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# The regulation of trefoil factor 2 expression by the transcription factor Sp3

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ABSTRACT

Trefoil factor family 2 (TFF2) participates in mucus stabilization and repair, apoptosis, and inflammatory responses. Previously published reports have indicated that several growth factors and basal transcription factors are associated with the expression of *TFF2*. However, the detailed mechanisms that regulate *TFF2* expression are not fully understood. The present study was designed to assess the essential role of the transcription factor SP3 with respect to *TFF2* expression. We first demonstrated that there was a negative correlation between the expression levels of SP3 and TFF2. Thus, in the examined cells, the overexpression of SP3 decreased the expression level of TFF2, whereas the inhibition of SP3 increased the expression level of TFF2. Moreover, we discovered two GC boxes in the *TFF2* promoter and confirmed the specific binding of SP3 to this promoter. On the whole, this study indicated that Sp3 was a major regulator of *TFF2* expression. This knowledge should contribute to our understanding of the role that is played by SP3 in the regulation of TFF2 expression.



## 1. Introduction

TFF peptides, including TFF1, TFF2, and TFF3, are secretory peptides of the mucous epithelia, the immune system, and the central nervous system. TFF2, which is also known as spasmolytic polypeptide, was the first member of this family to be identified. TFF2 has roles in gastric cytoprotection and repair [1] and is also thought to participate in the mucosal immune response [2]. The regulation of *TFF2* gene expression is of considerable interest with respect to the aforementioned functions. It has been reported that *TFF2* is an allergen-induced gene that is regulated by Th2 cytokines and STAT6 [3]. Moreover, investigations addressing the *TFF2* promoter have demonstrated that it is a complex promoter/enhancer that is responsive to several growth factors and basal transcription factors, such as GATA-6 and hepatocyte nuclear factor HNF-3 [4,5]. In addition, gastrin regulates the *TFF2* promoter through gastrin-responsive *cis*-acting elements [6]. The expression of *TFF2* may be upregulated as a result of diverse pathologic conditions of the gastrointestinal tract, including ulceration, inflammatory bowel disease, *Helicobacter pylori* infection, and drug-related effects [7,8]. Moreover, the expression of *TFF* genes is reported to be auto- and cross-inductive [9] and dependent on promoter methylation [10,11].

Sp3 is a zinc finger transcription factor that belongs to the Sp transcription factor family; this factor can act as either an activator or a repressor of transcription, depending on sumoylation processes [12]. Proteins from the Sp family of transcription factors contain a highly conserved DNA-binding domain (a zinc finger region) near their C termini, and there are two glutamine- and serine/threonine-rich amino acid stretches in the N-terminal portions of these peptides that are less conserved. Thus, these proteins bind and act through GC boxes to regulate gene expression [13]. A number of chromatin immunoprecipitation studies have demonstrated that Sp3 is associated in situ with the GC boxes of a variety of promoters [14–17]. It has previously been reported that Sp1, Sp3, HDAC1, and HDAC2 are associated with the *TFF1* promoter in MCF-7 cells that are grown under estrogen-depleted conditions [17]. However, although both Sp1 and Sp3 bind to the estrogen-responsive *TFF1* promoter in MCF-7 cells, these proteins do not simultaneously occupy the same promoter [18].

In the present study, we performed promoter analysis to elucidate the regulatory mechanisms of *TFF2* expression. We first demonstrated that *TFF2* gene expression was highly regulated by SP3 in human gastric epithelial cells. We also provided evidence that SP3 bound to the promoter of the *TFF2* gene. Our results strongly suggested that Sp3 acted as a negative regulator that attenuated the transcriptional activity of the *TFF2* gene.

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## 2. Materials and methods

### 2.1. Cell culture and transfection

GES-1 human gastric cells were cultured in DMEM (GIBCO, Rockville, MD, USA) that had been supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 Ag/ml), and 0.3% glucose. The cells were grown in a 37 °C humidified incubator with 5% $CO_2$.

The stably transfected si-SP3 cells were established in accordance with previously described procedures [19,20]. To silence the expression of *SP3*, the oligonucleotide siSP3 (5′-GGTGGAGCCTTCACTTCAACT-3′) was synthesized (Invitrogen, Carlsbad, CA).

