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Sp1-Induced SETDB1 Overexpression Transcriptionally Inhibits HPGD in a β -Catenin-Dependent Manner and Promotes the Proliferation and Metastasis of Gastric Cancer

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ABSTRACT


Purpose: Gastric cancer (GC) has high morbidity and mortality, the cure rate of surgical treatment and drug chemotherapy is not ideal. Therefore, development of new treatment strategies is necessary. We aimed to identify the mechanism underlying Sp1 regulation of GC progression.

Methods and Methods: The levels of Sp1, β -catenin, SET domain bifurcated 1 (SETDB1), and 15-hydroxyprostaglandin dehydrogenase (HPGD) were detected by quantitative reverse transcription polymerase chain reaction and western blot analysis. The targets of SETDB1 were predicted by AnimalTFDB, and dual-luciferase reporter assay was used for confirming the combination of Sp1, β -catenin, and SETDB1. HGC27 or AGS cells (1×10^6 cells/mouse) were injected into mice via the caudal vein for GC model establishment. The level of Ki67 was detected using immunohistochemistry, and hematoxylin and eosin staining was performed for evaluating tumor metastasis in mice with GC.

Results: HPGD was inhibited, while the protein levels of Sp1, β -catenin, and SETDB1 were up-regulated in GC tissues and cell lines. HPGD overexpression or SETDB1 silencing inhibited the proliferation, invasion, and migration of GC cells, and Sp1 regulated the proliferation, invasion, and migration of GC cells in a β -catenin-dependent manner. Furthermore, HPGD served as a target of SETDB1, and it was negatively regulated by SETDB1; additionally, Sp1 and β -catenin bound to the SETDB1 promoter and negatively regulated HPGD expression. We proved that Sp1 regulated GC progression via the SETDB1/HPGD axis.

Conclusions: Our findings revealed that Sp1 transcriptionally inhibited HPGD via SETDB1 in a β -catenin-dependent manner and promoted the proliferation and metastasis of GC cells.

Keywords: Gastric carcinoma; β -Catenin; 15-Hydroxyprostaglandin dehydrogenase; Sp1 transcription factor; SET domain bifurcated 1

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Conflict of Interest

No potential conflict of interest relevant to this article was reported.

Author Contributions

Conceptualization: F.Y., Y.L., ¹L.L., ²L.L.; Data curation: R.Y.; Formal analysis: W.Y.; Funding acquisition: ¹L.L., ²L.L.; Investigation: F.Y., Y.L.; Methodology: F.Y., Y.L.; Project administration: ¹L.L., ²L.L.; Resources: R.Y.; Software: W.Y.; Supervision: ¹L.L., ²L.L.; Validation: F.Y., Y.L.; Visualization: F.Y., Y.L.; Writing - original draft: F.Y., Y.L., ¹L.L., ²L.L.; Writing - review & editing: ¹L.L., ²L.L.
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INTRODUCTION

Gastric cancer (GC) is one of the most common cancers with high morbidity. At present, surgical resection and extra-operative chemotherapy are the main treatment methods for GC [1]. With medical advancements, GC treatment has shown improvement. However, the mortality rate of a large number of patients with advanced GC remains high [2]. Therefore, the search for new biological targets would be helpful for developing effective treatment strategies for GC. 15-Hydroxyprostaglandin dehydrogenase (HPGD) is a degradation enzyme involved in prostaglandin biosynthesis, and it was first detected in colorectal cancer (CC) [3]. HPGD has been reported to be down-regulated in CC; this may reduce the metastatic potential of the tumor through activation of matrix metalloproteinase-2 (MMP-2) [4]. At present, HPGD has been proved to be a tumor-inhibiting factor [5]. Wolf et al. [6] has shown that HPGD plays an anti-tumor role by regulating the estrogen receptor (ER), which can be used as a molecular target for the prevention and treatment of breast cancer. The mechanism of HPGD in GC has also been preliminarily reported. Thiel et al. [7] indicated that HPGD is decreased in GC, which accelerates GC progression. Further research shows that HPGD exerts its anti-tumor effect by arresting the cell cycle and inducing GC cell apoptosis [8]. Studies by Song et al. [9] suggest that the proliferation of GC cells is inhibited by HPGD overexpression. These reports indicate that, as an inhibitor of GC, HPGD can be used as a target for the prevention and treatment of this disease. Consistently, our previous studies have shown that HPGD expression is low or absent in GC tissues and that HPGD overexpression significantly inhibits the proliferation and angiogenesis of GC cells. Previous reports have indicated that the HPGD promoter region contains Sp1-binding sites [10]. Our preliminary experiments have shown that inhibition of Sp1 can upregulate the expression of HPGD. Thus, a negative correlation was found between the level of Sp1 and HPGD in GC. However, it is unclear whether HPGD participates in the regulation of GC progression by regulating Sp1.

Sp1 is a transcription factor that participates in the regulation of cell growth, differentiation, apoptosis, and canceration by activating the transcription of cytokines [11]. Besides, Sp1 has been widely reported to regulate cancer progression by regulating target genes. In ovarian cancer, Sp1 inhibits tumor cell migration by negatively regulating miR-335 [12]. In cervical cancer, Sp1 exerts an anti-tumor effect by targeting CDK1 to arrest the cell cycle [13]. Sp1 plays a key role in lung cancer progression by regulating transcription of PDSS2 [14]. Particularly, Sp1 is up-regulated in GC tissues and targeted by miR-22 to inhibit GC cell migration and invasion [15]. We used the AnimalTFDB software (<http://bioinfo.life.hust.edu.cn/AnimalTFDB/#/>) to predict that Sp1 bound to the SET domain bifurcated 1 (SETDB1) promoter region. However, it is unclear whether Sp1 affects HPGD expression by regulating SETDB1 in GC.

SETDB1 is a histone H3K9 methyltransferase that is regulated in various tumors and in cancer development [16]. SETDB1 overexpression is closely related to tumorigenesis. For example, Shang et al. [17] reported that SETDB1 is highly expressed in GC, promoting the occurrence and metastasis of GC by up-regulating CCND1 and MMP-9 levels [18]. Moreover, SETDB1 has been confirmed to promote the occurrence and metastasis of GC by regulating target genes [17]. Using the AnimalTFDB software (<http://bioinfo.life.hust.edu.cn/AnimalTFDB/#/>), we predicted that SETDB1 bound to the promoter region of HPGD. However, whether Sp1 up-regulates SETDB1 expression to transcriptionally suppress HPGD expression remains unclear. Furthermore, studies have shown that β -catenin up-regulates SETDB1 expression

after binding to TCF4 [19]. However, it is unclear whether Sp1 cooperates with β -catenin to up-regulate SETDB1 and transcriptionally inhibit HPGD expression.

The Wnt signaling cascade is a major regulatory factor in animal development, and abnormal Wnt pathway components are responsible for the occurrence of many diseases, especially cancer [20]. Increasing evidence shows that the abnormal regulation of Wnt/ β -catenin signal pathway contributes to the occurrence and progression of certain solid tumors and hematological malignant tumors [21]. Li et al. [22] showed that the Wnt/ β -catenin signal pathway is activated by c-Myb to promote the invasion and metastasis of breast cancer. In breast cancer, prodigiosin exerts its anti-tumor effect in advanced breast cancer by inhibiting the Wnt/ β -catenin signal pathway and reducing cyclin D1 expression [23]. The Wnt/ β -catenin signaling pathway has been confirmed to be involved in regulating GC progression [1]. For example, Tian et al. [24] showed that the Wnt/ β -catenin signal pathway is activated by SERPINH1 to promote epithelial-mesenchymal transition and GC metastasis. Research by Zhong et al. [25] indicated that lncRNAs accelerate GC progression by activating the Wnt/ β -catenin signal pathway. Furthermore, studies have shown that β -catenin transcriptionally suppresses HPGD expression [26]. Bioinformatics analysis indicates that β -catenin binds to the promoter region of SETDB1. The above studies have shown that the Wnt/ β -catenin signal pathway is involved in the regulation of GC progression; however, the specific mechanism underlying this process remains unclear.

In summary, previous findings suggest that Sp1 and SETDB1 are both highly expressed in GC, and overexpression of Sp1 and SETDB1 are closely related to the invasion and metastasis of GC. Inversely, HPGD expression is low or absent in GC, and HPGD overexpression significantly inhibits the proliferation and angiogenesis of GC cells. However, the potential mechanism by which they regulate GC progression has not been fully elucidated. We speculated that Sp1 cooperates with β -catenin to up-regulate SETDB1 and transcriptionally inhibit HPGD expression to promote the proliferation and metastasis of GC. In the present study, we aimed to identify new biomarkers for targeted treatment of GC.

MATERIALS AND METHODS

Patients and tissue specimen

From March 2020 to May 2021, after receiving the written informed consent, The First People's Hospital of Yunnan Province collected the information of 10 patients with GC (35–50 years old, including 5 males and 5 females) and surgical tumor samples and adjacent non-tumor tissues. The 10 selected patients did not receive chemotherapy and/or radiotherapy. The samples were immediately frozen in liquid nitrogen for verification. The Ethics Committee of the First People's Hospital of Yunnan Province approved this study, and the written informed consent of all patients was obtained. All the experiments were performed according to approved guidelines.

Cell lines and culture

GC cell lines including AGS, NCI-N87 and SNU-1 cell lines were obtained from American Type Culture Collection (Manassas, VA, USA); MKN45, MKN74, and MKN7, the JCRB Cell Bank (National Institute of Hygienic Sciences, Tokyo, Japan); HGC27 cells, the Cell Bank of the Shanghai Institute for Biological Sciences (Chinese Academy of Sciences, Shanghai, China); and the normal gastric epithelial cell line GES-1, Fengbio (Changsha, China).

The cells were cultivated in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) and incubated in a humid incubator at 37°C and under 5% CO₂.

Cell transfection

We used the following plasmids, which were synthesized by GenePharma (Shanghai, China): pcDNA3.1-HPGD, pcDNA3.1-Sp1, sh-Sp1, sh-SETDB1, sh- β -catenin, and the negative control (NC) plasmids pcDNA3.1-NC and sh-NC. GC cells including HGC27 and AGS cells were inoculated in a 6-well cell culture plate and incubated overnight in a serum-free medium. On the second day, after washing the cells with PBS, Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) was applied for transfecting the cells with the plasmids and their corresponding NCs. GC cells were used in subsequent experiments 24 hours following transfection.

