

## Original Article

# PLC $\epsilon$ knockdown prevents serine/glycine metabolism and proliferation of prostate cancer by suppressing YAP

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**Abstract:** The metabolic reprogramming is an important basis for the development of many tumors, including prostate cancer (PCa). Metabolic changes in many amino acids consist of serine and glycine affect the biological behavior of them. Phospholipase C epsilon (PLC $\epsilon$ ) plays an important role as an oncogene. However, its role in regulating amino acid metabolism remains unclear. In this study, results found significantly positive correlation between PLC $\epsilon$  and Yes-associated protein (YAP) in PCa tissues. LC-MS/MS and GC-MS results further displayed abnormally elevated levels of serine, glycine and its some downstream metabolites in the blood of PCa patients. Secondly, PLC $\epsilon$  knockdown can inhibit serine/glycine producing and proliferation of PCa both in vivo and in vitro. Mechanistically, PLC $\epsilon$  may affect the serine/glycine metabolism by regulating dephosphorylation and nuclear translocation of YAP. More interestingly, verteporfin (VP, a specific inhibitor of YAP) could effectively enhance the PLC $\epsilon$ -depletion induced inhibition of serine/glycine secretion and growth. Overall, this research revealed the possibility of anomalous serine/glycine levels in the blood for the diagnosis of PCa, identified the important role of the PLC $\epsilon$ /YAP axis in regulating serine/glycine metabolism, cell proliferation and tumor growth, and suggested the combination of VP with PLC $\epsilon$ -depletion may provide a new idea for the treatment of PCa.

**Keywords:** PLC $\epsilon$ , YAP, prostate cancer, serine/glycine metabolism, verteporfin, proliferation

## Introduction

Prostate cancer (PCa) is still one of the most important causes of cancer death in men in developed countries [1]. More urgent is the incidence of PCa in Chinese men dramatically increased in recent years, but the factors that cause it are not fully understood [2].

Metabolism reprogramming is the one of the most important features of tumors, including amino acids uptake disorder [3]. Excessive accumulation of glycine due to obstacles in the glycine deportation system [4], which provides raw materials for the synthesis of biological proteins, purines and nucleic acids [5]. In tumor cells, the intermediates of the glycolytic pathway first generate serine via the key enzymes phosphoserine aminotransferase1 (PSAT1) and phosphoserine phosphatase (PSPH), and then glycine is produced by serine hydroxymethyl-

transferase2 (SHMT2) [6, 7]. Additional, a variety of amino acids are closely related to the progression of PCa [8]. Many reports have pointed to some certain degree of metabolic disorders in PCa patients, including prostate specific antigen (PSA), sarcosine and many more [9-11]. Therefore, it is so necessary to explore the mechanism affects serine/glycine metabolism that provides some ideas for PCa diagnosis and treatment.

PLC $\epsilon$  is a new isozyme of the discovered PLC family. As an important member of the phospholipase family, it is not only an effector of Ras, Rho and Rap, but also mediates signals from G-protein coupled receptors (GPER) [12-14]. Researches found PLC $\epsilon$  is associated with many cancers including esophageal cancer, gastric cancer and so on [15-17]. Previous studies discovered that PLC $\epsilon$  can regulate mitochondrial metabolism of PCa and inducing inva-

sion and metastasis of castration-resistant prostate cancer (CRPC) [18, 19]. However, the regulation mechanism of PLC $\epsilon$  on serine/glycine metabolism in PCa is still unclear.

YAP, as a key effector molecule downstream of the Hippo signaling pathway [20], which is not only highly correlated with the homeostasis and development of organisms [21], but also been found to have significant biological properties in breast cancer, rectal cancer and the like [22, 23]. In recent years, accumulating evidences revealed that YAP is inextricably linked to tumor metabolisms such as glucose and fatty acid metabolism [24]. More importantly, YAP regulates the activity of key enzymes involved in the serine pathway in breast cancer [25]. Consequently, it is particularly essential to explore and confirm the relationship between YAP and tumor serine/glycine metabolism.

