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Sp1 transcription factor promotes *TMEPAI* gene expression and contributes to cell proliferation

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Abstract

Objectives: TMEPAI (transmembrane prostate androgen-induced protein) has been reported to be overexpressed during tumour progression; however, little is known concerning transcriptional mechanisms regulating *TMEPAI* gene expression.

Materials and methods: In this study, the *TMEPAI* gene promoter has been identified and characterized, and the effects of Sp1 on TMEPAI-induced viability of A549 cells were evaluated, using MTT and colony formation assays.

Results: We found that the sequence between −298 and +24 consists of basal promoter activity for *TMEPAI*. Further analysis indicated that two Sp1-binding sites are crucial for maintaining basal transcriptional activity of the *TMEPAI* promoter, and chromatin immunoprecipitation assays confirmed direct binding of Sp1 to the *TMEPAI* promoter. In addition, Sp1 up-regulated TMEPAI protein expression, as well as Sp1 promoting TMEPAI-induced cell proliferation.

Conclusions: These results indicate that the sequence between −298 and +24 consists of the basal promoter activity for *TMEPAI*. Sp1 promotes *TMEPAI* expression and contributes to cell proliferation.

1 | INTRODUCTION

TMEPAI (transmembrane prostate androgen-induced protein), also known as PMEPA1 (prostate transmembrane protein androgen induced 1), is overexpressed in many tumours, including prostate, breast, ovarian, kidney, stomach, colon and lung cancers.[1–8] *TMEPAI* was originally identified as an androgen-induced gene in the prostate cell and overexpressed in androgen independent xenografts.[1,4] Functional analysis of TMEPAI has revealed that it is an E3 ubiquitin ligase Nedd4-binding protein and plays a role in down-regulation of the androgen receptor through a negative feedback loop between androgen receptor and TMEPAI.[9] It has been reported that the overexpression of TMEPAI promotes androgen receptor-negative prostate cell proliferation and that decreased expression of TMEPAI inhibits cell growth and migration in breast cancer cells.[2,3] However, other studies have shown that TMEPAI has a tumour suppressor activity. For example, it has been reported that TMEPAI mediated p53-dependent apoptosis and inhibited prostate cancer cell colony formation in vitro.[1,9,10] Xu et al. reported a decrease or loss of *TMEPAI* mRNA expression in the tumour specimens of 62% of prostate patients.[9]

Specific protein-1 (Sp1) belongs to the specificity protein/Krüppel-like factor family and is widely expressed in many cells, and Sp1 binds to the GC-rich promoter element through the homologous sequence that the C-terminal of three Cys2His2 type zinc finger domain to regulate gene expression.[11] It has been reported that Sp1 contributes to tumorigenesis through regulating gene transcription.[12] In particular, in the process of tumour growth and metastasis, Sp1 plays an extremely important role in regulating the expression of oncogenes and tumour suppressor genes, cell cycle and growth-related signal transduction pathways, angiogenesis factors and apoptosis.[13–16] In addition, Sp1 is overexpressed in many tumours, including gastric, pancreatic, breast cancer and thyroid cancers.[17–19]

In this study, we have characterized the *TMEPAI* promoter region to analyse its transcriptional regulation mechanisms and revealed that Sp1 promotes *TMEPAI* expression and contribute to cell proliferation.

[#]These authors contributed equally to the study.

2 | MATERIALS AND METHODS

2.1 | Cell cultures

Human hepatic carcinoma cell line (HepG2), human alveolar epithelial lung cell line (A549), human breast cancer cell line (MDA-MB231), HeLa cells, human gastric cancer cell line (MGC-803) and human prostate cancer cell line (DU145) were obtained from the American Type Culture Collection (ATCC). HepG2 cells, HeLa cells, MGC-803 cells and DU145 cells were cultured in DMEM containing 10% foetal bovine serum (FBS), 100 U/mL penicillin and 100 U/mL streptomycin, at 37°C and 5% CO_2. A549 cells were cultured at 37°C and 5% CO_2 in F-12K medium, supplemented with 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin. MDA-MB231 cells were cultured in RPMI 1640 medium containing 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin, at 37°C and 5% CO_2.

2.2 | Genomic DNA isolation and cloning of the TMEPAI promoter region

Genomic DNA was isolated from the HeLa cells, and a 926-bp fragment from −902 to +24 in the 5′-flanking region of the *TMEPAI* (GenBank ID: 56937) gene was amplified by PCR. The numbering is relative to the transcriptional start site (TSS). The amplified DNA was cloned into a pUCm-T vector (Sangon, Shanghai, China) and sequenced. Other truncated fragments were amplified by PCR using the plasmid as a template. The PCR products were digested with *Kpn*I and *Xho*I, and then ligated into pGL4-basic vector (Qcbio Science and Technology, Shanghai, China), which contains the firefly luciferase reporter gene. All constructs were confirmed by DNA sequencing.

2.3 | Site-directed mutagenesis of the Sp1 recognition sites in TMEPAI promoter

TRANSFAC database (http://www.gene-regulation.com) was used to detect the binding sites for individual transcription factors in the *TMEPAI* promoter region. Mutations of the Sp1-binding sites in the *TMEPAI* promoter were generated by point mutations in the −298/+24 promoter region. The point mutations were generated by an overlapping PCR-based approach, the mutation of Sp1-A-binding site construct replaced the CCCGCCCC sequence by TTTGCCAT, and the mutation of Sp1-B-binding site construct replaced the CCCCGCCCC sequence by CCATGAAAT. All plasmid constructs were identified by DNA sequencing.

