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Original Article

EP300 regulates the expression of human survivin gene in esophageal squamous cell carcinoma

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Abstract: Survivin is selectively up-regulated in various cancers including esophageal squamous cell carcinoma (ESCC). The underlying mechanism of survivin overexpression in cancers is needed to be further studied. In this study, we investigated the effect of EP300, a well known transcriptional coactivator, on survivin gene expression in human esophageal squamous cancer cell lines. We found that overexpression of EP300 was associated with strong repression of survivin expression at the mRNA and protein levels. Knockdown of EP300 increased the survivin expression as indicated by western blotting and RT-PCR analysis. Furthermore, our results indicated that transcriptional repression mediated by EP300 regulates survivin expression levels via regulating the survivin promoter activity. Chromatin immunoprecipitation (ChIP) analysis revealed that EP300 was associated with survivin gene promoter. When EP300 was added to esophageal squamous cancer cells, increased EP300 association was observed at the survivin promoter. But the acetylation level of histone H3 at survivin promoter didn't change after RNAi-depletion of endogenous EP300 or after overexpression of EP300. These findings establish a negative regulatory role for EP300 in survivin expression.

Keywords: Survivin, EP300, transcription regulation, ESCC

Introduction

Survivin belongs to the inhibitor of apoptosis (IAP) gene family. It is involved in regulation of apoptosis, cell cycle progression, and microtubule stability. Survivin is ubiquitous in fetal tissues, but is negligible in the majority of terminally differentiated adult tissues [1, 2]. In various cancers including esophageal cancer, survivin is significantly overexpressed [3]. Interference survivin expression in cancer cells could increase the cells apoptosis and slower its growth [4]. Given that deregulation of survivin gene expression appears to be a common and significant event in carcinogenesis, it is crucial to understand the molecular mechanisms of survivin gene expression and regulation.

EP300 is a transcriptional factor which was implicated in a number of diverse biological functions including proliferation, differentiation, and DNA damage response [5]. As a transcriptional activator, EP300 promotes gene transcription by bridging between DNA-binding transcription factors and the basal transcription machinery, or by providing a scaffold for integrating a variety of different proteins [6]. It has also been shown to be able to acetylate histones and transcription factors through its HAT activity [7]. However, EP300 has also been shown to repress transcription of a number of genes, such as c-Myc and miR-142 [8, 9]. Nevertheless, whether EP300 participates in survivin transcriptional regulation has not been elucidated. EP300 activity is under aberrant control in human disease, particularly in tumourigenesis [10]. The EP300 gene is often found mutated or in a truncated form in various human cancers [11, 12]. Survivin overexpression and aberrant EP300 function are common events associated with neoplastic development, suggesting a functional link between survivin and EP300. Interestingly, bioinformatics analysis revealed that there was EP300 binding site located in the promoter region of survivin gene. So, we hypothesized that EP300 participates in survivin transcriptional regulation in esophageal cancers. And this study was aimed

to investigate whether EP300 could regulate survivin expression in esophageal cancer cells.

Materials and methods

Cell cultures

The human esophageal squamous cancer cell lines Eca109 and TE-1 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), where they were characterized by mycoplasma detection, DNA-Fingerprinting, isozyme detection and cell vitality detection. The cells were maintained in culture as an adherent monolayer in RPMI-1640 (GIBCO) medium supplemented with 10% FBS (50 mL FBS/500 mL medium). Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

For EP300 treated, an equal number of each esophageal cancer cell lines were plated in six-well cell culture plates at a density of 4×10^4 cells/ml. The media were removed 24 hours later, and the wells were treated with 2 ml of fresh media containing Human recombinant EP300 (Creative BioMart, USA) at a final concentration of 10 ng/ml or 20 ng/ml. Any concentration had three wells. The control wells only contained fresh media.

The EP300 gene silencing was performed using small-interfering RNA (siRNA) targeting EP300. The siRNA sequence was 5-TGACACAGGCAGGCTTGAC-3. siRNA was transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. The transfection was performed for 48 h. Specific silencing of EP300 was confirmed by Western blotting, as described below. A non-related, scrambled siRNA was used as a control.

Survivin promoter-reporter gene construction

Genomic DNA was isolated from one cancer tissues using Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacture's protocol. The DNA was used as template to amplify the survivin promoter region (nucleotides 2163-2798, GenBank Accession Number U75285). The sequence of the human survivin gene was used to engineer PCR cloning primers. The fragment containing the promoter region of survivin was amplified with the following primers: forward primer, 5'-GGGGTACCTTGTTCATTTGTCCTTC-3', reverse primer, 5'-GGAAGATCTACCTCTGCCAACGGGTC-3'. PrimeSTAR® HS DNA Polymerase (TaKaRa, Japan) was used to minimize error rate in DNA synthesis by the PCR. The survivin promoter fragments were inserted into the luciferase vector PGL3-basic (Promega, USA) at the Kpn I and Bgl II sites. These plasmids were confirmed by DNA sequencing.

