Clinical Cancer Research

A Positive Feedback Loop of lncRNA-*PVT1* and FOXM1 Facilitates Gastric Cancer Growth and Invasion

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Abstract

Purpose: The long, noncoding RNA (lncRNA) *PVT1* is an important epigenetic regulator with a critical role in human tumors. Here, we aimed to investigate the clinical application and the potential molecular mechanisms of *PVT1* in gastric cancer tumorigenesis and progression.

Experimental Design: The expression level of *PVT1* was determined by RT-qPCR analysis in 190 pairs of gastric cancer tissues and adjacent normal gastric mucosa tissues (ANT). The biologic functions of *PVT1* were assessed by *in vitro* and *in vivo* functional experiments. RNA protein pull-down assays and LS/MS mass spectrometry analysis were performed to detect and identify the *PVT1*-interacting protein FOXM1. Protein–RNA immunoprecipitation assays were conducted to examine the interaction of FOXM1 and *PVT1*. Chromatin immunoprecipitation (ChIP) and luciferase analyses were utilized to identify the binding site of FOXM1 on the *PVT1* promoter.

Results: The lncRNA *PVT1* was significantly upregulated in gastric cancer tissues compared with ANTs. High expression of *PVT1* predicted poor prognosis in patients with gastric cancer. *PVT1* enhanced gastric cancer cell proliferation and invasion *in vitro* and *in vivo*. *PVT1* directly bound FOXM1 protein and increased FOXM1 posttranslationally. Moreover, *PVT1* is also a FOXM1-responsive lncRNA, and FOXM1 directly binds to the *PVT1* promoter to activate its transcription. Finally, *PVT1* fulfilled its oncogenic functions in a FOXM1-mediated manner.

Conclusions: Our study suggests that *PVT1* promotes tumor progression by interacting with FOXM1. *PVT1* may be a valuable prognostic predictor for gastric cancer, and the positive feedback loop of *PVT1*-FOXM1 could be a therapeutic target in pharmacologic strategies. *Clin Cancer Res; 23(8); 2071–80. ©2016 AACR.*

Introduction

Despite a significant decrease in its incidence, gastric cancer is still the most common malignancy of the gastrointestinal system, with a high rate of recurrence and a substantially low 5-year survival rate (<10% at advanced stages; ref. 1). Although 5-fluorouracil (FU)- and capecitabine-based chemotherapy has improved the overall prognosis of gastric cancer, the survival rate of patients with advanced cancer remains dismal. The mortality rate of gastric cancer is especially high in China, with 498 deaths per 100,000 in 2015 (2). Molecular and genetic alterations in tumors have provided critical information regarding optimal timing and treatment regimens; thus, exploring the underlying molecular mechanism may be helpful for treatment strategies as well as monitoring the prognosis of gastric cancer (3).

Long, noncoding RNAs (lncRNAs, RNA gene products consisting of 200 to 100,000 nt) have been confirmed as a class of largely functional but mechanistically unexplored transcripts with a major role in human disease, including cancer (4). Our previous study used microarrays to identify the signature of lncRNAs dysregulated in gastric cancer, and we found that several lncRNAs were functionally implicated in carcinogenesis and progression (5, 6). LncRNA *PVT1* is abnormally upregulated in somatic malignancies and has been found to promote tumor growth in gastric cancer (7–9). Although several target modulators of *PVT1* have been reported, such as NOP2 (10) and c-MYC (11), the molecular mechanisms underlying the oncogenic functions of *PVT1* require further exploration. The identification of the upstream and downstream targets of *PVT1* would help elucidate its critical role in tumor progression.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-16-0742

Translational Relevance

Long, noncoding RNAs (lncRNA) have important regulatory roles in cancer development. The lncRNA *PVT1* is an important epigenetic regulator with a critical role in human tumors. However, the mechanisms underlying the *PVT1* oncogenic functions remain elusive. In this study, the authors demonstrate a reciprocal link between *PVT1* and FOXM1 in gastric cancer. *PVT1* binds to the FOXM1 protein and enhances its stability. *PVT1* fulfills its functions in a FOXM1-mediated manner. Moreover, FOXM1 binds directly to and constitutively transactivates the *PVT1* promoter. The authors report a novel mechanism by which transcript-induced lncRNAs facilitate mRNA function by stabilizing a transcript-coding protein posttranscriptionally. Clinically, *PVT1* expression may be a useful biomarker for gastric cancer prognosis, and the positive feedback loop of *PVT1*-FOXM1 could be a therapeutic target in pharmacologic strategies.

The forkhead box M1 (FOXM1) protein belongs to the family of forkhead box transcription factors. It has emerged as a master regulator of cell proliferation and metastasis in a variety of human cancers (12, 13). FOXM1 has been suggested to be important in tumor initiation, progression, metastasis, and cancer drug response in gastric cancer by regulating the transcription of its downstream targets, such as c-MYC, CCNB1, AURKB, and SKP2, suggesting the diversity and complexity of the regulatory network centered around FOXM1 in somatic cancers (14–16). Recently, it was reported that lncRNAs may be involved in the functions of FOXM1 (17). However, the molecular mechanisms underlying the FOXM1–lncRNA interaction remain unclear.

In this study, we identified a positive feedback loop between the lncRNA *PVT1* and FOXM1. We found that *PVT1* was a valuable prognostic predictor of gastric cancer and promoted cell proliferation and invasion in gastric cancer cell lines. *PVT1* directly bound to FOXM1 protein and enhanced its stability. *PVT1* is a FOXM1-responsive lncRNA and fulfills its oncogenic functions in a FOXM1-mediated manner. Taken together, these results suggest that *PVT1* is a valuable prognostic predictor of gastric cancer. The feedback loop of *PVT1*-FOXM1 promotes gastric cancer progression and appears to be a promising target for gastric cancer therapy.

Materials and Methods

Patient samples

This study was approved by The Clinical Research Ethics Committee of the Fudan University Shanghai Cancer Center (FUSCC; Shanghai, China). Written informed consent was obtained from all participants. A total of 190 gastric cancer samples collected during 2007 to 2010 were obtained from the biobank of FUSCC. None of patients had received preoperative chemotherapy. The clinicopathologic features of the patients are listed in Supplementary Table S1. All patients were staged on the basis of the criteria of the WHO Classification of Tumors of the Digestive System, 2010 edition (18). The follow-up interval was from the date of surgery to the date of disease progression, death, or the last clinical investigation. The average follow-up time was 32.43 months (median, 28.5 months; range, 1–85 months). A dataset from an independent cohort was utilized for the evaluation of *PVT1* (Affymetrix ID: 222087_at) expression as a prognostic factor of gastric cancer. The survival analysis results of 599 gastric cancer tissue samples from The Cancer Genome Atlas (TCGA) are available at the KMPlot database (http://kmplot.com).

