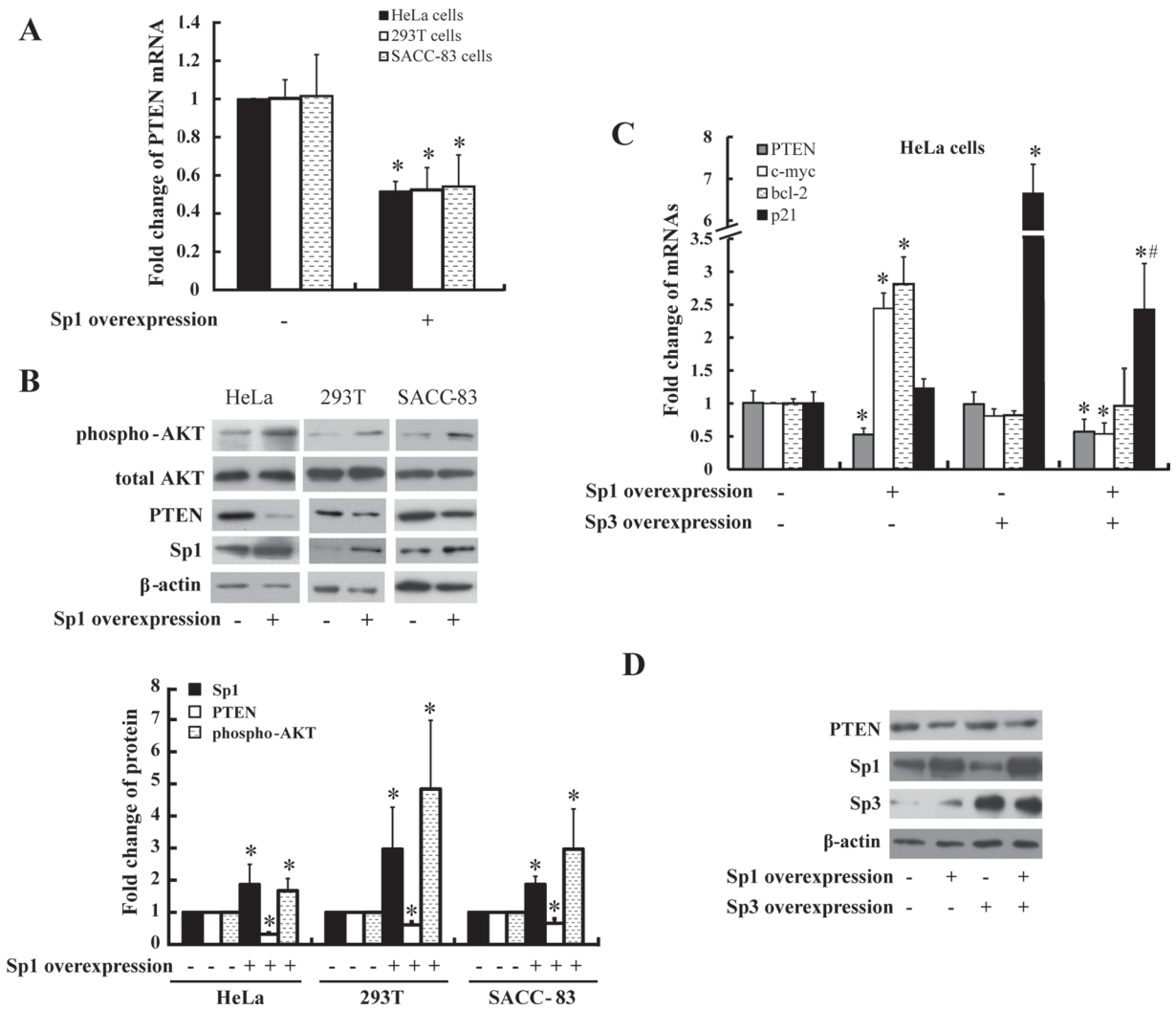

Fig. 1. Sp1 overexpression inhibited PTEN expression and upregulated AKT phosphorylation. (**A**) Sp1 overexpression inhibited PTEN mRNA expression. Cells were transfected with empty plasmids (control) or Sp1 expression plasmids for 48 h. The mRNA expression of PTEN was quantitated by real-time PCR. Data (mean ± SD of three separated experiments) were presented as fold of the control group. $^{*}P < 0.05$ versus the control ($n = 3$, one-way ANOVA). (**B**) Sp1 overexpression inhibited PTEN protein expression and upregulated AKT phosphorylation (Thr 308). Total AKT served as the control for AKT phosphorylation and β-actin served as the internal control for equal loading. Cells were transfected with empty plasmids or Sp1 expression plasmids for 48 h and whole cell lysates were subjected to western blot. The membrane was stripped for detection with antibodies as indicated. The protein levels of Sp1, PTEN and phosphor-AKT were quantitated (lower panel). The target bands on western blots were scanned and densitometry was performed. Data (mean ± SD of three separated experiments) were presented as folds of the control group. $^{*}P < 0.05$ versus the control ($n = 3$, one-way ANOVA). (**C**) Effects of the overexpression of Sp1, Sp3 or both on the mRNA expressions of PTEN, p21, c-myc and bcl-2. Cells were transfected with Sp1, Sp3 or both for 48 h. mRNA expressions were quantitated by real-time PCR. Data (mean ± SD of three separated experiments) were presented as folds of the control group. $^{*}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the Sp1 and Sp3 groups ($n = 3$, two-way ANOVA). (**D**) Expressions of PTEN, Sp1 and Sp3 after transfection of Sp1, Sp3 or both. 293T cells were transfected for 48 h and whole cell lysates were subjected to western blot.

