

PBOV1 promotes prostate cancer proliferation by promoting G₁/S transition

Tiejun Pan^{1,*}
 Rongpei Wu^{2,*}
 Bo Liu¹
 Handong Wen¹
 Zhong Tu¹
 Jun Guo¹
 Jiarong Yang¹
 Guoqiu Shen¹

¹Department of Urology, Wuhan General Hospital of Guangzhou Military Command, Wuhan, Hubei,
²Department of Urology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, People's Republic of China

*These authors contributed equally to this work

Abstract: Prostate cancer (PC) is one of the leading causes of cancer death in men, and thus, finding new regulators is critical for PC therapy. Prostate and breast cancer overexpressed 1 (PBOV1) is overexpressed in breast, prostate, and bladder cancers, as it is upregulated in the serum of patients with PC, but the role of PBOV1 in PC has not been studied. In this article, we found that PBOV1 was indeed overexpressed in PC cells; PBOV1 overexpression promoted cell proliferation and colony formation ability and arrested cell cycle in the G₀/G₁ phase and tumorigenicity ability in vitro, whereas knockdown of PBOV1 reduced these effects. Further analysis of PBOV1 overexpression inhibited cell cycle inhibitors, P21 and P27, and increased the phosphorylation level of Rb and cyclin D1 expression, suggesting that PBOV1 promoted cell proliferation through promoting G₁/S transition.

Keywords: prostate cancer, PBOV1, cell proliferation, G₁/S transition

Introduction

Prostate cancer (PC) is one of the most common malignancies in men; age, family, and race are the major risk factors. New cases were 1.1 million (15% of all cancers in males) and cancer deaths were 0.3% million (7% of all cancer deaths in males) in 2012.¹ A better understanding of the regulation of PC initiation and progression is critical for prevention or therapy. Some genes or mutations have been reported that could drive PC progression. For example, at least 22 unique somatic mutations of tumor suppressor gene ATBF1 (zinc finger homeobox 3), which inhibits AFP and MYB and transactivates CDKN1A, have been found in patients with PC.² Transcriptional corepressor C-terminal binding protein 1 (CtBP1) is overexpressed in metastatic PC cells, and the knockdown of CtBP1 reduces proliferation and invasion; further analysis finds that CtBP1 regulated invasion mediated by lipocalin 2.³

Prostate and breast cancer overexpressed 1 (PBOV1; also named as UROC28 or UC28), which contains 135 amino acids, was found in 2000, and it is significantly overexpressed in primary and metastatic PC tissues in both protein and mRNA levels and located in the glandular epithelium.⁴ Doak et al also confirmed that PBOV1, which is like BMP2, BMP5, and BMP7 expression, is higher in PC than prostatic intraepithelial neoplasia and adjacent benign epithelium.^{5,6} Samusik et al found that PBOV1 is poorly conserved in the mammalian evolution. It is overexpressed in several kinds of tumors, for example, breast, lung, and bladder cancers but is not expressed in the normal tissues. They thought that PBOV1 may be regulated by C/EBPβ, EP300, FOXA1, FOXA2, and Hedgehog signaling pathway through analyzing ChIP-seq data from the Encyclopedia of DNA Elements (ENCODE) project, but they do not confirm this result using experiments. They also analyzed the relationship between PBOV1 expression and the outcome and

Correspondence: Tiejun Pan
 Department of Urology, Wuhan General Hospital of Guangzhou Military Command, Wuhan, Hubei 430070, People's Republic of China
 Email mnwkptj@aliyun.com



found that high PBOV1 expression in breast cancer and glioma tissues correlates with poor outcome.⁷ Although PBOV1 has been demonstrated to be upregulated in PC cells and tissues, the role of PBOV1 in PC has not been reported.

In the present study, we used Western blot and real-time polymerase chain reaction (PCR) to determine the expression of PBOV1 in PC cell lines and then determined the role of PBOV1 in cellular proliferation, tumorigenicity ability in vitro, and cell cycle progression by modulating its expression. Finally, we analyzed whether key cell cycle regulatory proteins were regulated by PBOV1.

Material and methods

Cell lines and plasmids

The primary prostate epithelial cell line was constructed in our laboratory and indicated as N. PC cell lines, including PC3, 22Rv1, Tsu-Pr1, LNCaP, and DU145, were obtained from the American Type Culture Collection (ATCC), (Manassas, VA, USA). N was cultured in Ham's F12 medium (Thermo Fisher Scientific, Waltham, MA, USA) with 5% fetal bovine serum (Thermo Fisher Scientific), 5 µg/mL insulin, 10 ng/mL epidermal growth factor, and 1 µg/mL hydrocortisone (Sigma-Aldrich Co., St Louis, MO, USA). PC cell lines were maintained in RPMI1640 medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum.