Cell culture and treatment

Human gastric cancer cell lines AGS, HGC27, NCI-N87, MKN45, and MGC803 and human HEK-293T cells were purchased from the IBS Cell Bank of Fudan University and the Cell Bank of Shanghai Institute, Shanghai, China. Cell culture was performed as previously described (5). Stable AGS and MGC803 cells transfected with pHBLV-IRES-ZsGreen-PGK-puro constructs were grown in 2 μg/mL puromycin (Cat. No. 631306, Clontech), and the stable *PVT1*-overexpressing MKN45 and HGC27 cell lines transfected with pLVX-sh FOXM1-G418 were additionally maintained with 400 μg/mL G418 (Cat. No. 631308, Clontech).

Plasmid construction

The full-length *PVT1* sequence was amplified by PCR from cDNA of AGS cells and then subcloned into the pcDNA3.1 (+) vector (Invitrogen) and Expression Lentivectors (Transheep). The pcDNA3.1-antisense-*PVT1* was constructed by subcloning the antisense *PVT1* sequence into the pcDNA3.1 (−) vector (Invitrogen). The putative *PVT1* promoter was PCR-amplified and cloned into the *Sma*I/*Hin*dIII site of the pGL3-Control vector (Promega) to generate the pGL3-*PVT1*-3′UTR vector. All PCR products were verified by DNA sequencing.

Luciferase assays

Cells were transfected with the pGL3-based constructs containing the *PVT1* promoter (*PVT1*-WT, *PVT1*-Mut1, *PVT1*-Mut2, *PVT1*-Mut1+2) plus the *Renilla* luciferase plasmid (pRL-TK). Then, the cells were harvested after 48 hours for firefly/*Renilla* luciferase assays using the Dual-Luciferase Reporter Assay System (Promega). Luciferase activities were normalized to the cotransfected pRL-TK plasmid (mean ± SD).

Biotin pull-down assays and mass spectrometry

The *PVT1* and its antisense plasmid were linearly cut, transcribed, and biotin-labeled *in vitro* with Bio-16-UTP (Life Technologies) using a MAXIscript T7 Transcription Kit (Life Technologies). Protein–RNA interactions were carried out using a Pierce Magnetic RNA-Protein Pull-Down Kit (Life Technologies) with the lysates of H293T and HGC27 cells. Then, the retrieved proteins were detected by Western blot analysis or resolved by in-gradient gel electrophoresis followed by mass spectrometry (MS) identification.

Other methods used in this study were described in previous publications and are listed in the Supplementary Information (5, 19–21).

Reproducibility

Each experiment was performed in triplicate, and the data are presented as the mean ± SD. The Western blot, RNA immunoprecipitation (RIP), and chromatin immunoprecipitation (ChIP) results are representative of 3 independent experiments.

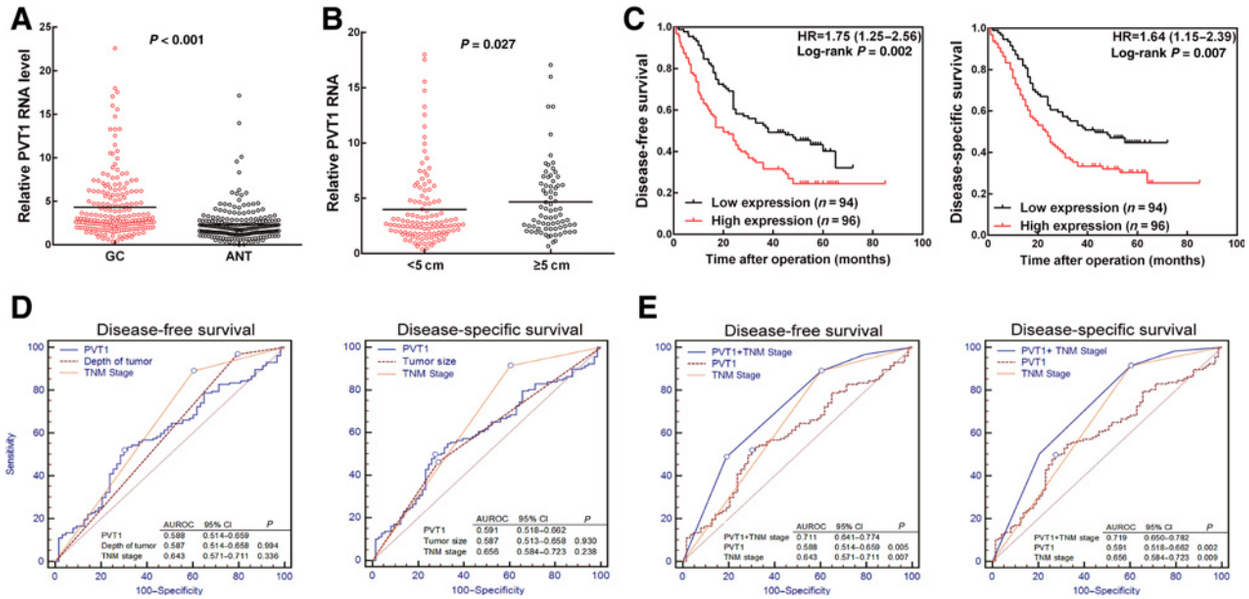

Figure 1.

PVT1 is upregulated in human gastric cancer tissues and associated with poor prognosis of gastric cancer. **A,** RT-qPCR results showed that the levels of *PVT1* in 190 pairs of gastric cancer tissues were significantly higher than those in ANTs. **B,** Stratified analysis showed that *PVT1* expression was significantly higher in patients with bigger tumor size. **C,** Kaplan–Meier survival curves showed poor DFS and DSS with high expression of PVT1 compared with those in the low expression group. **D,** ROC curves for DFS and DSS. *P* values show the area under the ROC (AUROC) of the *PVT1* signature versus the AUROC of TNM stage, depth, or size. **E,** ROC curves for DFS and DSS.*P* values show the AUROC of the combined *PVT1* expression and TNM stage model versus AUROCs of TNM stage alone or *PVT1* expression alone.

Statistical analysis

All statistical analyses were performed using SPSS 20.0 (IBM, SPSS) and GraphPad Prism. The Student *t* test and one-way ANOVA were used to analyze 2 or multiple groups, respectively, for statistical significance. Pearson correlation coefficient analysis was used to determine the correlations. Disease-free survival (DFS) and disease-specific survival (DSS) curves were calculated with the Kaplan–Meier method and were analyzed with the log-rank test. The DFS rate was calculated from the date of surgery to the date of progression (local and/or distal tumor recurrence) or to the date of death. The DSS rate was defined as the length of time between the diagnosis and death or last follow-up. Univariate analysis and multivariate models were fit using a Cox proportional hazards regression model. All tests were 2-sided, and $P < 0.05$ was considered statistically significant.

