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Sp1 and Sp3 regulate basal transcription of the *survivin* gene

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Abstract

Survivin, a unique member of the inhibitor of apoptosis protein family, is overexpressed in many cancers and considered to play an important role in oncogenesis. In this study, we cloned and identified the proximal 269 bp promoter of *survivin* gene, which exhibited strong promoter activity in HeLa cells. The TATA-less, GC-rich promoter contains 7 putative binding sites for Sp1, two of which (one at position −148 to −153, the other at position −127 to −140) are essential in regulating basal survivin promoter activity. Not only Sp1 but also Sp3 can activate the survivin promoter, which were proven by EMSA, blocking Sp1 or Sp3 using RNAi or mithramycin treatment of HeLa cells, and overexpression of Sp1 or Sp3. Our results collectively suggest that Sp1 cooperates with Sp3 to regulate survivin promoter activity.

Keywords: Survivin; Sp1; Sp3; GC-rich sequence; Promoter regulation; Transcriptional factor; Tumor

Survivin is a member of the inhibitor of apoptosis protein (IAP) family [1–3]. The human *survivin* gene is comprised of four exons with three introns spanning 14.7 kb on chromosome 17q25, encoding a 16.5 kDa protein. Survivin appears to be involved in regulation of apoptosis as well as cell division. This gene inhibits apoptosis induced by a variety of apoptotic triggers [4,5]. Survivin is expressed during fetal development and also in a wide range of tumors, including human cancers of the lung, colon, breast, brain, etc., but is rarely present in terminally differentiated tissues [3]. Current studies indicate that the expression of survivin in cancer cells is associated with cancer progression, poor prognosis, drug resistance, and shorter survival times [3,6]. It has been demonstrated that inhibition of survivin expression or interference of survivin function induces cancer cell death [3]. Additionally, analysis of the differences in gene expression between normal and tumor cells has revealed that survivin is one of the genes most consistently overexpressed in tumor cells relative to normal tissue.

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. Therefore, we attempted to elucidate the mechanisms involved in its transcriptional regulation.

Li and Altieri have characterized that the survivin promoter lacks a typical TATA or CCAAT box and contains several putative Sp1/Sp3 transcription factor-binding sites [2]. Sp1 and Sp3, which are ubiquitously expressed in mammalian cells, can bind with similar affinities for GC boxes and act through GC boxes to regulate gene expression of many housekeeping, tissue-specific, viral, and inducible genes [7–9]. The actions of Sp1 and Sp3 depend on promoter structure and cellular environment, as well as their potential interaction with other transcription factors. Sp1 acts mainly as a transcriptional activator, while Sp3 can act as an activator or repressor of transcription [10–12]. These published studies suggest that Sp1/Sp3 may regulate basal transcriptional expression of the human *survivin* gene.

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In this study, we cloned a 987 bp region of the survivin promoter. Mutational and functional analysis of the promoter revealed two important Sp1 sites in the survivin promoter. siRNA and co-transfection studies indicated that Sp1 and Sp3 can cooperatively regulate survivin promoter activity. Thus, our study established a molecular basis for further understanding the mechanisms governing *survivin* gene expression.

Materials and methods

Cell culture and reagents. The human epithelial carcinoma cell line, HeLa, was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). HeLa cells were cultured in Eagle's minimal essential medium (Gibco, USA) supplemented with 10% fetal bovine serum (Hyclone, USA), 1% L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Mithramycin (Sigma, St. Louis, MO) was added 24 h before harvesting the cells.