The retroviral vector pMSCV-PBOV1 was used to overexpress PBOV1, and the empty vector served as negative control (indicated as vector). PBOV1 short hairpin RNAs (shRNAs) were designed and cloned in the retroviral vector pSUPER. retro.puro obtained from Oligoengine (Seattle, WA, USA).⁸ The sequence of shRNA was as follows: PBOV1 shRNA#1: CCAGCCAAGTAACTGAACCAT and PBOV1 shRNA#2: GCAGACACACTTGACCATGAA. pMSCV-vector and scramble plasmids were used as negative controls (indicated as vector and scramble, respectively). Then, 1.5 µg vector and 4.5 µL FuGENE HD (Hoffman-La Roche Ltd, Basel, Switzerland) were used for transfection; puromycin was used to screen the transfected cells to generate stable cell lines.

RNA extraction, cDNA synthesis, and real-time PCR

RNA extraction, cDNA synthesis, and real-time PCR were performed according to the manufacturer's protocol. Trizol (Thermo Fisher Scientific) was used to extract RNA. HiScript 1st Strand cDNA Synthesis Kit (R111-02; Vazyme Biotech Co., Ltd, Nanjing, People's Republic of China) was used to generate mRNA. Real-time PCR was carried out on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories

Inc., Hercules, CA, USA). AceQ® qPCR SYBR® Green Master Mix (Q111-02; Vazyme Biotech Co., Ltd) was used to determine gene expression changes. The gene expression was defined based on the threshold cycle (C_T); glyceraldehyde 3-phosphate dehydrogenase was used to normalize the measured transcript, and samples were run in triplicate. The relative expression was calculated as:

$$2^{-[(C_{T, \text{of PBOV1, p21p27, ki67}}) - (C_{T, \text{of GAPDH}})]} \quad (1)$$

Western blot assay

Western blot assay was performed according to the methods as described previously⁹ using anti-PBOV1 (orb101850; Biorbyt Ltd, Cambridge, UK), anti-cyclin D1 (SC-253, 1:3,000; Santa Cruz Biotechnology Inc, Dallas, TX, USA), anti-P21 (SC-397, 1:1,000), anti-P27 (SC-528, 1:2,500), anti-Rb (#9309, 1:1,000; Cell Signaling Technology, Inc, Danvers, MA, USA), anti-phosphorylated Rb (ab47763, 1:500; Abcam, Cambridge Science Park, Cambridge, UK), and anti-Ki67 (SC-15402, 1:1,000) antibodies. The membranes were stripped and reprobed with anti- α -tubulin antibody (SC-8035, 1:1,000) as a loading control.

MTT assay

The effect of PBOV1 on cell proliferation was measured using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells, seeded on 96-well plates, were stained at the indicated time point with 100 µL sterile MTT dye (0.5 mg/mL; Sigma-Aldrich Co.) for 4 hours at 37°C, followed by the removal of the culture medium and addition of 150 µL of dimethyl sulfoxide (Sigma-Aldrich Co.). The absorbance was measured at 570 nm. All experiments were performed in triplicates.

Colony formation analysis

Cells were seeded in six-well plates at a density of 500 cells/well and cultured for 10 days. Colonies were fixed with 10% formaldehyde for 5 minutes followed by staining with 1.0% crystal violet for 30 seconds. The cells were photographed and counted.

Bromodeoxyuridine labeling and immunofluorescence

Cells were grown on cover slips (Thermo Fisher Scientific) and incubated with bromodeoxyuridine (BrdU) for 1 hour; anti-BrdU antibody (Upstate, Temecula, CA, USA) was then stained according to the manufacturer's instructions. Images were acquired using a Carl Zeiss fluorescence microscope.

Cell cycle assay

Cells were harvested and washed using cold phosphate-buffered saline (PBS) followed by fixation in 70% ethanol for at least 30 minutes at 4°C. After washing in cold PBS twice, cells were resuspended in PBS containing 2 µg/mL RNase (Sigma-Aldrich Co) and incubated at 37°C for 30 minutes, and then 20 µg/mL of propidium iodide was added (Sigma-Aldrich Co) for 20 minutes at room temperature. Therefore, 5×10⁴ cells were analyzed on a FACSCalibur cytometer (BD Biosciences, San Jose, CA, USA).

Soft agar growth analysis

A total of 500 cells were resuspended in a 2 mL complete medium with 0.3% agar (Sigma-Aldrich Co.) after the cells were trypsinized. The agar–cell mixture was placed on the top of a bottom layer in which 1% agar and complete medium were mixed. After 10 days, visible colonies that contained more than 50 cells or were larger than 0.1 mm were measured. The size of colonies larger than 0.1 mm in diameter was counted using an ocular micrometer. All experiments were performed in triplicates.

Replicates and statistical analysis

All experiments were performed three times, and the results are presented as the mean ± standard deviation using SPSS Version 10.0 Software (SPSS Inc., Chicago, IL, USA). Statistical

differences were determined by Student's *t*-test (two-tailed). *P*<0.05 was considered statistically significant.