Western blot

Total cell lysates were prepared with RIPA lysate buffer (Beyotime, Nanjing, China). The protein was isolated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and then transferred to polyvinylidene fluoride (PVDF) membranes. TBST and 5% skimmed milk powder were used for sealing the PVDF membrane. Next, the PVDF membrane was incubated overnight with specific antibodies (1:1,000; Abcam, Cambridge, UK), including rabbit anti-Sp1, anti- β -catenin, anti-SETDB1, and anti-HPGD at 4°C. On the second day, a secondary anti-rabbit immunoglobulin G (IgG) antibody coupled with horseradish peroxidase was incubated at room temperature for 2 hours, and the bands were detected by immobilized Western chemiluminescence instrument. β -Actin was used as the endogenous control. Finally, the ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used for quantifying the strength of the bands obtained from western blot analysis.

Detection of cell viability, proliferation, migration, and invasion

The pcDNA3.1-Sp1, sh-SETDB1, and pcDNA3.1-HPGD plasmids and the corresponding negativecontrol plasmids pcDNA3.1-NC and sh-NC were co-transfected into HGC27 and AGS cells for Sp1 and HPGD overexpression and SETDB1 inhibition. Subsequently, CCK-8 assay, colony-formation assay, and Transwell assay were performed for evaluating the viability, proliferation, migration, and invasion, respectively, of HGC27 and AGS cells, in accordance with previous reports [27,28].

SETDB1 target selection and dual-luciferase reporter assay

First, the AnimalTFDB bioinformatics software (<http://bioinfo.life.hust.edu.cn/AnimalTFDB/#/>) was applied for predicting the potential binding sites of SETDB1. The binding site between HPGD and SETDB1 was identified, and the physical interaction between HPGD and SETDB1 was confirmed by a dual-luciferase reporter assay. MUT HPGD or WT HPGD was subcloned to pGL3 luciferase reporter vectors (Promega, Madison, WI, USA) for constructing HPGD-WT and HPGD-MUT. Subsequently, GC cells were co-transfected with HPGD-WT, HPGD-MUT, sh-SETDB1, and simulated NC using Lipofectamine™ 3000 (Takara, Dalian, China). Luciferase activity was detected 48 hours following transfection.

Co-IP assay

The corresponding plasmids were transfected into HGC27 and MKN28 cells; subsequently, the sample lysates were added to 1.5 mL centrifuge tubes containing magnetic beads with RNase, IgG or Ago2 antibodies and incubated overnight at 4°C. Later, the proteins were washed with Co-IP cleaning solution. Finally, Sp1 and β -catenin protein levels were detected by western blot analysis.

Table 1. Primer sequences

Primer name	(5'-3') Primer sequences
F-Sp1	5'-GCGAGAGGCCATTATGTGTAC-3'
R-Sp1	5'-CAAATTTCTCTCACCTGTGTGT-3'
F-β-actin	5'-TACATGGCTGGGGTGTGAA-3'
R-β-actin	5'-AAGAGAGGCATCCTACCCT-3'

ChIP-reChIP assay

Auto iDeal ChIP-seq kit for Histones (C01010171; Diagenode, Seraing, Belgium) was used for extracting chromatin from GC cells, in accordance with previous reports [29]. Subsequently, the extracted chromatin was lysed using a Bioruptor™ sonicator at 4°C. We used 20 µg of anti-Sp1 and non-immune rabbit IgG as the NC. ChIP was performed using the SX-8X IP-Star Compact Automated System, in accordance with previous reports [30].

Re-ChIP assay was performed by immunoprecipitation of deoxyribonucleic acid, which was used in the first ChIP detection. Subsequently, 20 µg of anti-β-catenin and non-immune rabbit IgG were used as the NC. Finally, the immunoprecipitated DNA and total DNA (input) were purified on a MicroChip DiaPure column (C03040001) and analyzed using real-time quantitative polymerase chain reaction (PCR), in accordance with previous reports [5].

Quantitative reverse transcription PCR (qRT-PCR)

After total RNA was extracted with TRIzol reagent, Sp1 gene level was detected using a one-step SYBR Prime Script Plus RT-PCR kit. Data were analyzed using β-actin as the endogenous control. The crease change was calculated using the $2^{-\Delta\Delta Ct}$ method. The entire process was repeated 3 times. The primers used in this study are shown in **Table 1**.

Animal model

Eight-week-old BALB/c mice were purchased from Hunan slake Jingda company (Changsha, China). The mice were kept in standard cages with 4 mice per cage at approximately 25°C and 60% humidity and under a 12-hour light-dark cycle; adequate food and water were provided every day. Subsequently, the mice were randomly divided into 6 groups: NC group, 0.2 mL PBS was injected under the subcutaneous or caudal vein of each mouse; Sp1 group, 5×10^6 Sp1-overexpressing HGC27 and MKN28 cells were suspended in 0.2 mL and injected under the subcutaneous or caudal vein of each mouse; sh-SETDB1 group, 5×10^6 SETDB1-silenced HGC27 and MKN28 cells were suspended in 0.2 mL and injected under the subcutaneous or caudal vein of each mouse; Sp1+sh-SETDB1 group, 5×10^6 Sp1-overexpressing and SETDB1-silenced HGC27 and MKN28 cells were suspended in 0.2 mL and injected under the subcutaneous or caudal vein of each mouse; HPGD group, 5×10^6 HPGD-overexpressing HGC27 and MKN28 cells were suspended in 0.2 mL and injected under the subcutaneous or caudal vein of each mouse; Sp1+HPGD group, 5×10^6 Sp1- and HPGD-overexpressing HGC27 and MKN28 cells were suspended in 0.2 mL and injected under the subcutaneous or caudal vein of each mouse. Tumor growth was monitored once a week. Mice were sacrificed 30 days after injection, tumor specimens were weighed, and immunohistochemistry (IHC) and hematoxylin and eosin (H&E) staining was performed after fixation.

IHC

First, the tumor tissue was cut into 4 µm thick sections, fixed with formalin, and embedded in paraffin. The sections were rehydrated in graded ethanol series and treated with 3% hydrogen peroxide in methanol for 10 minutes to inhibit endogenous peroxidase, followed by antigen repair with microwave for 10 minutes. Sections were incubated with normal goat

serum at 37°C for 30 minutes and then incubated with rabbit anti-Ki67 antibody (1:500; Abcam) at 37°C for 1 hour. Subsequently, the sections were incubated with 1:100 dilution of biotin-labeled sheep anti-mouse immunoglobulin secondary antibody at 37°C for 1 hour. Finally, color was developed using 3,3-diaminobenzidine tetrachloride (Abcam).

H&E staining

H&E staining was used for evaluating lung tissue injury in mice. In brief, the GC tissues were cut into 5 µm thick blocks and fixed overnight with 10% neutral buffer formalin at 4°C. The next day, the tissue sections were dehydrated, washed, and embedded in paraffin with graded ethanol series. Subsequently, H&E staining was performed for 10 minutes, and eosin staining was performed for 5 minutes. Finally, the bone tissue sections were imaged using an optical microscope (BD Pharmingen, San Diego, CA, USA).

Statistical analysis

The data from 3 repeated experiments were expressed as the mean±standard deviation. Student t-test or analysis of variance was used for determining the differences among groups, and the random post-Bonferroni test was performed. $P < 0.05$ was considered to be statistically significant.

RESULTS

The levels of HPGD, Sp1, β-catenin, and SETDB1 genes in GC tissues and cell lines

We analyzed surgical tumor samples and adjacent non-tumor tissues of 10 patients with GC (clinicopathological features of 10 patients with gastric cancer were shown in **Table 2**). Western blot and IHC analysis indicated that HPGD was significantly inhibited in all 4 tumor tissues compared with that in matched non-tumor tissues. In contrast, the levels of Sp1, β-catenin, and SETDB1 proteins were significantly increased in the 4 tumor tissues compared with that in the non-tumor tissues (**Fig. 1A**, **Supplementary Fig. 1**). Additionally, the results of analysis in GC cell lines were consistent (**Fig. 1B**). Because HPGD expression was the lowest in the HGC27 and MKN28 cells, we selected HGC27 and MKN28 cells for follow-up

Table 2. Clinicopathological features of 10 patients with gastric cancer

Characteristics	Cases
All cases	10
Age (yr)	
≤60	4
>60	6
Sex	
Male	3
Female	7
T stage	
T1–T2	2
T3–T4	8
N stage	
N0–N1	5
N2–N3	5
M stage	
M0	7
M1	3
Tumor size (cm)	
≤5	6
>5	4