Results

PVT1 is upregulated in human gastric cancer tissues and is associated with poor prognosis of gastric cancer

To investigate the expression and the clinical significance of *PVT1* in gastric cancer, we first measured the mRNA levels of *PVT1* in 190 pairs of gastric cancer tissues and the corresponding adjacent normal gastric mucosa tissues (ANT). The *PVT1* expression level was significantly elevated in 76.8% (146 in 190) of the tumors compared with that of the ANTs (Fig. 1A), which was confirmed by data analysis of 2 independent cohorts from TCGA in cBioPortal (refs. 22, 23; Supplementary Fig. S1A). Next, we examined the *PVT1* expression level in gastric cancer patients with different clinicopathologic factors. As shown in Fig. 1B, the *PVT1* levels were substantially increased in patients with larger tumor sizes ($P = 0.027$). We also found that the high *PVT1* expression group ($n = 94$) divided by the mean value (24) showed a greater tumor size ($P = 0.025$) and depth ($P = 0.025$) and increased distant metastasis ($P = 0.021$) compared with those of the low expression group (Supplementary Table S1, Fig. 1B). High *PVT1* expression was also associated with a significantly poorer DFS ($P = 0.002$, Fig. 1C) and DSS ($P = 0.007$, Fig. 1D) than those of the low *PVT1* expression group. High *PVT1* expression resulted in a significantly poorer DFS ($n = 359$, $P = 6.8e\text{-}07$, Supplementary Fig. S1B) and DSS ($n = 593$, $P = 0.022$, Supplementary Fig. S1B) in another independent cohort available at the KMPlot database (http://kmplot.com). Univariate and multivariate Cox proportional hazards analyses showed that *PVT1*, tumor size, and tumor–node–metastasis ()TNM stage were independent prognostic factors for DFS in patients with gastric cancer (Supplementary Table S2), and *PVT1*, in addition to tumor depth and TNM stage, was identified as an independent prognostic factor for DSS in patients with gastric cancer (Supplementary Table S3).

We also performed stratified analyses of patients with lymphatic metastasis from our cohort and the independent cohort. Compared with patients with low *PVT1* expression, those with lymphatic metastasis (N1) and high *PVT1* expression had a decreased DFS ($P < 0.001$, Supplementary Fig. S1C) and DSS ($P = 0.002$, Supplementary Fig. S1C). Moreover, an exploratory subset analysis stratified by distant metastasis status indicated that patients without distant metastasis (M0) and high *PVT1* expression had a reduced DFS ($P = 0.001$, Supplementary Fig. S1C) and DSS ($P = 0.005$, Supplementary Fig. S1C). Supplementary Figure S1 shows

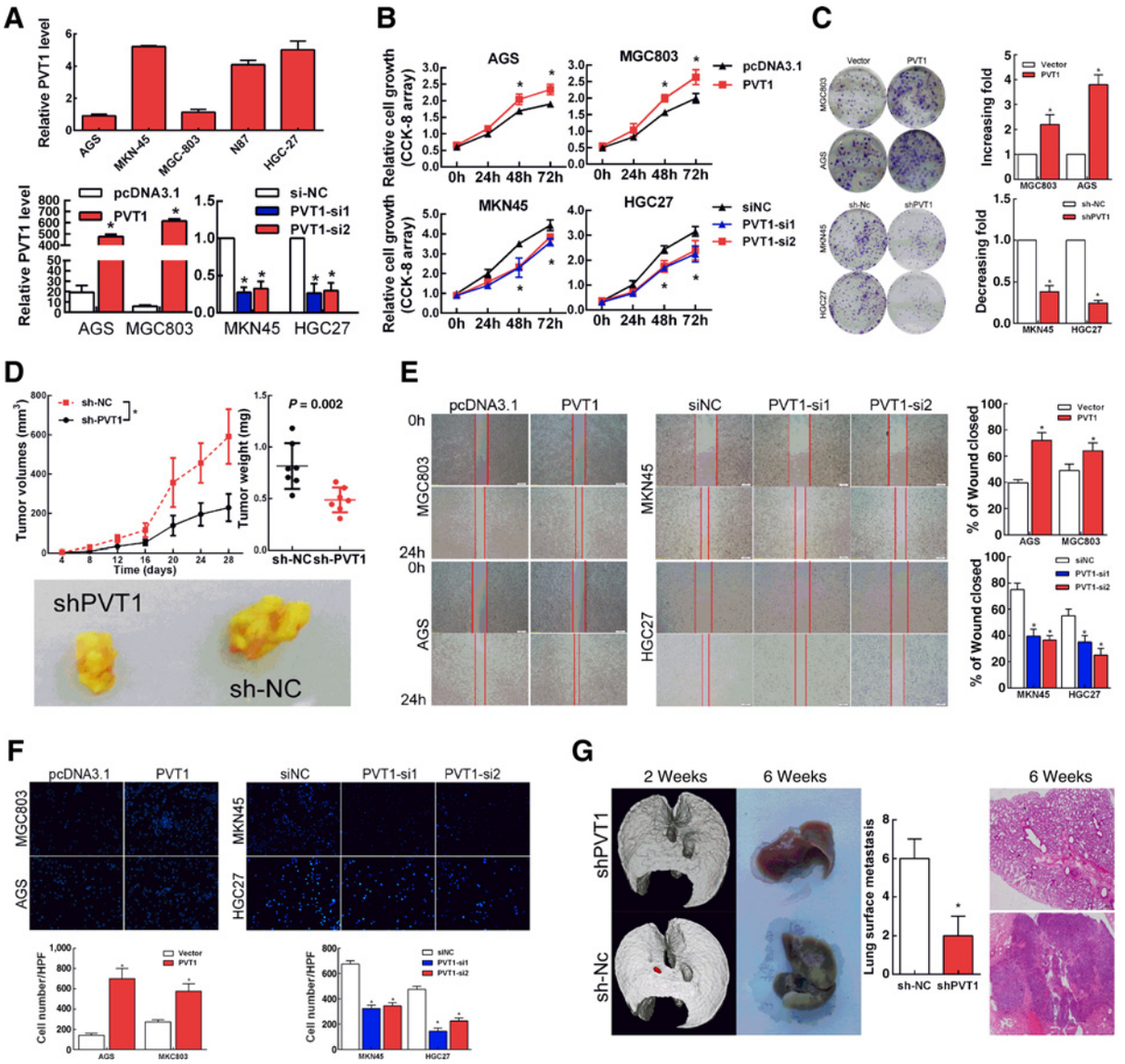

Figure 2.

