LncRNA PVT1 Promotes Bladder Cancer Progression by Forming a Positive Feedback Loop With STAT5B

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Abstract

Background: Plasmacytoma Variant Translocation 1 (LncRNA PVT1) and signal transducer and activator of transcription 5B (STAT5B) have been reported to play important roles in various cancers, but their interaction in bladder cancer (BC) remains unclear.

Purpose: In this study, we aimed to explore the interaction between lncRNA PVT1 and STAT5B in BC tumorigenesis.

Methods: The association of the expression of the lncRNA PVT1 and STAT5B to the prognosis of patient with BC was evaluated via bioinformatic analysis. Loss- and gain-of-function assays were performed to determine the biological functions of lncRNA PVT1 and STAT5B in BC cells. Quantitative real time polymerase chain reaction, Western blot, immunohistochemistry, and immunofluorescence were used to detect lncRNA PVT1 and STAT5B expression. Fluorescence in situ hybridization, RNA pull-down and RNA immunoprecipitation assays were conducted to determine the regulatory effect of lncRNA PVT1 on STAT5B. The transcriptional effect of STAT5B on lncRNA PVT1 gene was determined using luciferase reporter assay, chromatin immunoprecipitation and DNA-affinity precipitation assays.

Results: We found that lncRNA PVT1 and STAT5B enhance the expression of each other and promote the malignant phenotypes in BC, including cell viability and invasion. lncRNA PVT1 stabilizes STAT5B by decreasing ubiquitination, enhances STAT5B phosphorylation, and promotes the translocation to the nucleus of STAT5B to trigger further carcinogenesis activities. In the nucleus, STAT5B activates the transcription of lncRNA PVT1 by binding directly to its promoter region, leading to a positive feedback.

Conclusions: We first identified the lncRNA PVT1/STAT5B positive feedback loop for bladder carcinogenesis, which may provide new molecular targets for interventions of BC.

Introduction

Bladder cancer (BC) is the ninth most common malignant disease worldwide, and it ranks fourteenth in cancer mortality worldwide (1). There are two types of BC: muscular invasive BC and non-muscular invasive BC. Present investigations indicate that one-quarter of patients with muscle-invasive BC have a poor prognosis in despite of the effectiveness of radiotherapy and radical surgery. Patients with non-muscular invasive BC have up to 50% risk of recurrence and 20% risk of progression within 5 years (2). BC is a heterogeneous disease and its natural history exhibits significant variability. Low-grade bladder tumors present a low progression rate and require initial endoscopic treatment and surveillance but rarely pose a threat to the patients. At the other extreme, high-grade bladder tumors have a high malignant potential to lead to significant progression and cancer death rates (3).

Plasmacytoma Variant Translocation 1 (lncRNA PVT1),a lncRNA longer than 500 nucleotides, first reported in mouse plasmacytoma and has been found to participate in the oncogenesis of various cancers (4). The main molecular mechanism by which lncRNA PVT1 exerts its cancer-promoting role is

“the ceRNA molecular sponge mechanism”, indicating that lncRNA PVT1 acts as sponge to regulate the expression of certain oncogenes by binding and degrading microRNAs (5; 6). Apart from the ceRNA mechanism, lncRNA PVT1 has been reported to trigger the modification of target proteins such as ubiquitination, by binding to the protein molecules in angiogenesis of tumor (7). Although there have been literatures revealing that lncRNA PVT1 was expressed at high levels in BC tissues and correlates with clinical progression and poor prognosis in BC patients, the intrinsic molecular mechanism of lncRNA PVT1 in BC still remains unclear (8).

Signal transducer and activator of transcription (STAT) family are a class of potential transcription factors activated by cytokines and growth factors. Mammals have 7 STAT proteins: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6 (9). The STAT5 proteins, containing the closely related members STAT5A and STAT5B that share 96% homology at the amino acid level, is frequently constitutively active in solid cancers and inflammation. Phosphorylated STATs (p-STATs) form dimers, translocate to the nucleus, and bind to specific sites and regulate transcription (10). Essentially, STAT5 plays a critical role in the function and development of Tregs and consistently activated STAT5 is associated with a suppression in antitumor immunity and an increase in cell viability, invasion and survival of tumor cells (11). In BC, STAT3 has been reported to exert pro-oncogenic effects by phosphorylation and activation. However, there have been few literatures reporting the interaction between STAT5 and the pathogenesis of BC so far. In our survival analysis of 6 STAT genes, only high levels of STAT5B gene had a significant poorer prognosis for BC. Nevertheless, the STAT5B gene in GSE databases presented a slightly lower expression in BC tissues. This indicated that post-translational modifications (PTMs) may participate in the regulation of STAT5B intracellular levels and affect the function of STAT5B in BC carcinogenesis.

There have been few reports revealing the interaction between lncRNA PVT1 and STAT5B in the pathogenesis of BC so far. Thus, in this article, we confirmed the positive feedback loop related to lncRNA PVT1 and STAT5B in BC, and elucidated their interaction in regulating the malignant phenotype and progression of BC.

Materials And Methods

Identification of BC associated genes

The expression datasets for genes in bladder tissues of BC patients and normal bladder tissues were obtained from the GEO databases (GSE13507 and GSE40355) and the Cancer RNA sequencing (RNA-Seq) Nexus database. To obtain more accurate results, the differentially expressed genes were obtained based on the intersection of the three databases. Differential expression analysis was performed between the normal colon and BC tissues using a *t*-test with False Discovery Rate (FDR)-adjusted *P*-value less than 0.05.

Next, the seven intersected RNAs obtained from the intersection of GSE40355, GSE13507 and the Cancer RNA-Seq Nexus database, in addition to the six STAT genes including STAT1, STAT2, STAT3, STAT4,

STAT5B and STAT6 genes, were analyzed for survival analysis. Survival analysis was performed with Kaplan-Meier survival curves on the website of http://kmplot.com/analysis/index.php?p=service.

Cell culture

The human BC cell lines (5637, T24, J82, EJ and RT4) and normal bladder uroepithelial cell line SV-HUV-1 were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 1 mmol/L glutamine, and 100 U/mL penicillin at 37°C in an incubator with 5% CO_2. The expression of lncRNA PVT1 and STAT5B in the above cells was examined in pre-experiments, and the T24 and EJ cell lines with the highest lncRNA PVT1 and STAT5B expression levels were selected for the formal experiments.

Western blot (WB) and antibodies

Cell lysates were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (GE Healthcare, USA). The membrane was blocked with 5% skim milk for 2 h at room temperature in TBS (20 mM Tris-HCl, 137mM NaCl, pH 7.6), and incubated overnight at 4°C with specific primary antibodies, followed by the secondary antibodies at room temperature for 2 h. WB analysis was performed using the Bio-Rad Western blotting system (Bio-Rad, USA). β-actin was used as an internal control. The specific antibodies used for WB were as follows: anti-STAT5B (phospho Y705) antibody (EP2147Y, ab76315) and anti-STAT5B antibody (E121-21, ab32500).

Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from samples using Trizol reagent according to the manufacturer's protocol instructions (Invitrogen, CA, USA). Approximately 30 mg of each sample was grinded thoroughly with a homogenizer. After centrifugation and the treatment of Trizol and chloroform, the upper suspension was removed, and the RNA precipitate was washed with 1 mL of 75% ethanol. RNase-free water was added to the dried RNA precipitate to dissolve the RNA completely. qRT-PCR was performed with SYBR® Premix Ex Taq™ II kit (RR820A, TaKaRa, Dalian, China) in ABI 7500 Real Time PCR System (ABI Company, Oyster Bay, NY, USA). All samples were done in triplicate and normalized by the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences used for qRT-PCR and promoter were listed in **Table 1**.

Immunohistochemistry (IHC)

The paraffin-embedded tissues sections were dewaxed in xylene and rehydrated in ethanol gradient. Then the tissues were incubated in 1% hydrogen peroxide and boiled in citrate buffer (10mM, pH = 6.0) for 15min. Later, following incubation with the primary antibodies at 4°C overnight, horseradish peroxidase

(HRP)-conjugated goat anti-rabbit secondary antibodies (zsbio, Beijing, China) were used for incubation with the tissues. IHC staining was conducted with triplicate sections for each sample.