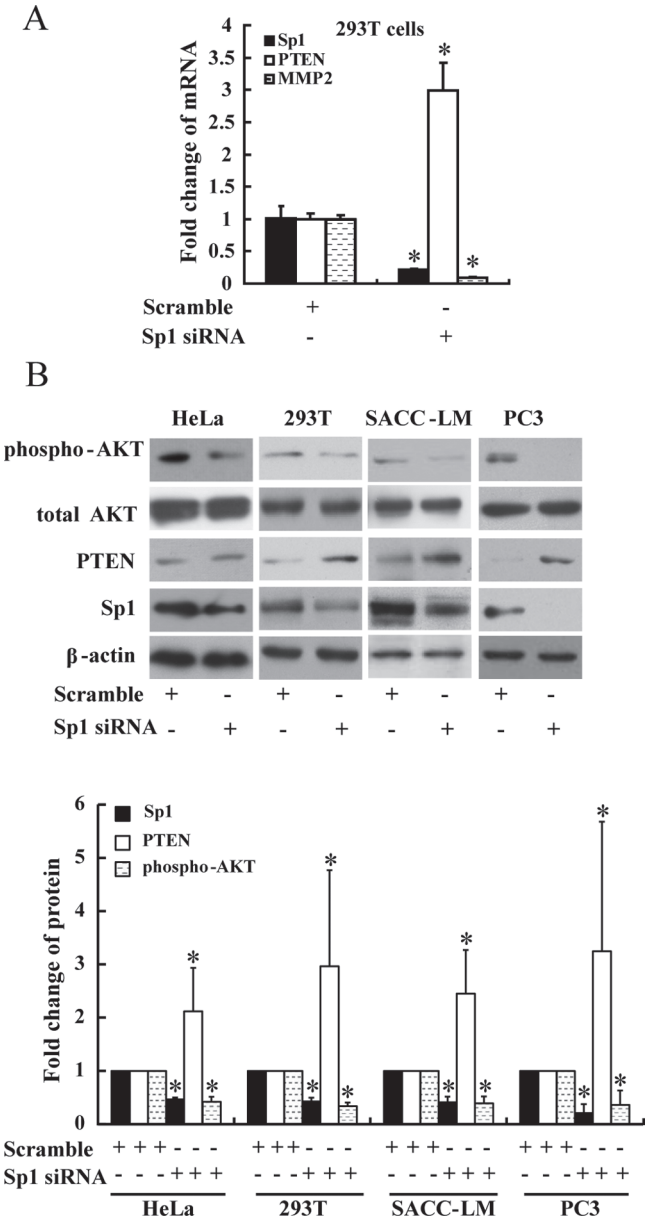

Fig. 2. Sp1 knockdown upregulated PTEN expression and downregulated AKT phosphorylation. (**A**) Sp1 knockdown specifically upregulated PTEN mRNA expression. Cells were transfected with scramble siRNA or Sp1 siRNA for 48 h. mRNA expressions of PTEN, Sp1 and MMP2 were quantitated by real-time PCR. Data (mean ± SD of three separated experiments) were presented as fold of the control group. MMP2 served as the positive control. *$P < 0.05$ versus the control group ($n = 3$, one-way ANOVA). (**B**) Sp1 knockdown inhibited PTEN protein expression and upregulated AKT phosphorylation. Cells were transfected with scramble siRNA or Sp1 siRNA for 48 h and whole cell lysates were subjected to western blot. β-actin served as the internal control for equal loading. The membrane was stripped for detection with antibodies as indicated. The protein levels of Sp1, PTEN and phosphor-AKT were quantitated (lower panel). The target bands on western blots were scanned and densitometry was performed. Data (mean ± SD of three separated experiments) were presented as fold of the control group. *$P < 0.05$ versus the control group ($n = 3$, one-way ANOVA).

significantly affected cell proliferation in the transient transfection assay (data not shown).

Identification of the PTEN core promoter

To test whether the Sp1-mediated inhibition of PTEN expression was at the transcription level, we examined whether PTEN promoter