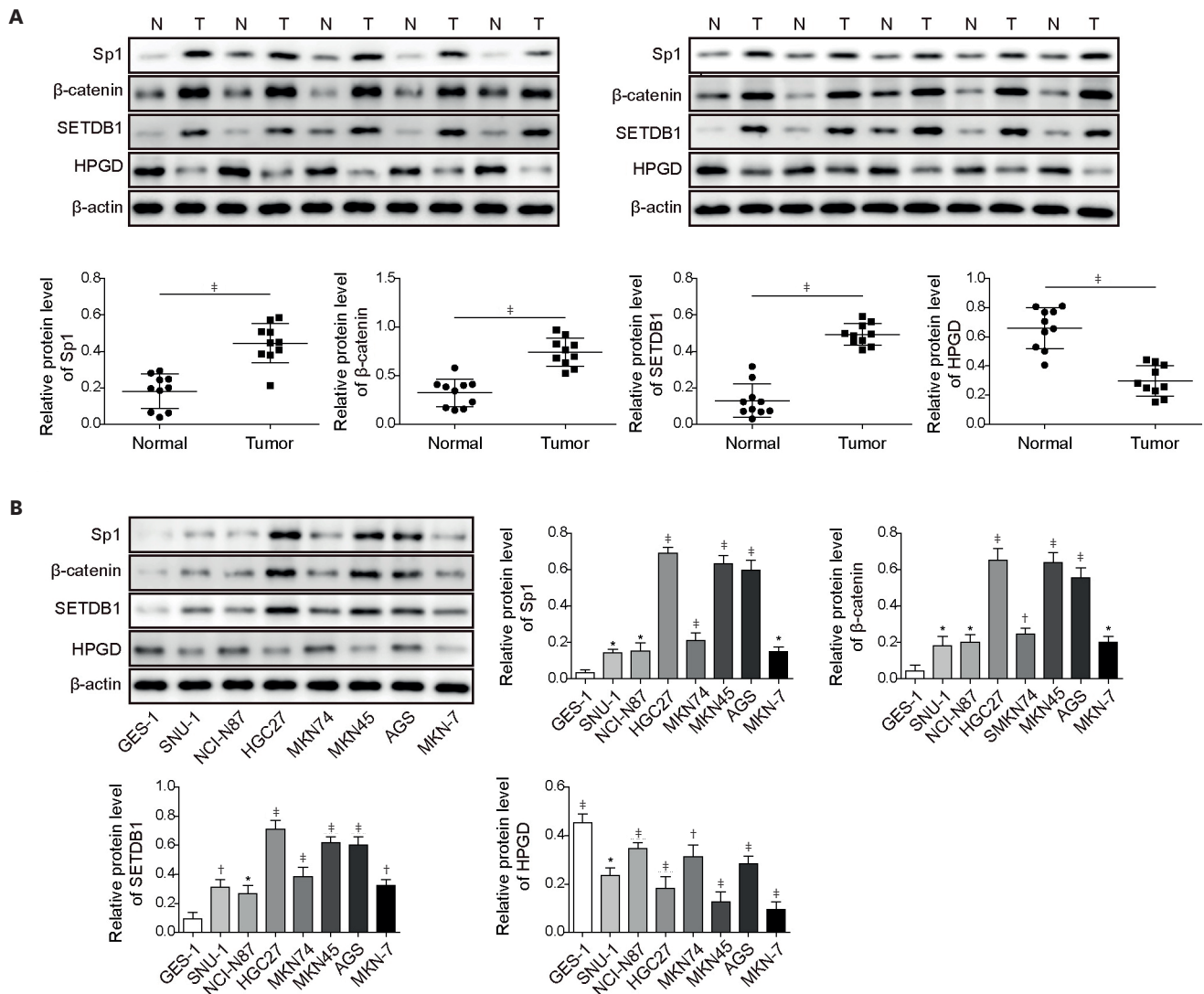


Fig. 1. The gene expression levels of HPGD, Sp1, β-catenin, and SETDB1 in GC tissues and cell lines. Surgical tumor samples and adjacent non-tumor tissues were collected from 10 patients with GC, and total protein was extracted from the patients' tissues and GC cell lines, including AGS, HGC27, NCI-N87, MKN74, MKN45, SNU-1, and MKN7 cells. GES-1 cells served as the negative control. (A) Western blot showed that HPGD was significantly inhibited in all 4 tumor tissues compared with that in the matched non-tumor tissues, and the levels of Sp1, β-catenin, and SETDB1 proteins were significantly increased in the 4 tumor tissues compared with that in the non-tumor tissues. (B) Western blot indicated that HPGD was significantly inhibited in GC cell lines compared with that in GES-1 cells. In contrast, the levels of Sp1, β-catenin, and SETDB1 proteins were significantly increased in GC cell lines compared with that in GES-1 cells. HPGD = 15-hydroxyprostaglandin dehydrogenase; SETDB1 = SET domain bifurcated 1; GC = gastric cancer. *P<0.05; †P<0.01; ‡P<0.001.

functional studies. Moreover, the fragments per kilobase million values of Sp1, β-catenin, SETDB1, and HPGD were examined on the basis of the StarBase (TCGA) database; the data demonstrated that Sp1, β-catenin, and SETDB1 were highly expressed and that HPGD showed low expression, consistent with the results of our study (**Supplementary Fig. 2A**). Besides, we analyzed the correlation between the HPGD, SETDB1, and Sp1 levels and the overall survival rate of patients with GC, using the TCGA database. The results showed that the Sp1 level was negatively correlated with the survival rate of patients with GC (**Supplementary Fig. 2B**). Consistently, the SETDB1 level was negatively correlated with the survival rate of patients with GC (**Supplementary Fig. 2B**). Inversely, the HPGD level was positively correlated with the survival rate of patients with GC (**Supplementary Fig. 2B**).

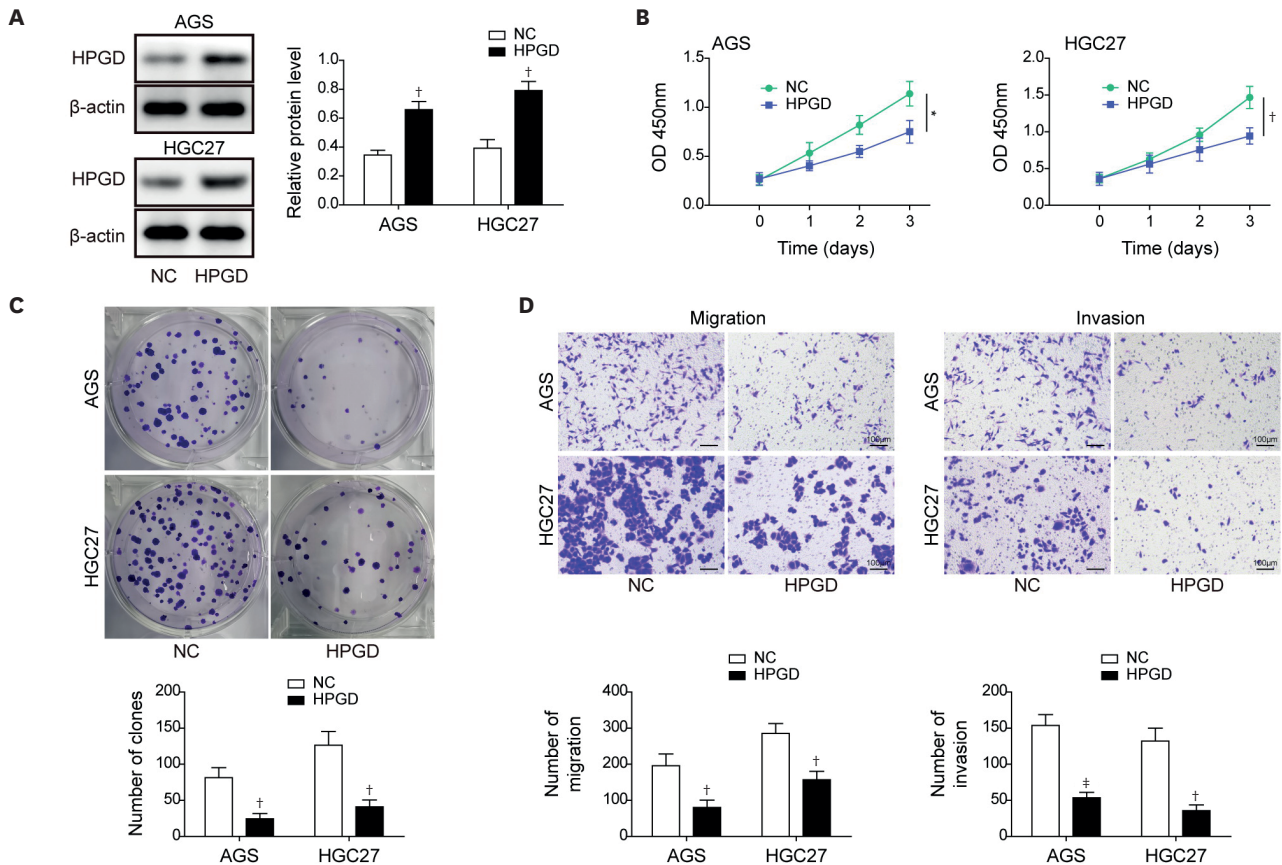


Fig. 2. HPGD overexpression inhibited the proliferation, invasion, and migration of GC cells. pcDNA3.1-HPGD and its NC pcDNA3.1-NC were transfected into HGC27 and AGS cells for inducing HPGD overexpression. (A) Western blot analysis indicated that compared with the control cells, cells transfected with the pcDNA3.1-HPGD plasmid showed significant overexpression of HPGD. (B-D) CCK-8 assay, colony-formation assay, and Transwell assay analysis showed that HPGD-overexpressing HGC27 and MKN28 cells showed significant inhibition of proliferation, migration, and invasion compared with the control cells. HPGD = 15-hydroxyprostaglandin dehydrogenase; GC = gastric cancer; NC = negative control; SETDB1 = SET domain bifurcated 1. * $P < 0.05$; [†] $P < 0.01$; [‡] $P < 0.001$.

HPGD overexpression inhibited the proliferation, invasion, and migration of GC cells

To explore the role of HPGD and its mechanism in GC progression, pcDNA3.1-HPGD plasmid (pcDNA3.1-HPGD NC as the NC) was transfected into HGC27 and MKN28 cells for HPGD overexpression. Western blot analysis indicated that compared with the control cells, the cells transfected with pcDNA3.1-HPGD plasmid showed significant overexpression of HPGD (Fig. 2A). Subsequently, cell proliferation, migration, and invasion were significantly inhibited in HPGD-overexpressing HGC27 and MKN28 cells, compared with the control cells (Fig. 2B-D).