2.4 | Construction of Sp1 expression plasmids

Total RNA was extracted from HepG2 cells and reverse transcribed to obtain the cDNA according to the manufacturer's protocol. The following primers were used to amplify the Sp1 (GenBank accession no. NM 138473) gene sequence: forward, 5′-CGGAATTCATGAGCGACC AAGATCACTC-3′, and reverse, 5′-GCTCTAGATCAGAAGCCATTGCC ACTGATAT-3′. The PCR products were digested with *Eco*RI and *Xba*I and then ligated into pcDNA-HA vector, which were digested with *EcoRI* and *XbaI*. All constructs were confirmed by DNA sequencing.

2.5 | Transfections and luciferase report assay

Cell transfection was performed with TurboFect (Thermo Scientific, Waltham, MA, USA) according to the manufactures' instructions. Cells were seeded in 24-well plate for 24 hours before transfection. The cells were transfected with 1 μg of various *TMEPAI* promoter constructs or pGL4-basic vector, with pRL-TK Renilla luciferase vector (Promega, Madison, WI, USA) as the control for transfection efficiency. The cells were harvested 24 hours after transfection and luciferase activity was determined with the Promega Dual Reporter Assay according to the manufacturer's instructions. Relative luciferase activity was calculated as the ratio of firefly luciferase activity to Renilla (transfection control) luciferase activity. All experiments were performed in triplicates.

2.6 | RNA interference

Lentiviral vectors expressing Sp1 shRNAs (TRCN0000020448:5′-CCGGGCTGGTGGTGATGGAATACATCTCGAGATGTATTCCATCAC CACCAGCTTTTTG-3′, TRC: 5′-CCGGATCACTCCATGGATGAAATG ACTCGAGTCATTTCATCCATGGAGTGATTTTTG-3′); TMEPAI shRNAs (TRCN0000000332: 5′-CCGGATCACGGAGCTGGAGTTTGTTCT CGAGAACAAACTCCAGCTCCGTGATTTTTT-3′, TRCN0000000335: 5′-CCGGGAGTTTGTTCAGATCATCATCCTCGAGGATGATGATCT GAACAAACTCTTTTT-3′) and the non-target shRNA control vector (SHC002) were obtained from Sigma (St. Louis, MO, USA) and the knockdown level was tested by Sigma. The lentiviruses were produced according to the manufacturers' manual. Cells were infected with lentiviruses for 3 days before experiments were performed.

2.7 | Semi-quantitative RT-PCR

Total RNA was isolated and reverse transcribed to obtain the cDNA according to the manufacturer's protocol. RT-PCR was amplified using primers specific for *TMEPAI*, *Sp1* and β-*actin* genes. The following primer sets were used: *TMEPAI* forward, 5′-CCTGCCACCATCTCGCTGTC-3′, reverse, 5′-CGCTGATGCCCGAGTTACTGCTGG-3′; *Sp1* forward, 5′-ACGCTTC ACACGTTCGGATGAG-3′, reverse, 5′-TGACAGGTGGTCACTCCTC ATG-3′; β-*actin* forward, 5′-CACGATGGAGGGGCCGGACTCATC-3′, reverse, 5′-TAAAGACCTCTATGCCAACACAGT-3′. The amplified products were separated by 2% agarose gel electrophoresis.

2.8 | Chromatin immunoprecipitation (ChIP) assays

HeLa cells were cultured in 10-cm dishes and respectively transfected with pcDNA-HA-Sp1 plasmid. The transiently transfected cells were processed for ChIP assay. Cells were fixed 15 min by adding formaldehyde to the medium to a final concentration of 1% at room temperature. Then, glycine was added to a final concentration of 125 mM, and then the cells were washed with cold PBS, pelleted and

resuspended in lysis buffer, then sonicated to generate 200–1000 bp of DNA fragments. The cleaved chromatin was treated with proteinase K and RNase and measured by 0.8% agarose gel electrophoresis. Ten per cent of the chromatin was used as an input. Then, the control IgG or anti-HA (Abgent, San Diego, CA, USA) was used for the immunoprecipitation. The immunoprecipitated DNAs were purified using a purification kit (Manufacturer). The specific primers for detection of the *TMEPAI* promoter DNA sequence containing Sp1-binding elements were used for PCR amplification. Amplification of promoter DNA was assessed by PCR using the following primers: *Sp1*, forward, 5′-CGGGTCTACGTGGGCCGCCTAGC-3′; reverse, 5′-AGGTTCCCCCGCACCCCCTCC-3′. Analysis of the PCR products was performed on a standard 2% agarose gel electrophoresis.

2.9 | Western blot

Protein were extracted from cells using RIPA buffer (1% NP-40, 0.05 M Tris-HCl, 0.1% SDS, 150 mM NaCl, 1% sodium deoxycholate) containing a cocktail of protease inhibitors and separated in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Then, the proteins were transferred from the gels onto polyvinylidene fluoride (PVDF) membranes. The PVDF membranes were blocked by non-fat milk followed by incubation with primary anti-TMEPAI, anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-HA (Abgent) antibodies at 4°C overnight. After incubating with the corresponding secondary antibodies, membranes were visualized by Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, NE, USA).

2.10 | Bioinformatics analysis of TMEPAI promoter region

The transcription start sites (TSS) were identified by using the database of DBTSS (http://www.biomedsearch.com). The *TMEPAI* promoter region was analysed by using online analysis tools, Promoter Scan (http://www-bimas.cit.nih.gov/molbio/proscan/), Promoter 2.0 Prediction Server (http://www.cbs.dtu.dk/services/Promoter/) and BDGP (http://fruitfly.org:9005/seq_tools/promoter.html). The putative binding sites for transcription factors with 5′-upstream sequence of *TMEPAI* gene were analysed with TRANSFAC database (http://www.gene-regulation.com).