Survivin promoter–reporter gene constructs. The sequence of the human survivin gene (GenBank Accession No. U75285) was used to engineer PCR cloning primers. Numbering begins at the initiation site, ATG [2,6]. The reverse primer (−27 to −1: 5′CCCAAGCTTGCCGCCGCCGCCAC CTCTGCCAACGGG3′) was paired with the following forward primers: (−987 to −970: 5′GGAAGATCTGCCATAGAACCAGAGAAG3′); (−596 to −578: 5′CGTGCTAGCCCGCACCCTGTAAAGCTCTCC3′); (−269 to −246: 5′GCACGCGTTCTTTGAAAGCAGTCGAG3′); (−158 to −142: 5′GGAAGATCTCAACTCCCGGCACACCC3′); (−60 to −41: 5′GGGAGCTCTTACGCGCCATTAACCGCCAGAT3′). PCR-amplified products, with pSRVN-Luc vector DNA serving as the template, were isolated on a 2% agarose gel (pSRVN-Luc, containing *survivin* gene −987 to −11, was kindly provided by Dr. Mien-Chie Hung, The University of Texas, MD, Anderson Cancer Center, Houston, TX, USA [13]), digested with enzymes (*Bgl*II–*Hin*dIII, *Nhe*I–*Hin*dIII, *Mlu*I–*Hin*dIII, *Bgl*II–*Hin*dIII, and *Sac*I–*Hin*dIII) and cloned into the corresponding sites of the pGL3-basic firefly luciferase expression plasmid (Promega) to generate the plasmids pLuc-surP-987, pLuc-surP-596, pLuc-surP-269, pLuc-surP-158, and pLuc-surP-60 for use in transfections and luciferase assays. DNA sequence analysis confirmed these sequences.

Mutagenesis of the Sp1/Sp3 recognition sites on the survivin promoter. Mutation of the Sp1/Sp3 binding sites found in the survivin promoter was performed by the generation of point mutations from the wild-type promoter region. The point mutations were generated using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). Oligonucleotides with site-specific mutations at the critical nucleotides necessary for transcription factor binding to the GC boxes [Sp1 (A)–(G)] are listed in Table 1. The mutations were confirmed by sequencing.

Transient transfection and reporter gene assays. Cells were plated at a density of 5×10^5 cells per well in 6-well dishes and allowed to settle overnight. The following morning, cells were transfected with 4 μg firefly luciferase reporter construct and 0.04 μg *Renilla* luciferase construct (pRL-SV40, Promega) using lipofectamine2000 (Invitrogen, Carsbad, CA, USA), according to the manufacturer's protocols. In some experiments, reporter plasmids were also co-transfected with Sp1 and/or Sp3 expression vectors (pN3-Sp1 and pN3-Sp3 were kindly provided by Dr. Guntram Suske, Philipps-University, Marburg, Germany [14,15]). The corresponding empty vector, pN3, was used in control transfections. In these experiments, the pRL-SV40 construct was not co-transfected, as Sp1 is able to increase SV40 promoter activity, due to the presence of several Sp1 DNA-binding sites in this promoter [10,11]. After 48 h, the cells were harvested and lysed, and dual luciferase assays were performed, according to the manufacturer's (Promega) protocol, on a Monolight 2010 luminometer (Analytical Luminescence Laboratory). Protein was normalized using the Bio-Rad protein assay (Bio-Rad). Luciferase activity was normalized to total protein levels, as well as to *Renilla* luciferase activity.

Table 1
Sequences of oligonucleotides used in site-directed mutagenesis and EMSA probes

Name	Sequence
Site-directed mutagenesis	
MutA	gcgctaggtgtggTTagggacgagctggc
MutB	gcaccgcgaccacTgTTagagccacgcggcg
MutC	cgggcagagccacgcATTgggaggactacaactc
MutD	ggactacaactccTTgcacaccccgcgcc
MutE	cccggcacaccccCcAAcAAcccAActctactcccagaaggccgc
MutF	cgggggtggaccAActaagagggcgtg
MutG	accgcctaagagggAAAgcgctcccgac
EMSA analysis	
SpD	ggactacaactcccggcacaccccgcgc
SpDmut	ggactacaactccTTgcacaccccgcgc
SpE	acaccccgcgccgccccgcctctactcc
SpEmut	acaccccCcAAcAAcccAActctactcc
Con-Sp1	attcgatcggggcggggcgagc
NS	tgtcgaatgcaaatcactagaa