SETDB1 silencing inhibited the proliferation, invasion, and migration of GC cells

To investigate the specific mechanism through which SETDB1 regulates GC progression, sh-SETDB1-1, sh-SETDB1-2, and their NC plasmids sh-NC were transfected into HGC27 and MKN28 cells for SETDB1 silencing. Compared with the control cells, the cells transfected with sh-SETDB1-1 and sh-SETDB1-2 plasmids showed a significantly lower expression of SETDB1 (Fig. 3A). Subsequently, the results of HGC27 and MKN28 cell bioactivity analysis showed that proliferation, migration, and invasion were significantly inhibited by SETDB1

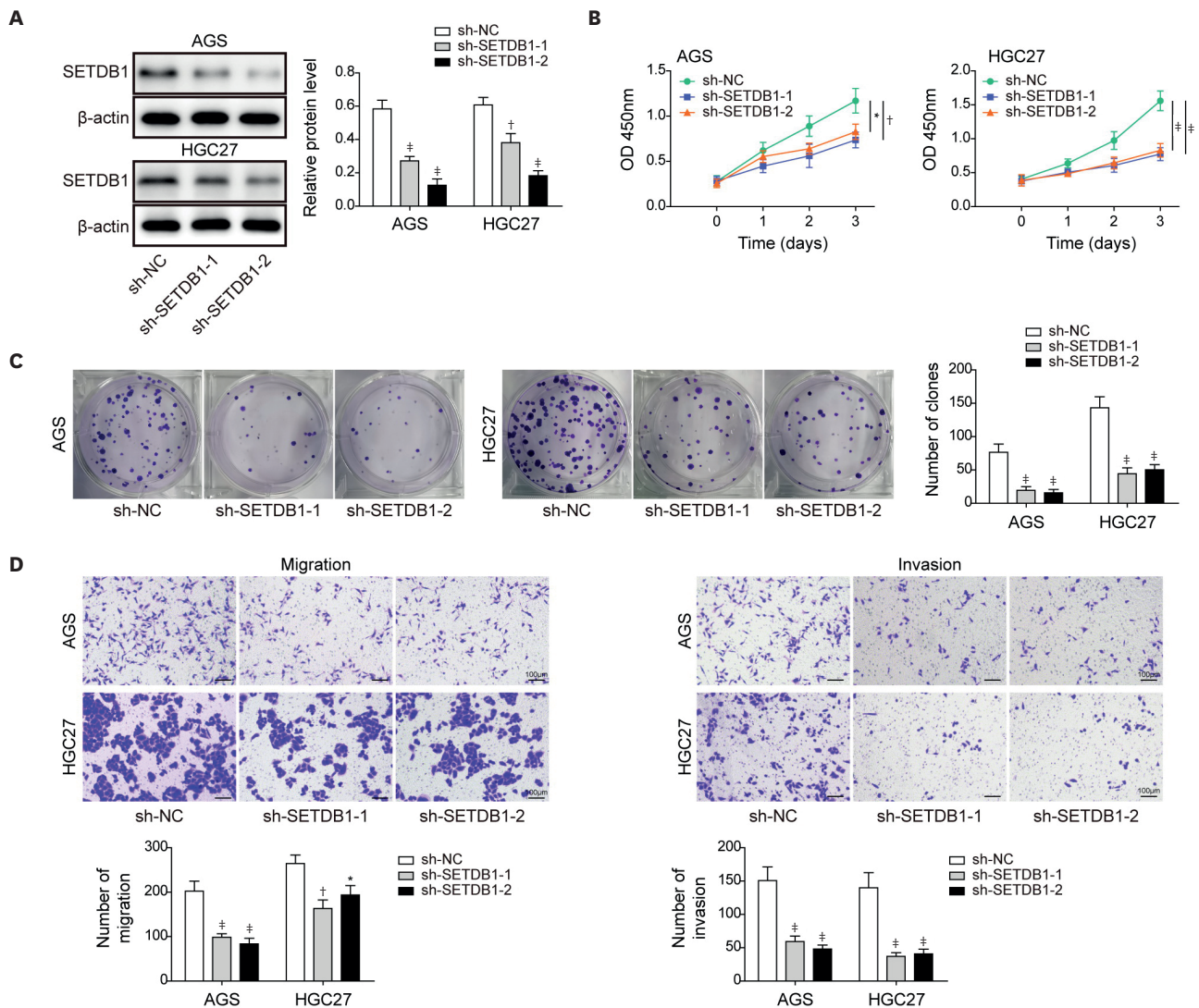


Fig. 3. SETDB1 silencing inhibited the proliferation, invasion, and migration of GC cells. sh-SETDB1-1, sh-SETDB1-2, and their NC plasmid sh-NC were transfected into HGC27 and AGS cells for SETDB1 silencing. (A) Western blot analysis showed that compared with the control cells, cells transfected with the sh-SETDB1-1 and sh-SETDB1-2 plasmids showed significantly lower SETDB1 expression. (B-D) CCK-8 assay, colony formation assay and Transwell assay analysis indicated that HGC27 and MKN28 cell proliferation, migration, and invasion were significantly inhibited in cells subjected to SETDB1 silencing compared with that in the control cells. HPGD = 15-hydroxyprostaglandin dehydrogenase; GC = gastric cancer; NC = negative control.

* $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$.

silencing in HGC27 and MKN28 cells, compared with that in the control cells (**Fig. 3B-D**), indicating that SETDB1 plays a positive role in GC progression.

HPGD served as a target for SETDB1, and it was negatively regulated by SETDB1

To further explore the interaction between SETDB1 and tumor suppressor gene HPGD in GC, sh-SETDB1-1 and its NC plasmids sh-NC were transfected into HGC27 and MKN28 cells for SETDB1 silencing. Compared with that in the control cells, SETDB1 expression was significantly inhibited and HPGD expression was significantly increased in the cells transfected with sh-SETDB1-1 plasmid (**Fig. 4A**). The AnimalTFDB software was applied for predicting the potential targets of SETDB1; 3 binding sites (BS1, BS2, and BS3) between

SETDB1 and HPGD promoter were found (Fig. 4B). Subsequently, ChIP assay analysis showed that physical interaction existed between SETDB1 and HPGD promoter only in BS3 (Fig. 4C). Dual-luciferase reporter assays further verified the interaction between SETDB1 and HPGD promoter. The luciferase activity of HPGD-WT reported gene was increased by co-transfection with sh-SETDB1; however, co-transfection with sh-SETDB1 did not affect the luciferase activity of HPGD-MUT reported gene (Fig. 4D). Moreover, we analyzed the correlation between SETDB1 and HPGD in GC using the StarBase (TCGA) database. Results showed a negative correlation between the levels of SETDB1 and HPGD in GC (Supplementary Fig. 2C).

Sp1 regulated the proliferation, invasion, and migration of GC cells in a β -catenin-dependent manner

To further explore whether Sp1 regulates GC progression via the Wnt/ β -catenin signal pathway, pcDNA3.1-Sp1 and its NC plasmid pcDNA3.1-NC were transfected into HGC27 and MKN28 cells for Sp1 overexpression, and Sp1-overexpressing HGC27 and MKN28 cells were treated with JW 55, a β -catenin inhibitor, for 24 hours. Western blot analysis indicated that JW 55 treatment had no effect on Sp1 expression; Sp1 overexpression rescued JW 55-inhibited β -catenin expression. Consistently, JW 55-inhibited SETDB1 expression was rescued by Sp1 overexpression. Sp1 overexpression inhibited HPGD expression, whereas JW 55 increased HPGD expression, and Sp1 overexpression inhibited the effect of JW 55 on HPGD (Fig. 5A). Further functional research showed that Sp1 overexpression accelerated HGC27 and MKN28 cell viability, proliferation, migration, and invasion; however, HGC27 and MKN28 cell viability, proliferation, migration, and invasion were inhibited by JW 55 treatment, and Sp1 overexpression alleviated the effect of JW 55 on HGC27 and MKN28 cells (Fig. 5B-D).

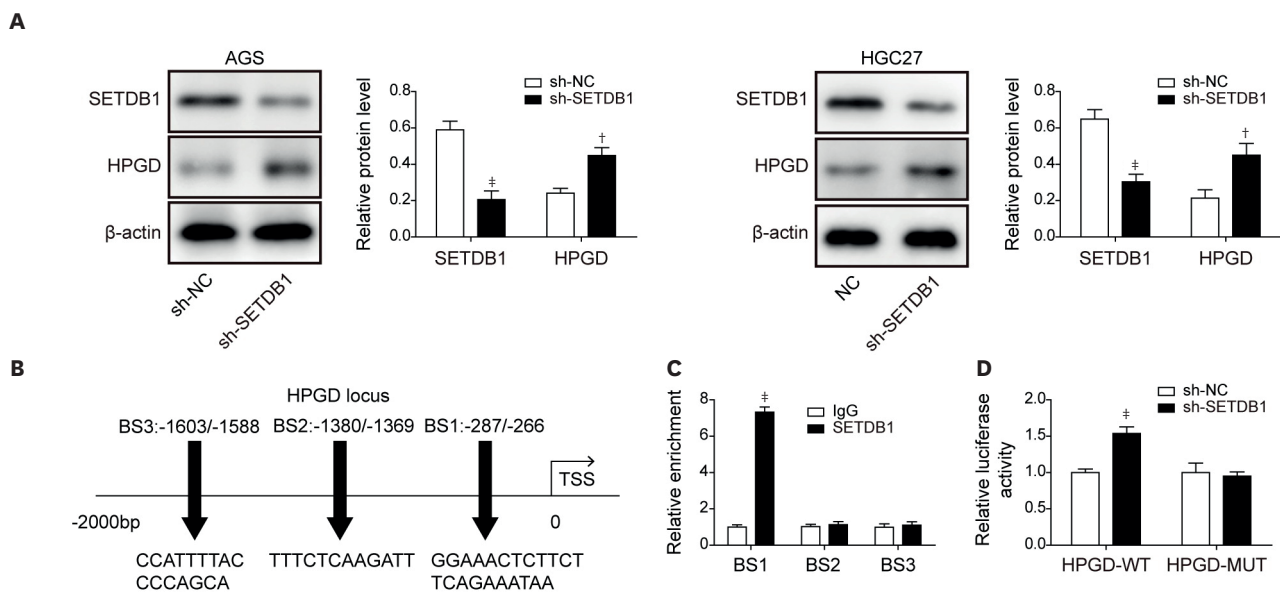


Fig. 4. HPGD served as a target for SETDB1, and it was negatively regulated by SETDB1. sh-SETDB1-1 and its NC plasmid sh-NC were transfected into HGC27 and AGS cells for SETDB1 silencing. (A) Western blot analysis indicated that compared with the control cells, cells transfected with the sh-SETDB1-1 plasmid showed significant inhibition of SETDB1 expression and a significant increase in HPGD expression. (B) Three binding sites (BS1, BS2, BS3) between SETDB1 and HPGD promoter were identified using the AnimalTFDB software. (C, D) The direct binding relationship between SETDB1 and HPGD promoter was confirmed using ChIP and dual-luciferase reporter assays.