2.11 | Cell viability assay

Cell proliferation was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded in 96-well plates (8000 cells/well). At the end of the incubation period, 20 μL 5 mg/mL MTT was added into each well for 4 hours, then 200 μL DMSO was added to dissolve the purple crystals. The optical densities at 490 nm were measured using a microplate reader.[20]

2.12 | Colony formation assay

Different recombinant cells were collected and seeded at 400 cells/well into six-well culture plates and cultured for 14 days. Then, the colonies were stained with 5 mg/mL MTT (Sigma) and photographed, and three independent soft agar plating experiments were performed for statistical analysis. Plates were scored for the number of visible colonies. The colony forming rate was estimated as follow: (colony number/cell number) × 100%.

2.13 | Statistical analysis

Data are presented as the mean ± standard deviation (SD). All the data were analysed with GRAPHPAD PRISM 5. Results were analysed using the Student's *t*-test. A value of $P<.05$ was considered significant.

3 | RESULTS

3.1 | Identification of the TMEPAI promoter region

The *TMEPAI* promoter region was firstly analysed by using online analysis tools, then the DNA fragment that spans from −902 to +24 relative to the transcription start site of the *TMEPAI* promoter was amplified by PCR and then cloned into the pGL4 basic vector to produce construct **pGL4 −902/+24**. To choose the cell lines suitable for *TMEPAI* promoter function analysis, **pGL4 −902/+24** was transfected into six different cell lines, and the highest *TMEPAI* promoter activity was found in the transfected HeLa cells (Fig. 1a). The basal mRNA and TMEPAI protein levels were also tested in the six cell lines, as shown in Fig. 1b–e; *TMEPAI* is expressed in all cancer cell lines and with higher expression in HeLa and A549 cells. Therefore, the HeLa and A549 cells were selected for further analysis of the regulation of *TMEPAI* expression.

To identify the core promoter region of the *TMEPAI* gene, we generated six promoter truncated constructs: **pGL4 −902/+24**, **pGL4 −902/−298**, **pGL4 −902/−588**, **pGL4 −588/+24**, **pGL4 −588/−298** and **pGL4 −298/+24**. These constructs were transfected into HeLa cells to determine the DNA sequence element required for *TMEPAI* promoter activity. As shown in Fig. 2, compared with the basal luciferase activity of pGL4 basic, the longest construct, **pGL4 −902/+24**, gave about 80-fold activation, which indicates the presence of the *TMEPAI* promoter activity. To investigate the 3′-truncated constructs and the 5′-truncated constructs, the construct **pGL4 −298/+24** showed the highest activity among the *TMEPAI* promoter constructs, which was approximately 110 times compared to the pGL4 basic. These results indicate that the promoter region between −298 and +24 is required for the basal transcriptional activity of the *TMEPAI* gene.

3.2 | Two Sp1-binding sites are essential for maintaining the basal transcriptional activity of the TMEPAI promoter

Since the deletion analysis has shown that the regulatory elements required for the promoter activity of the *TMEPAI* gene were mainly located in the −298 to +24, we then investigated which domains within this region were required to the activation of *TMEPAI* promoter. The *TMEPAI* promoter region between −298 and +24, along