Immunofluorescence (IF)

Deparaffinization and rehydration were performed following standard procedures using xylene and graded solutions. Tissues were then blocked with Protein Blocking Solution (Dako) and incubated for 60 min at room temperature followed by incubation with antibodies. The images were obtained with fluorescence microscope (Olympus) and measured using ImageJ 2.0 software. IF staining was conducted with triplicate sections for each sample.

Fluorescence in situ hybridization assay (FISH)

To identify the subcellular localization of lncRNA PVT1 in BC cells, FISH assay was conducted using a FISH kit (Ribo Bio, Guangzhou, China) in accordance with the manufacturer's instructions. The cells were fixed with 4% paraformaldehyde and treated with 0.5% Triton in PBS. After pre-hybridization, they were then hybridized at 5 μM probe concentration overnight. Ribosomal RNA (18S) was used as the reference gene. Streptavidin-biotin system labeled PVT1 RNA probe was used as the probe mix. Samples were visualized by a confocal microscope (Zeiss).

Cell counting test

Cell counting test was performed to detect cell proliferation using Cell Counting Kit-8(CCK-8) (Beyotime, Shanghai, China). Each well (96-well plate) was filled with approximately 1×10^5 cells in a culture volume of 100 μL. Each group was set with 3 parallel wells for the assay. Cell-free wells with complete medium and CCK-8 solution were set as background control wells. A total of 10 μL CCK-8 solution was added, followed by incubation for 1h. Mix it thoroughly and the absorbance was measured at 50 nm.

Wound healing assay

Wound healing assay was performed to detect cell migration. Approximately 1×10^5 cells/mL were added to each well and cells grew to more than 90% the next day. Cell scratch wounds were created using a sterile 20 μL pipette tip, after which the dislodged cells were washed out with fresh medium. Wound healing was assessed at 0 h and 48 h by measuring the wound areas on digitized photographs using ImageJ software. The wound healing assay was performed in triplicate.

Invasion assay

The invasion assay was performed using transwell membranes precoated with DMEM-diluted Matrigel® (BD Biosciences) for 2 h at 37°C. About 2×10^4 Cells were seeded into the upper chamber in serum free medium in triplicate. A total of 500 μL DMEM/F-12 medium supplemented with 10% FBS was plated in the lower chambers. Cells were allowed to migrate towards the lower chamber. After 72 hours, the

invasive cells were fixed in methanol for 15 min at room temperature and were stained using hematoxylin for 10 min. The invasion was examined with light microscopy according to the manufacturer's protocols.

RNA immunoprecipitation (RIP) assay

RIP assay was performed using EZ-Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Catalog No. 17-701) (Millipore, Burlington, MA, USA) according to the instructions. Briefly, cell lysates were collected and incubated with magnetic beads conjugated with antibodies. RIP was performed using either anti-SNRNP70 (Cat. # CS203216) or Normal Rabbit IgG (Cat. # PP64B) as the immunoprecipitating positive or negative antibody. The relative abundance of lncRNA PVT1was normalized to the amount of enriched U1 snRNA (Cat. # CS203215) via qRT-PCR.

RNA pull-down assay

RNA pull-down assay was performed using the PierceTM Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, the cell lysates were incubated with control or biotinylated lncRNA PVT1 at room temperature for 4 h, followed by the addition of streptavidin magnetic beads (Thermo Fisher Scientific, Waltham, MA, USA) at 4°C for 60 min with rotation. After three washing steps with washing buffer, the RNA-binding proteins were eluted using 50 μL elution buffer and analyzed via WB. The BIO-PVT1 probe sequence was designed by the prediction of catRAPID fragments for a possible PVT1-STAT5B binding interval of 40-119bp. BIO-PVT1 probe sequence was: 5'-GTCCCGGGGCACATCTTTGCTCGCAGCTCGTCGT-3'.

si-RNA and overexpressed plasmid construction

si-RNA transfection was performed using Lipofectamine™ 3000 Reagent. Dilute 7.5 μL reagents and si-RNA with 125 μL Opti-MEM® I. Mix the diluted siRNA with reagents and incubate for 5 minutes at room temperature. Add them to each well containing cells and culture medium. And then gently mix it up and incubate for 4 hours at 37°C. The target sequence for si-PVT1 #1 was 5'-GAGCTGCGAGCAAAGATGT-3', and for si-PVT1 #2 was 5'-ACTTTAAGTGGAGGCTGAATCATCT-3'. The target sequence for si-STAT5B was 5'-GTGTCAAGGCAGATTAGTTAT-3'. Overexpressed plasmids were performed using Lipofectamine™ 3000 Reagent. Dilute 7.5 μL reagents and 5 μL plasmid reagents with 125 μL Opti-MEM® I. Mix the diluted plasmids with reagents and incubate for 5 minutes at room temperature. Add them to each well containing cells and culture medium. And then gently mix it up and incubate for 4 hours at 37°C.

Cell transfection

Using a lentiviral system, stably transfected cells were created. Lentiviral vectors encoding the human STAT5B gene and lncRNA PVT1 and its siRNAs, or empty vector were transfected into T24/EJ cells with a multiplicity of infection of 40 to 50 in the presence of polybrene (5 μg/mL). Transfected cells were selected at 48 h after transfection. Transfection efficiency was confirmed by qRT-PCR. Pooled

populations of knockdown cells and overexpressed cells, which were obtained 2 weeks after selection without subcloning, were used in both in vitro and in vivo experiments.

Luciferase reporter assay

Wild-type or mutant lncRNA PVT1 promoter plasmids were cotransfected into T24/EJ cells with STAT5B overexpressed plasmids to detect the effect of STAT5B on the activation for lncRNA PVT1 promoter. The high scoring predicted lncRNA PVT1-STAT5B binding sites was 5'- ATTTCACTGAA -3' of 1532-1542bp. T24/EJ cells were plated at a density of 2×10^4 cells per well in 24-well plates for 24 h. After incubation, 4 μg of plasmids were transfected into T24/EJ cells. The cells were collected after about 48 h in culture for luciferase reporter assays using the Dual-Luciferase® Reporter (DLR™) Assay System (Promega) according to the manufacturer's instructions. A fluorescence signal was generated by the addition of Photinus pyralis and the signal lasted for 1 min. After quantifying the fluorescence intensity, the Stop & Glo® reagent was added to quench the above reaction and simultaneously initiate the Renilla reniformis reaction, while a second measurement was performed. The luciferase activity was determined by the relative light unit value of the two measurements. The assay was performed in triplicate.

Chromatin immunoprecipitation (ChIP)

ChIP assay was performed using the EZ-ChIP™ Kit (Millipore). Firstly, break up the DNA by sonication on crushed ice and centrifuge at 4°C at 12,000 rpm for 10 min. Then remove the precipitates and leave the supernatant. Take 100 μL of chromatin solution from the previous step. Add 900 μL of dilution and 60 μL of Protein G Agarose to each tube. After incubation at 4°C for 1h and centrifugation at 3000 rpm for 1 min, add IP antibody to supernatant and set up each control as follows: positive control: anti-RNA polymerase; negative control: normal rat IgG. After incubation overnight at 4°C and adding Protein G Agarose to per tube, wash it with Wash Resuspend and Wash Buffer. Then add 5 M NaCl and incubate at 65°C for 4-5 h to release the Protein/DNA crosslink. Finally, add sample/Bind Reagent to purify the DNA.

DNA-affinity Precipitation Assay (DAPA)

Based on the high scoring predicted lncRNA PVT1-STAT5B binding sites 5'- ATTTCACTGAA -3' of 1532-1542bp, we designed 5'-biotinylated-wt-Sense oligonucleotide and the Anti-sense oligonucleotide for DAPA to detect STAT5B expression levels. Briefly, following annealing of Sense and Anti-sense oligonucleotdies for 1 h at 95°C, 4 μg of annealed biotinylated probe was incubated with 500 μg of nuclear cell lysate, and 40 μL of streptavidin-agarose bead was suspended in diluted PBS for 2–3 h rocking at room temperature. The streptavidin-agarose beads were pelleted by centrifugation and washed 5 times with PBS. The captured probe-binding proteins were eluted from the streptavidin-agarose beads by boiling in 2×SDS sample buffer for 5 min and subjected to immunoblot analysis.