Sequences listed above are from 5′ to 3′. The regions of capitalized oligonucleotides indicate the positions that carry mutations.

siRNAs. Small interfering RNAs (siRNAs) were prepared by Shanghai GenePharma Co., and targeted coding regions of Sp1 and Sp3. The targeted sequences used to silence transcription of Sp1 and Sp3 were, 5′-AUCACUCCAUGGAUGAAAUGATT-3′, and 5′-GCGGCAGG UGGAGCCUUCACUTT-3′, respectively [16,17]. siRNAs were transfected into cells using lipofectamine2000 according to the manufacturer's instructions. For transactivation studies, cells were co-transfected with pLuc-surP3 and siRNAs for 48 h, and then luciferase activity was determined as described above. Western blot analysis was performed on whole-cell lysates from transfected cells.

Immunoblotting. Subconfluent cells were harvested and lysed in RIPA buffer supplemented with protease inhibitors. Protein concentrations were determined using the Bio-Rad Protein assay. Equal amounts of protein (between 30 μg and 60 μg) were run on 10 or 12% SDS–polyacrylamide gels and transferred to a nitrocellulose membrane (Bio-Rad) using a Bio-Rad semidry transfer system. The membrane was blocked in 20 mM Tris-buffered saline (pH 7.6) with 0.1% Tween 20 (TBST) containing 5% (w/v) nonfat dry milk at 4 °C overnight. The membrane was incubated with primary antibody at a 1:200 dilution (survivin, FL-142; Sp1, PEP2; Sp3, and D-20; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. Membranes were washed with TBST and incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody at a 1:5000 dilution (Jackson ImmunoResearch Laboratories) for 1 h at room temperature. After TBST washes, the blot was incubated in detection reagent (ECL™ Advance Western blotting detection kit) and exposed to a Hyperfilm™ ECL™ film (Pierce). β-Actin was served as a loading control and was detected with mouse monoclonal anti-β-actin antibody (Sigma).

Electrophoretic gel mobility shift assay (EMSA). Oligonucleotides (probes) were labeled at the 5′ end with [γ-^{32}P]ATP using T4 polynucleotide kinase. The proteins Sp1 (Promega) or Sp3 (Panomics), (100–300 ng) were pre-incubated in the binding buffer (5× binding buffer, Promega) for 20 min at 25 °C in a volume of 19 μl with and without an excess of unlabeled oligonucleotide competitors. After addition of 1 μl of labeled DNA ($1–2 \times 10^5$ cpm), the mixture was incubated for 60–90 min at 4 °C. Each reaction mixture was then loaded into the well of a 4% nondenaturing polyacrylamide gel and electrophoresed at 100 V in 0.5× TBE buffer at 4 °C for 1–2 h. Gels were dried and visualized by autoradiography.

Results and discussion

Cloning and identification of the human survivin gene promoter

The survivin promoter has been characterized previously [2]. In our work, a 987 bp genomic DNA fragment of the survivin promoter (GenBank Accession No. U75285), which lacks a typical TATA or CCAAT box and contains a highly GC-rich content in its proximal promoter region, was cloned.

To determine the probable minimal promoter region of the *survivin* gene, we conducted functional analysis on this 987 bp of the survivin promoter. Five luciferase reporter constructs, containing 987, 596, 269, 158, and 60 bp fragments of the survivin promoter, respectively, were transfected into HeLa cells. As shown in Fig. 1A, cells transfected with the 987 bp survivin promoter-driven luciferase reporter construct expressed a high-level of luciferase activity. Deletion of a portion of the survivin promoter, up to 269 bp upstream of the ATG translation initiation site, did not reduce luciferase activity, but rather increased it. Further truncation to 158 bp from the ATG site reduced luciferase activity compared with that observed in cells transfected with the 269 bp promoter-driven luciferase reporter gene. Transfection of the 60 bp construct into HeLa cells almost abolished the reporter activity. These results suggest that the cis-regulatory elements required