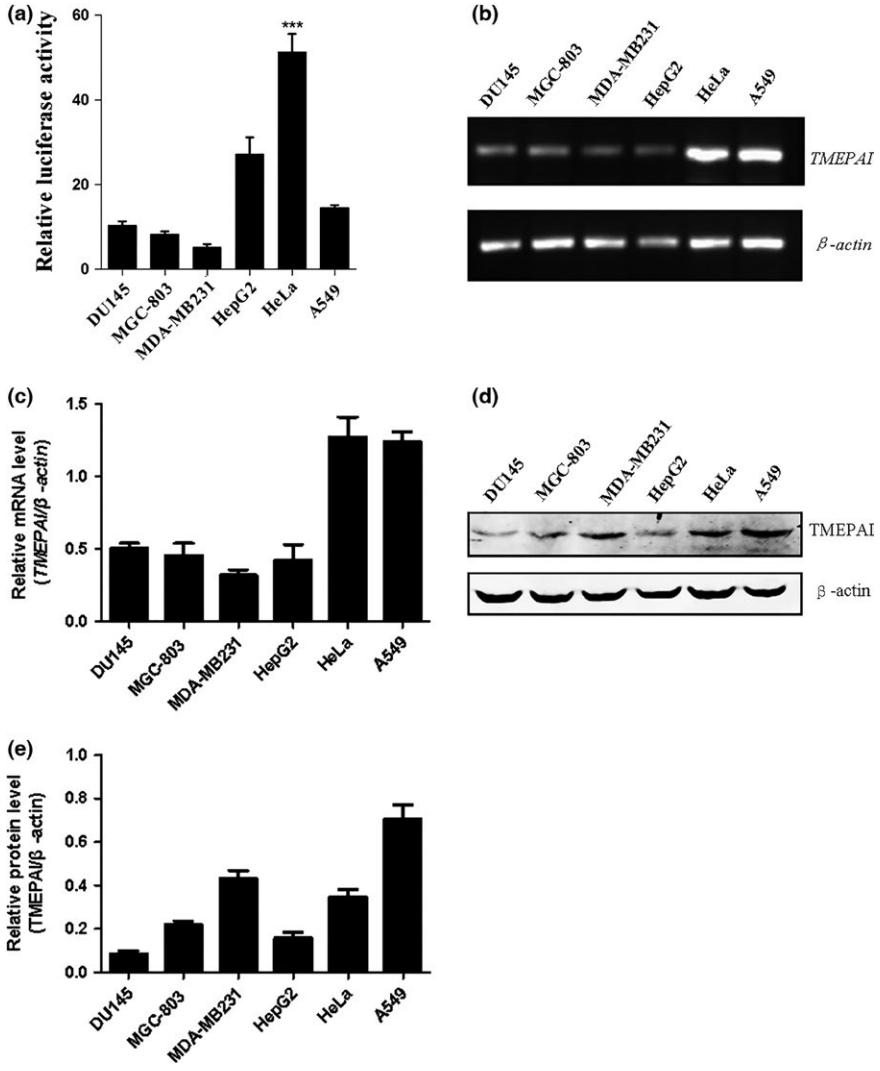

FIGURE 1 Determination of *TMEPAI* promoter activity and endogenous mRNA and protein levels of TMEPAI in different cancer cell lines. (a) The **pGL4 −902/+24** and pGL4 basic vector were co-transfected into six different cell lines (DU145, MGC803, MDA-MB231, HepG2, HeLa and A549) with pRL-TK plasmids. The luciferase activities were measured 24 h after transfection. Data were mean ± SD from three independent experiments (****P*<.001 vs pGL4 basic). (b) Semi-quantitative PCR was performed for analysis of *TMEPAI* mRNA in the cell lines, including DU145, MGC803, MDA-MB231, HepG2, HeLa and A549, and β-*actin* mRNA was used as the control. (c) Quantification of semi-quantitative PCR for *TMEPAI* mRNA levels using IMAGEJ software is shown. (d) Western blot tested the TMEPAI protein expression in the cell lines using antibodies against TMEPAI and β-actin. (e) Quantification of WB for TMEPAI protein levels using IMAGEJ software is shown

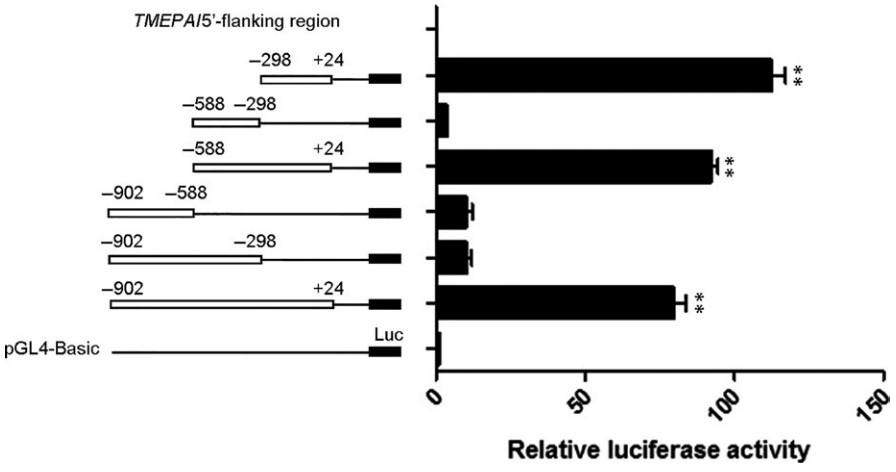

FIGURE 2 Deletion analysis of the *TMEPAI* promoter in HeLa cells. Schematic diagram represents the structure of the different constructs containing various lengths of the *TMEPAI* promoter. The numbering is relative to the TSS. Each promoter construct or the pGL4-basic vector was transfected into HeLa cells along with pRL-TK control. The luciferase activities were measured 24 h after transfection to determine the *TMEPAI* promoter activity. The luciferase activity is relative to the pGL4 basic activity. Data were mean ± SD from three independent experiments (***P*<.01 vspGL4-Basic)

with some putative binding sites for transcriptional regulator, was predicated using TRANSFAC database analysis. This promoter region contains GC box, which could be a potential binding site for Sp1 transcription factor binding site (Fig. 3a). To evaluate the contribution of the putative binding sites to regulation of the *TMEPAI* promoter, the point mutations at the binding sites were generated to measure the mutated promoter activities. As shown in Fig. 3b, mutation of the Sp1-A- or Sp1-B-binding site reduced 33% and 45% *TMEPAI*