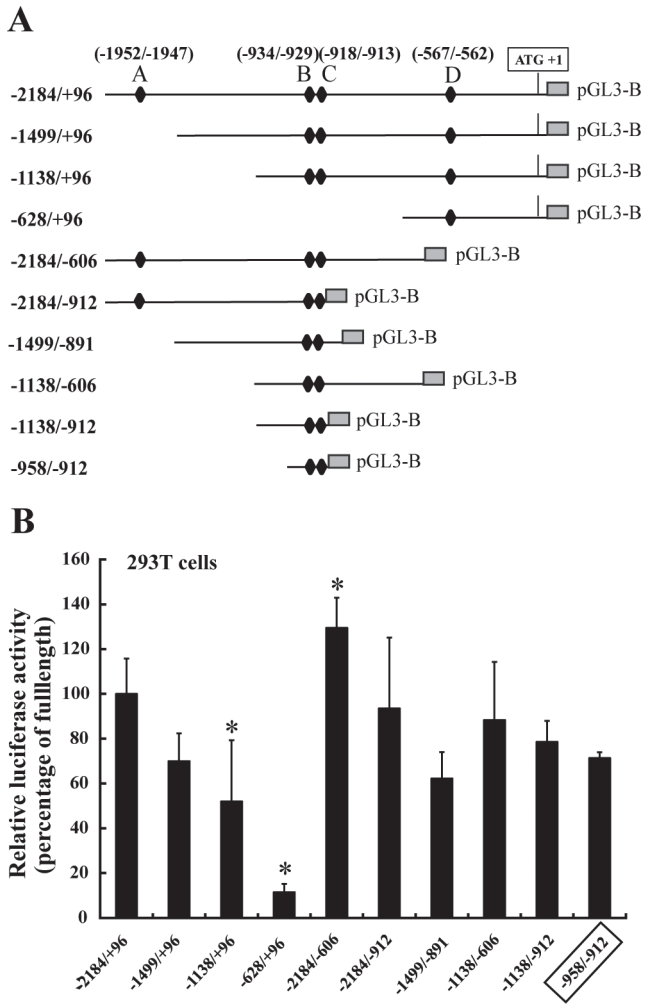

Fig. 3. Identification of PTEN core promoter. (**A**) Schematic maps of deletion mutants of the PTEN promoter. The PTEN promoter was simulated by a straight line. The typical consensus of Sp1-binding sites were represented by filled diamond shapes and designated as A, B, C and D. The translational start site was defined as (+1) because multiple transcriptional start sites between −958 and −821 prior to the initiation codon ATG were reported. The gray boxes represent luciferase reporter constructs (pGL3-B, pGL3-Basic plasmid). The same schematic map was used in the other figures. (**B**) Activities of deletion mutants of PTEN promoter. The shortest mutant (−958/−912) denoted by a rectangle was identified as the core promoter of PTEN. PTEN promoter-reporter constructs were transfected into 293T cells for 24 h and luciferase activity was measured and normalized by total protein concentration. Data (mean ± SD of five separated experiments) were presented as percentage of the full-length promoter (−2184/+96) activity. *$P < 0.05$ versus the other groups ($n = 5$, two-way ANOVA).

activity was affected by Sp1. The PTEN full-length promoter-pGL3-Basic reporter construct showed a luciferase activity about 20-fold that of pGL3-Basic, which does not possess a promoter or an enhancer, and 25% that of pGL3-Control, which possesses an SV40 promoter and enhancer (data not shown). We then performed a series of deletion mutations of the PTEN promoter in the reporter construct and examined the promoter activities of the deletion mutants. According to the Sp1-binding consensus, four putative typical Sp1-binding sites located at −1952/−1947 bp, −934/−929 bp, −918/−913 bp and −567/−562 bp of the PTEN full-length promoter region (−2184/+96) were designated as A, B, C and D, respectively (Figure 3A). As shown in Figure 3B, the −2184/−606 region showed the maximum promoter activity, whereas the −628/+96 region lacked an efficient promoter activity, as compared with that of the full-length promoter. This finding suggests that the −628/+96 region could possess a negative regulatory region or a silencer. Furthermore, the activity of the