Results

PBOV1 overexpression promotes proliferation and tumorigenicity of PC cells

To explore the role of PBOV1 in the progression of PC, we first determined the expression of PBOV1 between PC cells and primary prostate epithelial cells, and found that PBOV1 expression in PC cells was higher than that in primary prostate epithelial cells detected by both real-time PCR (Figure S1A) and Western blot analyses (Figure S1B). This result was in agreement with the previous report⁴ that PBOV1 was overexpressed in PC cells.

Given that PBOV1 was highly expressed in PC cells, it might promote PC progression. We overexpressed PBOV1 in PC cells, PC3 and DU145, to determine its effect on cell proliferation (Figure S2). MTT assay found that PBOV1 overexpression increased cell proliferation rate in both indicated cells compared with empty vector control cells (Figure 1A). Colony formation assay also found that PBOV1 overexpression significantly increased the colony numbers of indicated cells (Figure 1B). Soft agar growth assay found that PBOV1 overexpression significantly increased their anchorage-independent growth ability (Figure 1C), as shown

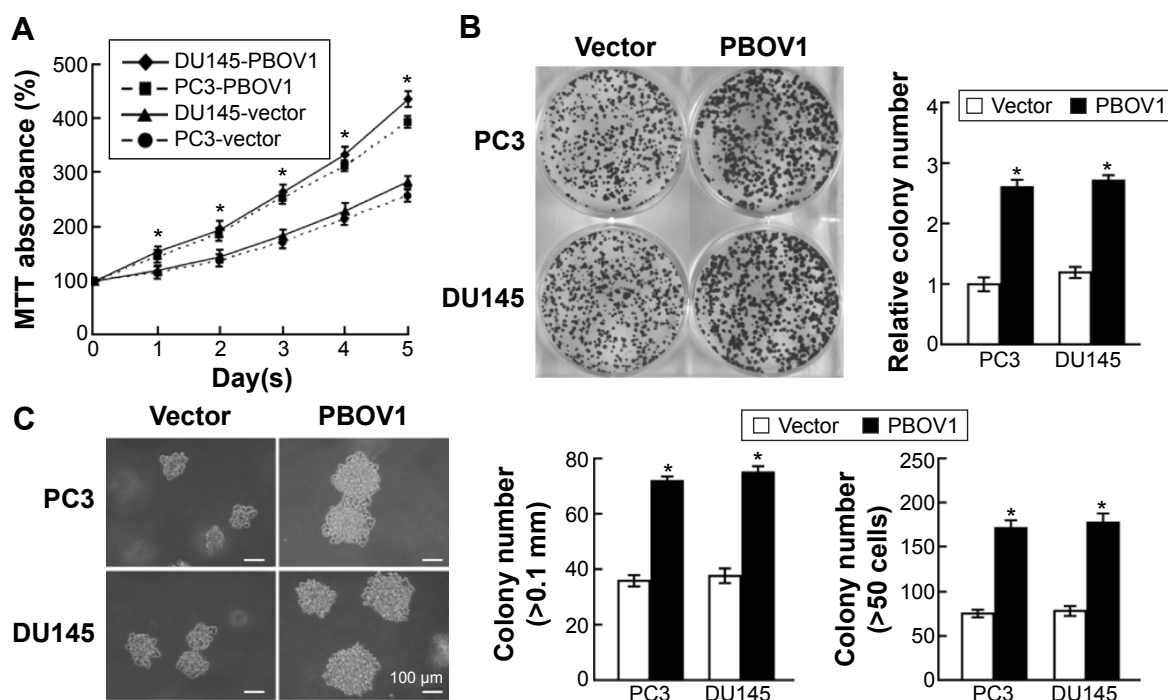


Figure 1 (Continued)