Transient transfection and reporter assays

To normalize transfection efficiency, we used the Dual-Luciferase reporter assay system according to the manufacturer's instructions (Promega, USA). The dual luciferase assay is a widely used technique. In this system, the plasmid pRL-TK that contains the Renilla luciferase gene under the control of the thymidine kinase promoter is cotransfected as an internal control. Cells were grown in 24-well plates with the suggested medium until 60-80% confluent. Then, the cells were transiently transfected with 1 μg of tested plasmid DNA along with 0.1 μg of pRL-TK using 2 μl of lipofectamine 2000 in 1 ml of Opti-MEM® I (GIBCO) per well for 4-6 hours. PGL3-Basic vector was used as an empty experimental control plasmid. Cell lysates were prepared 48 hours after transfection for the dual luciferase assay by following the manufacturer's instructions (Promega, USA) and using a GloMax-Multi Jr Single-Tube Multimode Reader (Promega, USA). The dual luciferase ratio was defined as the luciferase activity of the tested plasmids divided by the luciferase activity of pRL-TK.

Detection of survivin and EP300 transcripts using RT-PCR

Total RNA from esophageal squamous cancer cells and tissues was isolated using TRIpure Reagent (Aidlab, China) according to the manufacturer's protocol. One mg of the total RNA was reversely transcribed using PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Japan). PCR was performed for all cDNA samples with 35 (EP300), 30 (survivin) or 25 cycles (β-actin), which did not reach the saturation phase and was eligible for the densitometric quantification and comparison of amplification products. Primer sequences were as follows: survivin: sense 5' CAGCCCTTTCTCAAGGACCAC 3', antisense 5' TTTCTCCGCAGTTTCCTCAAA 3'; EP300: sense: 5' TCCTTTCCATACCGAACC 3', antisense: 5' GGACAATACGCTCTGATACA 3'; β-actin: sense 5' GTTGCGTTACACCCTTTCTTGACA 3', antisense 5' GCACGAAGGCTCATCATTCAAAA 3'.