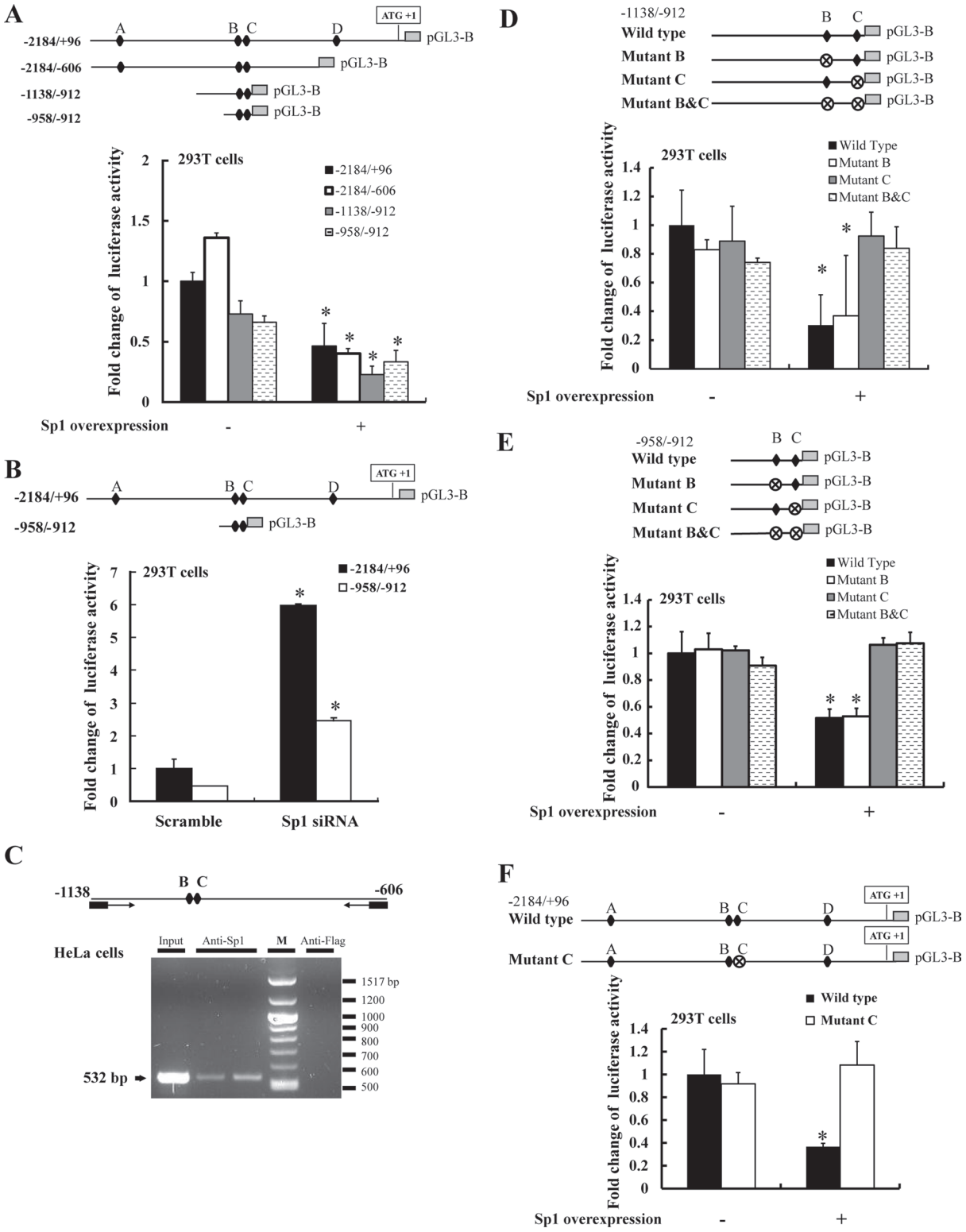

Fig. 4. Sp1 overexpression inhibited PTEN promoter activity through a specific Sp1-binding site at the PTEN core promoter. (**A**) Narrowing down of the Sp1-targeted region of the PTEN promoter. Data (mean ± SD of five separated experiments) were presented as folds of the full-length promoter (−2184/+96) in the control group. *$P < 0.05$ versus the control group ($n = 5$, one-way ANOVA). (**B**) Sp1 knockdown upregulated the activities of PTEN full-length promoter and core promoter. After transfection with scramble siRNA or Sp1 siRNA for 24 h, cells were transfected with the reporter constructs for another 24 h. Data (mean ± SD of five separated experiments) were presented as folds of the full-length promoter (−2184/+96) in the control group. *$P < 0.05$ versus the control group ($n = 5$, one-way ANOVA). (**C**) Sp1 bound to PTEN promoter *in vivo*. ChIP assays were performed in HeLa cells with anti-Sp1 or anti-FLAG antibodies and with primers amplifying the −1138/−606 region of the PTEN promoter containing Sp1-binding sites B and C. The two lanes of anti-Sp1 are separated samples. (**D–F**) Mutation of Sp1-binding site C (−918/−913) abolished the inhibitory effects of Sp1 on the activities of the −1138/−912 region, core promoter and full-length PTEN promoter. Mutated Sp1-binding site was represented by crossed circle. Cells were transfected with reporter constructs alone or in combination with Sp1 expression plasmids for 24 h. Data (mean ± SD of five separated experiments) were presented as folds of the wild type in the control group. *$P < 0.05$ versus the control groups or mutant C or mutant B and C groups ($n = 5$, two-way ANOVA).