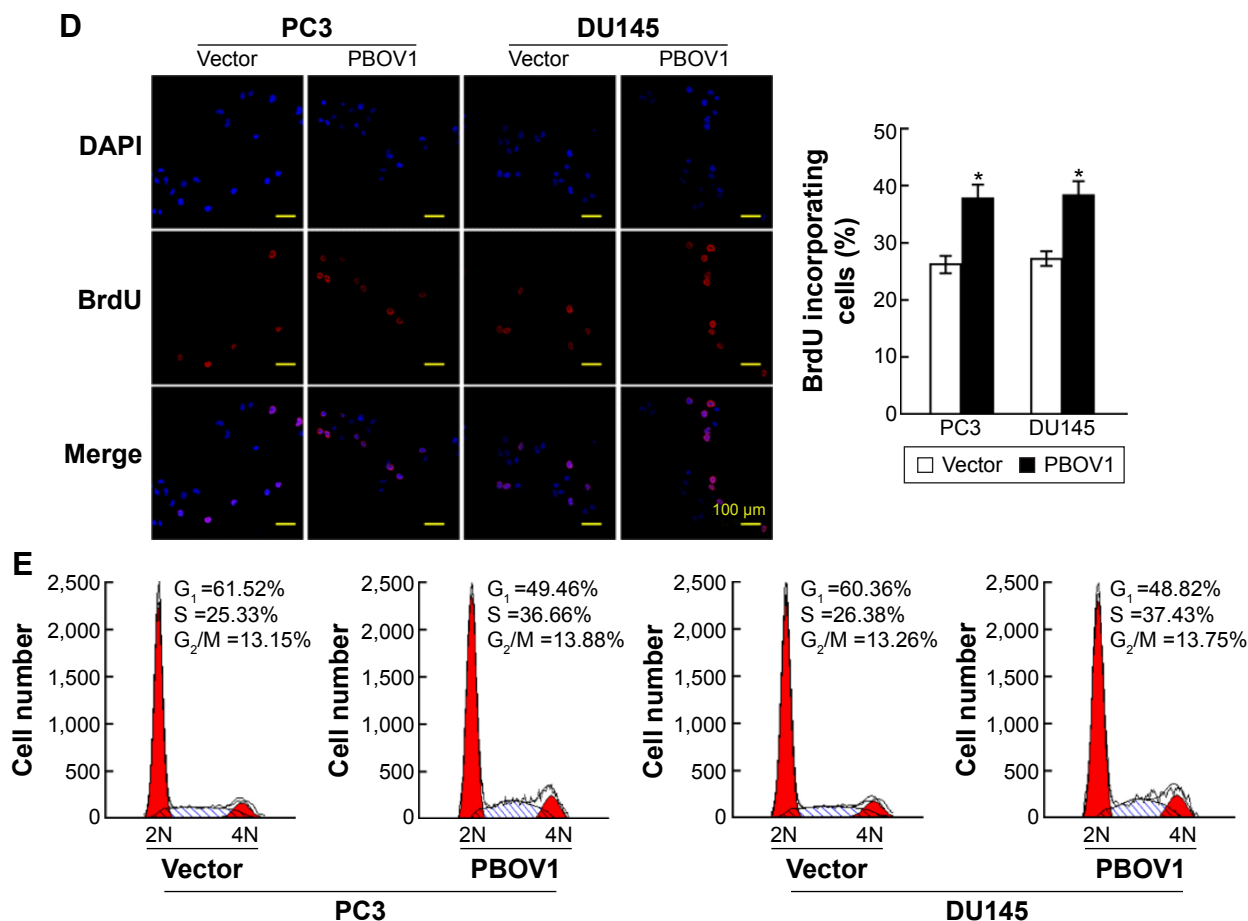


Figure 1 Overexpression of PBOV1 promotes prostate cancer cell proliferation.

Notes: (A) MTT assay found that PBOV1 overexpression induced the growth of DU145 and PC3 prostate cancer cell lines. (B) Colony formation assay revealed that PBOV1 overexpression promoted prostate cancer cell proliferation, representative micrographs (left), and quantification (right) of crystal violet-stained cell colonies. (C) PBOV1 overexpression promoted the anchorage-independent growth of prostate cancer cells, representative micrographs (left), and quantification of colonies that were >0.1 mm and >50 cells (right). Each scale bar represents 100 μ m. (D) BrdU incorporation assay found that PBOV1 overexpression promoted cell proliferation. Representative micrographs (left) and quantification of BrdU-incorporating cells (right). Each scale bar represents 100 μ m. (E) Flow cytometric analysis found that PBOV1 overexpression induced the percentage of increased G₁ phase cells. Each bar represents the mean of three independent experiments. * $P < 0.05$.

Abbreviations: BrdU, bromodeoxyuridine; DAPI, 4',6-diamidino-2-phenylindole; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBOV1, prostate and breast cancer overexpressed 1.

by increased colony numbers and sizes, this result suggested that PBOV1 overexpression enhanced PC tumorigenicity *in vitro*.

To address the mechanism responsible for the effects of the overexpression on the accumulation of PC cells in culture, we determined whether PBOV1 overexpression influenced cell cycle progression. The BrdU incorporation assay found that the percentage of S phase cells were significantly increased in PBOV1 overexpression of PC cells, as compared with that of empty vector control cells (Figure 1D). Cell cycle analysis used flow cytometry to further reveal that PBOV1 overexpression induced the percentage of increased G₁ phase cells and the percentage of S phase increased in two indicated cells (Figure 1E); these data suggested that PBOV1 overexpression promoted cell cycle progression and contributed to progression from G₁ to S.

Knockdown of PBOV1 inhibits proliferation and tumorigenicity of PC cells

To confirm the role of PBOV1 in anchorage-dependent and anchorage-independent growth, we used two shRNAs to deplete PBOV1 expression, respectively (Figure S2). MTT assay found that the knockdown of PBOV1 in PC3 and DU145 reduced the proliferation rate, as compared with that of scramble control cells (Figure 2A), and colony formation assay also found that the colony numbers significantly reduced in indicated prostate cells with PBOV1 knockdown, as compared with that of scramble control cells (Figure 2B). Soft agar assay found that the knockdown of PBOV1 significantly decreased its anchorage-independent growth ability, as shown by reduced colony numbers and sizes; this suggested