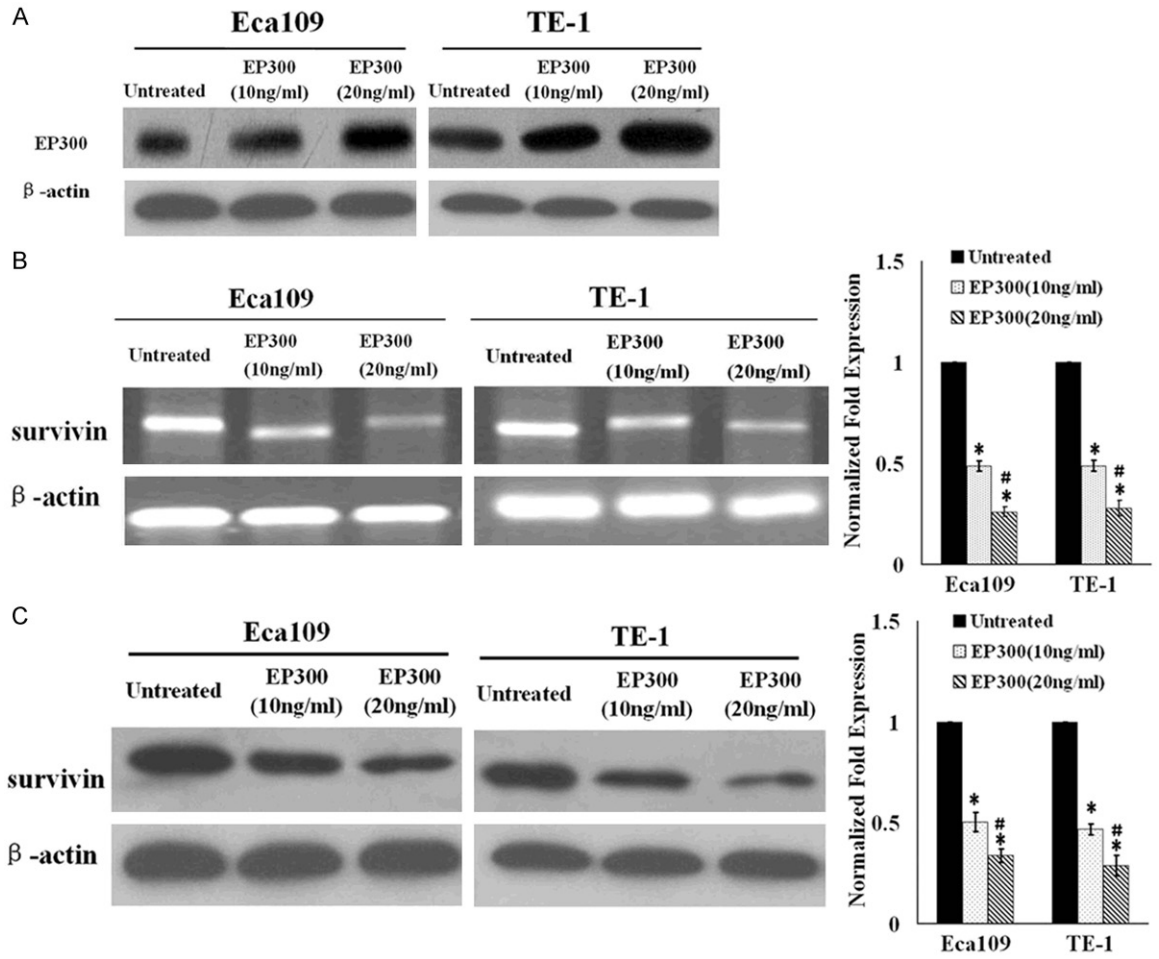

Figure 1. Survivin expression was repressed by EP300. A: Western blotting analyses showed EP300 protein levels. B: Survivin mRNA was down-regulated following EP300 induction in human ESCC cell lines Eca109 and TE-1. C: Western blot analyses of survivin protein in Eca109 and TE-1 cell lines. EP300 notably decreased the survivin protein expression both in Eca109 and TE-1 cancer cells.

Quantitative analyses were performed using a Gel Doc 2000 scanner system and Quantity One image analysis software (Bio-Rad).

Western blotting

Equal amounts of whole cell lysates were resolved by SDS-PAGE and electrotransferred on a PVDF membrane. Primary antibody [Anti-EP300 CT Antibody (1:1000; Millipore, USA); Survivin (6E4) mAb (1:2000; Cell Signaling, USA); Actin (1:1000; Dingguo, China)] incubation was carried out overnight at 4°C. The immunoreactive signals were detected with an enhanced chemiluminescence kit (Millipore). Quantitative analyses were performed using a Gel Doc 2000 scanner system and Quantity One image analysis software (Bio-Rad).

Chromatin immunoprecipitation (ChIP) assay

ChIP was performed with a commercially available Chromatin Immuno-precipitation Kit EZ-ChIP™ (Upstate, Milipore, USA) according to the manufacturer's instructions. Immunoprecipitation was performed with anti-EP300 (Millipore, USA) antibody and anti-acetylated histone H3 (Milipore, USA) antibody. Mouse IgG (Millipore, USA), Rabbit IgG (Millipore, USA) and no antibody were added as negative controls. The captured immunocomplex containing bound transcriptional DNA fragments were eluted. The eluted DNA was subjected to PCR amplification for the survivin gene promoter region. Following primers were used in PCR assay: P1: F, 5'-CCCCTGTTCATTTGTCCTT-3', R,