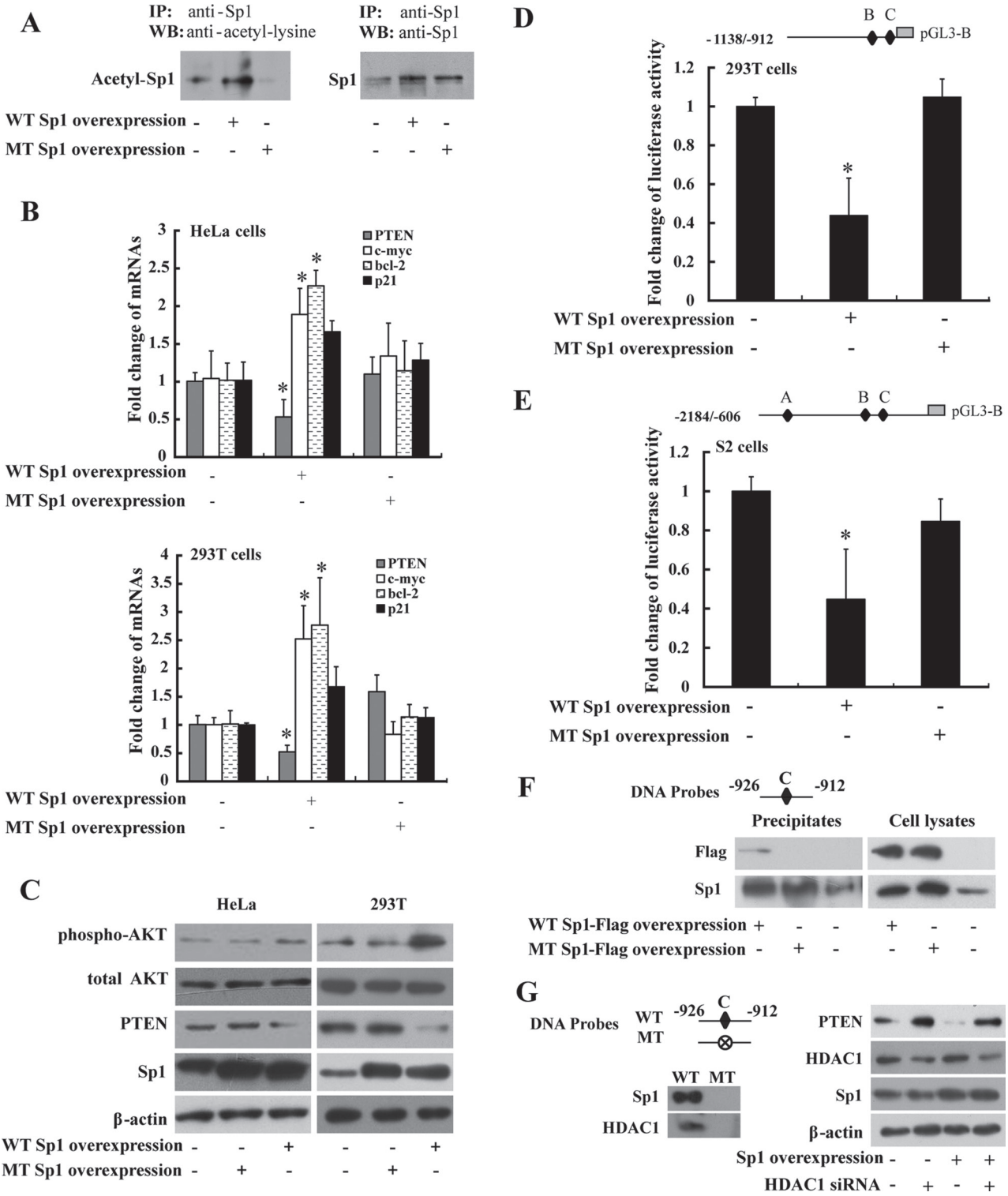

Fig. 5. Requirement of acetylation and HDAC1 for Sp1 to inhibit PTEN expression. (**A**) Mutation of the unique acetylation site K703 of Sp1 abolished Sp1 acetylation. 293T cells were transfected with wild-type Sp1 and non-acetylation Sp1 mutant for 48 h. Whole cell lysates were immunoprecipitated with anti-Sp1 antibody. The immunocomplexes were subjected to western blot with anti-acetyl-lysine antibody (left panel). The membrane was stripped for detection with anti-Sp1 antibody (right panel). IP, immunoprecipitation; WB, western blot; WT, wild type; MT, mutant. (**B**) Overexpression of non-acetylated Sp1 did not inhibit PTEN mRNA expression and did not upregulate bcl-2 and p21 mRNA expressions. Cells were transfected with wild-type Sp1 or non-acetylated Sp1 mutant for 48 h. The mRNA expressions of genes were quantitated by real-time PCR. Data (mean ± SD of three separated experiments) were presented as folds of the control group. $*P < 0.05$ versus the control group ($n = 3$, one-way ANOVA). (**C**) Overexpression of non-acetylated Sp1 failed to inhibit PTEN protein expression. Cells were transfected with wild-type Sp1 and non-acetylated Sp1 mutant for 48 h, and whole cell lysates were subjected to western blot. The membrane was stripped for detection with antibodies as indicated. β-actin served as the internal control for equal loading. (**D**, **E**) Overexpression of non-acetylated Sp1 failed to inhibit PTEN promoter activity in cells with or without endogenous Sp1. Cells were transfected with a combination of PTEN promoter-reporter constructs and wild-type Sp1 expression plasmid or non-acetylated Sp1 mutant expression plasmid for 24 h. Data (mean ± SD of five separated experiments) were presented as folds of the control group. $*P < 0.05$ versus the other groups ($n = 5$, two-way ANOVA). (**F**) Non-acetylated Sp1 failed to bind to the PTEN promoter. The Flag

–958/–912 region (47 bp) was approximately 70% that of the full-length promoter. Based on the definition of a core promoter, this finding indicates that the –958/–912 region (containing Sp1-binding sites B and C) is the core promoter of PTEN (43).

Specific Sp1-binding site of PTEN core promoter responsible for Sp1-mediated inhibition of PTEN promoter activity

As shown in Figure 4A, Sp1 overexpression inhibited PTEN promoter activities at different lengths, including the core promoter. As shown in Figure 4B, Sp1 knockdown by Sp1 siRNA enhanced the activities of both the full-length and the core promoter. These results suggest that Sp1 targets the core promoter to inhibit PTEN promoter activity.

To examine whether Sp1 could bind to the region containing Sp1-binding sites B and C *in vivo*, we performed ChIP assays in HeLa cells using the primers amplifying for the –1138/–606 (532 bp) region that contains Sp1-binding sites B and C. As shown in Figure 4C, an expected DNA fragment of 532 bp was specifically amplified from the chromatin pulled down by anti-Sp1 antibody but not by anti-FLAG antibody.

Moreover, mutation of Sp1-binding site C alone or both sites B and C but not site B alone completely abolished the inhibitory effect of Sp1 on the –1138/–912 region or the core promoter compared with the wild type (Figure 4D and 4E). Hence, the mutation of site C in the full-length promoter confirmed that only site C was responsible for the Sp1-mediated inhibition of PTEN promoter activity (Figure 4F).

Requirement of acetylation and HDAC1 for Sp1-mediated inhibition of PTEN expression

We determined whether the acetylation of Sp1 affects its inhibitory effect on PTEN expression by mutating a Sp1 unique acetylation site (K703) to abolish Sp1 acetylation. As shown in Figure 5A, the overexpression of wild-type Sp1 increased the acetylation of precipitated Sp1 compared with that of the control group (vehicle transfected). In contrast, the overexpression of the non-acetylated Sp1 mutant decreased the acetylation of the precipitated Sp1 compared with that of the control. This result may be attributed to the predomination of ectopically overexpressed non-acetylated Sp1 in the cell lysates, which correspondingly decreased the proportion of endogenous Sp1 (including acetylated and non-acetylated Sp1) in the precipitate. The amounts of precipitated Sp1 in the cells transfected with wild-type Sp1 or non-acetylated Sp1 mutant were almost equal and both significantly higher than the control cells, implying that the difference in acetylation level of the wild-type and mutant was not due to the difference in Sp1 precipitation (Figure 5A). These results confirm that the K703 mutation of Sp1 successfully abolished Sp1 acetylation.

Compared with the wild-type Sp1, the overexpression of non-acetylated Sp1 mutant did not inhibit the mRNA and protein expressions of PTEN, did not induce the mRNA expressions of c-myc and bcl-2 and did not affect the mRNA expression of p21 (Figure 5B and 5C). In addition, the overexpression of non-acetylated Sp1 mutant failed to inhibit PTEN promoter (–1138/–912 region) activity in 293T cells and -2184/-606 region promoter activity in S2 cells, which lack endogenous Sp1 expression, compared with the wild-type Sp1 (Figure 5D and 5E).

We further examined whether acetylation could affect the DNA binding activity of Sp1 using DAPA. As shown in Figure 5F, the Flag fusion protein was only detected in the precipitates pulled down by DNA from the cells transfected with the wild-type Sp1-Flag but not from the cells transfected with the non-acetylated Sp1-Flag mutant. However, Sp1 was detected in all the precipitates from the transfected and control cells. Moreover, the Sp1-Flag fusion protein could be detected in the lysates of the cells transfected with the wild-type Sp1-Flag or non-acetylated Sp1-Flag.