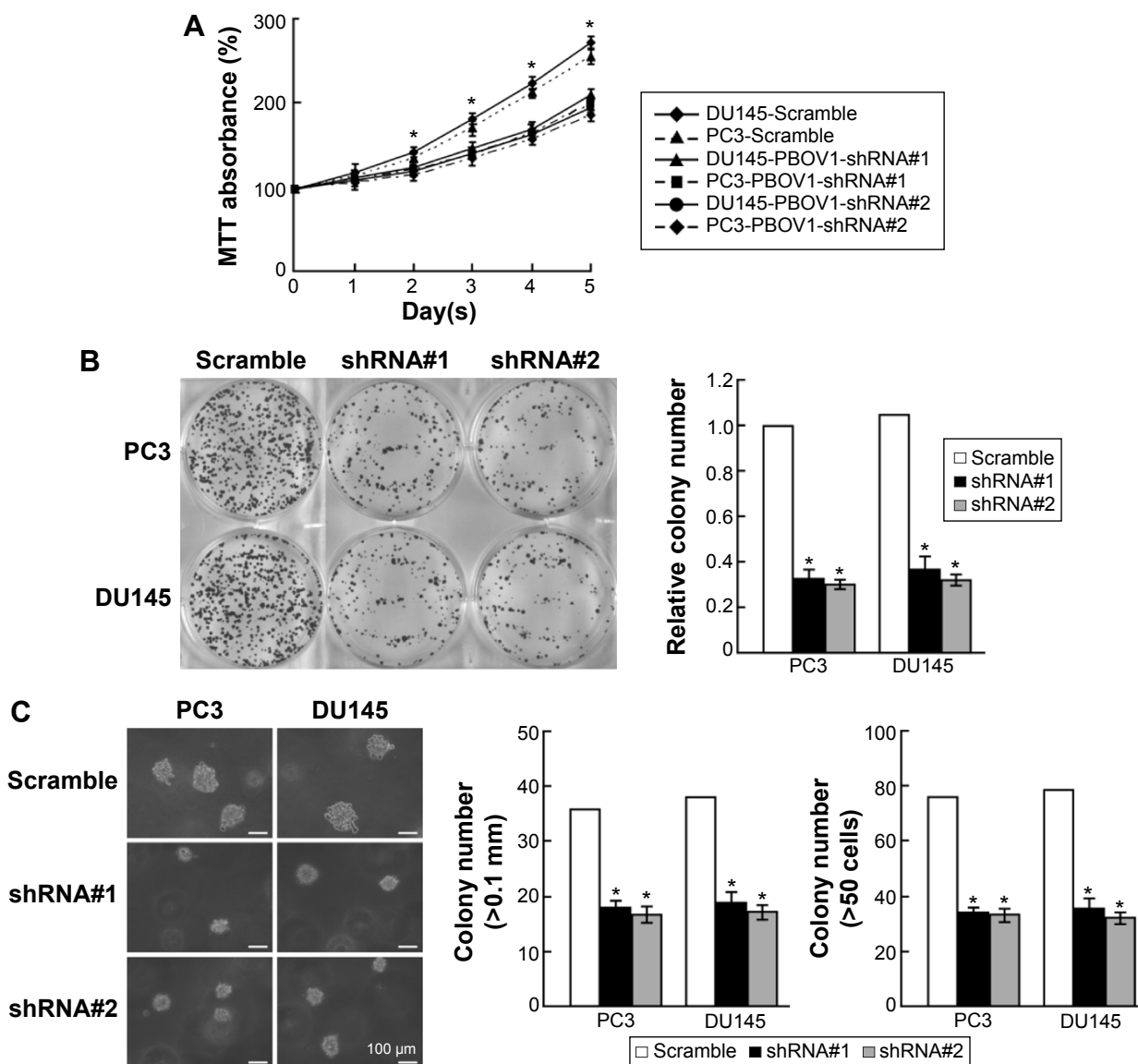


Figure 2 Knockdown of PBOV1 suppresses prostate cancer cell proliferation.

Notes: (A) MTT assay found that PBOV1 knockdown inhibited the growth of DU145 and PC3 prostate cancer cell lines. (B) Colony formation assay revealed that PBOV1 knockdown inhibited prostate cancer cells proliferation, representative micrographs (left), and quantification (right) of crystal violet-stained cell colonies. (C) PBOV1 knockdown inhibited the anchorage-independent growth of prostate cancer cells, representative micrographs (left), and quantification of colonies that were >0.1 mm and >50 cells (right). Each bar represents the mean of three independent experiments. $*P < 0.05$. Each scale bar represents 100 μ m.

Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBOV1, prostate and breast cancer overexpressed I; shRNA, short hairpin RNA.

that the knockdown of PBOV1 inhibited PC tumorigenicity in vitro (Figure 2C).

Therefore, we further confirmed the effects of PBOV1 on cell cycle progression. The BrdU incorporation assay found that the percentage of S phase cells was significantly decreased in PC cells with PBOV1 knockdown as compared with that of scramble control cells (Figure 3A). Cell cycle analysis used flow cytometry to reveal that the knockdown of PBOV1 induced a partial G_1 arrest in two indicated cells (Figure 3B); these further demonstrated that PBOV1 contributed to the transition from G_1 to S.