HPGD = 15-hydroxyprostaglandin dehydrogenase; SETDB1 = SET domain bifurcated 1; GC = gastric cancer; NC = negative control.

* $P < 0.01$; * $P < 0.001$.

Sp1 and β -catenin bound to the SETDB1 promoter and negatively regulated HPGD expression

To further explore the roles of Sp1 and β -catenin in GC progression and their specific mechanisms, Co-IP assay was performed for evaluating the relationship between Sp1 and β -catenin in GC cells. The results indicated physical interaction between Sp1 and β -catenin in the HGC27 and MKN28 nuclei (**Fig. 6A**); Sp1 and β -catenin were co-localized in the HGC27 and MKN28 nucleus (**Fig. 6B**). Subsequently, sh- β -catenin and its NC plasmid sh-NC were transfected into HGC27 and MKN28 cells for silencing β -catenin. Western blot analysis indicated that compared with that in the control cells, β -catenin expression was significantly inhibited in HGC27 and MKN28 cells transfected with sh- β -catenin plasmid. β -catenin silencing significantly inhibited the levels of Sp1 and SETDB1 proteins but significantly up-regulated HPGD expression (**Fig. 6C**). qRT-PCR analysis further revealed that β -catenin silencing had no effect on Sp1 mRNA level (**Fig. 6D**), indicating that β -catenin only affected the protein level of Sp1. Confocal microscopy showed that after silencing β -catenin in the HGC27 and MKN28 nucleus, the fluorescence intensity of β -catenin and Sp1 both decreased (**Fig. 6E**). Furthermore, sh-Sp1 and its NC plasmids sh-NC were transfected into HGC27 and MKN28 cells for Sp1 silencing. After transfection of sh-Sp1 plasmid, Sp1 expression was significantly inhibited, compared with that in the control cells (**Fig. 6F**). Sp1 silencing significantly inhibited the levels of β -catenin and SETDB1 but significantly increased HPGD expression. Additionally, the correlation between β -catenin and HPGD in GC was analyzed using the StarBase (TCGA) database. The findings indicated a negative correlation between the levels of β -catenin and HPGD in GC (**Supplementary Fig. 2C**). Confocal microscopy showed that after silencing Sp1 in the HGC27 and MKN28 nuclei, the fluorescence intensity of β -catenin and Sp1 both decreased (**Fig. 6G**). Consistently, ChIP-reChIP assay analysis indicated that Sp1 and β -catenin bound to the SETDB1 promoter (**Fig. 6H**). These findings suggest that Sp1 and β -catenin bound to the SETDB1 promoter and negatively regulated HPGD expression.

Sp1 regulated GC progression via SETDB1/HPGD axis

Finally, to determine whether Sp1 regulates GC progression via the SETDB1/HPGD axis, we transfected the HGC27 and MKN28 cells with pcDNA3.1-Sp1, pcDNA3.1-HPGD, and their NC pcDNA3.1-NC for Sp1 and HPGD overexpression and sh-SETDB1 and its NC sh-NC for SETDB1 silencing. Western blot analysis showed that SETDB1 expression inhibited by sh-SETDB1 plasmid transfection was rescued by Sp1 overexpression, and HPGD overexpression further increased SETDB1 expression. Inversely, HPGD expression up-regulated by SETDB1 silencing was antagonized by Sp1 overexpression, and HPGD overexpression partly alleviated the effect of Sp1 overexpression on HPGD (**Fig. 7A**). Subsequently, the analysis of GC cell bioactivity indicated that Sp1 overexpression significantly accelerated HGC27 and MKN28 cell viability, proliferation, migration, and invasion; however, SETDB1 silencing or HPGD overexpression inhibited HGC27 and MKN28 cell viability, proliferation, migration, and invasion and partly reversed the effect of Sp1 overexpression on HGC27 and MKN28 cell bioactivity (**Fig. 7B-7D**). Statistical analysis showed that Sp1 overexpression promoted tumor enlargement and aggravation, while SETDB1 silencing or HPGD overexpression led to tumor reduction and lightening and partially reversed the trend of Sp1 overexpression (**Fig. 7F-H**). In the tumor tissue, HPGD overexpression or SETDB1 silencing partly alleviated the effect of Sp1 overexpression on HPGD (**Fig. 7I**). Sp1 overexpression aggravated liver injury and tumor metastasis in mice with GC; however, SETDB1 silencing or HPGD overexpression alleviated liver injury and tumor metastasis and partially reversed the trend of Sp1 overexpression (**Fig. 7J and K**). These findings suggest that Sp1 regulated GC progression via the SETDB1/HPGD axis.

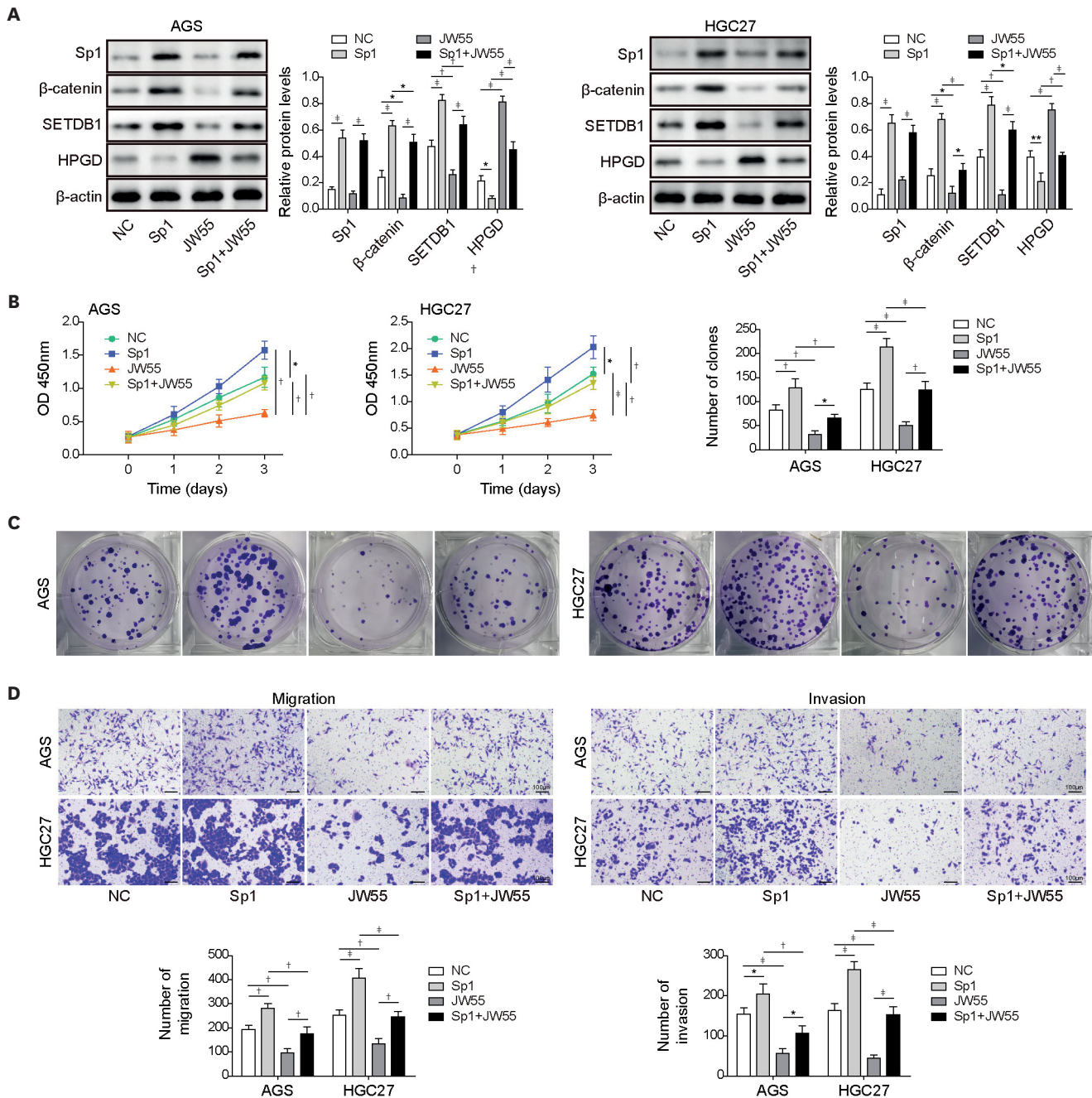


Fig. 5. Sp1 regulated the proliferation, invasion, and migration of GC cells in a β -catenin-dependent manner. pcDNA3.1-Sp1 and its NC plasmid pcDNA3.1-NC were transfected into HGC27 and AGS cells for inducing Sp1 overexpression, and the Sp1-overexpressing HGC27 and AGS cells were treated with JW 55, an inhibitor of β -catenin, for 24 hours. (A) Western blot analysis indicated that JW 55 treatment had no effect on Sp1 expression; Sp1 overexpression inhibited the expression levels of β -catenin and SETDB1 that were inhibited by JW 55. Sp1 overexpression inhibited HPGD expression, whereas JW 55 increased HPGD expression, and Sp1 overexpression produced an antagonistic effect on JW 55-induced HPGD expression. (B-D) CCK-8 assay, colony formation assay, and Transwell assay analysis indicated that HGC27 and MKN28 cell viability, proliferation, migration, and invasion were increased by Sp1 overexpression but inhibited by JW 55 treatment and that Sp1 overexpression alleviated the effect of JW 55 on HGC27 and MKN28 cells.

HPGD = 15-hydroxyprostaglandin dehydrogenase; SETDB1 = SET domain bifurcated 1; GC = gastric cancer; NC = negative control.

* $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$.