PVT1 promotes gastric cancer cell growth and metastasis *in vitro* and *in vivo*. **A,** RT-qPCR results show the baseline mRNA levels of *PVT1* in ANTs and 5 gastric cancer cell lines and the efficiencies of *PVT1* overexpression in AGS and MG803 cells and *PVT1* knockdown in HGC27 and MKN45 cells. *, $P < 0.01$. **B,** CCK-8 assays showed that overexpression of *PVT1* promoted cell proliferation in AGS and MGC803 cells, whereas knockdown of *PVT1* suppressed cell proliferation in HGC27 and MKN45 cells. *, $P < 0.01$. **C,** Colony-forming assays showed that overexpression of PVT1 promoted cell proliferation in AGS and MGC803 cells, whereas knockdown of PVT1 suppressed cell proliferation in MKN45 and HGC27 cells. *, $P < 0.01$. **D,** *PVT1*-knockdown HGC27 or Nc-shRNA–transfected HGC27 cells were injected into nude mice ($n = 7$) subcutaneously (3×10^6 per mouse). The nude mouse xenograft model showed that knockdown of *PVT1* decreased tumor growth (top left) and reduced tumor weights (top right) compared with the Nc-shRNA groups. *, $P < 0.01$. The representative images of tumors were graphed (below). **E,** Representative images (left) and the number of migratory cells (right) per high-power field showed that the flattening and spreading of cells were promoted by overexpression of *PVT1* in AGS and MGC803 cells but attenuated by knockdown of *PVT1* in HGC27 and MKN45 cells. *, $P < 0.01$. **F,** Representative images (left) and the number of invasive cells (right) per high-power field showed that cell invasiveness was promoted by overexpression of *PVT1* in AGS and MGC803 cells but suppressed by *PVT1* knockdown in HGC27 and MKN45 cells. *, $P < 0.01$. **G,** *PVT1*-overexpressing HGC27 or vector-transfected HGC27 cells were injected into the caudal vein of nude mice ($n = 5$, 1×10^6 per mouse). The CT scan results (left), the gross lung metastatic tumors (middle), and hematoxylin and eosin (H&E)-stained sections of lungs (right) showed that overexpression of *PVT1* strongly promoted the metastasis of gastric cancer in the nude mouse metastasis model. *, $P < 0.01$.

similar results in the independent cohort from the TCGA dataset (Supplementary Fig. S1D).

Furthermore, we compared the prognostic value of PVT1 for DFS and DSS with that of other independent prognostic factors using receiver operating characteristics (ROC) curves, which showed that the *PVT1* expression was not different from tumor depth and TNM stage with regard to DFS (Fig. 1D) and tumor size and TNM stage with regard to DSS (Fig. 1D). Interestingly, when

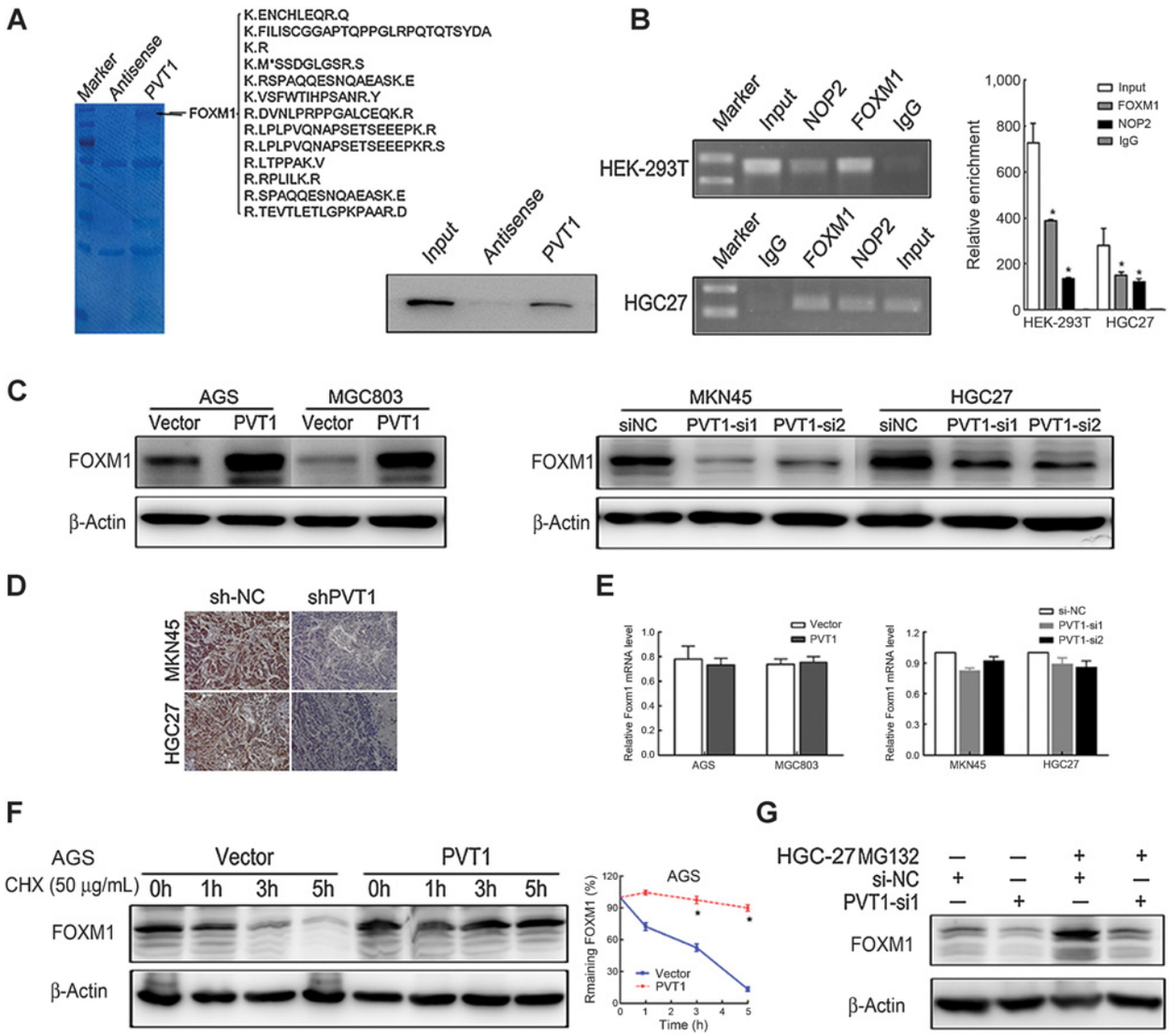

Figure 3.