PBOV1 regulates key regulators about G_1/S transition

We found that PBOV1 regulated cell cycle by regulating G_1/S transition, so we determined the expression of key proteins concerning this process to confirm our results. P21 and P27 are key cell cycle inhibitors,^{10,11} they were downregulated once in PBOV1 overexpression, as compared with that of vector control cells (Figure 4A and B), suggesting PBOV1 overexpression inhibited the expression of cell cycle inhibitors. The expression of cell proliferation marker Ki67 was also increased (Figure 4A and B). Ki67 is a nuclear antigen;

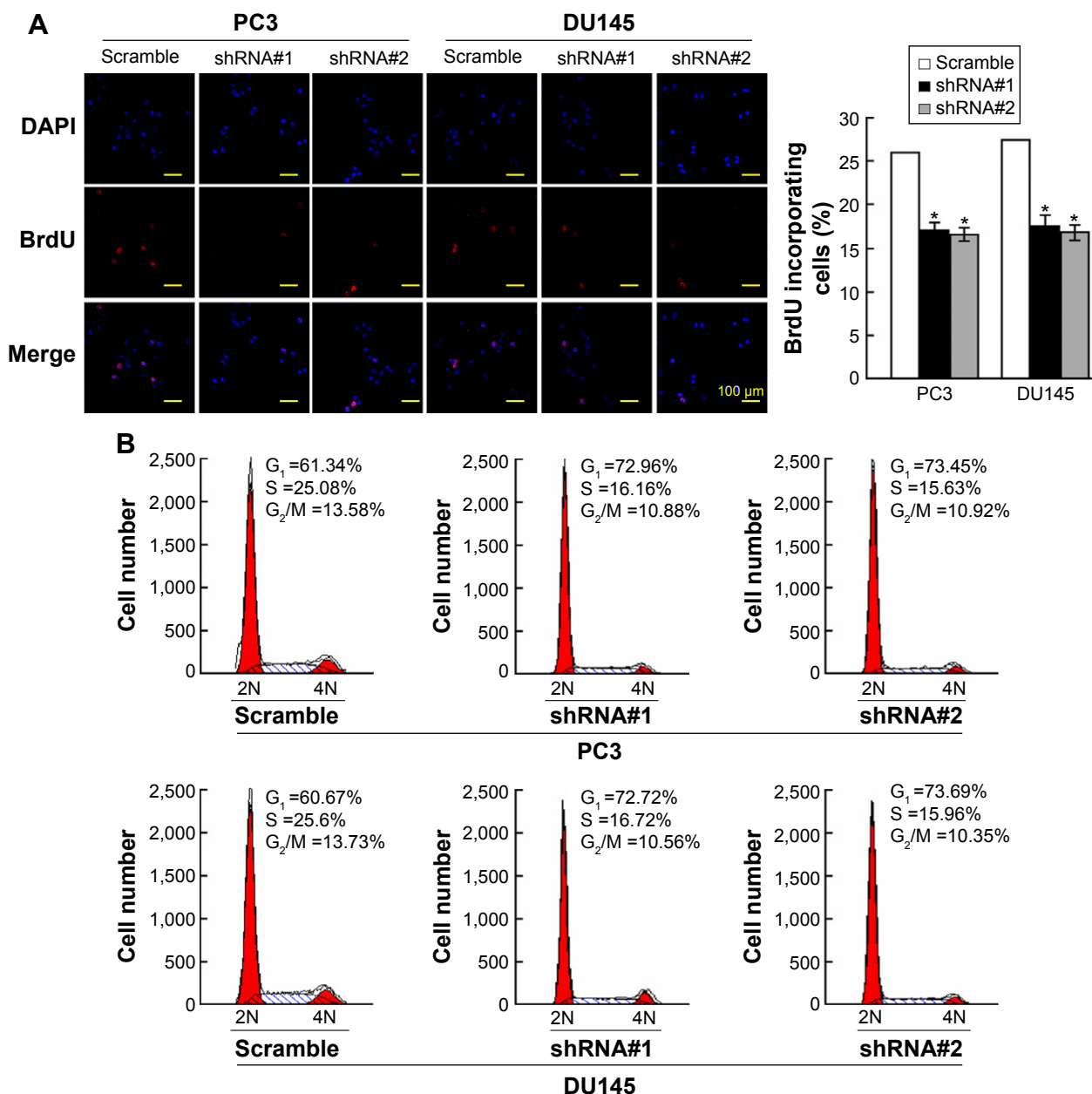


Figure 3 Knockdown of PBOV1 suppresses cell cycle of prostate cancer cell progression.

Notes: (A) BrdU incorporation assay found that PBOV1 knockdown inhibited cell proliferation. Representative micrographs (left) and quantification of BrdU-incorporating cells (right). Each scale bar represents 100 μ m. (B) Flow cytometric analysis found that PBOV1 knockdown induced the percentage of reduced G₁ phase cells. Each bar represents the mean of three independent experiments. * $P < 0.05$.