Nude mice tumorigenesis

Every experimental group had 4 mice. Male BALB/c nude mice (4-6 weeks of age) were injected with 1×10^6 cells in a total volume of 0.2 mL per injection site selected subcutaneously near the extremities. Tumor sizes were measured according to the longest diameter (a) and shortest diameter (b) after feeding nude mice every 3 days for 6 consecutive times until the tumor was visible to the naked eyes (about one week). Tumor growth curves were plotted using time as the horizontal coordinate and tumor volume as the vertical coordinate. Tumor tissues were taken and photographed for IHC testing. Tumor volume was calculated according to the formula: $V = a \times b^2 \times 0.52$ (mm^3).

Statistical analysis

All statistical analysis was performed using the GraphPad Prism Software, Version 8 (GraphPad Software, San Diego, CA, USA). Significant differences were analyzed using two-tailed Student's *t*-test or the one-way ANOVA. The value of $P < 0.05$ was considered statistically significant.

Results

LncRNA PVT1 and STAT5B are upregulated in BC tissues

We made the intersection analysis of GSE13507, GSE40355 and the Cancer RNA-Seq Nexus database, and obtained 7 up-regulated genes for BC. Interestingly, we found lncRNA PVT1 was one of the 4 upregulated non-coding RNAs (PDXDC2P, LINC00839, PVT1 and LINC01658) in multiple BC tumor tissues compared with the normal tissues in the three databases (**Fig. 1A-C**). Meanwhile, the survival analysis showed that the higher lncRNA PVT1 expression levels were associated with a shorter survival time (**Fig. 1D**). In addition, the lncRNA PVT1 expression levels of tumor tissues from BC patients were significantly higher than that of their adjacent normal tissues (**Fig. 1E**). Taken together, these data indicated that lncRNA PVT1 is highly expressed in BC tissues and it may serve as a potential prognostic biomarker of BC.

The survival analysis of six STAT (STAT1, STAT2, STAT3, STAT4, STAT5B and STAT6) genes was performed with survival curves. Among the six STAT gene candidates, only higher levels of STAT5B gene were significantly associated with poorer prognosis (**Fig. 1F**). Thus, we next did functional experiments to explore the effect of lncRNA PVT1 and STAT5B on the BC progression.

Effect of lncRNA PVT1 level on BC tumor growth and STAT5B level in vivo

Taking into consideration that the expression levels of lncRNA PVT1 and STAT5B were the highest in T24 and EJ cells among five BC cell lines (5637, T24, J82, EJ and RT4) (**Fig. 2A-B**), these two cell lines were selected for the following experiments. To explore the effect of lncRNA PVT1 on the proliferation and growth of BC cells, loss- and gain-of-function assays were performed. Firstly, si-RNA targeting lncRNA PVT1 or a lncRNA PVT1-overexpressed plasmid was transfected into T24 and EJ cells. As a result, lncRNA PVT1 knockdown significantly inhibited cell proliferation (**Fig. 2C**), migration (**Fig. 2D**) and invasion (**Fig. 2E**), whereas the overexpression of lncRNA PVT1 resulted in the opposite effects.

Meanwhile, we found that the STAT5B and p-STAT5B protein levels showed the same trend in loss- and gain-of-lncRNA PVT1 function assays, both increasing with lncRNA PVT1 (**Fig. 2F**). This indicated that lncRNA PVT1 could improve the levels of STAT5B and promote the phosphorylation of STAT5B. Interestingly, though STAT5B protein levels were positively associated with lncRNA PVT1 levels, the overexpression or knockdown of lncRNA PVT1 had no significant impact on the STAT5B RNA expression (**Fig. 2G**). In our analysis of GSE databases, the STAT5B gene in BC tumor tissues even presented slightly lower levels (**Fig. 2H**). This suggested that the lncRNA PVT1-mediated STAT5B regulation for BC tumorigenesis may involve post-translational modifications. Overall, lncRNA PVT1 promotes STAT5B phosphorylation, improves STAT5B expression levels to play an oncogenic role in BC.

LncRNA PVT1 directly binds to STAT5B and stabilizes STAT5B via deubiquitination

In FISH experiments, lncRNA PVT1 was predominantly distributed in the cytoplasm of T24/EJ cells (**Fig. 3A**), indicating that lncRNA PVT1 might participate in STAT5B translational regulation. Furthermore, degradation is an important factor affecting total protein levels, so we explored the effect of lncRNA PVT1 on the degradation of STAT5B. The STAT5B levels were detected using WB at different times in negative control T24/EJ cells, lncRNA PVT1 knockdown and lncRNA PVT1 knockdown with proteasome inhibitor groups. We found that the lncRNA PVT1 knockdown increased the degradation rates of STAT5B. However, this accelerating STAT5B protein degradation triggered by lncRNA PVT1 knockdown was significantly inhibited with the addition of the proteasome inhibitor MG132 (**Fig. 3B**). This implied that lncRNA PVT1 regulates STAT5B levels via the protein stabilization.

Considering that ubiquitination plays crucial part in protein degradation, we designed the follow-up experiments to further confirm whether it was by mediating STAT5B-deubiquitination that lncRNA PVT1 maintained STAT5B stability. We compared STAT5B-ubiquitination modification levels and total STAT5B levels in lncRNA PVT1 overexpressed, knockdown and negative control groups. As expected, lncRNA PVT1 overexpression attenuated STAT5B-ubiquitination modification and increased the total STAT5B protein levels compared to negative control group, while the lncRNA PVT1 knockdown group showed the contrast results (**Fig. 3C**). Therefore, we concluded that lncRNA PVT1 stabilizes STAT5B via decreasing STAT5B ubiquitination.

Next, we conducted RNA pull-down assay to investigate whether lncRNA PVT1 could interact with STAT5B. A possible interaction region between lncRNA PVT1 and STAT5B was predicted by catRAPID. The results showed that the lncRNA PVT1 probe pulled down more STAT5B than the oligo control (**Fig. 3D-E**). Similarly, RIP assay demonstrated that STAT5B remarkably immunoprecipitated lncRNA PVT1, particularly after the lncRNA PVT1 overexpression in T24/EJ cells (**Fig. 3F**). Taken together, these findings showed that STAT5B was abundantly enriched in the lncRNA PVT1 probe, suggesting that lncRNA PVT1 probe could directly interact with STAT5B via binding to it.

si-STAT5B reverses the lncRNA PVT1-induced tumorigenesis in BC

WB and IHC results implied that the expression of STAT5B and p-STAT5B protein was upregulated in BC tissues compared with the adjacent normal tissues (**Fig. 4A-B**), revealing that higher levels of STAT5B expression was associated with tumorigenesis in BC.

Additionally, we observed that the overexpression of STAT5B upregulated the lncRNA PVT1 level (**Fig. 4C**). So far, we have confirmed that the overexpression of either lncRNA PVT1 or STAT5B could promote the BC progression. Moreover, the overexpression of lncRNA PVT1 or STAT5B could upregulate the level of each other. Taken together, we hypothesized that lncRNA PVT1 promotes BC progression via STAT5B. To prove this theory, we transfected si-STAT5B plasmid into lncRNA PVT1 overexpressed BC cells and performed WB assay, cell counting test, wound healing assay and invasion assay to observe the tumor cell growth. As expected, the silencing of STAT5B in BC cells significantly reversed the promotion of cell proliferation (**Fig. 4D**), migration (**Fig. 4E**) and invasion (**Fig. 4F**) induced by overexpressed lncRNA PVT1. Besides, the expression of STAT5B and p-STAT5B was also obviously inhibited by si-STAT5B (**Fig. 4G**). Altogether, our data supported that lncRNA PVT1 exerts its tumor-promoting actions in BC oncogenesis and progression via STAT5B in vitro.