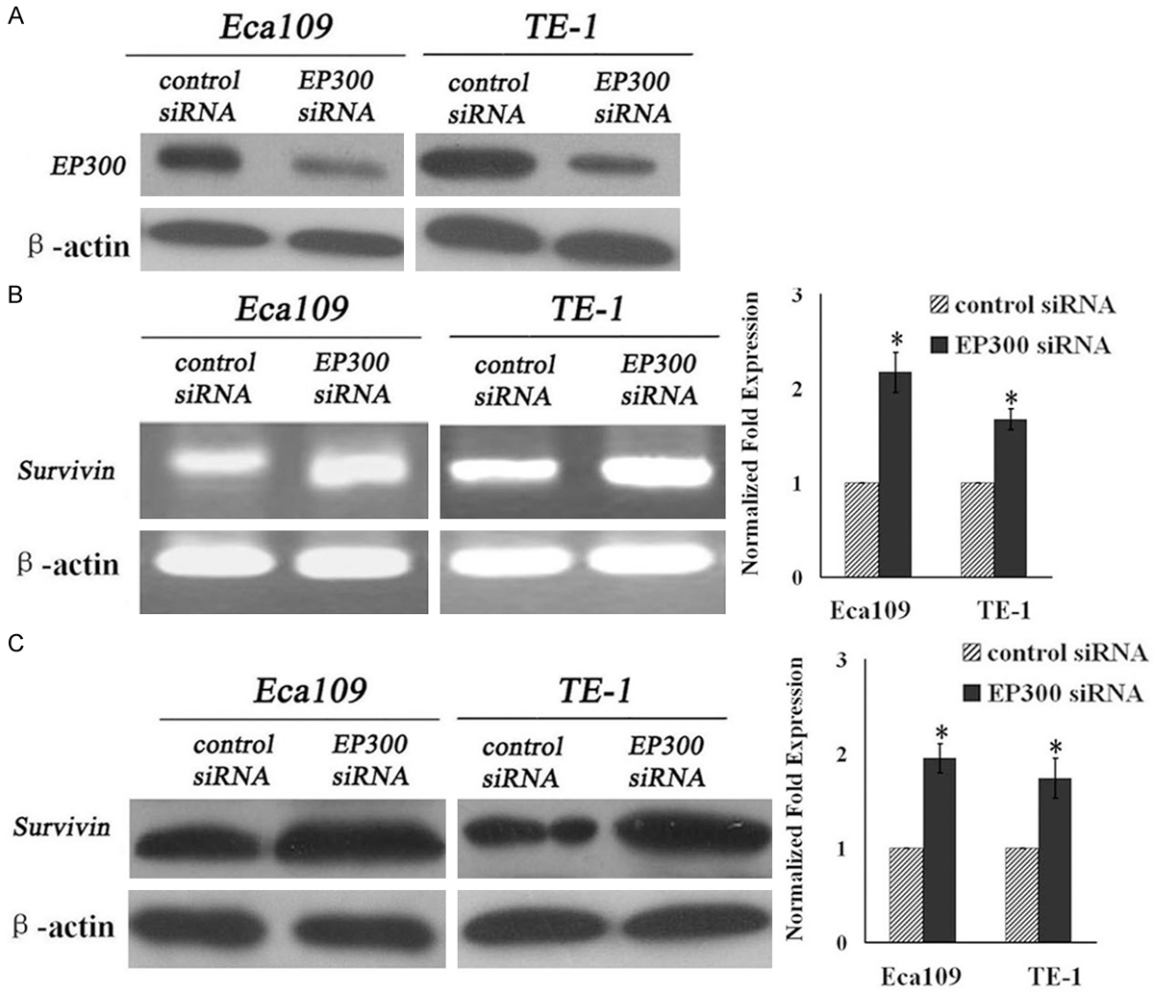

Figure 2. Survivin expression was increased when EP300 was silenced in ESCC cells. EP300 siRNAi was used to knock down EP300. A: Western blotting analyses showed that the EP300 siRNA was effective in decreasing the respective protein levels. B: Survivin mRNA was increased in EP300-knockdown ESCC cells. C: Western blot analyses showed that silencing of EP300 in Eca109 or TE-1 cells by EP300 siRNA increased survivin protein expression.

5'-ATTAGCTGGGCGTGGTGG-3'; P2: F, 5'-GCGGCGGGAGGACTACAACT-3', R, 5'-CGATTCAAATCTGGCGGTTA-3'.

Quantitative and statistical analysis

Quantitative data are expressed as the mean ± SEM. Differences between groups were determined using statistics including Student's t test and one-way ANOVA using SPSS 13.0 software. *P* values less than 0.05 were considered statistically significant.

Results

EP300 decreased survivin expression in esophageal squamous cancer cells

In order to study whether EP300 could regulate survivin expression, two different approaches were used to alter EP300 function in two kinds of esophageal squamous cancer cell lines, Eca109 and TE-1 cell lines. First, we enhanced the EP300 level by added recombinant EP300 protein to Eca109 and TE-1 cells, cells with overexpression EP300 exhibited a decrease of survivin levels compared with the control cells in both mRNA and protein levels (**Figure 1**). Furthermore, the inhibitory effect of EP300 to survivin is dose dependent. Next, we decreased EP300 protein levels in esophageal squamous cancer cells using synthesized siRNA. We found that silencing of EP300 in Esophageal cancer cells (Eca109 or TE-1 cells) by EP300 siRNA increased survivin mRNA and protein expression as determined by RT-PCR and Western blotting analysis (**Figure 2**). These results suggested that EP300 was involved in the survivin