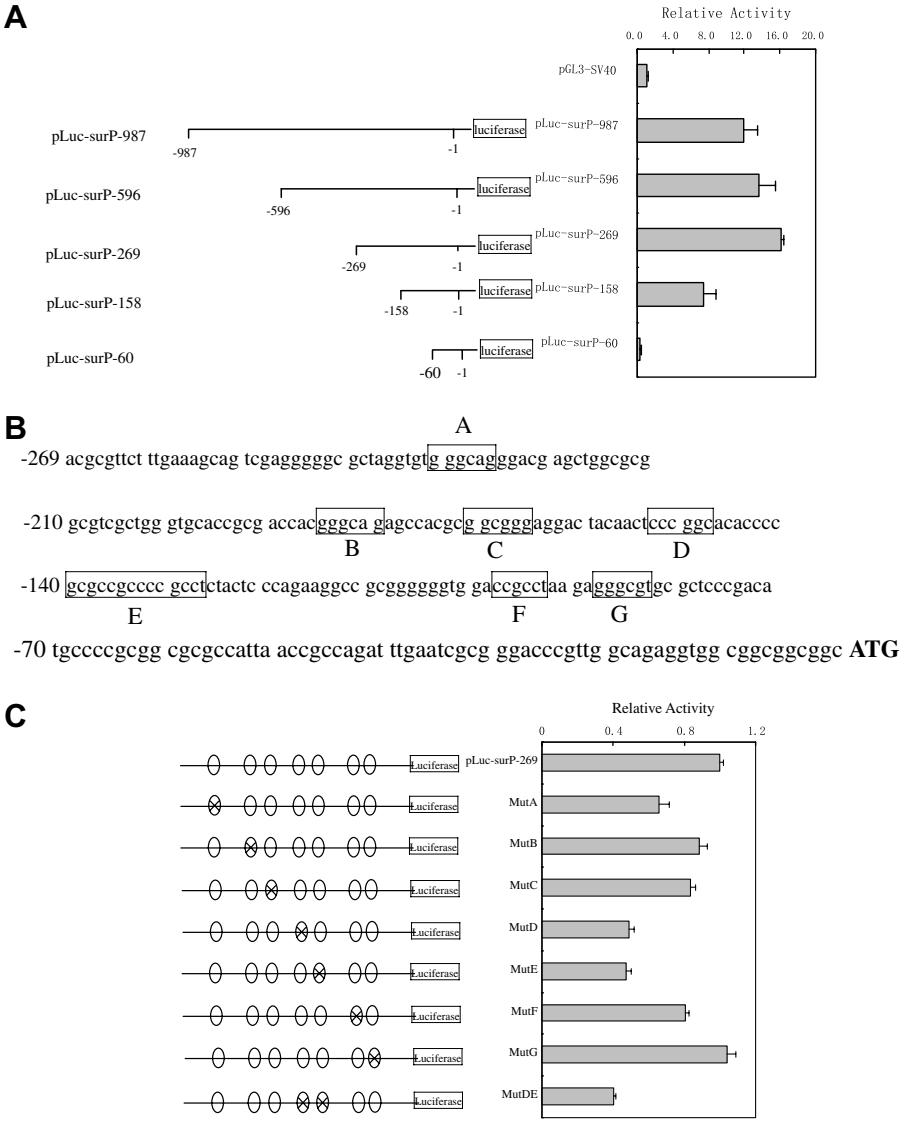

Fig. 1. Function analysis of the survivin promoter. (A) Deletion analysis of the survivin promoter. Schematic diagram shows the structure of reporter construct containing various lengths of the survivin promoter. Numbering is from the initiating ATG. The reporter construct was transfected to HeLa cells and the reporter activity was measured. (B) Nucleotide sequence of the survivin promoter. The putative binding sites for Sp1 are boxed in. The ATG translation start site is shown in boldface. (C) Mutation analysis of the Sp1-binding sites in the survivin promoter. A series of mutants were constructed and used for transient promoter assays. The reporter activity is expressed as the fold increases in relative promoter activities compared with that in the pLuc-surP-269. Data for (A,C) were means ± SD from three independent experiments, with each experiment carried out in triplicate.

for the core promoter activity of the *survivin* gene are mainly located in the 269 bp region upstream of the ATG initiation site. Sequence analysis revealed that 7 putative binding sites for Sp1 are present in this 269 bp survivin promoter region (Fig. 1B) [2].