STAT5B activates the transcription of lncRNA PVT1 in BC cells

IF assay was performed to detect the fluorescence intensity of STAT5B in lncRNA PVT1 knockdown and control group. Interestingly, we found that lncRNA PVT1 knockdown reduced the total STAT5B level and impaired the translocation of STAT5B to the nucleus (**Fig. 5A**), implying that lncRNA PVT1 could promote the translocation of STAT5B to the nucleus in BC. Inspired by this phenomenon, we wondered whether STAT5B could activate the transcription of lncRNA PVT1 to promote BC progression. To figure out it, we designed primers according to the prediction for the promoter region of lncRNA PVT1. Importantly, this prediction was validated via luciferase reporter assays **(Fig. 5B)**. The overexpression of STAT5B dramatically enhanced the luciferase activity of the wild-type lncRNA PVT1 promoter but did not affect the transcriptional activity of the mutant lncRNA PVT1 promoter. Moreover, the ChIP assay demonstrated that STAT5B was remarkably enriched in the lncRNA PVT1 promoter region with IgG as a control, especially in the STAT5B overexpressed cells (**Fig. 5C**). Consistently, DAPA analysis showed that the wild-type probe pulled down more STAT5B protein than the mutant probe did, especially in STAT5B overexpressed group (**Fig. 5D**). Overall, these results indicated that STAT5B could bind to the promoter of lncRNA PVT1 and activate its transcription.

LncRNA PVT1 exerts its oncogenic effect for BC via STAT5B in vivo

The in vivo experiments were conducted to validate the oncogenic effect of lncRNA PVT1 via STAT5B in BC. The nude mice were divided into three groups: negative control group, lncRNA PVT1 overexpressed group, and lncRNA PVT1 overexpression with STAT5B knockdown group. Male BALB/c nude mice were injected with transfected cells and were measured for tumor size every 3 days. Tumor growth curves demonstrated that the overexpression of lncRNA PVT1 significantly promoted the tumor growth

compared to the negative controls, whereas the knockdown of STAT5B remarkably reversed the above oncogenic effect (**Fig. 6A**). Meanwhile, WB and IHC assays showed that STAT5B and p-STAT5B expression levels were significantly increased by the overexpression of lncRNA PVT1 and decreased by the knockdown of STAT5B (**Fig. 6B-C**). Collectively, the above findings suggested that lncRNA PVT1 regulates the BC-associated tumor growth through STAT5B in vivo.

Discussion

LncRNAs play crucial roles in tumor development and progression by interacting with DNA, RNA, and protein molecules to modulate local or global gene expression (7). As a lncRNA longer than 500 nucleotides, lncRNA PVT1 exerts its cancer-promoting role by acting as sponge binding and degrading microRNAs to regulate the expression of certain oncogenes in various cancers (5; 6). In this study, we observed that lncRNA PVT1 was overexpressed in BC tissues and BC cell lines, and promoted the proliferation, migration and invasion in BC cells, which was in accordance with previous literature reports (8; 12).

STAT5 was involved in the function of Tregs and consistently activated STAT5 can lead to a suppression in antitumor immunity and an increase in viability of tumor cells (11). Although the roles of STAT5 in BC was rarely studied in literatures, STAT3 was considered to play a critical role in BC tumorigenesis. STAT3 enhances the growth of BC cells via phosphorylation, inhibiting apoptosis and cell cycle arrest or interacting with other molecules (13-15). In this paper, we validated the pro-cancer roles in BC tumorigenesis of STAT5B by functional experiments. STAT5B was overexpressed in BC tissues and BC cell lines, and promoted the proliferation, migration and invasion in BC cells. Besides, we found that the inhibition of STAT5B expression could significantly reverse the oncogenesis effect of lncRNA PVT in BC, indicating this may serve as a new molecular target in the interventions of BC.

LncRNAs mediate cell proliferation by regulating the expression of downstream molecules in various cancers: lncRNA NEAT1 promotes the progression of non-small cell lung cancer by up-regulating E2F3 expression (16); lncRNA FTH1P3 contributes to melanoma progression and invasion by targeting miR-224-5p (17); lncRNA LINC00673 promotes hepatocellular carcinoma cell proliferation via down-regulation of miR-205 (18). In BC progression, lncRNA PVT1 sponges miR-194-5p to promote b-cells lymphoma-2-associated transcription factor 1 (BCLAF1) expression and subsequently accelerates the malignant BC cells' phenotypes (19). The interaction between lncRNA PVT1 and proteins, however, was rarely reported in BC progression. In this report, we confirmed the mutual function between lncRNA PVT1 and STAT5B in bladder carcinomas. Both overexpressed lncRNA PVT1 and STAT5B could promote the viability and survival of BC cells. Besides, they were upregulated by the other, showing that there is a positive feedback loop of lncRNA PVT1 and STAT5B exerting cancer-promoting effects in BC.

We noticed lncRNA PVT1 promoted STAT5B phosphorylation in BC cells. STATs are post-translationally modified by acetylation, ubiquitylation, ISGylation, sumoylation and the most common being phosphorylation (20). Interestingly, in our study, the STAT5B protein and STAT5B gene levels did not

present a consistent association with lncRNA PVT1. This suggested that the lncRNA PVT1-mediated STAT5B regulation for BC tumorigenesis is likely to occur at the posttranscriptional level. Besides by promoting phosphorylation, lncRNA PVT1 also regulated STAT5B by decreasing STAT5B ubiquitination to protect it from degradation. Our functional experiments confirmed that lncRNA PVT1 stabilized STAT5B via decreasing STAT5B ubiquitination to protect it from proteasome-dependent degradation, further promoting the accumulation of STAT5B and p-STAT5B. Protein ubiquitination is a crucial and reversible post-translational protein modification process for protein degradation (21). Generally, the protein ubiquitination and deubiquitination constitute a dynamic equilibrium in vivo (22). Indeed, examples of STATs being protected from degradation and stabilized by lncRNAs can be found in other tumorigenesis. It was documented that STAT3 was protected from proteasome-mediated degradation by lncRNA TNK2-AS1 in non-small cell lung cancer, and was protected from poly-ubiquitination by lncRNA PVT1 in gastric cancer, further elevating VEGFA expression to stimulate angiogenesis (7; 23).

In this study, STAT5B also exerts a positive feedback effect on lncRNA PVT1. With luciferase reporter assay, ChIP and DAPA assays, we confirmed that STAT5B bound to the promoter region of lncRNA PVT1 and improved the lncRNA PVT1 level, indicating STAT5B can activate the transcription of lncRNA PVT1 in BC cells. The direct binding between lncRNA PVT1 and STAT5B was further confirmed by RNA pull-down and RIP assays. Interestingly, we noticed that lncRNA PVT1 knockdown impaired the translocation of STAT5B to the nucleus by IF assay. Due to this, it can be speculated that STAT5B is translocated to the nucleus in the presence of lncRNA PVT1 and further activates the transcription of lncRNA PVT1 in turn to promote BC progression. Coincidentally, in Zhao's article, lncRNA PVT1 was reported to act as a STAT5B-responsive lncRNA, as STAT5B could occupy the lncRNA PVT1 promoter to facilitate its transcription in gastric cancer (7).

In recent years, some tumor-related proteins were described to form a positive feedback loop with their relevant upstream RNAs in cancer progression. Su et al. verified that E2F1 binds to the FOXD2-AS1 promoter region and subsequently enhances its transcriptional activity in BC progression and recurrence (24). Liu's paper showed that nuclear factor kappa B (NF-κB) and Yin Yang 1 (YY1) directly binds to the LINC01578 promoter and activates LINC01578 expression in colon cancer (25). Xu et al. revealed a positive feedback loop of lncRNA PVT1 and FOXM1 facilitates gastric cancer growth and invasion (26). More recently, an article clarified the positive feedback interaction of lncRNA PVT1 and STAT3 in gastric cancer (7). To our knowledge, the interaction between lncRNA PVT1 and STAT5B in BC progression has not been investigated yet. In this paper, we revealed the existence of positive feedback loop involving lncRNA PVT1 and STAT5B in BC progression. We found that lncRNA PVT1 or STAT5B could enhance the expression of each other and promote the malignant phenotype in BC. Meanwhile, lncRNA PVT1 enhances the STAT5B phosphorylation, stabilizes STAT5B by decreasing STAT5B ubiquitination level and promotes the translocation to the nucleus of STAT5B. In return, STAT5B activates the transcription of lncRNA PVT1 by binding to its promoter region, to trigger further carcinogenesis activities.