Abbreviations: BrdU, bromodeoxyuridine; DAPI, 4',6-diamidino-2-phenylindole; PBOV1, prostate and breast cancer overexpressed 1; shRNA, short hairpin RNA.

it only expresses in G₁, S, and G₂ phases but not in G₀ phase, and it has been used as a prognosis factor in breast cancer.^{12,13} Cyclin D1 can interact with Cdk4 and Cdk6 to phosphorylate Rb; the phosphorylation of Rb causes E2F to dissociate from Rb-E2F complex, and E2Fs transactivate genes, which are essential for DNA replication and subsequent events, for example, MCMs and cyclin B.^{14,15} Therefore, the increase in cyclin D1 expression and phosphorylation of Rb are essential for G₁/S transition. From our results, we found that cyclin D1 expression and phosphorylation level of Rb were

increased (Figure 4A and B). These suggested that PBOV1 promoted cell cycle progression by regulating key proteins about G₁/S transition.

Discussion

In the present study, we determined the role of PBOV1 in PC and also found that PBOV1 was overexpressed in PC cells; the overexpression of PBOV1 promoted cell proliferation, cell cycle progression, and tumorigenicity in vitro, whereas the knockdown of PBOV1 reduced these effects.

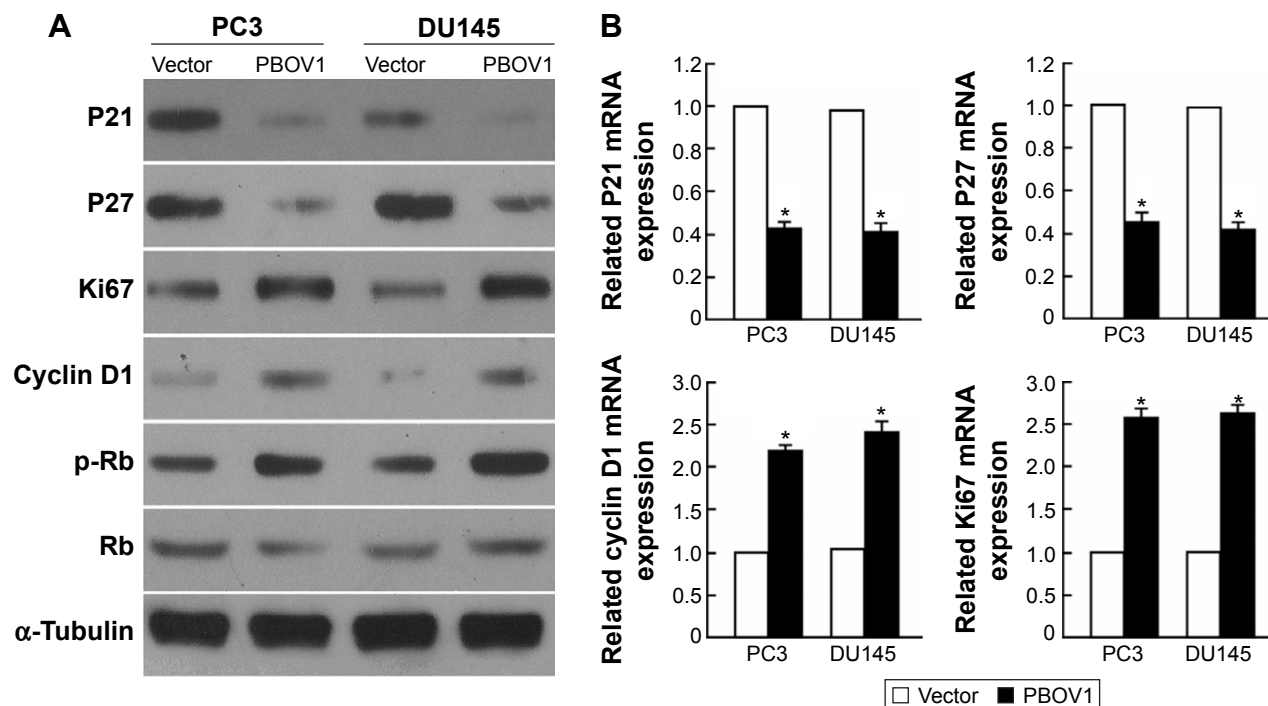


Figure 4 PBOV1 regulates key regulators about G₁/S transition.

Notes: (A) Western blot assay found that the expression of P21, P27, Ki67, cyclin D1, Rb and p-Rb, and α -Tubulin was used as the loading control. (B) Real-time PCR analysis determined the expression of P21, P27, cyclin D1, and Ki67 after the overexpression of PBOV1. Each bar represents the mean of three independent experiments. * $P < 0.05$. **Abbreviations:** PBOV1, prostate and breast cancer overexpressed 1; PCR, polymerase chain reaction.