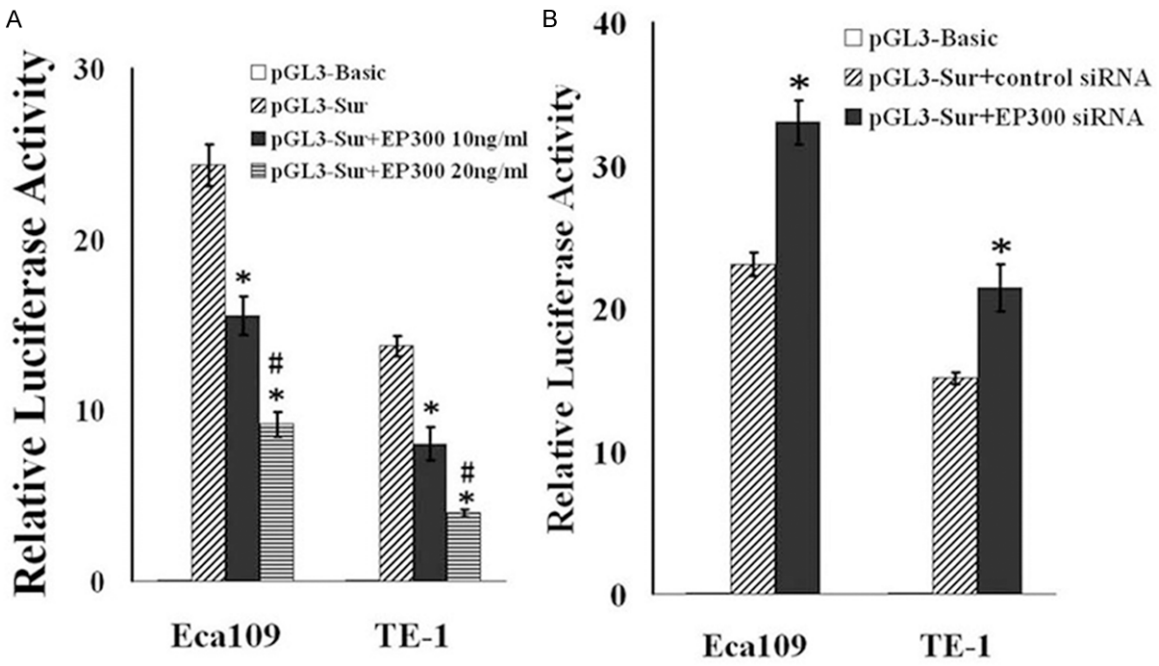

Figure 3. EP300 repressed the activity of survivin promoter in ESCC cells. A: Eca109 cells or TE-1 cells were transiently transfected with survivin promoter constructs, together with different amounts (10 ng/ml and 20 ng/ml) of recombinant EP300 protein. After 48 h of transfection, cells were harvested and the activity of survivin promoter was measured. B: Eca109 cells or TE-1 cells were transiently transfected with survivin promoter reporter construct and EP300 siRNA. After 48 h of transfection, cells were harvested, and the activity of survivin promoter was measured. *$P < 0.05$.

expression regulation. Activation of EP300 signal could down-regulate survivin expression levels in esophageal squamous cancer cells.

EP300 affects survivin promoter activity

Because the expression levels are driven in part by promoter activity, we next want to determine whether exposure to or inhibition of EP300 effects survivin promoter activity. A luciferase reporter construct was made to study survivin promoter. We used the survivin proximal promoter region containing the transcription start sites fused to the luciferase reporter gene to construct the promoter reporter. The promoter construct showed clearly detectable and measurable activity in the cells confirming that we cloned a functional promoter of the human survivin gene. Survivin promoter construct was transfected into the esophageal cancer cell lines. The empty luciferase reporter vector (Basic) was used as a control. The esophageal cancer cell lines were pretreated with recombinant EP300 protein or EP300 siRNA. We found that luciferase activity significantly increased in the EP300 knockdown cells (**Figure 3B**). When EP300 was introduced into Eca109 or TE-1 cells, reduction of survivin promoter activity was observed (**Figure 3A**). Thus, EP300 repressed transcriptional activity of survivin gene promoter.

EP300 associate with the human survivin promoter

Since EP300 could affect survivin promoter activity, we next investigated whether EP300 could directly bind to the promoter region. ChIP assay was performed. ChIP assay can monitor protein-DNA binding. The cross-linked DNA-protein complexes were immunoprecipitated with monoclonal antibody to EP300. The DNA in the complexes was analyzed by PCR for the enrichment of the survivin promoter sequence. According to the bioinformatics analysis (TFSearch, http://www.cbrc.jp/research/db/TFSEARCH.html), three putative EP300 binding sites were existed in survivin promoter region. We designed 2 pairs of primers on survivin promoter for ChIP experiments (**Figure 4A**): one flanking the first EP300 site in survivin promoter region (P1), another flanking the other two