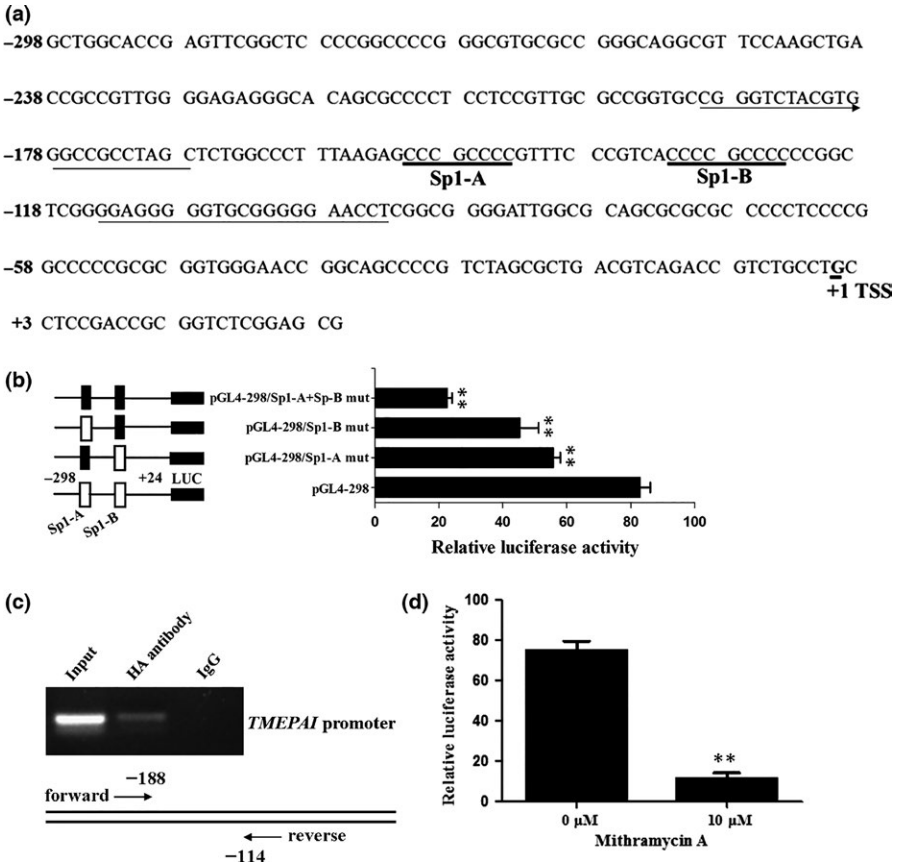

FIGURE 3 Function analysis of the *TMEPAI* promoter. (a) Analysis of the regulatory elements of the *TMEPAI* promoter by bioinformatics analysis. The transcription start site (TSS) is marked by ahorizontal line and numbered as +1. The consensus sequence for the putative binding sites of transcription factors are underlined, and the names are indicated down the sequence. (b) The binding sites for Sp1-A and Sp1-B transcription factors are indicated with opening square. Mutations are shown in bold. A series of mutants were constructed and transfected into HeLa cells. The luciferase activities were measured 24 h after transfection to determine the *TMEPAI* promoter activity. The luciferase activity is relative to the pGL4 basic activity (**P<.01 vs **pGL4 −298**). (c) Chromatin fragments isolated from the HA-Sp1 plasmid-transfected HeLa cells were subjected to immunoprecipitation with an HA antibody and IgG antibody as a negative control, binding of Sp1 with the *TMEPAI* promoter was assessed by PCR amplification. (d) The HeLa cells were transfected with **pGL4 −298/+24** plasmid and then treated with mithramycin A for 24 h to determine the *TMEPAI* promoter activity. The luciferase activities were measured 24 h after transfection. Data were mean ± SD from three independent experiments (**P<0.01 vs 0 μM mithramycin A)

promoter activity, respectively, compared with the wild-type promoter activity, and the double Sp1-A and Sp1-B mutant reduced 72% *TMEPAI* promoter activity. These results indicate that two Sp1 sites (at position −150 to −144 and −132 to −122) are essential for the basal transcriptional activity of the *TMEPAI* promoter.

ChIP assays were performed to determine the Sp1 occupancy in the *TMEPAI* promoter. The chromatins in HA-Sp1 plasmid-transfected HeLa cells were sheared into 200- to 1000-bp-sized fragments. As demonstrated in Fig. 3c, the anti-HA antibody, but not the control antibody IgG, precipitated the *TMEPAI* promoter region containing the Sp1 sites in HA-Sp1 plasmid-transfected HeLa cells, respectively.

Mithramycin A is a specific inhibitor of the Sp1 transcription factor, and it blocks Sp1-mediated transcriptional activity (Sangon, Shanghai, China).[21] HeLa cells were transfected with **pGL4 −298/+24** and then treated with mithramycin A for 24 hours, and the luciferase assay showed that mithramycin A significantly inhibited the transcriptional activity of the human *TMEPAI* promoter (Fig. 3d).

3.3 | Sp1 up-regulates the expression of TMEPAI

The basal mRNA levels of *Sp1* in the cell lines were tested, as shown in Fig. 4a. *Sp1* was highly expressed in HeLa and A549 cells, which were consistent with the high expression of *TMEPAI* in HeLa and A549 cells (Fig. 1b). To further examine whether expression of Sp1 has an impact on the transcriptional activity of the human *TMEPAI* promoter, HeLa cells were co-transfected with **pGL4 −298/+24** and Sp1 expression vector. Compared to the cells co-transfected with the pcDNA-HA empty vectors, with increasing dose of the Sp1, the *TMEPAI* promoter showed higher luciferase activity (Fig. 4b,c). These results indicate that Sp1 can up-regulate the *TMEPAI* promoter activity. Since the increased Sp1 levels could show higher transcriptional activity of the **pGL4 −298/+24**, we supposed that the expression of the endogenous *TMEPAI* gene would be increased. Furthermore, Western blot analysis showed that overexpression of Sp1 increased the expression of endogenous TMEPAI protein (Fig. 4d). These results indicate that