### 2.2. The RT-PCR assays

RNA was isolated using the Qiagen RNeasy Kit (Qiagen, Valencia, CA). RevertAid M-MuLV Reverse Transcriptase (MBI Fermentas, Vilnius, Lithuania) was then used to synthesize cDNA from this RNA. The PCR amplification was performed with appropriate primers to detect TFF2, GAPDH, and SP3. Quantitative real-time PCR was performed using the SYBR Green Master Mix (Fermentas). The primers were designed as follows: the *TFF2* primers were 5′-TCGTCCTGGGGCTATGTGC-3′ and 5′-TGAGACCTCCATGACGCACTG-3′; the *SP3* primers were 5′-GCTGCTACTTCAAGTGGGCAGTATG-3′ and 5′-CTGCTGACTGGATCTGTGGTATCAC-3′ and the *GAPDH* primers were 5′-CACCAGGGCTGCTTTTAACTCTG-3′ and 5′-G ATGACAAGCTTCCCGTTCTCA-3′.

### 2.3. The electrophoretic mobility shift assay (EMSA)

The EMSA procedure used a LightShift Chemiluminescent EMSA Kit (Pierce Chemical Co., Rockland, IL) with biotin-labeled oligonucleotides (Invitrogen). Nuclear extracts from GES-1 cells were prepared in accordance with previously described procedures [21]. The binding reaction mixture contained 25 mM HEPES (pH 7.9), 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.1 μg of poly(dI–dC), and 10 μg of nuclear extracts. After the samples were incubated at room temperature for 20 min, they were loaded onto an 8% nondenaturing polyacrylamide gel. Different sequences of oligonucleotides are: 5′-CCTTCCTCAGGGAGGGGACTTTTCCATGCT-3′ (WT) and 5′-CCTTCCTCAGACTGCGGACTTTTCCATGCT-3′ (mut).

### 2.4. The chromatin immunoprecipitation (ChIP) assay

Cells were sonicated after they had been cross-linked in 1% formaldehyde. An incubation with salmon sperm DNA was used to preclear the cell lysates, and these lysates were then incubated for 6 h at 4 °C with 100 μL of protein A-agarose beads and either anti-Sp3 antibody or rabbit IgG. The resulting DNA fragments were extracted and subsequently amplified by PCR. The primers spanning the two GC boxes region are: 5′-CTTCCGGGCCAGGGTGACT-3′ and 5′-ATTCCTGTGGTCCCTGCCC-3′; and the primers spanning the proximal GC box region are: 5′-ACCGAGCAGGGAGAGAGTCA-3′ and 5′-CCTTTCTTCTCTGCCCCGT-3′.

### 2.5. The construction of the plasmids and the luciferase reporter assay

The plasmids were constructed in accordance with previously described procedures. The −100 to +40 and −1950 to +40 regions of the *TFF2* promoter were subcloned into the pGL3 basic luciferase reporter vector (Promega, Heidelberg, Germany). Cells were then transfected as necessary with β-galactosidase (β-gal), luciferase reporter plasmids and other expression vectors. The relative luciferase activity was measured in accordance with previously established procedures [22].

## 3. Results

### 3.1. The expression levels of SP3 and TFF2 are negatively correlated

To determine the relationship between SP3 and TFF2, we first examined their expression levels. As depicted in Fig. 1A, we confirmed by RT-PCR that *SP3* mRNA expression was significantly lower in immortalized human gastric epithelial cells than in gastric cancer cell lines. Interestingly, compared with gastric cancer cells, the human gastric epithelial cell line GES-1 displayed elevated levels of *TFF2* mRNA. Western blot analyses indicated a pattern of SP3 and TFF2 expression that was consistent with these PCR results (Fig. 1B). These data suggested that there was a negative correlation between the expression levels of SP3 and TFF2.

### 3.2. SP3 affects the expression of TFF2

Subsequently, to elucidate the essential role of SP3 with respect to TFF2 expression, we overexpressed SP3 by transient transfection in one group of GES-1 cells and stably knocked down the expression of *SP3* with a specific siRNA against *SP3* in another group of GES-1 cells. Using RT-PCR and qRT-PCR assays, we discovered that *TFF2* expression is up-regulated by approximately threefold in the absence of *SP3*, whereas the overexpression of *SP3* greatly inhibited the expression of *TFF2* (Fig. 2A and B). In addition, we examined the effect of SP3 on the protein expression level of TFF2, and Western blot assay results demonstrated a pattern of TFF2 expression (Fig. 2C) that was consistent with the RT-PCR results. Thus, these data revealed that SP3 negatively regulated the expression of TFF2.