PVT1 binds to the FOXM1 protein and increases its protein level by enhancing its stability. **A,** SDS-PAGE with Coomassie brilliant blue staining (left) showed the proteins pulled down by *PVT1* or its antisense RNA from H293T cells. The arrow indicates the possible FOXM1 in the *PVT1*-bound complex, the peptides of which were detected by subsequent LS/MS mass spectrometry. The FOXM1 protein was detected by Western blotting using the corresponding antibody in another independent RNA pull-down assay (right). **B,** RIP assays showed that FOXM1 interacted with PVT1 in H293T and HGC27 cells. Fifteen percent of the cell lysates as input and NOP2 were used as positive controls, and IgG was used as a negative control. The RT-qPCR products were analyzed with RNA electrophoresis (left). The mRNA levels were graphed (right). *, $P < 0.01$. **C,** Western blot results suggested that the protein level of FOXM1 was elevated in *PVT1*-overexpressing AGS and MGC803 cells but reduced in *PVT1*-knockdown HGC27 and MKN45 cells. **D,** Representative images of the immunohistochemical (IHC) analysis of *PVT1*-knockdown MKN45 and HGC27 cells. **E,** RT-qPCR results showed that overexpression and knockdown of *PVT1* failed to affect the mRNA level of FOXM1 in gastric cancer cells. **F,** AGS cells were transfected with the indicated plasmids for 48 hours. Cycloheximide (CHX; 50 μg/mL) was then added at 0, 1, 3, and 5 hours. Lysates were collected at the indicated time points and immunoblotted with the indicated antibodies. The intensities of Western blot results that reflected the remaining protein levels of FOXM1 were quantified by ImageJ software and graphed. The experiments were performed in triplicates (right: *, $P < 0.01$). **G,** HGC27 cells were transfected with siNC or siPVT1 for 48 hours. MG132 (10 μmol/L) was added for 3 hours before harvesting. Lysates were immunoblotted with the indicated antibodies.

we combined *PVT1* expression with TNM stage, the combination had a better prognostic value than TNM stage and the *PVT1* expression alone (Fig. 1E), suggesting that combined detection of PVT1 and TNM stage will provide more accurate predictions in gastric cancer.

PVT1 promotes gastric cancer cell proliferation and metastasis *in vitro* and *in vivo*

To investigate the potential effect of *PVT1* on the pathogenesis of gastric cancer, we first measured the baseline levels of *PVT1* in 5 gastric cancer cell lines and compared then with those of the NTs.

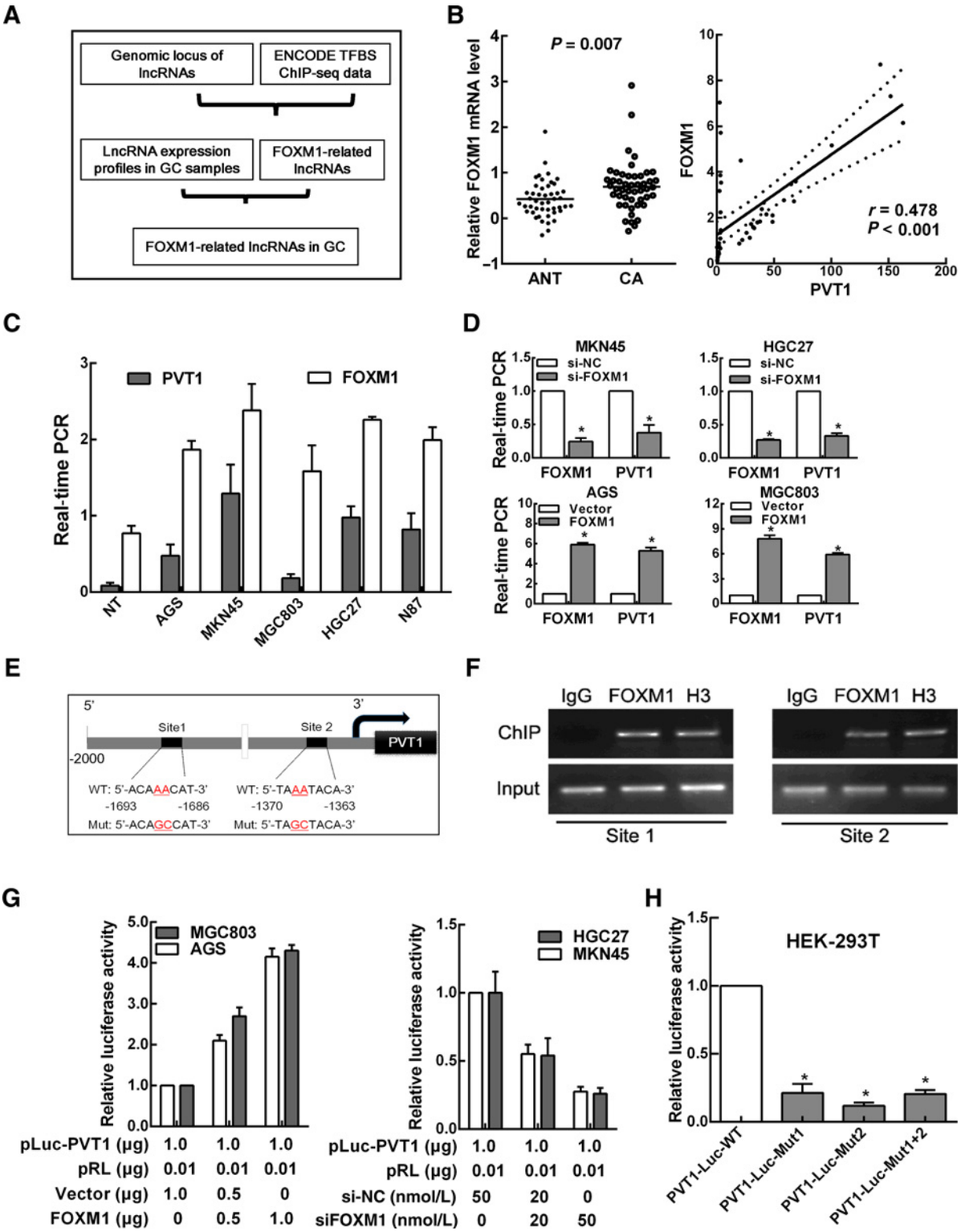

Figure 4.