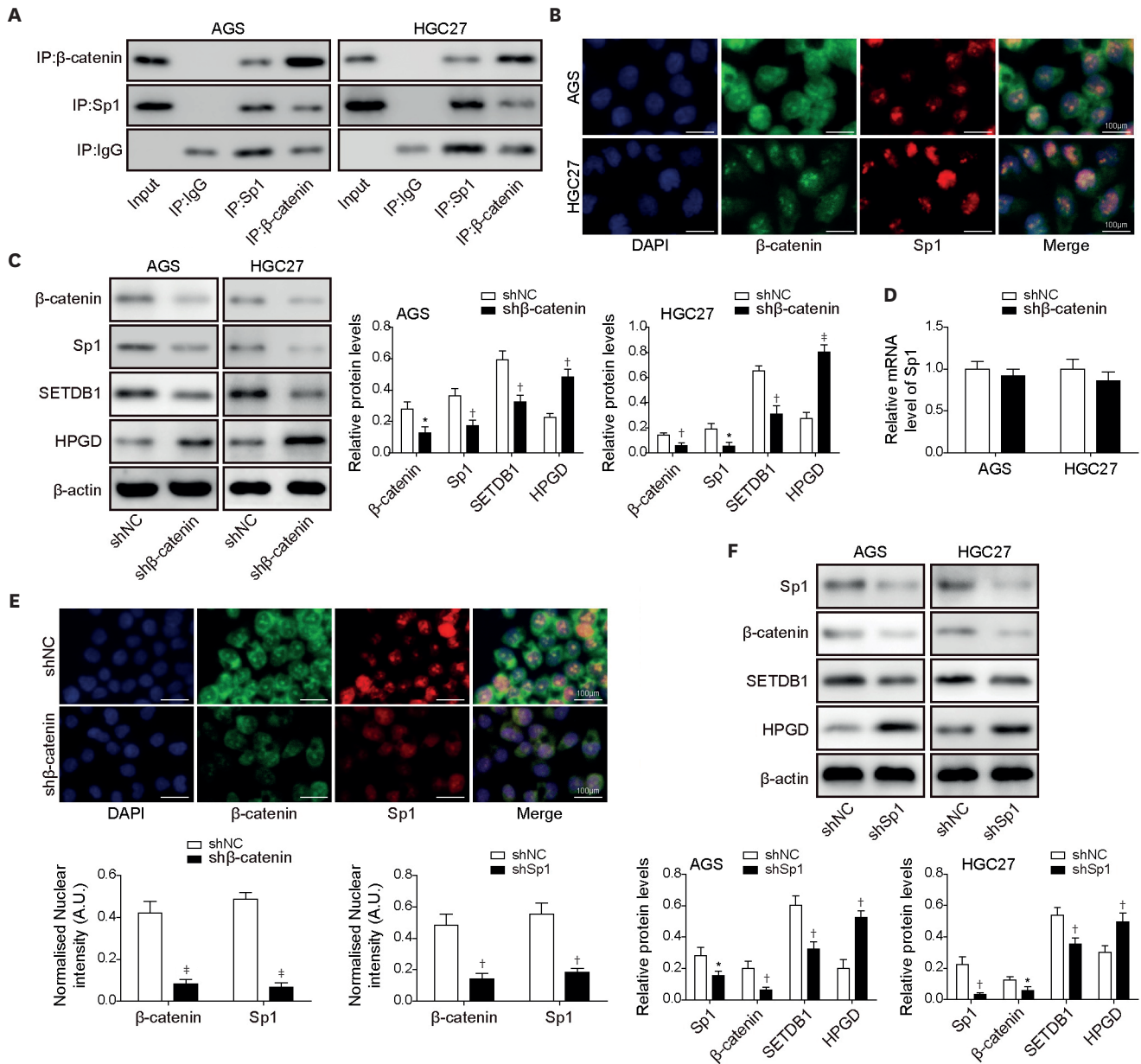


Fig. 6. Sp1 and β-catenin bound to the SETDB1 promoter and negatively regulated HPGD expression. (A, B) Co-IP assay and confocal microscope analysis showed a physical interaction between Sp1 and β-catenin in the HGC27 and MKN28 nuclei, and Sp1 and β-catenin were co-localized in the HGC27 and MKN28 nuclei. sh-β-catenin and its NC plasmid sh-NC were transfected into HGC27 and AGS cells for β-catenin silencing. (C) Western blot analysis indicated that compared with the control cells, HGC27 and MKN28 cells transfected with the sh-β-catenin plasmid showed significant inhibition of β-catenin expression. β-catenin silencing significantly inhibited the levels of Sp1 and SETDB1 proteins but significantly up-regulated HPGD expression. (D) qRT-PCR analysis furtherly revealed that β-catenin silencing had no effect on Sp1 mRNA levels. (E) Confocal microscopy showed that after silencing β-catenin in the HGC27 and MKN28 nuclei, the fluorescence intensity of β-catenin and Sp1 both decreased. sh-Sp1 and its NC plasmid sh-NC were transfected into HGC27 and AGS cells for Sp1 silencing. (F) Western blot analysis indicated that Sp1 expression in the cells transfected with the sh-Sp1 plasmid was significantly inhibited compared with the control cells. Sp1 silencing significantly inhibited the levels of β-catenin and SETDB1 but significantly increased HPGD expression. (G) Cells transfected with sh-Sp1 plasmid showed significant inhibition of Sp1 expression compared with the control cells. Sp1 silencing significantly inhibited the levels of β-catenin and SETDB1 but significantly increased HPGD expression. (H) ChIP-reChIP assay analysis indicated that Sp1 and β-catenin bound to the SETDB1 promoter. HPGD = 15-hydroxyprostaglandin dehydrogenase; SETDB1 = SET domain bifurcated 1; NC = negative control; qRT-PCR = quantitative reverse transcription polymerase chain reaction; IgG = immunoglobulin G; DAPI = 4',6-diamidino-2-phenylindole; A.U. = arbitrary unit.

*P<0.05, †P<0.01, and ‡P<0.001.

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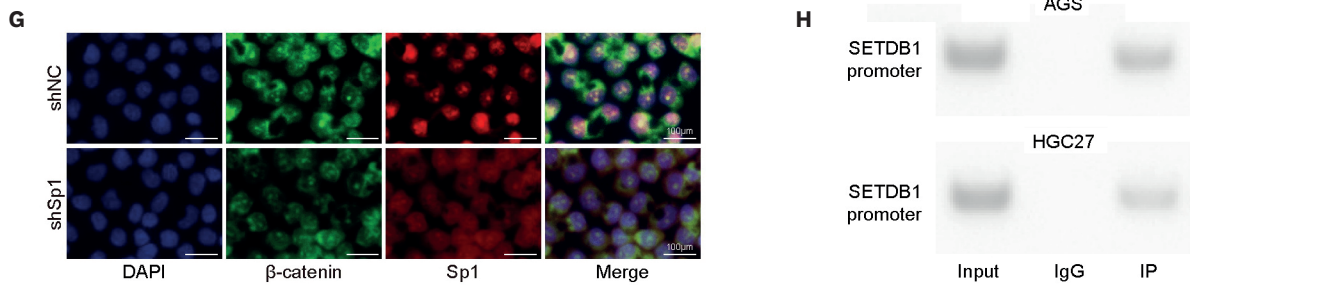


Fig. 6. (Continued) Sp1 and β -catenin bound to the SETDB1 promoter and negatively regulated HPGD expression. (A, B) Co-IP assay and confocal microscope analysis showed a physical interaction between Sp1 and β -catenin in the HGC27 and MKN28 nuclei, and Sp1 and β -catenin were co-localized in the HGC27 and MKN28 nuclei. sh- β -catenin and its NC plasmid sh-NC were transfected into HGC27 and AGS cells for β -catenin silencing. (C) Western blot analysis indicated that compared with the control cells, HGC27 and MKN28 cells transfected with the sh- β -catenin plasmid showed significant inhibition of β -catenin expression. β -catenin silencing significantly inhibited the levels of Sp1 and SETDB1 proteins but significantly up-regulated HPGD expression. (D) qRT-PCR analysis further revealed that β -catenin silencing had no effect on Sp1 mRNA levels. (E) Confocal microscopy showed that after silencing β -catenin in the HGC27 and MKN28 nuclei, the fluorescence intensity of β -catenin and Sp1 both decreased. sh-Sp1 and its NC plasmid sh-NC were transfected into HGC27 and AGS cells for Sp1 silencing. (F) Western blot analysis indicated that Sp1 expression in the cells transfected with the sh-Sp1 plasmid was significantly inhibited compared with the control cells. Sp1 silencing significantly inhibited the levels of β -catenin and SETDB1 but significantly increased HPGD expression. (G) Cells transfected with sh-Sp1 plasmid showed significant inhibition of Sp1 expression compared with the control cells. Sp1 silencing significantly inhibited the levels of β -catenin and SETDB1 but significantly increased HPGD expression. (H) ChIP-reChIP assay analysis indicated that Sp1 and β -catenin bound to the SETDB1 promoter. HPGD = 15-hydroxyprostaglandin dehydrogenase; SETDB1 = SET domain bifurcated 1; NC = negative control; qRT-PCR = quantitative reverse transcription polymerase chain reaction; IgG = immunoglobulin G; DAPI = 4',6-diamidino-2-phenylindole; A.U. = arbitrary unit. * $P < 0.05$, * $P < 0.01$, and * $P < 0.001$.