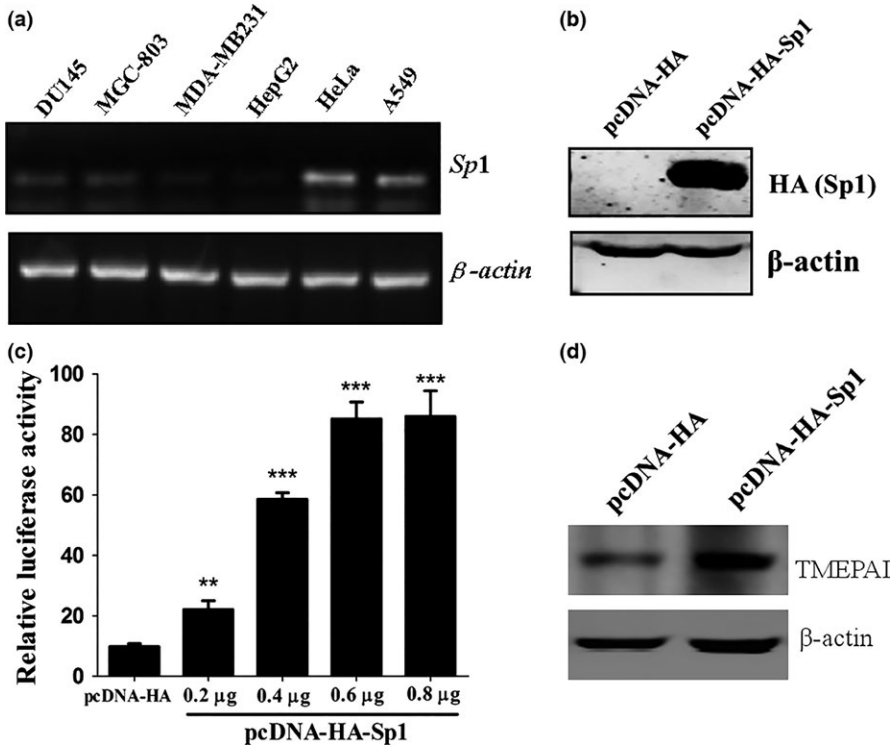

FIGURE 4 Overexpression of Sp1 transactivates the *TMEPAI* promoter. (a) Semi-quantitative PCR was performed for analysis of *SP1* mRNA, respectively, in the cell lines, including DU145, MGC803, MDA-MB231, HepG2, HeLa and A549, and β-*actin* mRNA was used as the control. (b, c) HeLa cells were transfected with the pcDNA-HA or pcDNA-HA-Sp1 expressing plasmids, Western blots were performed to test the levels of Sp1 proteins, and the luciferase activities were measured 24 h after transfection of **pGL4 −298/+24** with the empty vector pcDNA-HA or pcDNA-HA-Sp1 to determine the *TMEPAI* promoter activity. Data were mean ± SD from three independent experiments (**P<.01, ***P<.001 vs pcDNA-HA). (d) HeLa cells were transiently transfected with the pcDNA-HA, pcDNA-HA-Sp1 expressing plasmid, and the expression of TMEPAI was analysed by Western blot using antibodies against TMEPAI and control β-actin

Sp1 can up-regulate the *TMEPAI* promoter activity and endogenous TMEPAI protein expression.

To further confirm the role of Sp1 in the regulating of the TMEPAI expression, RNA interference was used to knock down Sp1 expression. Lentiviruses expressing Sp1-shRNAs were used to infect HeLa cells, and inhibition of Sp1 mRNA expression was confirmed by semi-quantitative RT-PCR and real-time PCR (Fig. 5a–c). Depletion of Sp1 in HeLa cells resulted in a significant decreasing *TMEPAI* promoter activity (Fig. 5d), and Western blot also showed the decreased protein expression of TMEPAI in the Sp1-shRNAs expressed cells (Fig. 5e,f). These results further indicate that Sp1 directly up-regulate TMEPAI expression through their transcriptional activity on the *TMEPAI* promoter.

3.4 | Sp1 promotes TMEPAI-induced cell proliferation of A549 cells

TMEPAI has been reported to be overexpressed in many cancer cells,[2–7] and TMEPAI promotes cell proliferation, migration and invasion.[2,3,12] We then examined whether Sp1 affects TMEPAI-induced cell viability of A549 cells using the MTT assay and colony formation assay. As shown in Fig. 6a, overexpression of Sp1 promoted A549 cell viability. Colony formation assay confirmed that overexpression of Sp1 promotes A549 proliferation, whereas Sp1 induced cell proliferation less significantly after the depletion of TMEPAI (Fig. 6a–c). Furthermore, we detected the protein expression level of TMEPAI in A549 cells which were overexpressed with Sp1 with or without TMEPAI depletion. The data demonstrated that the results shown in Fig. 6a–c were dependent on the protein expression level of TMEPAI (Fig. 6d,e). These results indicate that Sp1 promotes cell proliferation partly through regulating *TMEPAI* expression.

4 | DISCUSSION

TMEPAI was originally identified as an androgen-induced gene by analysis of gene expression in prostate cancer cells, and it has been reported that TMEPAI protein is overexpressed in many tumours.[8] However, little is known about how the transcriptional of the *TMEPAI* gene is regulated. To study the transcriptional mechanisms of the *TMEPAI* gene, the 926-bp DNA fragment of the *TMEPAI* promoter region was constructed and the activities of *TMEPAI* promoter constructs were measured by transient transfection assays. The luciferase reporter system driven by the *TMEPAI* promoter constructs showed a higher activity in HeLa and A549 cells. To identify the core region in the *TMEPAI* promoter, we generated a series of truncated constructs containing the 5′-truncated and the 3′-truncated, and the luciferase activity results show that the region from −298 to +24 bp relative to the TSS is necessary for the basal transcriptional activity of the *TMEPAI* gene. Transcriptional regulation is the result of the balance between RNA polymerase activity on regulatory regions and chromatin remodelling,[22] and the processes regulating gene transcription directly influences the genome organization. Although we have identified a core promoter region mainly relying on basal transcription factors, the transcription of the *TMEPAI* gene could be regulated by other pathways, including cell differentiation or cell-cycle progression.

The TRANSFAC database has been used to detect the consensus recognition sequences for transcription factors involving in regulation