PVT1 is the FOXM1-responsive lncRNA in gastric cancer. **A,** Schematic diagram shows the combined analysis of the ENCODE TFBS ChIP-seq data and the genomic locus information of lncRNAs from UCSC (http://genome.ucsc.edu) and our previously identified lncRNAs from microarray analysis of gastric cancer and adjacent normal samples. (*Continued on the following page.*)

The PVT1 level was significantly elevated in gastric cancer cells compared with that in the NTs (all $P < 0.01$, Fig. 2A). The AGS and MGC803 gastric cancer cell lines were selected for overexpression; the HGC27 and MKN45 cells were selected for knockdown of *PVT1*. The efficiencies of overexpression and interference were confirmed by RT-qPCR ($P < 0.01$, Fig. 2A). Next, we investigated the potential effect of *PVT1* on gastric cancer *in vitro* and *in vivo*. The results showed that overexpression of *PVT1* accelerated cell growth in AGS and MGC803 cells, whereas knockdown of *PVT1* suppressed cell proliferation in HGC27 and MKN45 cells as determined by CCK-8 assays ($P < 0.01$, Fig. 2B), colony-forming assays ($P < 0.01$, Fig. 2C), and mouse xenograft models ($P < 0.01$, Fig. 2D).

Similarly, overexpression of *PVT1* enhanced the flattening and spreading of AGS and MGC803 cells, whereas knockdown of *PVT1* strongly inhibited the flattening and spreading of MKN45 and HGC27 cells ($P < 0.01$, Fig. 2E). Transwell assays showed that overexpression of *PVT1* enhanced cell invasion in AGS and MGC803 cells, whereas knockdown of *PVT1* decreased the cell invasiveness in HGC27 and MKN45 cells ($P < 0.01$, Fig. 2F). Moreover, the mouse metastatic models showed that the overexpression of *PVT1* significantly promoted lung metastases (Fig. 2G). The metastatic lesions of *PVT1*-overexpressing MGC803 cells could already be observed in the lungs using a computed tomographic (CT) scan 2 weeks after tumor cell injection (Fig. 2G left), and 6 weeks later, the metastatic lesions of *PVT1*-overexpressing MGC803 cells surpassed those of the controls in both size and weight (Fig. 2G, middle and right). Taken together, these results indicate that *PVT1* contributes to the metastasis of gastric cancer.

PVT1 interacts with FOXM1 and elevates its protein expression in gastric cancer cells

Because lncRNAs can function by interacting with proteins (25), we performed biotin RNA–protein pull-down assays to identify potential proteins binding to *PVT1*. The *PVT1*-bound complex was then analyzed using SDS-PAGE, and the gel was stained with Coomassie blue. The lane with the PVT1-bound complex was excised and subjected to mass spectrometry (Fig. 3A, left). LS/MS mass spectrometric analysis identified FOXM1 as one of the binding targets of *PVT1* (Supplementary Table S4), which was further confirmed by Western blotting in 3 independent RNA pull-down assays (Fig. 3A, right). We also performed RIP using antibodies against FOXM1 and NOP2 in cell extracts from H293T and HGC27 cells. The RIP assays showed that *PVT1* was detected by RT-qPCR in FOXM1-immunoprecipitated RNAs in both H293T and HGC27 cell lines (Fig. 3B). Taken together, these results indicated that *PVT1* interacted directly with FOXM1.

Next, we explored the regulatory effects of *PVT1* on FOXM1. We found that overexpression of *PVT1* elevated FOXM1 protein levels (Fig. 3C). In addition, immunohistochemical analysis of xenografts showed that lower expression of FOXM1 was found in the *PVT1*-knockdown group compared with that of the controls (Fig. 3D). However, overexpression of PVT1 did not affect the mRNA level of FOXM1 in HGC27 cells (Fig. 3E), suggesting that FOXM1 might elevate PVT1 at the posttranscriptional level. Therefore, we used the protein synthesis inhibitor cycloheximide to observe the effect of PVT1 on degradation of FOXM1. The results showed that overexpression of *PVT1* in AGS cells moderately prolonged the half-life of FOXM1 in the presence of cycloheximide ($P < 0.01$, Fig. 3F). Moreover, the 26S proteasome inhibitor MG132 rescued the reduction of FOXM1 caused by knockdown of *PVT1* in HGC27 cells, suggesting that PVT1 promotes accumulation of FOXM1 by inhibiting its degradation (Fig. 3F). Taken together, our data suggest that *PVT1* elevates FOXM1 protein by reducing its degradation and enhancing its stability in gastric cancer cells.

FOXM1 regulates the expression of *PVT1* in gastric cancer

Considering that FOXM1 is a potent transcription factor that fulfill its oncogenic functions by activating the transcription of downstream targets in tumors, we identified a group of FOXM1-related lncRNAs by analyzed the ENCODE TFBS ChIP-seq data and the genomic locus information of lncRNAs from UCSC (http://genome.ucsc.edu). Through a conjoint analysis with our previously identified lncRNAs from the microarray profile analysis of gastric cancer and adjacent normal samples (5), we unexpectedly found that PVT1 was one of the gastric cancer–specific FOXM1-related lncRNAs (Fig. 4A; Supplementary Table S5). The mRNA levels of FOXM1 were significantly correlated with those of PVT1 in 48 pairs of gastric cancer and corresponding ANTs (Fig. 4B) and 5 gastric cancer cell lines (Fig. 4C). Then, we observed that the mRNA levels of PVT1 were increased in FOXM1-overexpressing AGS and MGC803 cells, whereas they were reduced in FOXM1-knockdown HGC27 and MKN45 cells (Fig. 4D). Taken together, these data suggest that FOXM1 may be a transcriptional regulator of *PVT*1 in gastric cancer.

FOXM1 directly binds to the promoter regions of *PVT1*

To further investigate the regulatory mechanism underlying the correlation between FOXM1 and *PVT1*, we searched for possible transcription factor–binding sites for FOXM1 in *PVT1*. Two online software programs were used: PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) and TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html). As shown in Fig. 4E, we found 2 putative FOXM1-binding sites within 2,500 bp upstream of the transcriptional start of *PVT1* and constructed a 2,500-bp long PGL3-*PVT1*-promotor plasmid and mutants of the predicted FOXM1-binding sites (Fig. 4E). ChIP assays revealed that FOXM1 directly bound to both predicted binding sites of the PVT1 promoter (Fig. 4F). The dual-luciferase reporter assays indicated that overexpression of FOXM1 stimulated the *PVT1* promoter activity in AGS and MGC803 cells, which