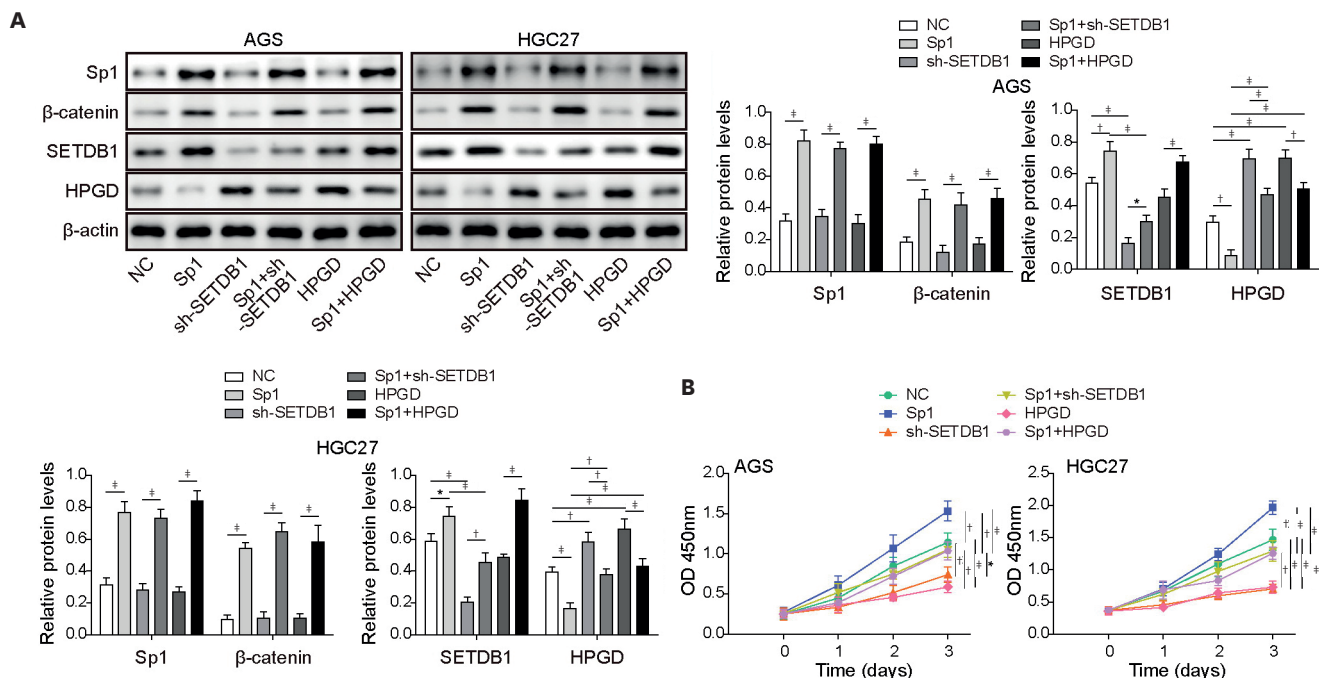


Fig. 7. Sp1 regulated GC progression via SETDB1/HPGD axis. HGC27 and AGS cells were transfected with pcDNA3.1-NC, pcDNA3.1-HPGD, and their NC plasmid pcDNA3.1-NC for inducing Sp1 and HPGD overexpression and with sh-SETDB1 and its NC plasmid sh-NC for SETDB1 silencing. (A) Western blot analysis showed that SETDB1 expression inhibited by sh-SETDB1 plasmid transfection was rescued by Sp1 overexpression, and HPGD overexpression further increased SETDB1 expression. Inversely, HPGD expression up-regulated by SETDB1 silencing was inhibited by Sp1 overexpression, and HPGD overexpression partly alleviated the effect of Sp1 overexpression on HPGD. (B-D) CCK-8 assay, colony formation assay, and Transwell assay analysis indicated that Sp1 overexpression significantly accelerated HGC27 and MKN28 cell viability, proliferation, migration, and invasion; however, SETDB1 silencing or HPGD overexpression inhibited HGC27 and MKN28 cell viability, proliferation, migration, and invasion and partly reversed the effect of Sp1 overexpression on HGC27 and MKN28 cell bioactivity. (E, F) Statistical analysis showed that Sp1 overexpression promoted tumor enlargement and aggravation, while SETDB1 silencing or HPGD overexpression led to tumor reduction and lightening and partially reversed the trend of Sp1 overexpression. (G) Ki67 level was detected by immunohistochemistry. (H) Western blot analysis indicated that in the tumor tissue, HPGD overexpression or SETDB1 silencing partly alleviated the effect of Sp1 overexpression on HPGD. (I-K) Analysis of gastric injury and tumor metastasis in mice with GC showed that Sp1 overexpression aggravated liver injury and tumor metastasis; however, SETDB1 silencing or HPGD overexpression alleviated the liver injury and tumor metastasis and partially reversed the trend of Sp1 overexpression. HPGD = 15-hydroxyprostaglandin dehydrogenase; SETDB1 = SET domain bifurcated 1; NC = negative control; GC = gastric cancer; OD = optical density. * $P < 0.05$; * $P < 0.01$; * $P < 0.001$.

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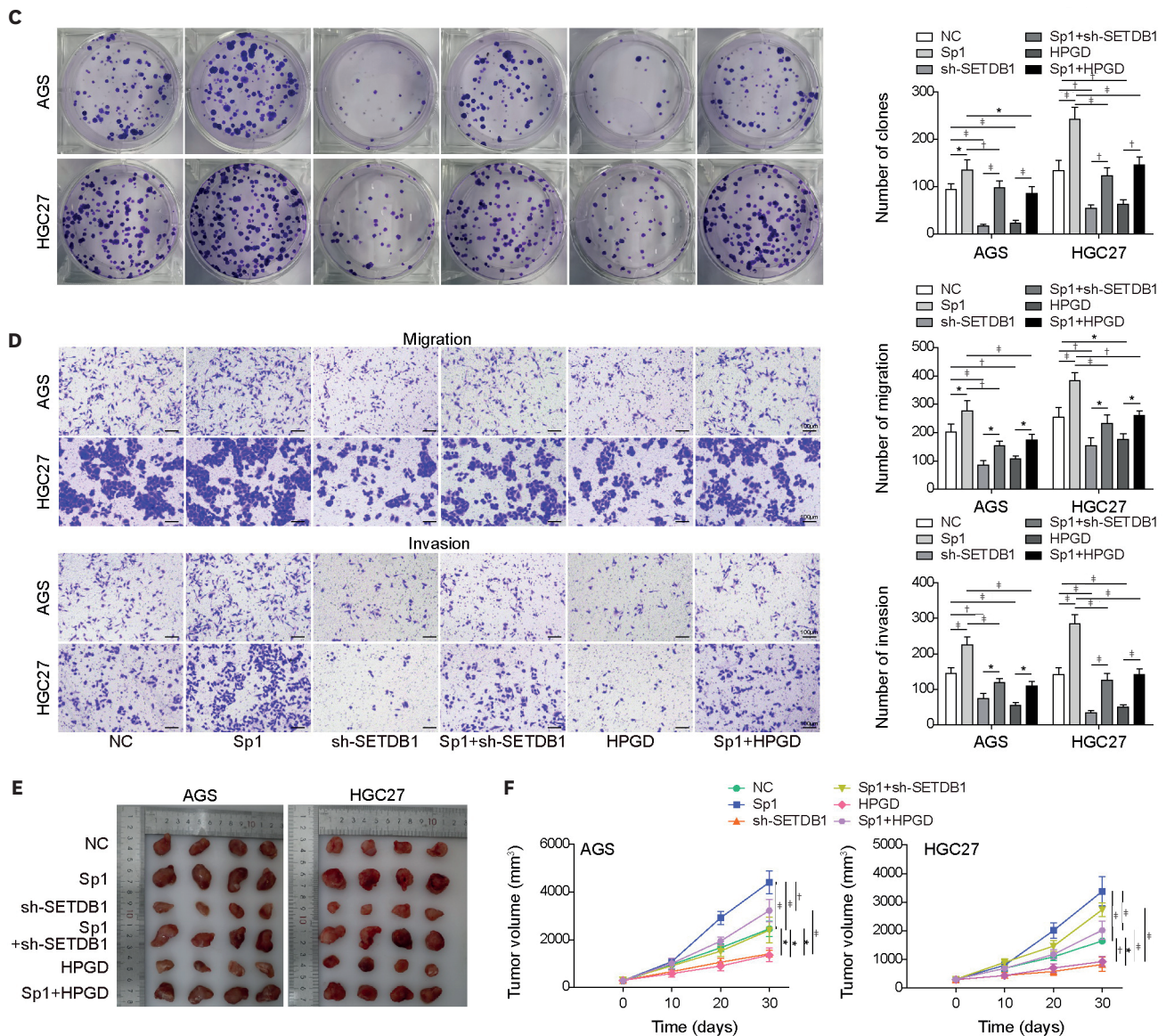


Fig. 7. (Continued) Sp1 regulated GC progression via SETDB1/HPGD axis. HGC27 and AGS cells were transfected with pcDNA3.1-Sp1, pcDNA3.1-HPGD, and their NC plasmid pcDNA3.1-NC for inducing Sp1 and HPGD overexpression and with sh-SETDB1 and its NC plasmid sh-NC for SETDB1 silencing. (A) Western blot analysis showed that SETDB1 expression inhibited by sh-SETDB1 plasmid transfection was rescued by Sp1 overexpression, and HPGD overexpression further increased SETDB1 expression. Inversely, HPGD expression up-regulated by SETDB1 silencing was inhibited by Sp1 overexpression, and HPGD overexpression partly alleviated the effect of Sp1 overexpression on HPGD. (B-D) CCK-8 assay, colony formation assay, and Transwell assay analysis indicated that Sp1 overexpression significantly accelerated HGC27 and MKN28 cell viability, proliferation, migration, and invasion; however, SETDB1 silencing or HPGD overexpression inhibited HGC27 and MKN28 cell viability, proliferation, migration, and invasion and partly reversed the effect of Sp1 overexpression on HGC27 and MKN28 cell bioactivity. (E, F) Statistical analysis showed that Sp1 overexpression promoted tumor enlargement and aggravation, while SETDB1 silencing or HPGD overexpression led to tumor reduction and lightening and partially reversed the trend of Sp1 overexpression. (G) Ki67 level was detected by immunohistochemistry. (H) Western blot analysis indicated that in the tumor tissue, HPGD overexpression or SETDB1 silencing partly alleviated the effect of Sp1 overexpression on HPGD. (I-K) Analysis of gastric injury and tumor metastasis in mice with GC showed that Sp1 overexpression aggravated liver injury and tumor metastasis; however, SETDB1 silencing or HPGD overexpression alleviated the liver injury and tumor metastasis and partially reversed the trend of Sp1 overexpression. HPGD = 15-hydroxyprostaglandin dehydrogenase; SETDB1 = SET domain bifurcated 1; NC = negative control; GC = gastric cancer; OD = optical density. * $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$.