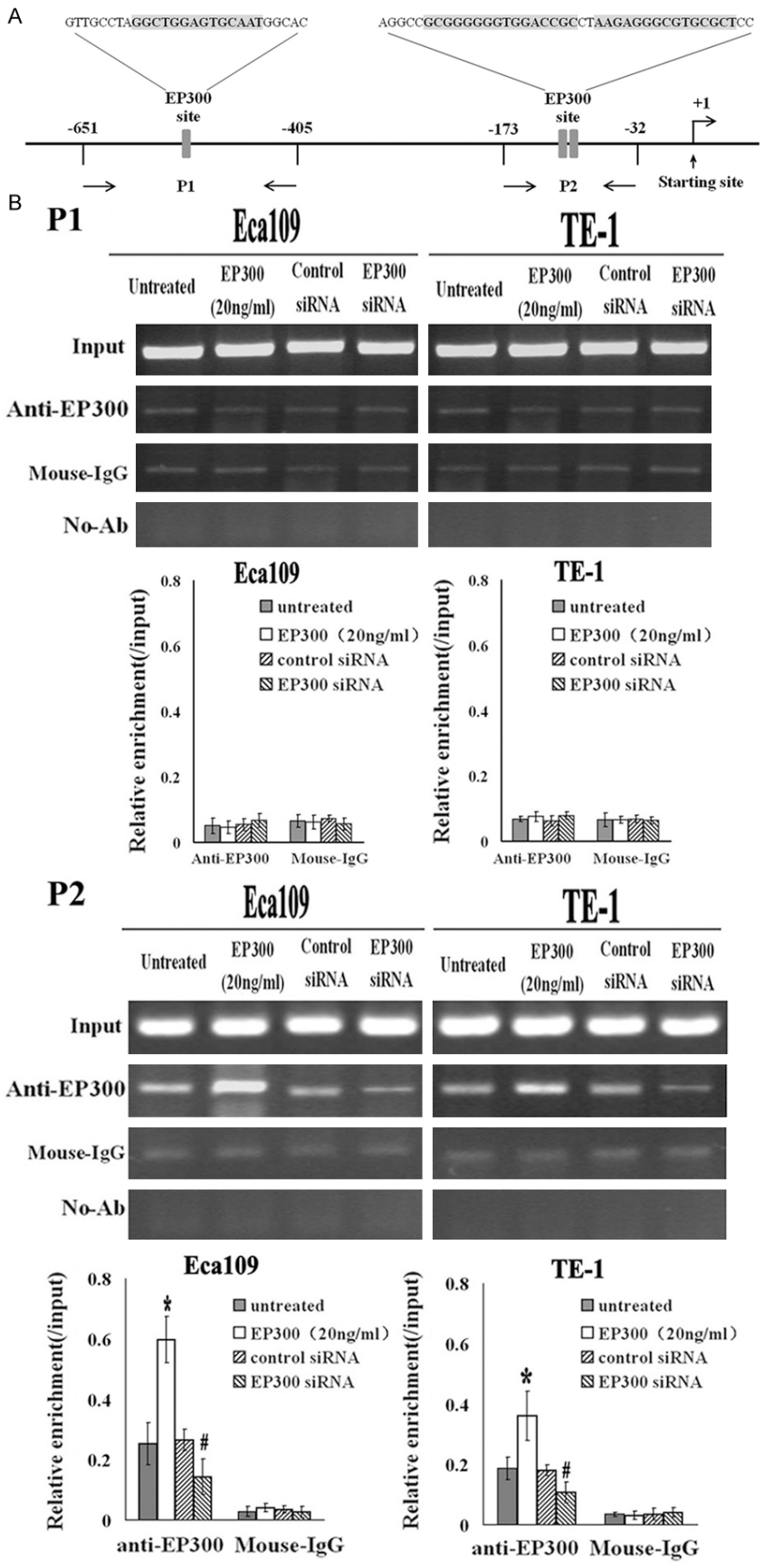

Figure 4. EP300 associate with the human survivin promoter. A: A schematic diagram of the survivin promoter showing the three putative EP300 binding sites. B: The promoter region flanking the first EP300 site had no obvious enrichment of EP300 protein. Binding of EP300 to the promoter region containing the second and the third EP300 sites was boosted in cells with overexpressed EP300. When EP300 was depleted, the recruitment of EP300 to survivin promoter is obviously decreased.

EP300 sites (P2). As shown in **Figure 4B**, recruitment of EP300 to the promoter region containing the second and the third EP300 sites was observed in Eca109 and TE-1 cells. However, promoter region flanking the first EP300 site had no obvious enrichment of EP300 protein. Furthermore, binding of EP300 in esophageal carcinoma cells transfected with EP300 siRNA or added with recombinant EP300 protein was also detected. As shown in **Figure 4B**, binding of EP300 to the promoter region containing the second and the third EP300 sites was boosted in cells with overexpressed EP300. No obvious enrichment in the first EP300 site was detected in cells with added recombinant EP300. And when EP300 was depleted by EP300 siRNA, the recruit-