### 3.3. Sp3 regulates the transcription level of the TFF2 promoter

The sequence analysis of the *TFF2* promoter revealed several potential nuclear factor binding sites, including two GC boxes for

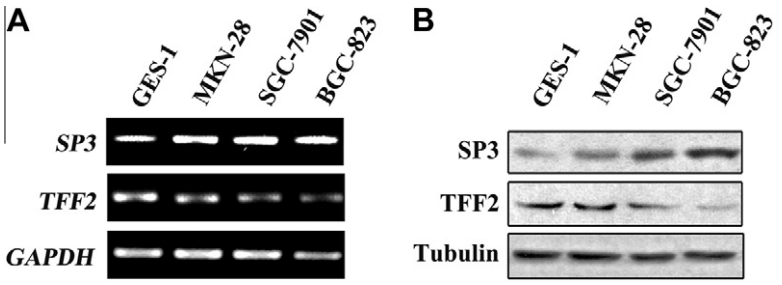


**Fig. 1.** The expression levels of SP3 and TFF2 in different gastric cell lines. (A) The mRNA levels of SP3 and TFF2 in different gastric cancer cell lines and in GES-1 cells were determined by RT-PCR analysis. GAPDH was used as a loading control. (B) The Western immunoblot analyses of protein levels of SP3 and TFF2 in GES-1 cells and three gastric cancer cell lines. For these immunoblots, β-tubulin was used as a loading control.

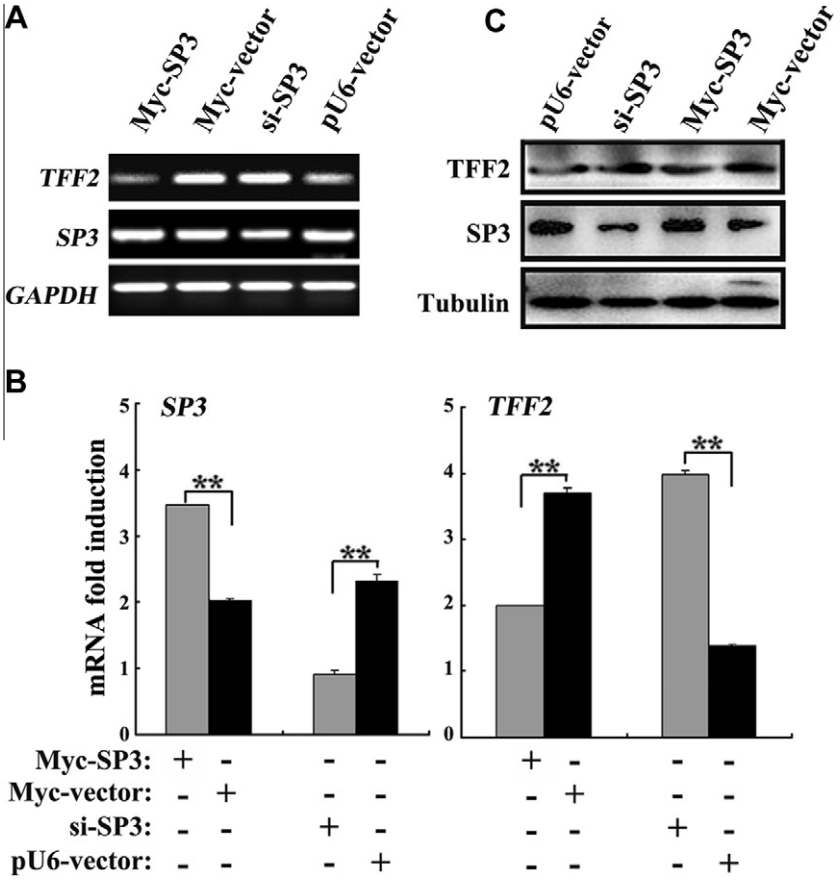


**Fig. 2.** SP3 controls TFF2 expression. Ges-1 cells were stably transfected with si-SP3 or transiently transfected for 24 h with an over-expression SP3 plasmid. The resulting RNA expression levels were determined by (A) RT-PCR analysis and (B) real-time RT-PCR analysis. For the real-time RT-PCR analysis, the numerical values are presented as the means ± SEM. A paired *t*-test was performed; ** indicates that $P < 0.01$. (C) The Western immunoblot analysis of the SP3-regulated expression of TFF2. For these immunoblotsβ-tubulin was used as a loading control.