Further analysis found that the overexpression of PBOV1 suppressed cell cycle inhibitors, P21 and P27, and increased Rb phosphorylation levels and cyclin D1 expression and suggested that PBOV1 contributed to G₁/S transition. It has been reported that PBOV1 is overexpressed in PC and our results are consistent with this conclusion. Hedgehog signaling pathway plays a critical role in PC; it is required for PC cell proliferation and tumor growth and is considered a characteristic feature of PC. Hedgehog signaling also promotes self-renewal of PC stem cells.^{16,17} Inhibition of hedgehog signaling can suppress the progression of PC; for example, LKB1 can inhibit PC proliferation by suppressing hedgehog signaling.¹⁸ Hedgehog signaling inhibitor GDC-0449 plus docetaxel inhibit PC proliferation effectively.¹⁹ Samusik et al found that Hedgehog signaling is one of the drivers of PBOV1 activation;⁷ this suggests that PBOV1 may be a downstream effector molecule of Hedgehog signaling, but whether PBOV1 activates Hedgehog signaling is still to be explored. We believed the proteins that interact with PBOV1 are important for explaining its regulation mechanism.

Conclusion

In summary, we found that PBOV1 is an oncogene and promotes PC proliferation and tumorigenicity. Nevertheless,

its regulatory mechanism and other functions remain to be elucidated.

Acknowledgments

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The research was approved by the ethics committee of Wuhan General Hospital of Guangzhou Military Command.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

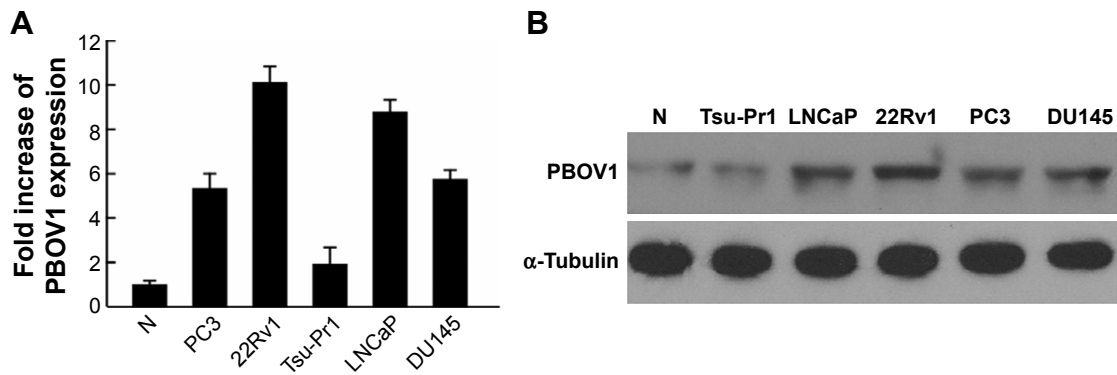


Figure S1 Upregulation of PBOV1 in human prostate cancer cell lines.

Notes: (A) Real-time PCR determined the expression of PBOV1 in primary prostate epithelial cell lines (indicated as N) and prostate cancer cell lines, including PC3, 22Rv1, Tsu-Pr1, LNCaP, and DU145. (B) Western blot assay determined PBOV1 expression in primary prostate epithelial cell lines and prostate cancer cell lines. Each bar represents the mean of three independent experiments.

Abbreviations: PBOV1, prostate and breast cancer overexpressed I; PCR, polymerase chain reaction.

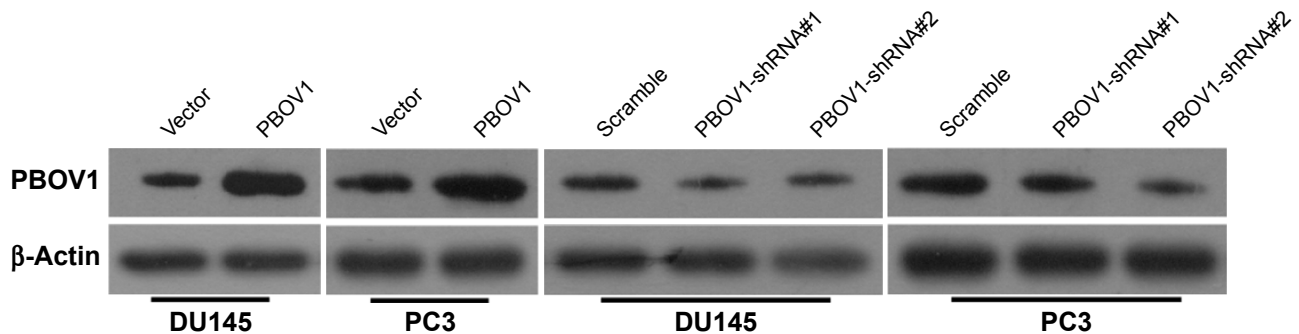


Figure S2 Determination of the effect of PBOV1 overexpression and knockdown in PC3 and DU145 using Western blot analysis.

Abbreviations: PBOV1, prostate and breast cancer overexpressed I; shRNAs, short hairpin RNAs.

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