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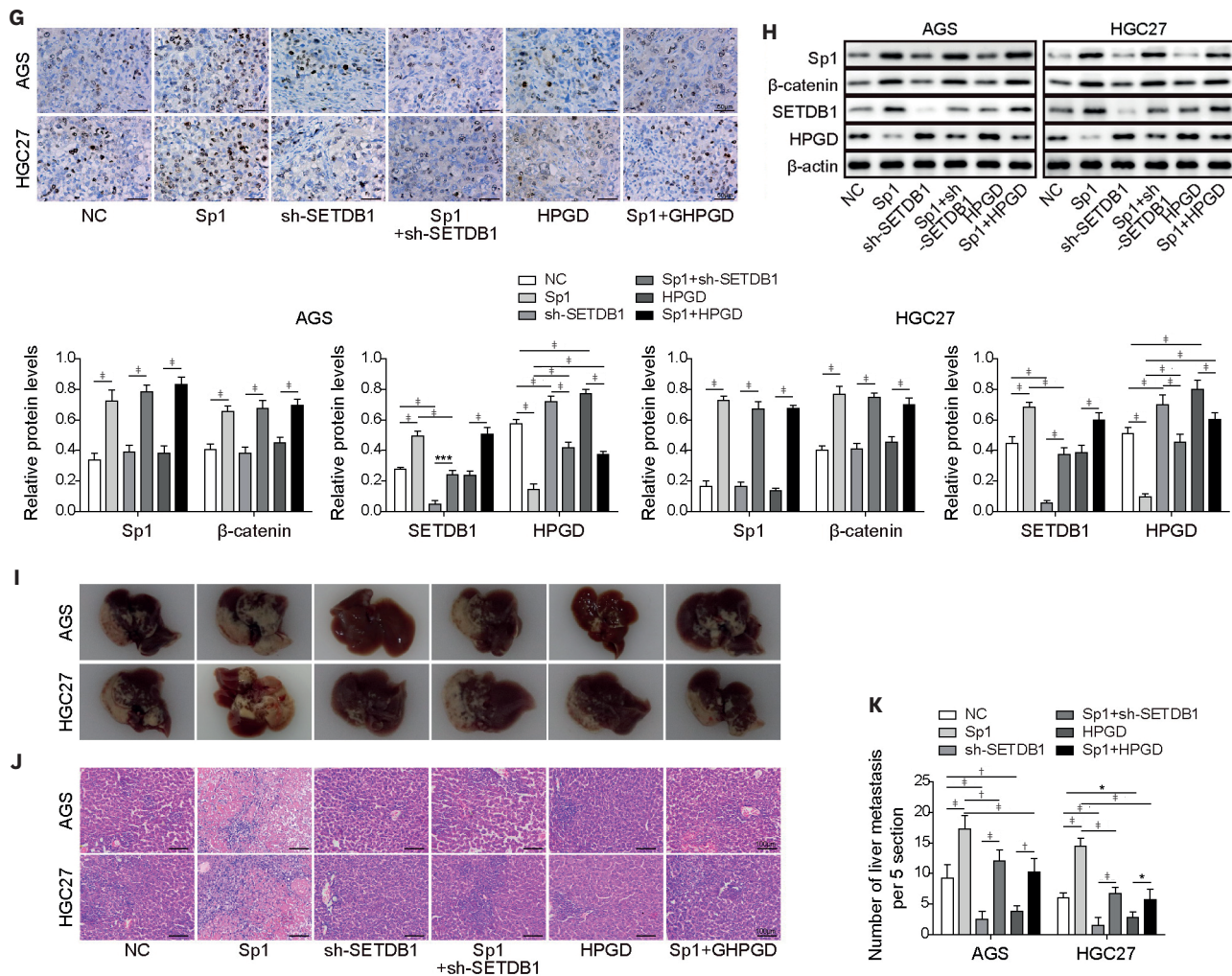


Fig. 7. (Continued) Sp1 regulated GC progression via SETDB1/HPGD axis. HGC27 and AGS cells were transfected with pcDNA3.1-Sp1, pcDNA3.1-HPGD, and their NC plasmid pcDNA3.1-NC for inducing Sp1 and HPGD overexpression and with sh-SETDB1 and its NC plasmid sh-NC for SETDB1 silencing. (A) Western blot analysis showed that SETDB1 expression inhibited by sh-SETDB1 plasmid transfection was rescued by Sp1 overexpression, and HPGD overexpression further increased SETDB1 expression. Inversely, HPGD expression up-regulated by SETDB1 silencing was inhibited by Sp1 overexpression, and HPGD overexpression partly alleviated the effect of Sp1 overexpression on HPGD. (B-D) CCK-8 assay, colony formation assay, and Transwell assay analysis indicated that Sp1 overexpression significantly accelerated HGC27 and MKN28 cell viability, proliferation, migration, and invasion; however, SETDB1 silencing or HPGD overexpression inhibited HGC27 and MKN28 cell bioactivity. (E, F) Statistical analysis showed that Sp1 overexpression promoted tumor enlargement and aggravation, while SETDB1 silencing or HPGD overexpression led to tumor reduction and lightening and partially reversed the trend of Sp1 overexpression. (G) Ki67 level was detected by immunohistochemistry. (H) Western blot analysis indicated that in the tumor tissue, HPGD overexpression or SETDB1 silencing partly alleviated the effect of Sp1 overexpression on HPGD. (I-K) Analysis of gastric injury and tumor metastasis in mice with GC showed that Sp1 overexpression aggravated liver injury and tumor metastasis; however, SETDB1 silencing or HPGD overexpression alleviated the liver injury and tumor metastasis and partially reversed the trend of Sp1 overexpression.

HPGD = 15-hydroxyprostaglandin dehydrogenase; SETDB1 = SET domain bifurcated 1; NC = negative control; GC = gastric cancer; OD = optical density.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

DISCUSSION

Understanding the pathogenesis of GC contributed to seeking novel therapeutic methods. Our findings showed that Sp1 transcriptionally inhibited HPGD via SETDB1 regulation in a β -catenin-dependent manner and promoted the proliferation and metastasis of GC cells. Our research resulted in the identification of new biomarkers for the treatment of GC.

Sp1, a zinc finger transcription factor, plays a key role in the transcriptional regulation of viral genes. It regulates the survival and growth of tumor cells and angiogenesis, and Sp1 activation may be involved in the occurrence and development of human tumors [31]. Studies have shown that Sp1 overactivation occurs in various tumors, including pancreatic adenocarcinoma, GC, and fibrosarcoma [32-34]. Consistently, our results showed that Sp1 expression was up-regulated in GC tissues and cell lines and that Sp1 accelerated the proliferation, migration, and invasion of GC cells in a β -catenin-dependent manner. The β -catenin signaling pathway is widely involved in the regulation of cancer progression, including in GC [24,35]. These findings suggest that Sp1 is abnormally activated in GC, which contributes to the invasion and metastasis of GC.

A growing number of studies have shown that SETDB1 is overexpressed in various cancers and is associated with poor prognosis in patients [36]. Karanth et al. [37] showed that SETDB1 is highly expressed in the tumor environment, and it leads to tumor growth and metastasis. Consistently, our results indicated that SETDB1 expression was up-regulated in GC tissues and cell lines and that SETDB1 silencing inhibited GC cell proliferation, migration, and invasion. These findings suggest that SETDB1 plays a positive role in GC progression; SETDB1 silencing may help alleviate the disease. Furthermore, it has been reported that SETDB1 promotes tumorigenesis and metastasis by regulating tumor-related genes. Shang et al. [17] showed that SETDB1 accelerates GC progression by regulating the levels of CCND1 and MMP-9. Our results indicated that SETDB1 targeted HPGD, which was negatively regulated by SETDB1, indicating that SETDB1 may regulate GC progression via HPGD.

HPGD has been reported to show low expression in various cancers and has been regarded as a tumor suppressor [38]. In prostate cancer, HPGD shows low expression, and it may exert an anti-tumor effect by regulating the ER signal [39]. In CC, aspirin exerts its anti-tumor effect by activating HPGD expression [40]. In breast cancer, HPGD may serve as a new tumor suppressor gene via regulation of the ER pathway [6]. Consistently, our results indicated that HPGD was down-regulated in GC tissues and cells, and HPGD overexpression inhibited the proliferation, migration, and invasion of GC cells. These results suggest that HPGD plays a negative role in GC progression; HPGD overexpression may inhibit GC progression. Furthermore, our findings indicated that Sp1 and β -catenin bound to the SETDB1 promoter and negatively regulated HPGD expression. Finally, we proved Sp1 that regulated GC progression via the SETDB1/HPGD axis.

In summary, our findings revealed that Sp1 transcriptionally inhibited HPGD via SETDB1 regulation in a β -catenin-dependent manner and promoted the proliferation and metastasis of GC. Our study elucidated the potential mechanism of proliferation and metastasis in GC and identified new biomarkers for targeted therapy of GC. Additionally, it provided a lead for further studies on the mechanism through which Sp1 regulates SETDB1 in a β -catenin-dependent manner and then negatively regulates HPGD in GC. However, our study has multiple shortcomings. For example, our study lacked additional plausible bioinformatics evidence and/or literature references. According to the StarBase (TCGA) database, Sp1 is weakly correlated with HPGD1; however, our preliminary experiments confirmed that inhibition of Sp1 can upregulate the expression of HPGD. This inconsistent result may be caused by the limited number of GC samples in the clinical database. The exact relationship between Sp1 and HPGD needs to be further studied in the future. Besides, further studies need to be conducted for determining whether other mechanisms are involved in regulating the role of HPGD in GC.

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SUPPLEMENTARY MATERIALS

Supplementary Fig. 1

Levels of HPGD, SETDB1, and Sp1 in clinical GC and paracancerous tissues as determined using IHC (200×).

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Supplementary Fig. 2

(A) The FPKM level of Sp1, CTNNB (β -catenin), SETDB1 and HPGD in STAD. (B) The level of Sp1, SETDB1 and HPGD is correlated with the survival rate of patients with GC. (C) A negative correlation is observed between the levels of SETDB1/ β -catenin and HPGD in GC.

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