We then examined whether HDAC1 was recruited and required for the Sp1-mediated inhibition of PTEN expression. As shown in Figure 5G, the wild-type DNA probes pulled down Sp1 and HDAC1 (left panel), whereas the mutant DNA probes did not. The knockdown of HDAC1 upregulated the PTEN expression and abolished the Sp1-mediated inhibition of PTEN expression (right panel). These findings indicate that Sp1 inhibited PTEN expression by recruiting HDAC1 into the PTEN promoter.

Sp1 upregulation and PTEN downregulation in clinical specimens of tongue cancers

To understand further the clinicopathological significance of Sp1 overexpression, we first examined whether an inverse expression of Sp1 and PTEN exists among different cancer cell lines. Generally, a clear inverse expression of Sp1 and PTEN was hardly found among the different cancer cells. 293T and HeLa cells had relatively higher expressions of both Sp1 and PTEN, whereas U251 and PC3 cells had relatively lower expressions of both Sp1 and PTEN. However, an inverse expression of Sp1 and PTEN was observed in both SACC-LM and SACC-83 cells (Figure 6A). We next examined whether an inverse expression of Sp1 and PTEN exists in clinical specimens of tongue cancers. As shown in Figure 6B, Sp1 mRNA expression was upregulated but PTEN mRNA expression was downregulated in the tongue cancer specimens compared with the adjacent normal tissue.

Sp1 knockdown inhibited migration and invasion of SACC-LM cells, whereas Sp1 overexpression promoted migration and invasion of SACC-83 cells

We further confirmed the inverse expression of Sp1 and PTEN with the corresponding AKT phosphorylation changes in SACC-LM cells and SACC-83 cells (Figure 6C). We also examined whether Sp1 could be responsible for the differences in the cell migration and invasion of the two cell lines. As shown in Figure 6D and 6E, SACC-LM cells had higher migration and invasion abilities than SACC-83 cells. Sp1 knockdown in SACC-LM cells decreased cell migration and invasion to the comparable level of SACC-83, whereas Sp1 overexpression in SACC-83 cells promoted cell migration and invasion to the comparable level of SACC-LM (Figure 6).

Discussion

In the present study, we provided substantial evidence to prove for the first time that Sp1 can promote the development and invasiveness of cancer by inhibiting PTEN expression. First, the overexpression of Sp1 decreased the mRNA and protein expressions of PTEN and upregulated AKT phosphorylation. Second, Sp1 inhibited PTEN promoter activity through a specific Sp1-binding site at the PTEN core promoter. Third, acetylation was required for Sp1 to bind to the PTEN core promoter, with HDAC1 recruitment to inhibit PTEN expression. Fourth, Sp1 was upregulated in the tongue cancer specimens compared with the adjacent normal tissue. Fifth, Sp1 knockdown

←
fusion protein was only detected in the precipitates of cells transfected with the wild-type Sp1-Flag, whereas Sp1 was detected in all the samples of precipitates (left panel). DAPA was performed using PTEN promoter –926/–912 region containing Sp1-binding site C after 293T cells were transfected with the wild-type Sp1-Flag or non-acetylated Sp1-Flag mutant for 48 h. The membrane was stripped for Sp1 detection. The Flag fusion protein was comparably detected in both lysates of the cells transfected with the wild-type Sp1-Flag or non-acetylated Sp1-Flag mutant, whereas Sp1 was detected in all cell lysates. The membrane was stripped for Sp1 detection. (**G**) HDAC1 was recruited and required for Sp1 to inhibit PTEN expression. Sp1 and HDAC1 were both pulled down by the wild-type probes, whereas neither Sp1 nor HDAC1 was pulled down by the mutant probes (left panel). DAPA was performed using PTEN promoter –926/–912 region with Sp1-binding site C intact or mutated as DNA probes. The membrane was stripped for HDAC1 detection. WT, wild type; MT, mutant. HDAC1 knockdown abolished the inhibitory effect of Sp1 on PTEN expression (right panel). Western blot was performed after 293T cells were transfected with Sp1 or HDAC1 siRNA for 48 h. The membrane was stripped for detection with antibodies as indicated.