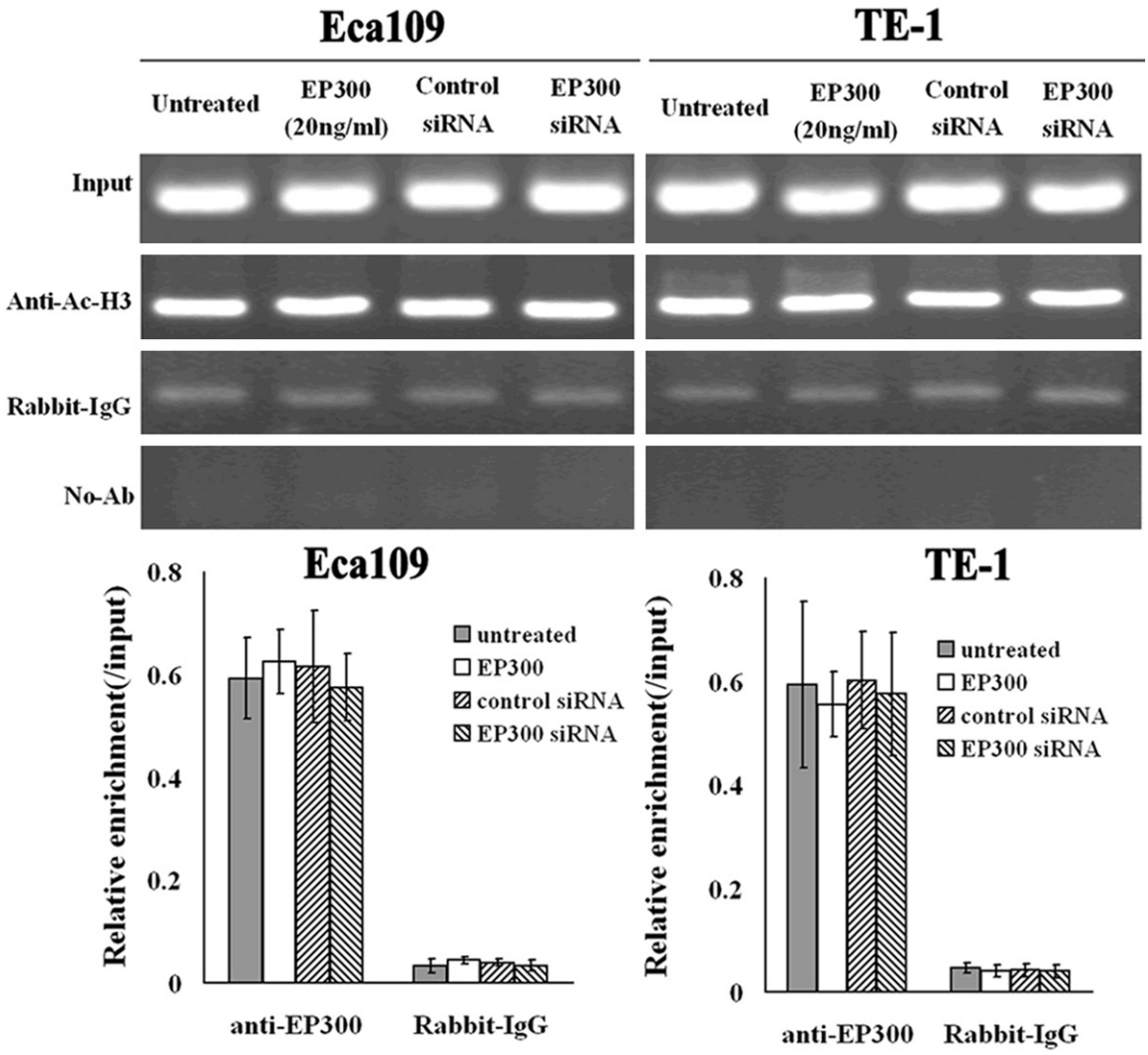

Figure 5. Ac-H3 binds to the survivin promoter by ChIP Assay. Eca109 cells or TE-1 cells were transiently transfected with EP300 siRNA or added with 20 ng/ml recombinant EP300 protein. After 24 h of transfection, ChIP assays were performed using specific antibodies against acetylated histone H3 (anti-Ac-H3) to detect occupancy of acetylated histone H3 at the survivin promoter in ESCC cells. (Input, DNA prior to immunoprecipitation).

ment of EP300 to survivin promoter is obviously decreased. This data demonstrated that EP300 associate with the survivin gene promoter and suggested a role for EP300 in the regulation of survivin gene transcription.

Repression of survivin by EP300 is independent of its HAT activity

The key property of EP300 is its HAT activity. We next want to know whether the HAT activity of EP300 associated to the inhibition of survivin expression. To test this, we performed ChIP assay to investigate if the acetylation level of histone H3 at survivin promoter changed after the treatment of EP300 protein. As can be seen in **Figure 5**, the acetylation level of histone H3 at survivin promoter didn't change obviously after RNAi-depletion of endogenous EP300 or after overexpression of EP300 protein. These indicated that the HAT activity of EP300 did not contribute to the inhibition of survivin expression.