In this study, we explored the role of PLC $\epsilon$  in serine/glycine metabolism of PCa, and tried to find a potential relationship between PLC $\epsilon$  and YAP. Therefore, we hypothesized that PLC $\epsilon$  knocking-down would regulate the serine/glycine metabolism and proliferation of PCa by affecting YAP.

### Materials and methods

#### *Blood and tissue samples*

Tissue specimens (including 55 cases of benign prostatic hyperplasia (BPH) and 58 cases of PCa) from 2015 to 2017, blood specimens (including 43 cases of normal samples and 66 cases of PCa) from 2018 to 2019 were all collected from the Department of Urology, the First Affiliated Hospital of Chongqing Medical University. All specimens were histologically diagnosed as BPH or PCa, and the patient's informed consent was obtained before the experiment.

#### *Immunohistochemistry*

All the samples involved in the experiment, including human PCa and BPH specimens, nude mice xenograft tissues were cut into paraffin sections. Detection of PLC $\epsilon$ , YAP and CyclinD1 expression in PCa, BPH and nude mice xenograft tissue samples by immunohistochemical (IHC) staining procedure, antibodies were as follows: anti-PLC $\epsilon$  (dilution 1:50; Santa Cruz Biotechnology); anti-YAP (dilution 1:200;

CST). The standard is the same as we have published before [26].

#### *Cell culture and treatment*

Prostate cell lines PC3, LNCaP and RWPE-1 came from Shanghai Zhong Qiao Xin Zhou Biotechnology Company. DU145 purchased from Shanghai Biowing Applied Biotechnology Company. RWPE-1 cultured with K-SFM medium, and cancer cell lines with RPMI1640 (Gibco), supplemented with 10% of fetal bovine serum (FBS, Gibco), placed in 37°C incubator containing 5% CO<sub>2</sub>, 1% O<sub>2</sub> and 45%-65% humidity. The lentivirus infection method was same as that published in our previous study [27]. Cells (1 × 10<sup>5</sup>/well) were seeded in a six-well plate and incubated with 2 ml of complete medium for 12 hours, and then added with plasmids or VP (MedChemExpress, USA). After 48 hours of incubation, subsequent experiments were performed.

#### *Immunofluorescence*

The cells were cultured on 24-well plate coverslips. Different treatments were added and incubation was continued for 48 hours. The cells were then treated with 4% paraformaldehyde, 0.1-1% Triton and 5% goat blocking serum in sequence. Primary antibody anti-YAP (dilution 1:100; CST) and secondary antibody and 4,6-diamidino-2-phenylindole (Zhongshan Golden Bridge Biotechnology, Beijing, China) were incubated. Immunofluorescence (IF) photographs were taken by Nikon Eclipse 80i microscope (Eclipse 80i, Tokyo, Japan) at 400 × magnification.

#### *Reverse transcription-quantitative polymerase chain reaction*

RNA was extracted with Trizol (Takara, Tokyo, Japan), and PrimeScript RT reagent kits (Takara) was used to reverse RNA. The SYBR enzyme was used to quantify the polymerase chain reaction.  $\beta$ -actin was regarded as a standard reference. The comparative 2<sup>- $\Delta\Delta$ Ct</sup> method was served as calculating the relative expression level of relative messenger RNA (mRNA). Repeat each experiment at least three times. The primer sequences were as following:

PLC $\epsilon$ , Forward: GGAGAATCCTCGGTAG, Reverse: GGTGTGTCAGCGTATGTCC; YAP, Forward: TAGCCTGCGTAGCCAGTTA, Reverse: TCATGCTTAGT-

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CCACTGTCTGT; PSAT1, Forward: TGCCGCACTCAGTGTGTTAG, Reverse: GCAATTCCTCCGCAACAAGATTCT; PSPH, Forward: GAGGACGCGGTGTCAGAAAT, Reverse: GGTTGCTCTGCTATGAGTCTCT; SHMT2, Forward: CCCTTCTGCAACCTCACGAC, Reverse: TGAGCTTATAGGGCATAGACTCG; CyclinD1, Forward: GCTGGAGCCCGTGAAAAGA, Reverse: CTCCGCCTCTGGCATTGTTG; PCNA, Forward: TCAAGAAGGTGTTGGAGGCA, Reverse: CAGCGGTAGGTGTCAAGC;  $\beta$ -actin, Forward: GGGACCTGACTGACTACCTC, Reverse: ACGAGACCACCTTCAACTCCAC.