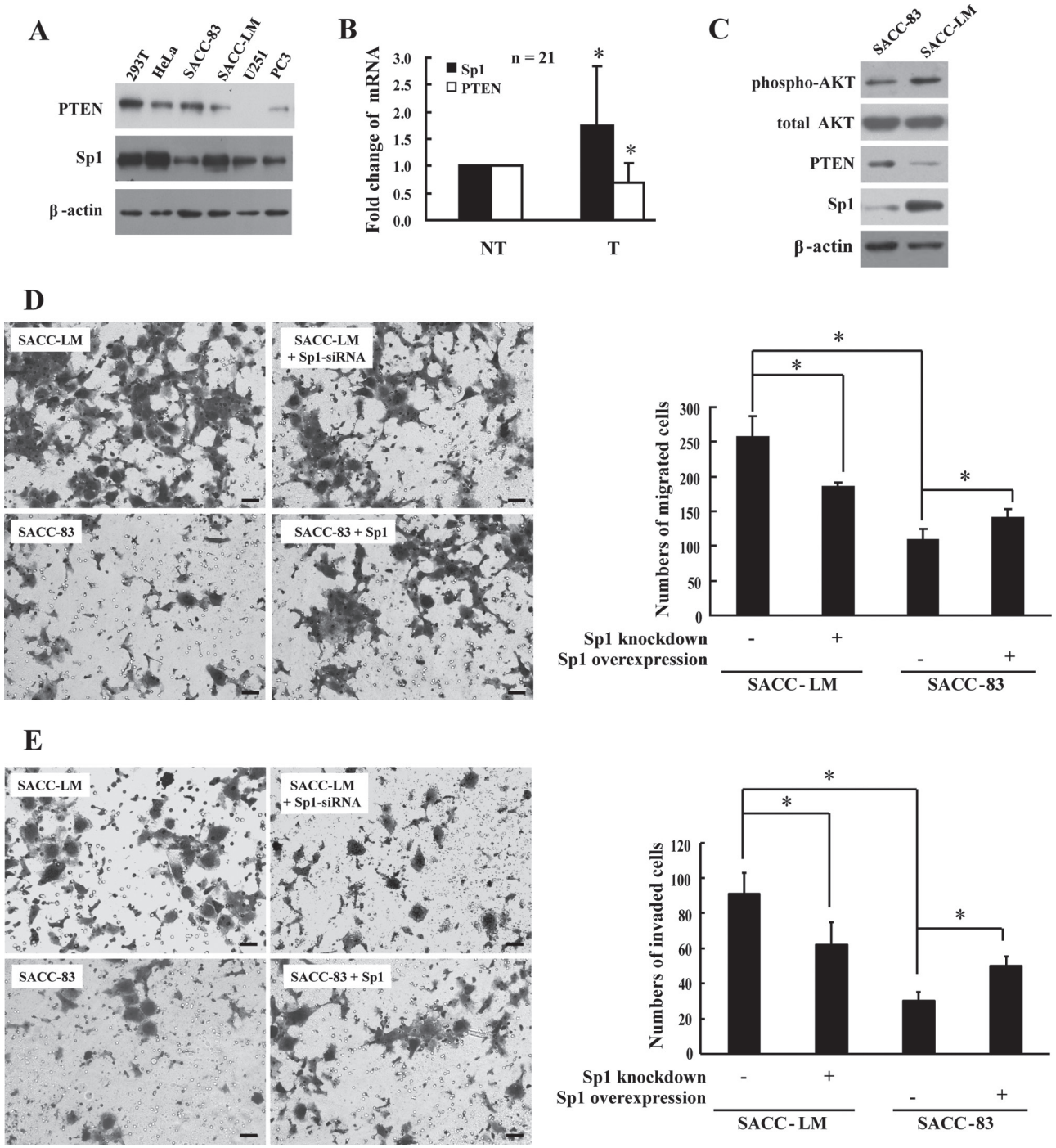

Fig. 6. Involvement of Sp1 in cancer development and invasiveness. (**A**) Protein expression of Sp1 and PTEN in cell lines. Generally, no typical inverse protein expression of Sp1 and PTEN was observed among the cell lines. The membrane was stripped for detection with antibodies as indicated. (**B**) Inverse mRNA expression of Sp1 and PTEN in tongue cancer specimens. The mRNA expressions of Sp1 and PTEN in the tumor tissue and adjacent normal tissue of 21 cases were quantitated by real-time PCR $*P < 0.05$ versus adjacent normal tissue ($n = 21$, one-way ANOVA). NT, adjacent normal tissue; T, tumor. (**C**) Inverse protein expression of Sp1 and PTEN in SACC-LM and SACC-83 cells. AKT phosphorylation was also inversely expressed with PTEN protein. The membrane was stripped for detection with antibodies as indicated. (**D**) Migrated cells decreased in SACC-LM cells with Sp1 knockdown and increased in SACC-83 cells with Sp1 overexpression. Microphotographs of transwell migration of SACC-LM cells with or without Sp1 knockdown and SACC-83 cells with or without Sp1 overexpression (left panel). Cell counting of migrated cells from 6 to 10 separated fields (right panel). Data were presented as mean ± SD. Bar = 200 μm. $*P < 0.05$ ($n = 6$–10, two-way ANOVA). (**E**) Invaded cells decreased in SACC-LM cells with Sp1 knockdown and increased in SACC-83 cells with Sp1 overexpression. Microphotographs of transwell invasion of SACC-LM cells with or without Sp1 knockdown and SACC-83 cells with or without Sp1 overexpression (left panel). Bar = 200 μm. Cell counting of invaded cells from 6 to 10 separated fields (right panel). Data were presented as mean ± SD. $*P < 0.05$ ($n = 6$–10, two-way ANOVA).

attenuated the migration and invasion of cancer cells and vice versa. The results of the present study demonstrated important clinicopathological significance and provided information to understand the regulation of PTEN in cancer development.

Sp1 is a negative regulator of PTEN. Sp1 is usually recognized as a transcriptional activator for various genes involved in almost all cellular processes in mammalian cells (2). However, Sp1 overexpression downregulated PTEN expression and upregulated AKT phosphorylation in all the three cell lines examined in the present study. Sp1 overexpression also induced bcl-2 and c-myc expressions. Conversely, Sp1 knockdown upregulated PTEN expression and downregulated AKT phosphorylation in all the four cell lines examined, including PC3 cells, which have relatively lower expressions of Sp1 and PTEN (Figures 2B and 6A). Our results showed that changes in the level of Sp1 in the cells via ectopic overexpression or knockdown by siRNA inversely changed PTEN expression and proportionally AKT phosphorylation in all the cell lines examined. Therefore, Sp1 negatively regulates PTEN expression. This finding is consistent with the data of previous studies (19,44). Sp3 and Sp1 have similar structures and the same binding sites; in some cases, Sp3 represses Sp1-mediated transcription (40,41). Sp3 overexpression showed no effect on PTEN expression but strongly induced p21 expression. These results are consistent with the results of previous studies (41,45). In the present study, the Sp1-mediated inhibition of PTEN expression was not reversed or enhanced by Sp3. Sp3 overexpression completely blocked the Sp1-mediated induction of bcl-2 and c-myc, whereas Sp1 overexpression blocked the Sp3-mediated induction of p21 by more than half. These results suggest that the reciprocal antagonism for Sp1 and Sp3 is not the Sp1-mediated inhibition of PTEN expression and that Sp1 specifically and negatively regulates PTEN expression.