Limitations, however, still existed in this study. Due to the low number of available clinical BC samples, we validated the clinical significance of lncRNA PVT1 utilizing the online database. Besides, the

mechanism of lncRNA PVT1/STAT5B axis may have different effects and oncogenesis features on different stages of BC, but this has not been further studied in our research. Nevertheless, we deem the trend would be analogical, which can be ascertained in years to come.

In summary, this study first put forward that the lncRNA PVT1/STAT5B positive feedback loop was involved in the tumorigenesis of BC. We implied the interaction between lncRNA PVT1 and STAT5B in BC cell growth, which could be a feasible target for therapies in the future.

Declarations

Conflict of interest

The authors declare that they have no conflict of interest.

Funds

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Authors' contributions

Li Zhuo and Jian Liu conceived and designed the study. Huifeng Fu, Yuanwei Li and Qaing Lu performed experiments. Wei Song collected the data. Jian Liu and Huifeng Fu interpreted the data. Jiansong Wang and Li Zhuo analyzed the data and wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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Tables

Table 1. Primers used for qRT-PCR and ChIP

Primers used for qRT-PCR	**5′- 3′**
STAT5B-F (Forward)	GAGGTGCGGCATTATTTATCCC
STAT5B-R (Reverse)	GCGGTCATACGTGTTCTGGAG
PVT1-F	AGCCAGTCTTGGTGCTCTGT
PVT1-R	AGTGAACTCCTCAGCCTCCA
β-actin-F	CATGTACGTTGCTATCCAGGC
β-actin-R	CTCCTTAATGTCACGCACGAT
18S-F	CAGCCACCCGAGATTGAGCA
18S-R	TAGTAGCGACGGGCGGTGTG
Primers for ChIP	**5′- 3′**
PVT1-F	CGTCCTTCTAGGGTCCATGA
PVT1-R	CACGCCCAGCTAATTTTTGT
GAPDH-F	TACTAGCGGTTTTACGGGCG
GAPDH-R	TCGAACAGGAGGAGCAGAGAGCGA

Figures

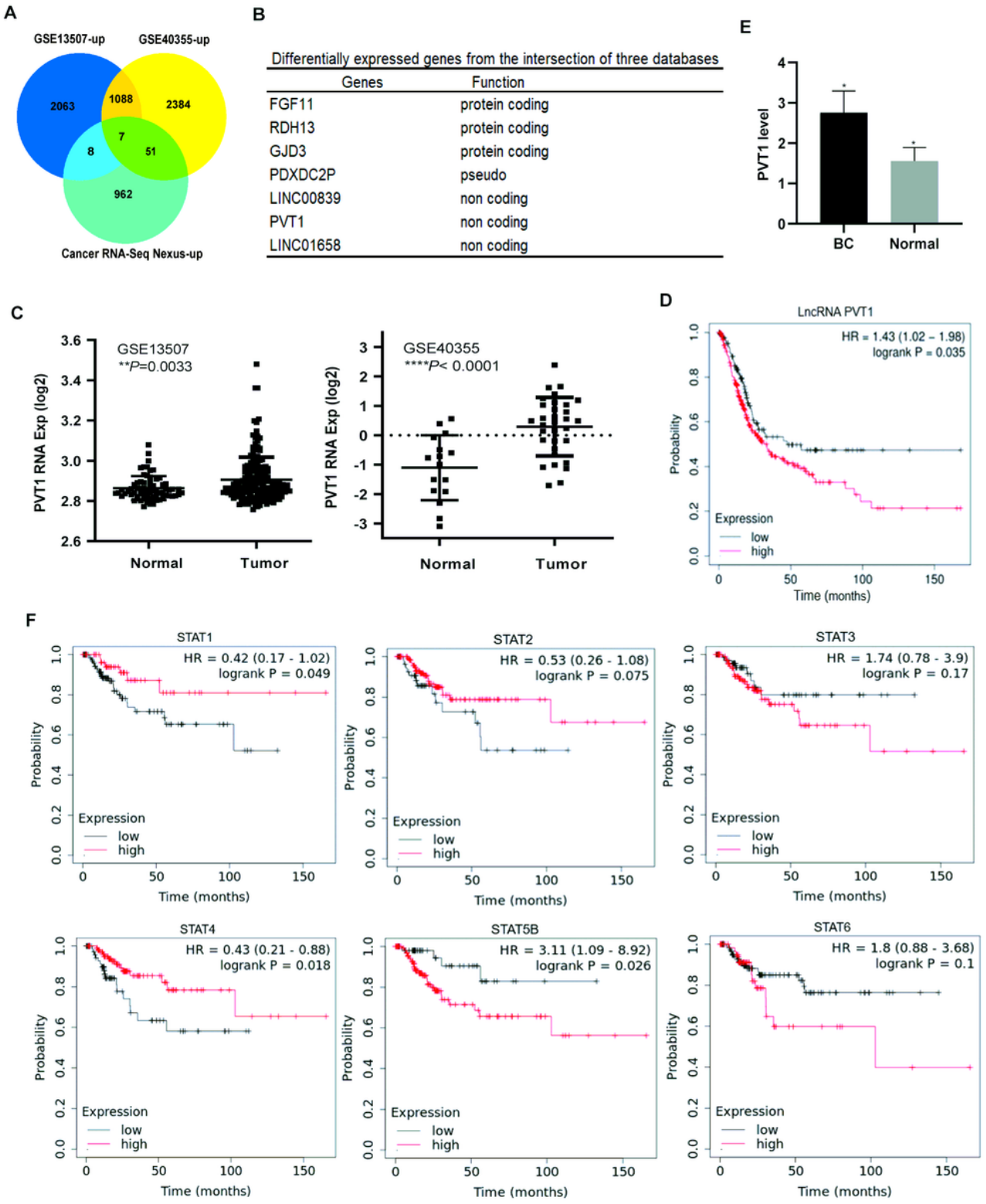


Figure 1

LncRNA PVT1 and STAT5B are upregulated in BC tissues. A. The number of intersected up-regulated genes in GSE13507, GSE40355 and the Cancer RNA-Seq Nexus database. B. Differentially expressed genes from the intersection of GSE13507, GSE40355 and the Cancer RNA-Seq Nexus database. C. LncRNA PVT1 levels in GSE13507 and GSE40355 database. D. Survival curves of lncRNA PVT1 in BC

database. E. The lncRNA PVT1 levels in 8 paired of BC tissues and adjacent normal tissues. F. Survival curves of 6 STATs in BC database. *P < 0.05.