### Western blot

Total proteins were extracted by RIPA lysate containing the protease inhibitor phenylmethane sulfonyl fluoride and phosphatase inhibitors. Nuclear and plasma proteins were extracted using nuclear and cytoplasmic proteins extraction reagent (Beyotime Institute of Biotechnology, Jiangsu, China). And the BCA kit (Beyotime Biotechnology, Shanghai, China) was used to detect protein concentration. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% gel) for protein separation (50  $\mu$ g). Antibodies were as following: anti-PLC $\epsilon$  (dilution 1:1000; Santa Cruz Biotechnology); anti-YAP (dilution 1:1000; CST); anti-PSAT1 (dilution 1:1000; Absin); anti-PSPH (dilution 1:1000; Absin); anti-SHMT2 (dilution 1:500; Novus); anti-CyclinD1 (dilution 1:500) and anti-PCNA (dilution 1:500) were from Wanlei-bio (Shenyang, China), anti- $\beta$ -actin (dilution 1:3000), and anti-H3 (dilution 1:2000) were purchased from Abcam (Cambridge, UK). Enhanced Chemiluminescence Detection Kit for exposure of protein strips.

### Cell Counting kit-8

Cells ( $3 \times 10^3$ /well) were cultured in 96-well plates overnight, then treated with VP. After 48 hours, 10  $\mu$ l of Cell Counting Kit-8 (CCK-8) reagent (Univ-bio, Shanghai, China) was added and continued for 1-4 hours in the container. Finally, the absorbance of cells was measured at 450 nm. Set 4 sub holes for each processing group.

### Colony formation assay

Cells (500/well) with different treatments were added into 6-well plates and cultured for about 7-14 days. Then, the cells were fixed with methanol and stained with crystal violet, and finally photographed. Each treatment group was set

three sub holes, and repeated trials three times independently for each treatment group.

### Flow cytometry

Cells were transferred to a six-well plate overnight and treated with VP. After 48 hours, cells were washed 2-3 times with PBS, then digested into single with 0.25% trypsin, PBS washed twice and transferred into EP tubes. Discarded the supernatant and resuspend with 100  $\mu$ l PBS, then 500  $\mu$ l cooled 75% ethanol were added into EP tubes after centrifugation. Cell cycle were detected by CytoFLEX (Beckman Coulter, Inc. California, USA), and the results were analyzed by ModFitLT5.

### Plasmid and lentiviral vector

PLC $\epsilon$  interference lentivirus (LV-sh-PLC $\epsilon$ ), and negative control (LV-sh-NC), interference YAP plasmid (vector-sh-YAP) and negative control (vector-sh-NC) were purchased from Gene Pharma Company (Shanghai, China). The sequences of the lentivirus have been shown in previous report [18]. The sequences of vector-sh-NC, vector-sh-YAP#1, vector-sh-YAP#2, vector-sh-YAP#3 are following:

vector-sh-YAP#1-F, CCGGCCAGTTAAATGTTCA-CCAATCTCGAGATTGGTGAACATTTAACTGGGTT-TTTG; vector-sh-YAP#1-R, GGCCGGTCAATTT-ACAAGTGGTTAGAGCTCTAACCCTTGTAAATTGACCCAAAAAC. vector-sh-YAP#2-F, GGCTGTTAG-AGAGATAATTGGAATTAATTTGACTGTAAACACA-AAGATATTAGTACAAAA; vector-sh-YAP#2-R, CC-GACAATCTCTCTATTAACCTTAATTAACCTGAC-ATTTGTGTTTCTATAATCATGTTTT. vector-sh-YAP#3-F, CACCGCCACCAAGCTAGATAAAGAATTCAAG-AGATTCTTTATCTAGCTTGGTGGCTTTTTTTG; vector-sh-YAP#3-R, GATCCAAAAAAGCCACCAAGC-TAGATAAAGAATCTCTTGAATTTCTTTATCTAGCTT-GGTGGC. YAP over-expression (vector-YAP) and negative control plasmid (vector-NC) were purchased from Addgene (<http://www.addgene.org/>). vector-YAP-F, GACGGATCGGGAGATCTCC-CGATCCCCTATGGTCGACTCTCAGTACAATCTG-CTCTGATG; vector-YAP-R, CTGCCTAGCCCTCTAG-AGGGCTAGGGGATACCAGCTGAGAGTCATGT-TAGACGAGACTAC.