Discussion

Survivin is completely repressed and undetectable in normal adult tissues but becomes prominently re-expressed in most human malignancies, including esophageal squamous

cancer. For survivin is dysregulated in many types of cancers, dedicated study on its regulation mechanism is important for exploring new strategies for cancer therapy. Many researches using RT-PCR showed a high level of survivin mRNA in cancer tissues compared with normal tissues [13, 14], indicating that the survivin over expression in cancers may arise predominately at the level of transcriptional regulation of the survivin gene.

EP300 is an important transcriptional factor involved in regulation of many genes. The EP300 gene is often found mutated in various human cancers [11, 12]. Whether EP300 participates in survivin transcriptional regulation has not been elucidated. In this study, we present experimental data showing that survivin expression is repressed by EP300 in esophageal cancer cells. Down regulation of EP300 in esophageal cancer cells increased survivin expression both in mRNA and protein levels.

Current evidence suggests that EP300 functions through multiple mechanisms. To date, two contradictory viewpoints can be found in the literature for depicting the effect of EP300. In most cases, EP300 is a transcriptional coactivator. EP300 can promote gene transcription by bridging between DNA-binding transcription factors and the basal transcription machinery, or by providing a scaffold for integrating a variety of different proteins [6]. The key property of EP300 is its HAT activity, enabling EP300 to influence chromatin activity by modulating nucleosomal histones and thus promoting gene transcription [7]. On the contrary, a role for EP300 as a corepressor in gene transcription, although not as well documented as its role in coactivation, is also described in other literatures. For instance, some studies provide direct experimental evidence to proof that EP300 negatively regulated c-Myc and miR-142 genes [8, 9]. In this study, we observed that in cells overexpressing EP300, survivin RNA and protein levels were reduced obviously. That is, EP300 is a repressor to survivin gene expression.

To assess the function of EP300 in controlling the survivin gene transcription in esophageal cancer cells, co-transfection studies was performed with the survivin promoter reporter construct. We found that RNAi-depletion of endogenous EP300 led to enhancement, while overexpression of EP300 led to partial repression of survivin promoter reporter activity. The promoter activity of survivin was repressed by EP300 in a dose-dependent manner. We concluded that EP300 inhibits survivin gene expression by modifying the survivin promoter activity.

Previous studies have indicated that the EP300 protein can bind DNA in a sequence-specific manner [15]. In order to investigated if EP300 binding to the human survivin promoter in esophageal cancer cells, we performed ChIP assay. The results revealed that EP300 was associated with the survivin promoter. We further found that when EP300 was added to esophageal cancer cells, increased EP300 association was observed at the survivin promoter. And when EP300 was depleted by siRNA, the recruitment of EP300 to survivin promoter is obviously decreased. The key property of EP300 is its HAT activity. EP300 can acetylate nucleosomal histones in the proximity of the promoter to which EP300 is recruited [16]. And the EP300 occupancy in general correlates with gene activation. Although EP300 acts as a repressor to survivin expression, we also want to know whether the HAT activity of EP300 associated to the inhibition of survivin expression. Our results showed that the acetylation level of histone H3 at survivin promoter didn't change after RNAi-depletion of endogenous EP300 or after overexpression of EP300. These indicated that the HAT activity of EP300 did not contribute to the inhibition of survivin expression.

Although the exact mechanisms by which EP300 may act to repress survivin gene transcription is still unclear, we proposed some hypotheses to explain the EP300's function in survivin repression. EP300 has been shown to downregulate gene expression by acetylating transcription factors. For example, acetylation of HMG(I)Y by EP300 shuts off IFN-β gene expression during the post induction period [17]. Thus, we considered it possible that EP300 could acetylate and down-regulate the DNA binding and/or the transcriptional activation activity of the survivin promoter-specific transcription factors that could contribute to survivin repression.

EP300 can influence gene transcription by bridging between DNA-binding transcription factors and the basal transcription machinery, or by providing a scaffold for integrating a vari-

ety of different proteins [6, 18]. We proposed that a possible mechanism contributing to EP300's transcriptional suppressor function is that EP300 recruitment is associated with the recruitment of additional repressive transcriptional elements. That is, recruitment of repressive elements to the survivin promoter by EP300 might associate with survivin transcriptional repression. Clearly, more studies will be needed to define the interaction between EP300 and other transcription repressive factors, whose binding-sites are located in the survivin promoter.

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Disclosure of conflict of interest

None.

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