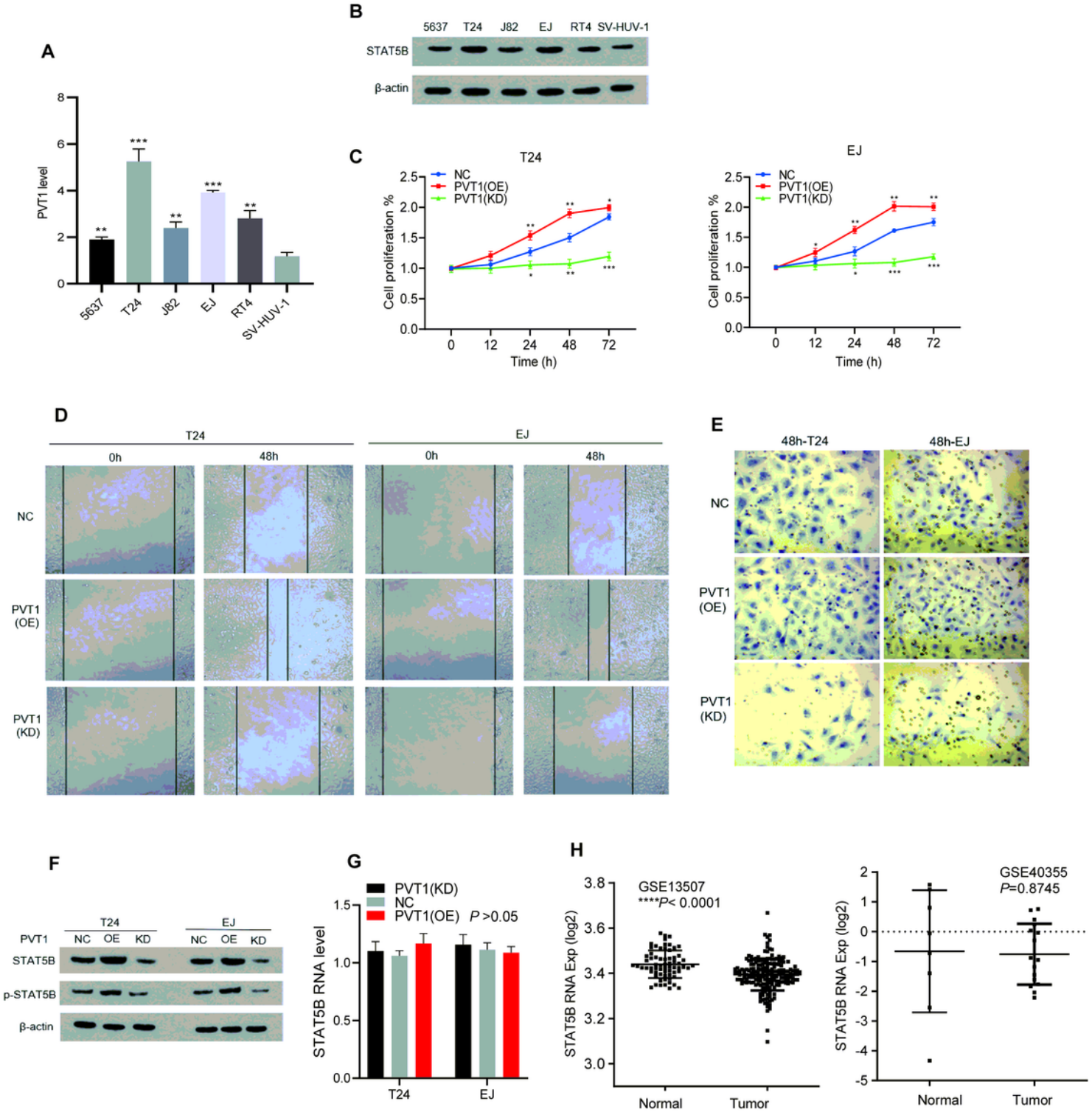
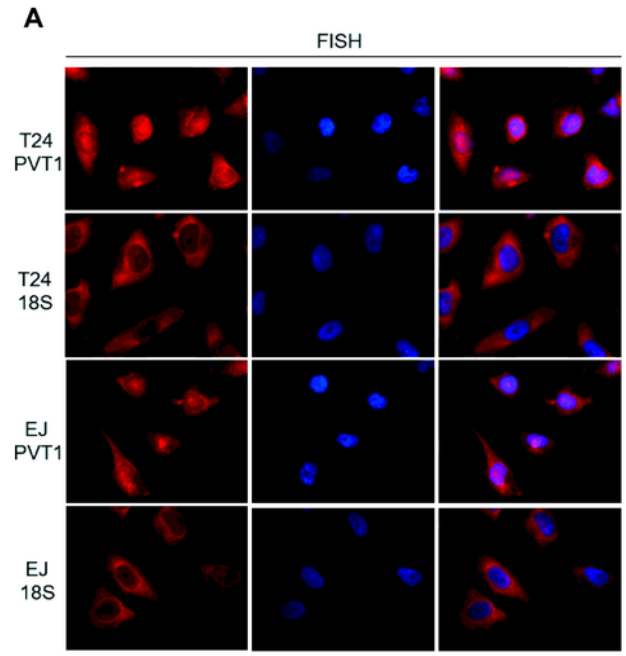
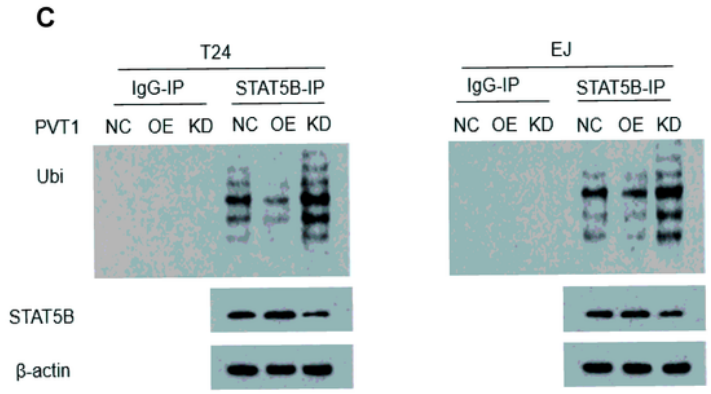


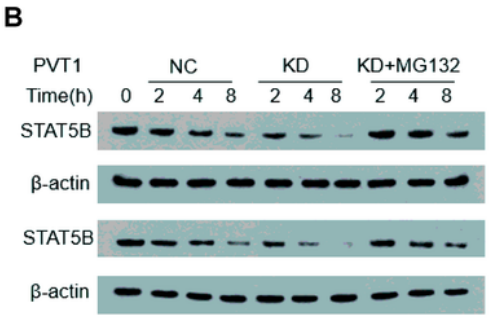
Figure 2

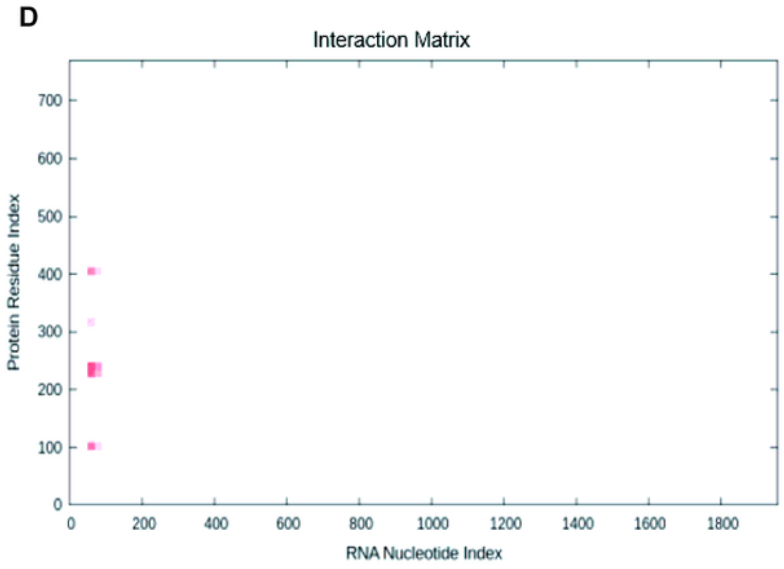
Effect of lncRNA PVT1 level on BC tumor growth and STAT5B level in vivo. A. LncRNA PVT1 levels of five BC cell lines (5637, T24, J82, EJ and RT4) and normal bladder uroepithelial cell lines SV-HUV-1. B. STAT5B expression levels of five BC cell lines (5637, T24, J82, EJ and RT4) and normal bladder

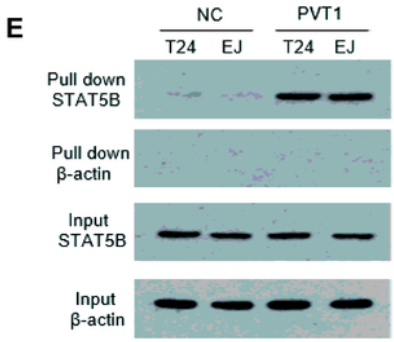
uroepithelial cell lines SV-HUV-1 by WB. C-E. The proliferation (C), migration (D) and invasion (E) ability of BC cells in negative control (NC) group, lncRNA PVT1 overexpressed (OE) group and lncRNA PVT1 knockdown (KD) group were evaluated by cell counting test, wound healing assay and invasion assay. F. STAT5B and p-STAT5B protein levels in negative controls (NC), lncRNA PVT1 overexpression (OE) and lncRNA PVT1 knockdown (KD) groups by WB. G. STAT5B RNA levels in negative control (NC) group, lncRNA PVT1 overexpressed (OE) group and lncRNA PVT1 knockdown (KD) group. H. The STAT5B RNA levels in GSE13507 and GSE40355 database. *P < 0.05, **P < 0.01, ***P < 0.001, ****P< 0.0001.