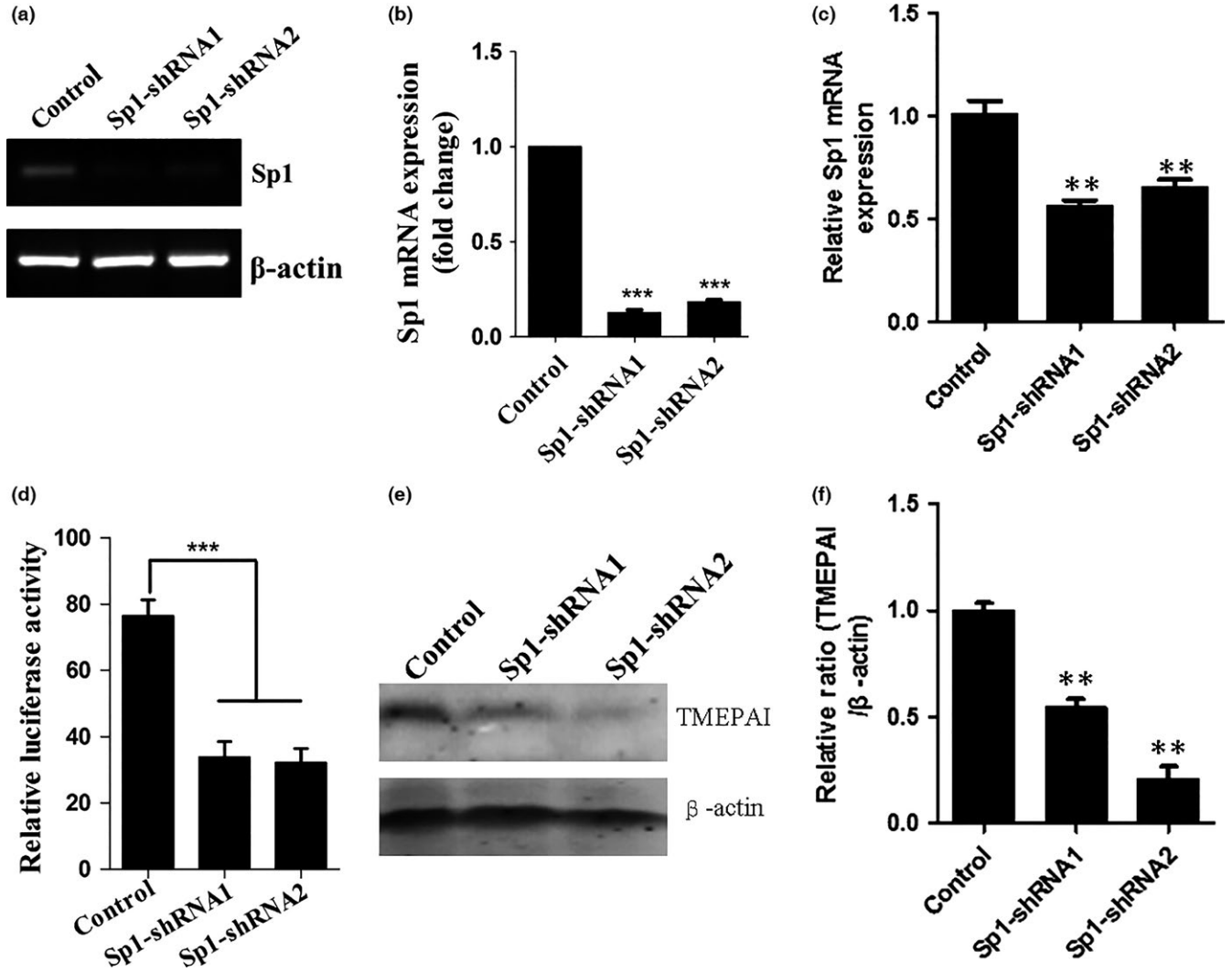

FIGURE 5 Depletion of Sp1 reduces the *TMEPAI* promoter activity. (a–c) Depletion of Sp1 protein in HeLa cells by infection of lentiviruses expressing Sp1-shRNAs or the control non-targeting shRNA was determined by semi-quantitative RT-PCR (a, b) or real-time PCR (c) after 3 d of infection. The IMAGE J software was used to analyse the results. (d) After 3 d of infection, the cells were transfected with **pGL4 −298/+24** constructs. The luciferase activities were measured 24 h after transfection to determine the *TMEPAI* promoter activity. Data were mean ± SD from three independent experiments (***P<.001). (e) HeLa cells were infected with lentiviruses expressing Sp1-shRNAs, or the control non-targeting shRNA, and the expression of TMEPAI was analysed by Western blot using antibodies against TMEPAI and β-actin. (f) Quantification of WB for TMEPAI protein levels using IMAGEJ software is shown. (**P<.01 vs control)

of gene expression. Sequence analysis revealed that the *TMEPAI* promoter region contains Sp1-binding sites by using the TRANSFAC. Sp1 binds to the GC-rich promoter element through the homologous sequence that binds the C-terminal of three Cys_2His_2 type zinc finger domain.[23] It has been reported that Sp1 regulates gene transcription related to cell proliferation, apoptosis, differentiation through Sp1-binding sites in the promoters,[24] and it binds GC-rich motifs and regulates the expression of genes by interacting with other transcription factors, such as Stat1[25] and c-myc,[26] and Sp1 is a typical activator of transcription.[27] Our data indicate that Sp1 binds to the *TMEPAI* promoter, and two Sp1-binding sites are crucial for the basal promoter activity.

Predicated using TRANSFAC database analysis, there is a CREB transcription factor–binding site (TGACGTCA), which located at −20 to −13 except Sp1 in the TMEPAI promoter region between −298 and +24. CREB (cAMP responsive element-binding protein) has been well known as one of the best studied inducible transcription factors and belongs to the leucine zipper transcription factor family. It has been reported that CREB correlates with various cancers, including prostate, breast, small-cell lung cancer and ovarian tumours. In addition, overexpression of CREB and its phosphorylation level play an important role in tumorigenesis, and the down-regulation of CREB by RNAi inhibits apoptosis in non–small-cell lung cancer cell lines. We will investigate CREB transcription factor on the regulation of TMEPAI gene expression in our further study.