SP family binding; one of these GC boxes was located in the −60 to −49 region of *TFF2*, whereas the other GC box was in the −311 to −306 region of *TFF2*. To better define the binding motif in the promoter, we constructed two luciferase reporters containing *TFF2* promoter sequences of different lengths; in particular, one of these reporters included the −1950 to +40 ($pGL3_{-1950/+40}$) region of *TFF2*, which contained both of the GC boxes in the *TFF2* promoter, whereas the other reporter included the −100 to +40 ($pGL3_{-100/+40}$) region of *TFF2*, which only contained the proximal GC box. We also generated three point mutants of these promoter reporters by mutating the Sp3 binding sites. One of these mutations involved changing the distal Sp3 binding site in the $pGL3_{-1950/+40}$ construct from GGGAGGGG to GACTGCGG, another of the mutations changed the Sp3 binding site in the $pGL3_{-100/+40}$ construct from GGCCCGG to GACTGCGG, and the third mutant was the $pGL3_{-1950/+40}$ construct containing both of the aforementioned binding site changes. The luciferase reporter assays demonstrated that the transcriptional activity of the reporter gene was more strongly suppressed by SP3 for the $pGL3_{-1950/+40}$ construct than for the $pGL3_{-100/+40}$ construct (Fig. 3A). Moreover, the mutation of the distal GC box element reduced the SP3-directed effect on the *TFF2* promoter by approximately 50%, and the point mutation of both GC boxes of the promoter nearly abolished the response to SP3. These results demonstrated that Sp3 could regulate the transcription level of the *TFF2* promoter and that the two GC-rich sequences in the −1950 to +40 region of *TFF2* were jointly responsible for the response of the *TFF2* promoter to SP3.

### 3.4. Sp3 binds to the TFF2 promoter

The *in vivo* binding status was assessed by ChIP and demonstrated that the human *TFF2* promoter was immunoprecipitated by a Sp3 antibody (Fig. 3B). To confirm the predicted binding of the Sp3 transcription factor to the *TFF2* promoter, an EMSA assay with double-stranded oligonucleotides was performed. The assay results indicated that DNA–protein complexes were strengthened by the addition of nuclear extract (Fig. 3C, *lanes 1–3*) and were supershifted by the addition of anti-SP3 antibody (Fig. 3C, *lane 6*). The binding specificity between Sp3 and the *TFF2* promoter was confirmed by the significant decrease in intensity that resulted from incubating the sample with a 100-fold excess of unlabeled probe and by the lack of a binding band in an incubation that used mutated probes (Fig. 3C, *lanes 4 and 5*). On the whole, these results confirmed that Sp3 is a major regulator of *TFF2* expression and directly binds to the promoter of *TFF2*.

### 3.5. TFF2 attenuates the transactivational functions of SP3

We have demonstrated that *TFF2* expression is regulated by SP3; thus, we subsequently explored whether TFF2 could affect SP3. Interestingly, through luciferase reporter assays, we found that TFF2 attenuated the transcriptional activity of SP3 on the *TFF2* promoter in a dose-dependent manner (Fig. 4). In addition, no significant reductions in this effect were observed in the reporter constructs that contained point mutants of the promoter. These