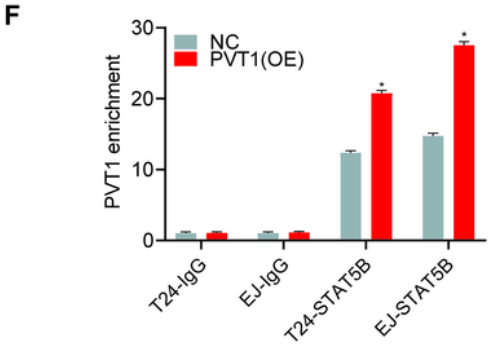


Figure 3

LncRNA PVT1 binds to STAT5B and stabilizes STAT5B via deubiquitination. A. FISH experiments suggested that lncRNA PVT1 was predominately expressed in the nucleus. Ribosomal RNA (18S) was used as the reference gene. B. STAT5B expression levels in negative control (NC) group, lncRNA PVT1 knockdown (KD) group and lncRNA PVT1 knockdown with MG132 (KD+MG132) group. C. The STAT5B-ubiquitination (Ubi) modification levels and total STAT5B levels of negative control (NC), lncRNA PVT1 overexpressed (OE) and lncRNA PVT1 knockdown (KD) group. D. The protein-RNA interaction matrix in RNA pull-down assay. E. RNA pull-down results of lncRNA PVT1 probe and negative control (NC) probe. F. RIP assay demonstrated that STAT5B remarkably immunoprecipitated lncRNA PVT1 in negative control (NC) group and lncRNA PVT1 overexpressed (OE) group. *P < 0.05.

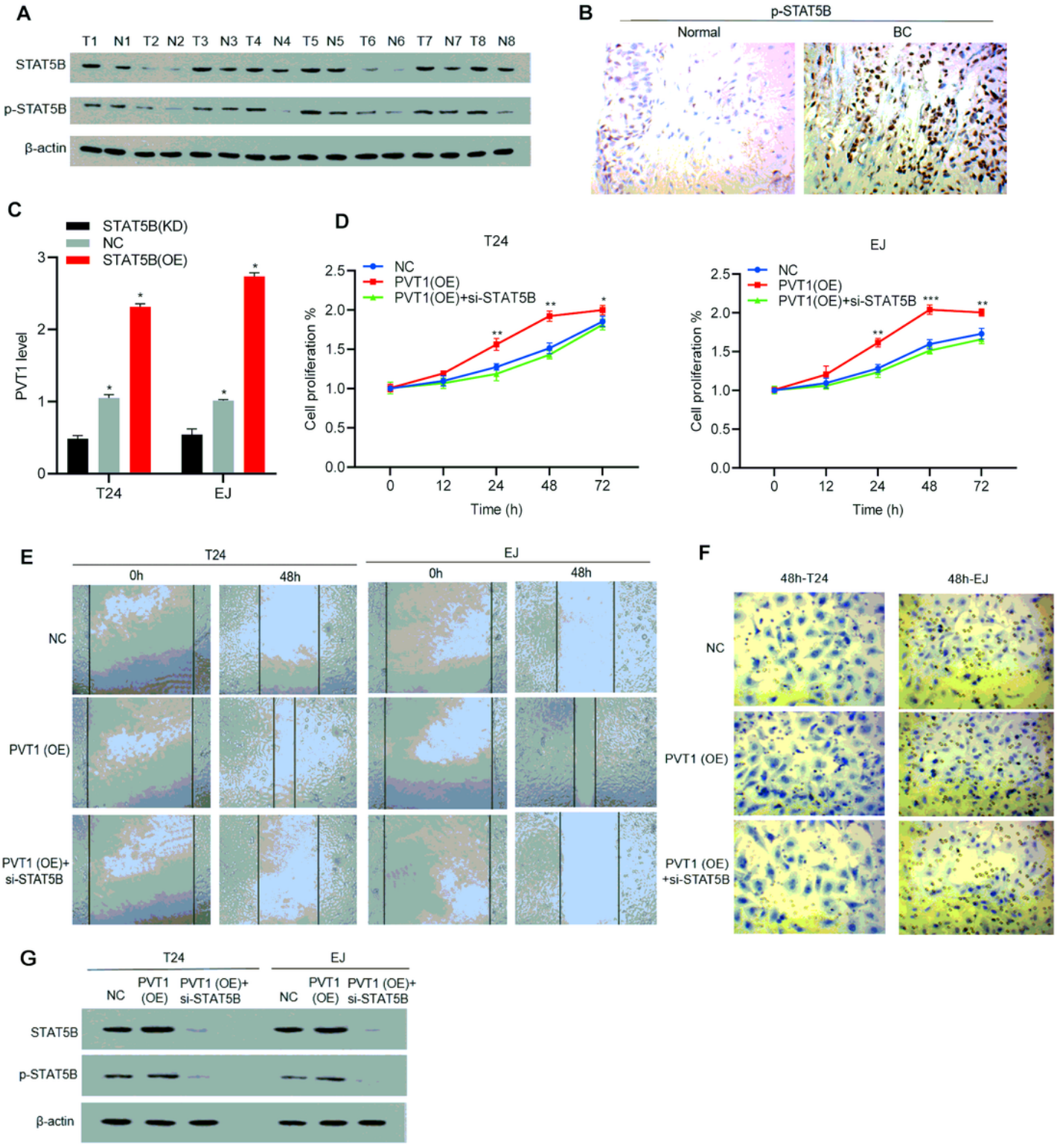


Figure 4

si-STAT5B reverses the lncRNA PVT1-induced tumorigenesis in BC. A. STAT5B and p-STAT5B expression levels of 8 paired of BC tissues (T) and adjacent normal tissues (N) by WB. B. p-STAT5B in BC tissues and adjacent normal tissues. C. LncRNA PVT1 levels in negative control (NC), STAT5B overexpressed (OE) and STAT5B knockdown (KD) groups. D-F. The proliferation (D), migration (E) and invasion (F) ability of BC cells in negative control (NC) group, lncRNA PVT1 overexpressed (OE) group and lncRNA

PVT1 overexpression with si-STAT5B transfection (PVT1(OE)+si-STAT5B) group were evaluated by cell counting test, wound healing assay and invasion assay. G. STAT5B and p-STAT5B levels in negative control (NC), lncRNA PVT1 overexpressed (OE) and lncRNA PVT1 overexpression with si-STAT5B transfection (PVT1 (OE)+si-STAT5B) groups. *P < 0.05, **P < 0.01, ***P < 0.001.