Two Sp1 sites are essential in modulating survivin activity

Sp1 is known to play a role in the regulation of genes lacking a functional TATA box. Examples of this are the neurogranin gene and myeloid Elf-1-like factor, each of which has a TATA-less promoter region in which Sp1 binds to a GC-box to activate transcription [18,19]. The presence of numerous Sp1 binding sites in the promoter region of survivin suggested that Sp1 might be involved in the regulation of survivin activity. To test whether these Sp1 sites have any functional role in regulating *survivin* gene expression, 7 Sp1 sites were individually or simultaneously mutated (Table 1 and Fig. 1C). A panel of the luciferase reporter constructs, driven by various mutated survivin promoters, was transfected into HeLa cells.

Compared with the activity of pLuc-surP-269 in HeLa cells, mutations of the Sp1-D site (MutD) or Sp1-E site (MutE) of the survivin promoter reduced luciferase activity by about 50% (Fig. 1C). Mutations of the Sp1-A site (MutA), the Sp1-C site (MutC), or the Sp1-F site (MutF) of the survivin promoter reduced luciferase activity by 15%, 16%, and 25%, respectively. Simultaneous mutations of the Sp1-D/E sites (MutDE) reduced luciferase activity by 60%. The mutations MutB and MutG of the survivin promoter had no effect on survivin promoter reporter activity. These results suggested that Sp1 binding sites D (at position −148 to −153) and E (at position −127 to −140) are important for the survivin promoter to attain maximum activity.

Li and Altieri made unbiased mutagenesis analysis of the human survivin promoter and revealed that, in HeLa cells, mutation of the Sp1-D site in the survivin promoter (CCCGGC→CCTGGC mutation at position Sp1-D) abolished basal transcriptional activity by about 60% [2], which is consistent with our results (CCCGGC→CCTTGC mutation at position Sp1-D). They also showed