(*Continued.*) **B,** RT-qPCR results showed that FOXM1 was upregulated in gastric cancer tissues compared with ANTs ($n = 48$, $P = 0.007$), and regression analysis identified a positive relationship between FOXM1 and PVT1 mRNA expression levels in gastric cancer tissues ($n = 48$, $r = 0.478$, $P < 0.001$). **C,** Expression level of PVT1 and FOXM1 was validated by the RT-qPCR analysis in ANTs and 5 gastric cancer cell lines (normalized by β-actin). **D,** RT-qPCR results showed that the mRNA level of *PVT1* was increased by overexpression of FOXM1 in AGS and MGC803 cells but reduced by knockdown of FOXM1 in HGC27 and MKN45 cells. *, $P < 0.01$. **E,** Schematic diagram showing the human *PVT1* upstream promoter region (top), including the predicted FOXM1-binding regions (site 1 and site 2), as well as the wild-type and 2 mutated (Mut) *PVT1* promoter luciferase (Luc) constructs. **F,** ChIP assays showed that endogenous FOXM1 bound to the upstream region of *PVT1* (site 1 and site 2). IgG served as a negative control, and H3 served as a positive control. **G,** Dual-reporter luciferase assays showed that overexpression of FOXM1 in AGS cells stimulated the promoter activity of *PVT1*, whereas knockdown of FOXM1 by siRNA in HGC27 cells suppressed the activity of the *PVT1* promoter reporter. *, $P < 0.05$. **H,** Dual-reporter luciferase assays showed that FOXM1 failed to stimulate the mutants of both predicted binding sites in the promoter region of *PVT1* in HEK-293T cells compared with the wild-type (WT). *, $P < 0.05$, compared with the pGL3-basic group.

was reduced by knockdown of FOXM1 in HGC27 and MKN45 cells (Fig. 4G), and mutation of both putative FOXM1-binding sites significantly reduced the transcriptional activity of *PVT1* regardless of overexpression of FOXM1 (Fig. 4H), suggesting that FOXM1 binds to both putative sites of the PVT1 promoter to activate its transcription.

PVT1 facilitates tumor proliferation and metastasis in a FOXM1-mediated manner

Finally, we conducted *in vitro* and *in vivo* experiments to investigate whether *PVT1* functioned in a FOXM1-mediated manner in gastric cancer. The CCK-8 assay results showed that overexpression of *PVT1* promoted the proliferation of AGC and MGC803 cells, which nevertheless was impaired by simultaneous knockdown of FOXM1 (Fig. 5A). Cell-wounding and Transwell assays also showed that FOXM1 knockdown partially attenuated the effects of overexpression of *PVT1* on gastric cancer cell metastasis compared with that of the controls (Fig. 5B and C). The *in vivo* xenograft experiments showed that knockdown of FOXM1 could partially abrogate the accelerated tumor growth as well as the increased tumor size/weight caused by overexpression of *PVT1* in mouse xenograft models (Fig. 5D). Our results indicated that *PVT1* promotes gastric cancer cell proliferation and metastasis in a FOXM1-mediated manner.

The effect of FOXM1-*PVT1* loop on EZH2, c-MYC, and NOP2 in gastric cancer

Previous studies indicated that *PVT1* can directly promote NOP2 (10), indirectly repression of p15 and p16, via EZH2 (9). Also, a similar loop was identified for c-myc/PVT1 (11). To detect the relationship between the FOXM1/*PVT1* loop and these proteins, we detected whether FOXM1 and *PVT1* affect the expression of c-Myc or EZH2 in gastric cancer cells, and our result showed that overexpression/knockdown of FOXM1 and PVT1 significantly upregulated/downregulated the expression of c-MYC and NOP2 protein in gastric cancer cells, and both FOXM1 and *PVT1* can rescue the effect of each other on c-MYC protein, whereas there was no significant change on the protein level of EZH2 (Supplementary Fig. S2).

Discussion

Here, we identified a positive feedback loop of *PVT1-FOXM1*, in which *PVT1* promotes gastric cancer tumor growth and metastasis by stabilizing FOXM1 protein and FOXM1 in turns stimulates the promoter activity of lncRNA *PVT1*. In addition, we provided the first evidence for FOXM1-regulating lncRNAs in gastric cancer using interaction analysis from an online database (UCSC) and our previous microarray analysis results (5). Therefore, our findings elucidate a hitherto unexplored mechanism for both FOXM1 and *PVT*1 in gastric cancer tumor progression.

We previously described the posttranslational modification of FOXM1, especially its degradation via the ubiquitin–proteasome system. Several proteins have been reported to influence its stability. However, no lncRNAs have been linked to the posttranslational modification of FOXM1. In our study, we revealed that *PVT1* directly bound to FOXM1 protein and regulated FOXM1 activity at the posttranslational level. The stabilization of FOXM1 inhibits its 26S proteasome–mediated degradation, resulting in accumulation of FOXM1 protein in cells, continuously activating its transcription-promoting functions. Because the 26S proteasome is strongly correlated with the mechanism of ubiquitination, whether *PVT1* stabilizes FOXM1 via the ubiquitin–proteasome system would be an interesting question that should be examined in the future. Furthermore, we discovered that several polyubiquitin chains are involved in the degradation of FOXM1, and the N-terminus as well as the KEN box of FOXM1 contributes to the ubiquitination of FOXM1 (26). It would be of interest to determine whether PVT1 interacts with FOXM1 by binding these ubiquitination-related domains of FOXM1 to affect its stability.

As a transcription factor, FOXM carries out its functions by binding to the promoter of downstream effector genes and stimulating their transcriptional activities. However, no lncRNAs have been reported to be the targets of FOXM1. We found 2 binding sites of FOXM1 on the promoter region of PVT1, and FOXM1 bound to these two sites to activate PVT1 transcription, which is similar to the findings of Carramusa and colleagues (27). They found that c-MYC regulates the promoter activity of *PVT1*. Moreover, Tseng and colleagues (11) reported that the mRNA level of *PVT1* was positively correlated with the protein level of c-MYC in cells. These results along with ours might reflect a reciprocal regulatory mechanism between transcriptional factors and lncRNAs, which consequently amplifies their mutual oncogenic functions in somatic malignancies. Interestingly, c-MYC has been reported to interact with FOXM1 (28, 29), and in our study, we found that overexpression of FOXM1 elevated the protein level of c-MYC in cells. We proposed that FOXM1 may stimulate the promoter activity of *PVT1* to elevate the protein level of c-MYC, and both FOXM1 and c-MYC bind to the promoter of *PVT1* to activate its transcription, thus forming a double positive feedback loop in this triangle.

In addition, *PVT1* has been suggested to interact with the epigenetic modifier enhancer of zeste homolog 2 (EZH2, the catalytic component of polycomb repressive complex 2), regulating the expression of p15 and p16 in gastric cancer (9). EZH2 has also been reported to function in a MAPK/FOXM1-dependent manner in human malignancies (30). However, in our study, we confirmed that both *PVT1* and FOXM1 had no effect on the protein level of EZH2, suggesting that the interaction between the FOXM1-*PVT1* loop and EZH2 may be complicated and regulated by multiple factors in cells.