Sp1 inhibited PTEN expression by repressing PTEN transcription. The overexpression or knockdown of Sp1 could inhibit or upregulate PTEN promoter activity. Thus, Sp1 inhibited the mRNA and protein expressions of PTEN by repressing PTEN transcription and not by destabilizing PTEN mRNA or protein. In addition, the Sp1-mediated inhibition of PTEN promoter activity was narrowed down to through the core promoter and Sp1 was further confirmed to bind to the promoter region containing the core promoter *in vivo*. The full-length PTEN promoter has four Sp1-binding sites, with sites B (–934/–929) and C (–918/–913) located in the core promoter. However, only site C was responsible for the Sp1-mediated inhibition of PTEN promoter activity because the mutation of site C abolished all the inhibitory effects of Sp1 on the activity of the core promoter, full-length promoter and –1138/–912 PTEN region. Site B may be too close to the Egr-1 (a strong PTEN activator) binding site (–947/–939 (33)) to be involved in the Sp1-mediated inhibition of PTEN because Egr-1 can inhibit Sp1 transactivation by overlapping Sp1/Egr-1 sites (46). The identification of the specific Sp1-binding site responsible for the Sp1-mediated inhibition of PTEN expression may be a target for the future development of anticancer drugs.

Acetylation was required for Sp1 to inhibit PTEN expression. After the mutation of the unique acetylation site K703, Sp1 was not acetylated and failed to inhibit the mRNA expression, protein expression and promoter activity of PTEN in cells with or without endogenous Sp1. Moreover, non-acetylated Sp1 failed to induce c-myc and bcl-2 expressions. Acetylation was required for Sp1 to inhibit PTEN expression because only acetylated Sp1 could bind to the PTEN promoter (Figure 5G). Therefore, acetylation is an important post-translational modification for Sp1 in cancer development.

HDAC1 was recruited and required for Sp1 to inhibit PTEN expression. HDAC1 could be recruited by Sp1 to form a complex with the core promoter of PTEN. Furthermore, the inhibitory effect of Sp1 on PTEN expression was abolished by HDAC1 knockdown. These results suggest that Sp1 inhibits PTEN expression by recruiting HDAC1 to PTEN core promoter to repress PTEN transcription. This recruitment of HDAC1 by Sp1 to repress transcription was also previously observed in many genes (17–20,38). In all these cases, Sp1 may serve as a target for HDAC1 to repress transcription. The recruitment of HDAC1 by Sp1 could be a universal mechanism for the specific repression of genes with core promoter containing Sp1 consensus. In this study, PTEN was used as an example. Further studies should be conducted to examine the role of other members of HDACs or histone acetyltransferases in the Sp1-mediated inhibition of PTEN expression.

Sp1 could be involved in cancer development. The existence of an inverse expression of Sp1 and PTEN in cancer cell lines or clinical specimens of cancers would be clinicopathologically significant. However, no such inverse expression of Sp1 and PTEN was found among the different cancer cell lines examined. Instead, an inverse expression of Sp1 and PTEN (i.e., upregulation of Sp1 and downregulation of PTEN) was observed in the specimens of tongue squamous cell carcinoma compared with the adjacent normal tissue. This finding is consistent with the results of a recent clinical observation in hepatocellular carcinoma (9). PTEN expression was inhibited by Sp1 in all the cell lines examined. Thus, the downregulation of PTEN in the tongue cancer specimens could be, to some extent, due to Sp1 upregulation. Insufficient PTEN is a key determinant in cancer progression (27). Hence, the inverse expression of Sp1 and PTEN in the tongue cancer specimens may contribute to tongue cancer progression. The current clinical results further support the recent proposal of developing antitumor drugs that target Sp1 (12). A pioneer study already showed that the downregulation of overexpressed Sp1 inhibits tumor formation (8).

Sp1 could promote cell migration and invasion. Interestingly, an inverse expression of Sp1 and PTEN and a corresponding change in AKT phosphorylation was observed in SACC-LM cells possessing higher potential for lung metastasis as compared with SACC-83 cells. Sp1 knockdown inhibited the migration and invasion of SACC-LM cells to the comparable level of SACC-83, whereas Sp1 overexpression promoted the cell migration and invasion of SACC-83 cells to the comparable level of SACC-LM cells. The present study showed that Sp1 could inhibit PTEN expression and upregulate the corresponding downstream AKT phosphorylation. Previous studies showed that PTEN knockdown enhances and AKT knockdown inhibits the migration and invasion of the cell lines (47,48). Thus, the higher expression of Sp1 in SACC-LM cells might contribute to its higher ability in cell migration and invasion through the PTEN/AKT signaling pathway.

The results of the present study showed that Sp1 inhibited PTEN and activated AKT, both playing strong roles in cell proliferation. These findings suggest that Sp1 overexpression inhibits cell viability but Sp1 knockdown enhances cell viability. However, neither the overexpression nor the knockdown of Sp1 significantly affected the viability of the cells (data not shown). This result may be attributed to the insufficient efficiency of the transient transfection. As a result, the proliferation of numerous non-transfected cells caused negligible Sp1-induced changes in the proliferation of the transfected cells. Future studies using Tet-on/off stable transfection may be conducted to confirm the changes in the viability of Sp1-overexpressed cells.

In conclusion, Sp1 negatively regulates PTEN expression through a specific Sp1-binding site at the PTEN core promoter, Sp1 acetylation and HDAC1 recruitment. The results of the present study show an important mechanism underlying the involvement of Sp1 in cancer progression and prove that Sp1 is an important regulator of PTEN. The development of antitumor drugs that target Sp1 may be a potential strategy for cancer therapy.

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