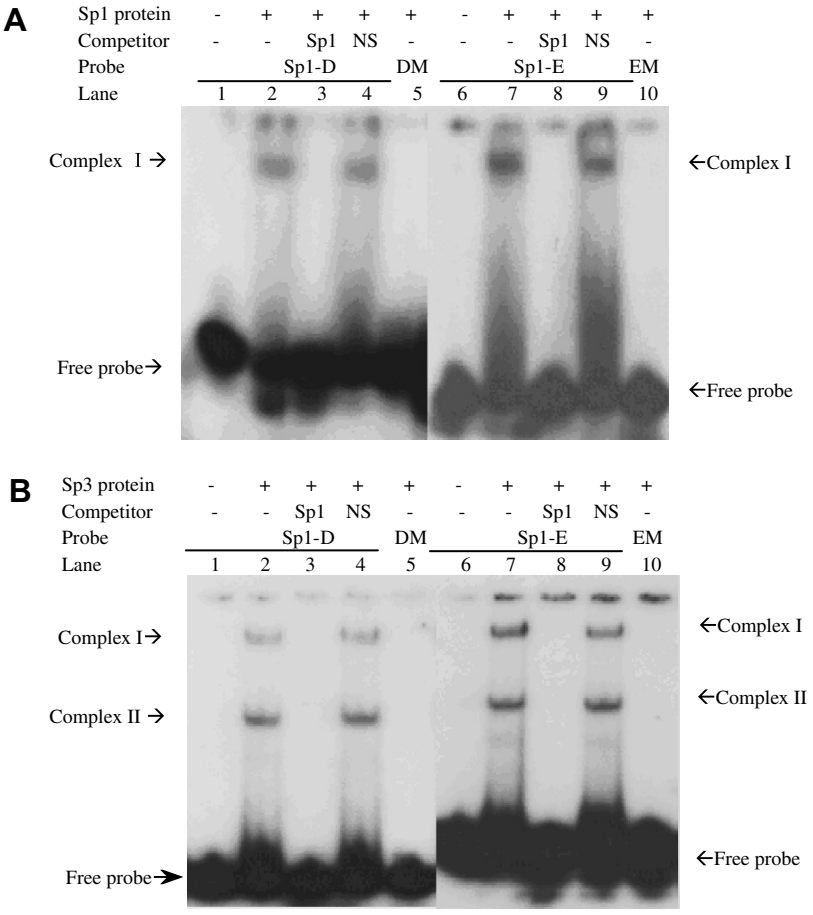

Fig. 2. Binding activity of the Sp1 D and E sites in the survivin promoter. Electrophoretic mobility shift assay experiments using radiolabeled double-strand oligonucleotides probes containing a putative or mutative Sp1 site with Sp1 or Sp3 protein. Competitive gel shift assay was done in the presence of 250-fold molar excess of unlabeled double-stranded oligonucleotide corresponding to the Sp1 consensus motif (Sp1) and nonspecific (NS). The arrows indicate specific binding. (A) EMSA experiments using probes with Sp1 protein. (B) EMSA experiments using probes with Sp3 protein.

that mutation of the Sp1-C site (GGCGGG→AGCGGG mutation at position Sp1-C) abolished basal transcriptional activity by about 80% [2]. In our study, however, the site mutation (GGCGGG→ATTGGG mutation at position Sp1-C) reduced luciferase activity by about 15%, which may be explained by different base mutation. Li and Altieri did not study the Sp1 binding sites A, B, and E∼G.

Sp1 and Sp3 bind to the survivin promoter

Having shown that Sp1 sites D and E were essential for the induction of transcriptional activity, we next conducted EMSA to examine the binding to these Sp1 sites by Sp1 and its closely related homologue, Sp3. As shown in Fig. 2, the Sp1 protein formed one complex with the SpD and SpE probes. The Sp3 protein formed two complexes with the SpD and SpE probes. To assess the specificity of Sp1 binding to the Sp1 sites in the survivin promoter, competition EMSA was conducted, using unlabeled consensus or nonspecific Sp1 probes, to compete with the ^{32}P-labeled probe for binding to either the Sp1 protein or the Sp3 protein. Mutation of the Sp1 site in the SpD or SpE probe eliminated the formation of all the complexes.

However, no DNA binding to the site D is detected with 293 nuclear extracts [20], suggesting that Sp1 interacts with other proteins and the resultant complexes collectively determine DNA binding selectivity and this site represents cell type-specific regulation.

Sp1/Sp3 transactivate the survivin promoter activity

The results suggested that Sp1 family members are important for the regulation of the survivin promoter; therefore, we conducted co-transfection experiments. Cells were co-transfected with the pLuc-surP-269 plasmid and either pN3-Sp1 or pN3-Sp3, or both expression plasmids simultaneously in cells (Fig. 3A). Compared with that cells co-transfected with the pN3 empty vectors, Sp1 transactivated the pLuc-surP-269 plasmid 3.33-fold and Sp3 up-regulated pLuc-surP-269 luciferase activity 3.86-fold. Moreover, transfection with both pN3-Sp1 and pN3-Sp3 activated the pLuc-surP-269 plasmid 8.66-fold, an effect higher than the sum of the levels observed for the two individual transactivations. We therefore conclude that Sp1 and Sp3 work cooperatively to activate the survivin promoter.

The increased Sp1 or Sp3 levels could induce higher transcription of the pLuc-surP-269 constructs, so we hypothesized that expression of the endogenous gene would also be increased. As expected, overexpression of Sp1 and Sp3 increased the expression of endogenous survivin RNA (data not shown) and survivin protein (Fig. 3B), indicating that Sp1/Sp3 expression positively effects endogenous survivin expression.