As shown in Fig. 2, the truncated promoter (−298 to +24) shows a higher activity than the longest promoter (−902 to +24). This may be because the longest promoter has some transcription factor–binding

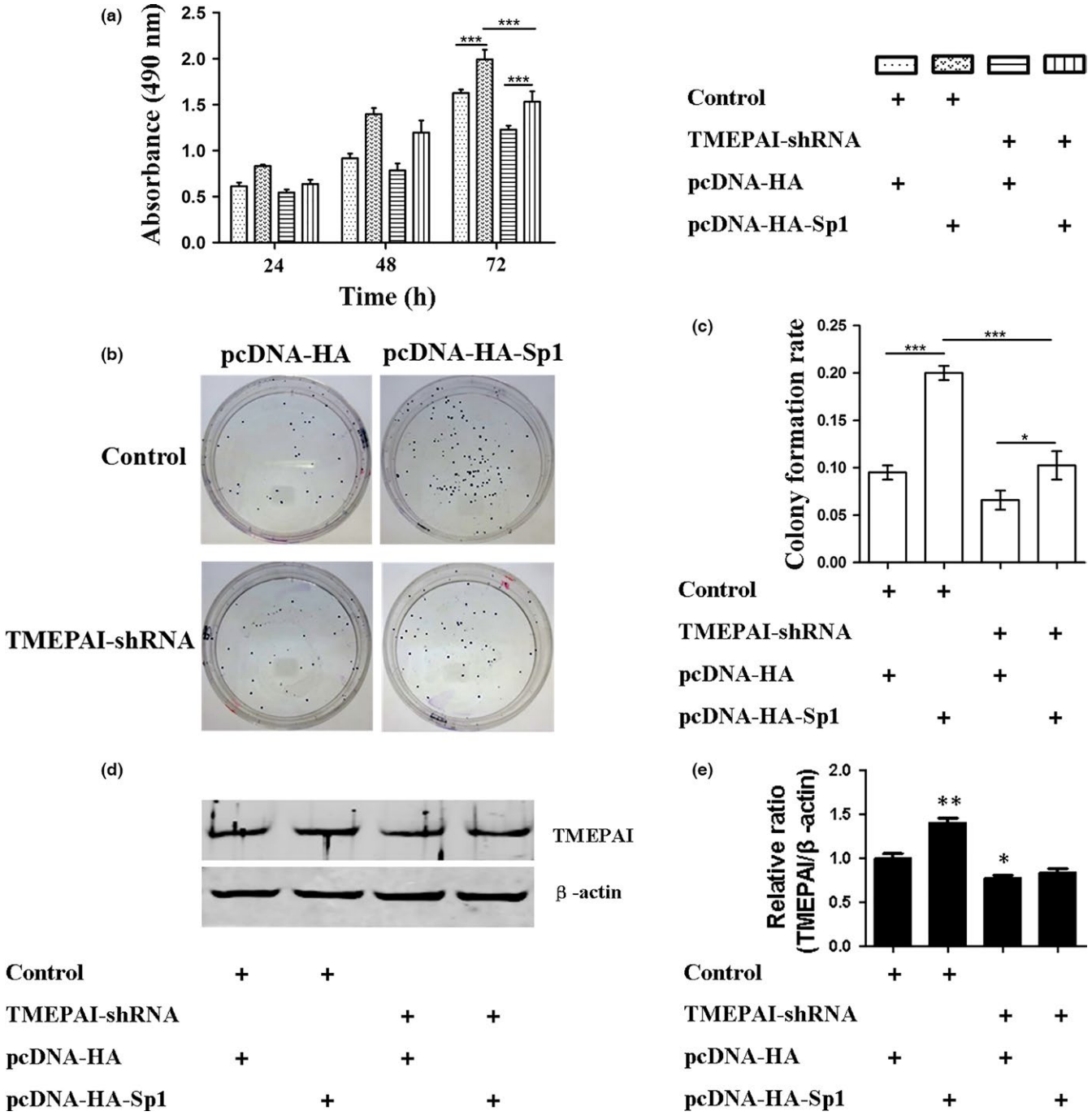

FIGURE 6 Sp1 promotes TMEPAI-induced cell proliferation. (a) A549 cells were overexpressed with Sp1 with or without TMEPAI depletion, and cell viability was measured by a MTT assay. (b, c) A549 cells were overexpressed with Sp1 with or without TMEPAI depletion, and cell proliferation was measured using a colony formation assay, quantifying the efficiency of colony formation by statistical analysis. Data were mean ± SD from three independent experiments (*P<.05, ***P<.001). (d) The protein expression level of TMEPAI in A549 cells which were overexpressed with Sp1 with or without TMEPAI depletion was detected by Western blot assay. (e) Quantification of WB for TMEPAI protein levels using ImageJ software is shown (**P<.01)

sites which inhibit the activity of the longest promoter. The in-depth mechanisms into this question will be covered in our future planned studies.

It has been reported that Sp1 plays an extremely important role in regulating the expression of oncogenes and tumour suppressor genes, cell cycle and growth-related signal transduction pathways, angiogenesis factors and apoptosis. Sp1 is known to play a role in the androgen induction of many genes, including p21, VEGF and NRIP.[28–30] In this study, we showed that Sp1 promotes cell proliferation partly through regulating TMEPAI expression (Fig. 6). The data would be helpful to understand the role of Sp1 in the regulating of cell proliferation.

TMEPAI expression is induced by TGF-β signalling, and in turn, the expression of TMEPAI negatively regulates TGF-β signalling.[31] TGF-β signalling plays important roles in cancer development by regulating cell growth, migration and differentiation.[10,32,33] Furthermore, the transcript of the TMEPAI gene has been reported to be induced by testosterone, its derivatives, or mutated p53 and to be implicated in tumorigenesis.[1,9,10,31,34,35] Our results show Sp1 transcription factor promotes *TMEPAI* gene expression and contribute to cell proliferation. Cell proliferation plays important roles in the process of tumorigenesis. It has been reported that the overexpression of TMEPAI can increase cell proliferation and migration of A549 cells.[36] In this study, we investigated the role of Sp1 in regulating TMEPAI-induced proliferation of lung cancer cells and found that Sp1 indeed promotes A549 cell proliferation. Further study will focus on the cross-talk among Sp1 and p53, TGF-β signalling, and their respect to the TMEPAI expression in malignant transformation.

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CONFLICT OF INTEREST

None declared.

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