Finally, our study investigated the prognostic potential of *PVT1* expression in survival of patients with gastric cancer independent of TNM stage, supporting the prognostic value of *PVT1* expression, which allows clinicians to potentially identify candidate patients for appropriate treatment to improve therapeutic outcomes. Moreover, we found that the combination of *PVT1* expression and TNM stage had a better prognostic value than TNM staging alone, suggesting that patients with gastric cancer classified in the same TNM stage might be stratified into different risk level groups according to the *PVT1* expression, thus indicating a wide clinical applicability. In addition, ROC analysis suggested that *PVT1* expression has a similar survival predictive ability as tumor size and TNM stage but provides more biologic characteristics of gastric cancer and extra information on molecular pathology, implying that identification of lncRNA in patients might be a more straightforward procedure.

Overall, our study identified the potential reciprocal link between *PVT1* and FOXM1, which may reflect the underlying molecular mechanism of their biologic functions. Our results also suggest that the *PVT1* expression may be a useful biomarker for prognosis, and targeting the *PVT1*-FOXM1 loop might be a useful strategy for future cancer treatment.

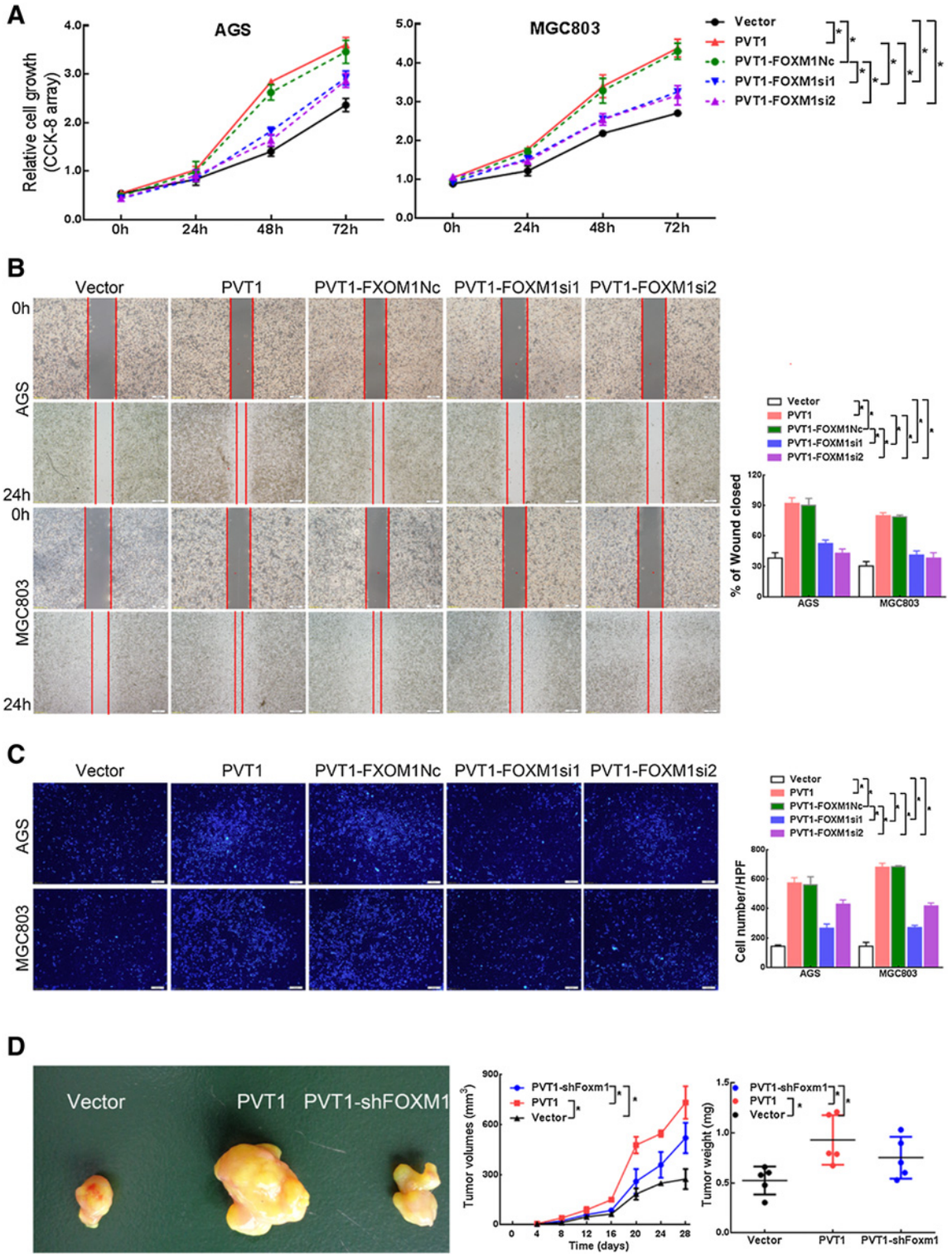

Figure 5.

PVT1 facilitated gastric cancer progression in a FOXM1-mediated manner. **A,** CCK-8 results showed that knockdown of FOXM1 partially attenuated the enhanced cell proliferation induced by overexpression of *PVT1* in AGS and MGC803 cells. *, $P < 0.01$. **B,** Representative images (left) and the number of migratory cells (right) per high-power field showed that knockdown of FOXM1 partially attenuated the enhanced migratory ability of AGC and MGC803 cells promoted by *PVT1* upregulation. *, $P < 0.01$. **C,** Representative images (left) and the number of invading cells (right) per high-power field showed that knockdown of FOXM1 partially attenuated the enhanced invasiveness of AGC and HGC27 cells promoted by *PVT1* upregulation. *, $P < 0.01$. **D,** Nude mouse xenograft model showed that knockdown of FOXM1 reduced the enhanced volume and weight of tumors by overexpression of PVT1 compared with overexpression of PVT1 only. *, $P < 0.01$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Xu, W. Weng, W. Lin, Q. Xu, Y. Ma, X. Du
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Acknowledgments

We thank Shenglin Huang of Fudan University Shanghai Cancer Center for helpful discussions and revises and Chen Wang of Life Technologies for technical assistance.

Grant Support

This study was supported by the grant from the Science and Technology Commission of Shanghai Municipality (No. 10DJ1400501), National Clinical Key Discipline (2011–2015), Priority of Shanghai key discipline of medicine (2013–2015), Shanghai R&D public service platform construction projects (12DZ2295100), National Natural Science Foundation of China (81602269, 81602078, 81472220, and 81272299), Hospital Foundation of Fudan University Shanghai Cancer Center (YJ201504), Science and Technology commission of Shanghai Municipality (15495810300), Shanghai Hospital Development Center Emerging Advanced Technology Joint Research Project (HDC12014105), Shanghai Key Developing Disciplines (2015ZB0201), Shanghai Science and Technology Development Fund (No. 15ZR 1407400), and Domestic Science and Technology Cooperation Project, No. 14495800300).

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Received March 22, 2016; revised September 16, 2016; accepted September 20, 2016; published OnlineFirst October 18, 2016.

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