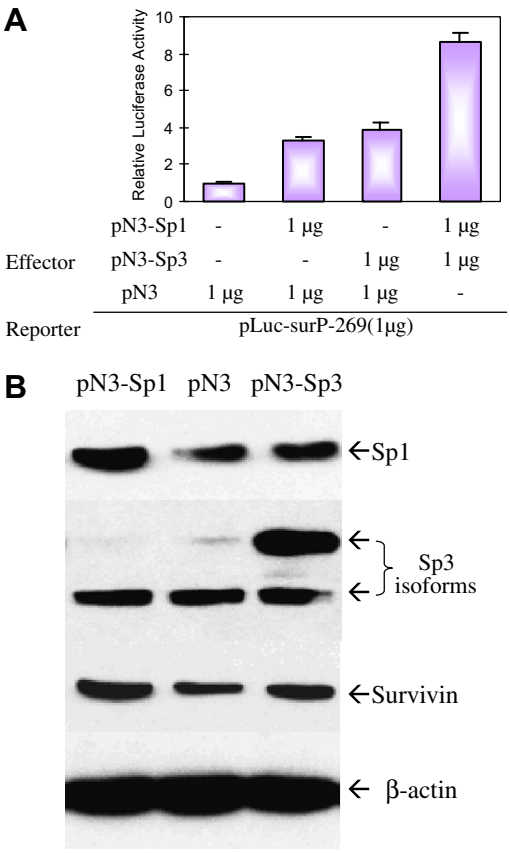

Fig. 3. Sp1/Sp3 transactivate the survivin promoter activity. (A) Induction of luciferase reporter gene expression by co-transfection with the Sp1 and Sp3 expression vectors. HeLa cells were transiently co-transfected with the pLuc-surP-269 and the indicated amounts empty vector (pN3) and/or pN3/Sp1 and/or pN3/Sp3. Luciferase activity was determined 48 h after transfection of plasmids and is expressed as fold activation over the empty vectors. Data were means ± SD from three independent experiments, with each experiment carried out in triplicate. (B) Overexpression of Sp1 or Sp3 lead to increased expression of the endogenous survivin. HeLa cells were transiently transfected with the pN3/Sp1 or pN3/Sp3 expression plasmid. Fourty-eight hours following transfection, cell lysates were prepared for Western blot analysis. Western blots showed levels of Sp1, Sp3, and survivin protein in the cells transfected with specific plasmids. β-Actin was used as a loading control.

Blocking Sp1 and Sp3 inhibits the activity of the survivin promoter

To further examine the role of Sp1 and Sp3 in regulating survivin promoter activity, we investigated the ability of mithramycin, a drug known to block activity of Sp1 family members by binding to GC-rich regions [16,21], to affect expression levels of survivin. Treatment of HeLa cells with mithramycin resulted in down-regulation of survivin mRNA transcription (data not shown) and decreased survivin protein level (Fig. 4A), suggesting a transcriptional mechanism for the modulation of survivin expression. Moreover, luciferase reporter assays revealed that mithramycin also decreased survivin promoter activity (Fig. 4B).

Mithramycin could potentially have nonspecific effects; therefore, we used siRNA to more selectively target Sp1