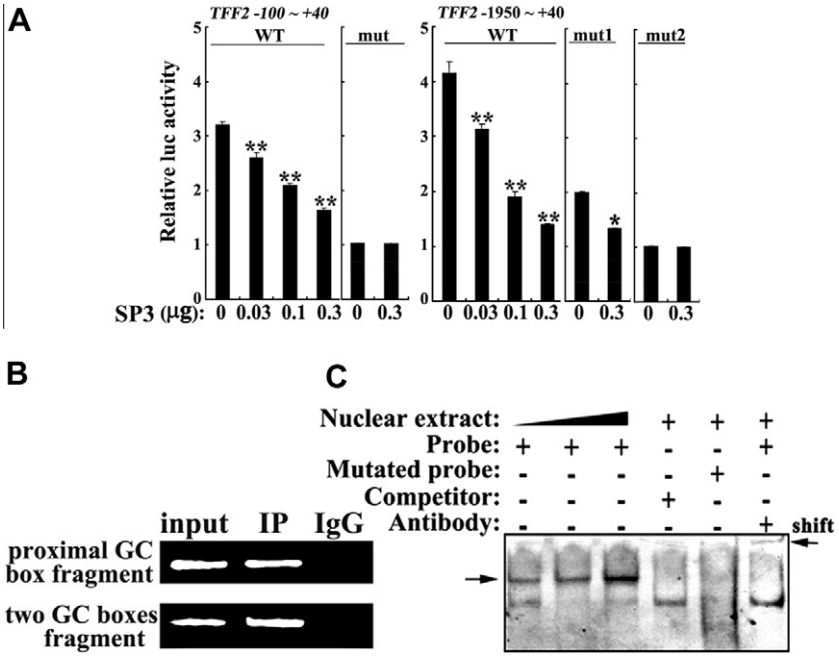


**Fig. 3.** Sp3 binds to the *TFF2* promoter and regulates its transcription level. (A) Promoter reporter plasmids including $pGL3_{-100/+40}$, $pGL3_{-1950/+40}$ and their mutants were constructed. Each plasmid was cotransfected with the SP3 expression plasmid and a β-galactosidase expression vector in HEK293 cells. The activity of the reporter gene was determined by a luciferase assay, and β-galactosidase activity levels were used to normalize for transfection efficiency. The bars indicate the SEM values; ** indicates that $P < 0.01$. (B) A ChIP assay was performed to monitor the chromatin that was immunoprecipitated by the Sp3 antibody. IgG was used as a negative control. The input indicates that similar DNA loading occurred throughout the assay. (C) EMSA was performed using an oligonucleotide that contained the distal GC box.

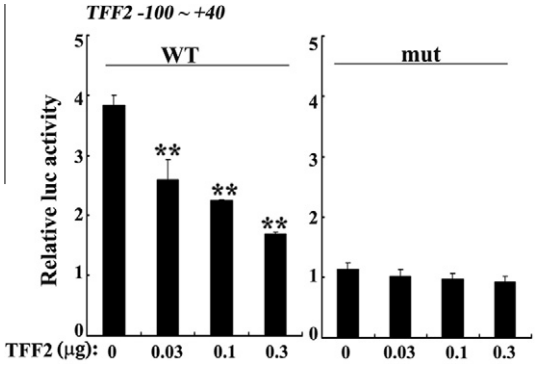


**Fig. 4.** TFF2 attenuates the transactivational functions of SP3. The TFF2 expression plasmid was cotransfected with either the promoter reporter plasmid $pGL3_{-100/+40}$ or its mutant plasmid in HEK293T cells. The luciferase activity measurements were normalized on the basis of β-galactosidase activity levels.

data suggested the existence of the autoregulation of *TFF2* expression by TFF2 itself.

## 4. Discussion

TFF2 is expressed specifically in the mucous neck cells of the stomach under normal conditions. It remains unclear whether TFF2 acts through a specific receptor, and several candidate molecules for this receptor have only recently been described [23,24]; however, TFF2 has been identified as an important promoter of cell migration [25,26], gastric cytoprotection and repair [1], and the mucosal immune response [2].

Several transcription factors, regulatory proteins and epigenetic factors regulate the cell-specific expression of TFF2. In addition, gastrin, short-chain fatty acids, and various inflammation mediators, including IL-1β, IL-6 and TNF-α, regulate the expression and secretion of TFF peptides [27,28]. In the current study, we first demonstrated that there was a negative correlation between the expression levels of SP3 and TFF2. The subsequent sequence analysis of the *TFF2* promoter revealed that the *TFF2* promoter contained several potential transcription factor binding sites. A consensus CACGTG E-box motif was found in the −150 and −145 region of *TFF2*, and two GC boxes were located in the −60 to −49 and the −311 to −306 regions of this gene. E-boxes are known to bind basic helix-loop-helix leucine zipper transcription factors, and GC-boxes are bound by the Sp family of nuclear zinc finger proteins. We used luciferase reporter assays to confirm that SP3 down-regulated the transcription level of *TFF2*; this result was consistent with the negative correlation between the expression levels of SP3 and TFF2. Using ChIP and EMSA assays, we also confirmed that this expression regulation was caused by the specific binding of SP3 to the *TFF2* promoter. Importantly, both of the GC-rich regions of the *TFF2* promoter were critical for the binding of SP3. In combination, we have demonstrated in this study that Sp3 is a significant regulator of *TFF2* expression.

Many SP3-targeting genes contain more than one GC box for binding, such as *SERCA3* (which contains 14 GC boxes), dihydrofolate reductase (4 GC boxes), and telomerase reverse transcriptase (5 GC boxes) [29–31]. Moreover, it has been demonstrated that the Sp1/SP3 transcription factors compete for binding sites; this competition creates a complex regulatory network [32,33]. In addition, other transcription factors might cooperate with Sp3 in the regulation of transcriptional activity. For example, the NF-κB/SP1 region is essential for the transcription of a human granulocyte–macrophage colony-stimulating factor transgene [34], and the estrogen receptor is associated with the SP3-mediated regulation of TFF1 expression [17]. Furthermore, we also discovered that TFF2 produces a feedback effect on the transactivational functions of SP3. Thus, further work is needed to define the ratio of Sp1 to Sp3 on the *TFF2* promoter and the precise mechanism by which the expression of trefoil factor family peptides is regulated.

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