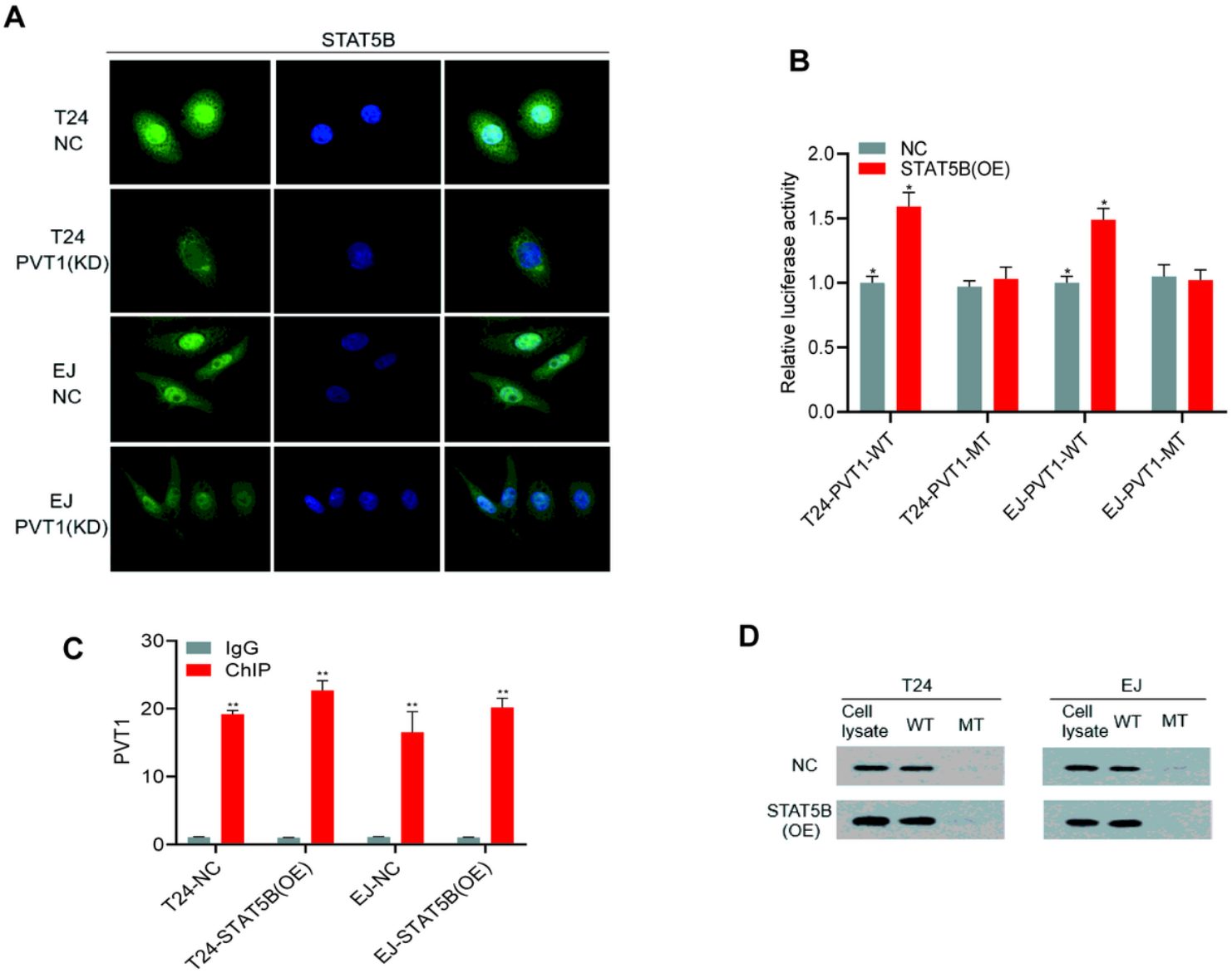


Figure 5

STAT5B activates the transcription of lncRNA PVT1 in BC cells. A. IF results of STAT5B in lncRNA PVT1 knockdown (KD) and negative control (NC) group. B. The relative luciferase activity of the wild-type (WT) lncRNA PVT1 promoter and the mutant (MT) lncRNA PVT1 promoter in negative control and STAT5B overexpressed (OE) groups. C. The ChIP results demonstrated that STAT5B was remarkably enriched in the lncRNA PVT1 promoter region with IgG as a control, especially in STAT5B overexpressed (OE) group. D. DAPA results showed that the wild-type (WT) probe pulled down more STAT5B protein than the mutant (MT) probe did, especially in STAT5B overexpressed (OE) group. *P < 0.05, **P < 0.01.

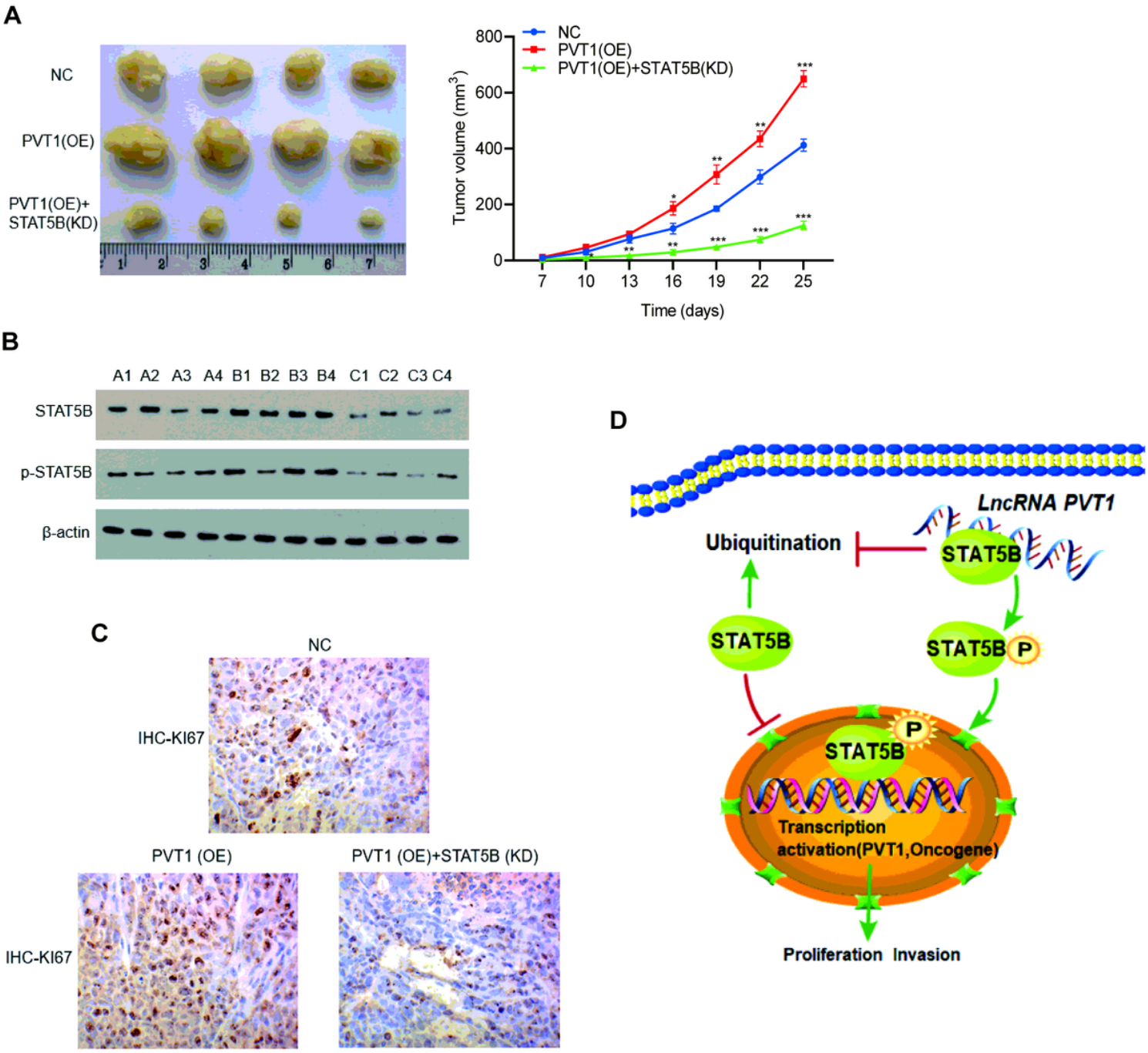


Figure 6

LncRNA PVT1 exerts its oncogenic effect for BC via STAT5B in vivo. A. Tumor growth of negative control (NC) mice, lncRNA PVT1 overexpressed (OE) mice and lncRNA PVT1 overexpression with STAT5B knockdown (PVT1 (OE)+STAT5B (KD)) mice. B. STAT5B and p-STAT5B expression levels of three groups of mice. A: negative control; B: lncRNA PVT1 overexpression; C: lncRNA PVT1 overexpression with STAT5B knockdown. C. IHC results in negative control (NC) mice, lncRNA PVT1 overexpressed (OE) mice and lncRNA PVT1 overexpression with STAT5B knockdown (PVT1 (OE)+STAT5B (KD)) mice. D. Schematic of the present study.