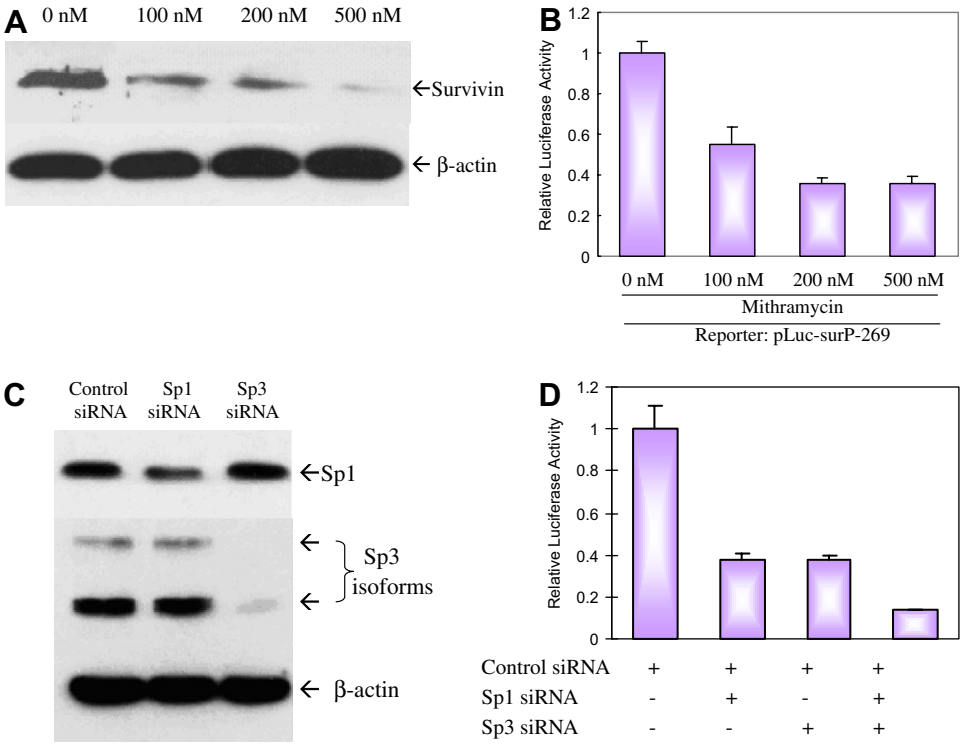

Fig. 4. Blocking Sp1 and Sp3 inhibit the activity of survivin promoter. (A) Mithramycin down-regulated survivin expression. Cells were treated with the indicated concentration of mithramycin. After 24 h, protein was harvested from cells. Western blots showed that mithramycin can decrease endogenous survivin expression. β-Actin was used as a loading control. (B) Mithramycin inhibited the activity of the survivin promoter. Cells were transfected with pLuc-surP-269 and 24 h after transfection, cells were treated with the indicated concentration of mithramycin for another 24 h. Luciferase activity of the lysates was normalized by lysate protein concentration and was shown as the fold increases in relative promoter activities compared with that in the cells without mithramycin treatment. (C) Cells were transfected with Sp1 siRNA or Sp3 siRNA or nonsilencing control siRNA, and 48 h later cells were harvested. Western blots showed levels of Sp1 and Sp3 protein in the cells transfected with specific siRNA. β-Actin was served as a loading control. (D) Cells were co-transfected with pLuc-surP-269 and the indicated siRNAs and 48 h later the samples were harvested. Luciferase activity of the lysate was normalized by lysate protein concentration and was shown as the fold increases in relative promoter activities compared with that in the control cells. Data for (B,D) were means ± SD from three independent experiments, each experiment was performed in triplicate.

and Sp3. We synthesized siRNAs to knock down Sp1 and Sp3, and RT-PCR (data not shown) and Western blotting analyses showed that these siRNAs were effective in decreasing the respective mRNA and protein levels (Fig. 4C). The results in Fig. 4D summarize the effects of Sp1 siRNA and Sp3 siRNA on luciferase activity in cells co-transfected with pLuc-surP-269 and the siRNAs. Sp1 siRNA and Sp3 siRNA both inhibited luciferase activity. These data demonstrated the involvement of Sp1 and Sp3 in regulating the human *survivin* gene.

In summary, we have elucidated the promoter of *survivin* gene expression and identified that not only Sp1 but also Sp3 play a critical role in regulating basal survivin promoter activity. They can activate the survivin promoter, which were proven by EMSA, blocking Sp1 or Sp3 using RNAi or mithramycin treatment of HeLa cells, and overexpression of Sp1 or Sp3, and they cooperatively regulate the survivin promoter activity. These data should be helpful in identifying the molecular basis of the dramatic overexpression of *survivin* in all the most common human cancers.

Acknowledgments

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