### Liquid chromatography-tandem mass spectrometry (LC-MS/MS) and gas chromatography-mass spectrometry (GC-MS) assay

The trypsin-digested cells ( $>10^6$ ) from different treatment groups were collected, and the su-

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**Table 1.** PLC $\epsilon$  and YAP in PCa tissues and clinicopathological parameters

Characteristics	No. specimens (%)	PLC $\epsilon$ staining			YAP staining		
		Positive	Negative	P value	Positive	Negative	P value
Total	58 (100)	43 (74.1)	15 (25.9)		48 (82.8)	10 (17.2)	
Age (year)							
<60	17 (29.3)	13 (22.4)	4 (6.9)	0.536	15 (25.9)	2 (3.4)	0.385
$\geq$ 60	41 (70.7)	30 (51.7)	11 (19.0)		33 (56.9)	8 (13.8)	
Histological stage							
Ta-T2	20 (34.5)	11 (19.0)	9 (15.5)	<b>0.019*</b>	13 (22.4)	7 (12.1)	<b>0.014*</b>
T3-T4	38 (65.5)	32 (55.1)	6 (10.4)		35 (60.4)	3 (5.1)	
Gleason score							
<7	25 (43.1)	15 (25.8)	10 (17.3)	<b>0.033*</b>	17 (29.3)	8 (13.8)	<b>0.012*</b>
$\geq$ 7	33 (56.9)	28 (48.3)	5 (8.6)		31 (53.5)	2 (3.4)	

Note. PCa: prostate cancer. Statistical method:  $\chi^2$  test. The bold entries represent statistically significant differences. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .

pernatant was discarded after centrifuged. Cells were washed three times with PBS, and then fully disrupted by sonicator. Finally, pre-cooled methanol was added for fixation. After being fixed, the lysed cells are suspended on a dried blood spot (DBS) to be sufficiently saturated. Fresh blood samples from different clinical patients were collected and the plasma was prepared in DBS format on filter paper and stored at 4°C. Standard testing techniques are described in the previous study [28].

### Animal experiment

PC3 ( $4 \times 10^6$  cells) infected with LV-sh-NC and LV-sh-PLC $\epsilon$  were implanted subcutaneously in 4-6 week old male nude mice (Chongqing Medical University Laboratory Animal Center). VP was injected into mice through the tail vein when tumors formed whose injection frequency was once every two days. Primary tumor growth was monitored every 2 to 3 days by an external caliper. Four weeks after giving drugs, all the mice were sacrificed and blood was taken for mass spectrometry experiments. Tumor tissues were removed, volume and weight measurements were taken and fixed for histological studies. Tumor volume ( $\text{mm}^3$ ) was calculated:  $\text{volume} (\text{mm}^3) = 1/2 \times \text{length} \times \text{width}^2$ . This animal experiment was carried out in strict accordance with the guidelines for animal experiments and was approved by the Ethics Committee of Chongqing Medical University (Chongqing, China).

### Statistical analysis

All experiments were repeated three times and above independently. SPSS17.0 was applied to

statistical analysis. All data were shown as the mean  $\pm$  SD. Student's t test, Pearson's analysis, the  $\chi^2$  test, Mann-Whitney test, one-way analysis of variance (ANOVA) and two-way ANOVA were used to evaluate significant differences between categorical variables. Value of  $P < 0.05$  was considered as statistically significant.

### Results

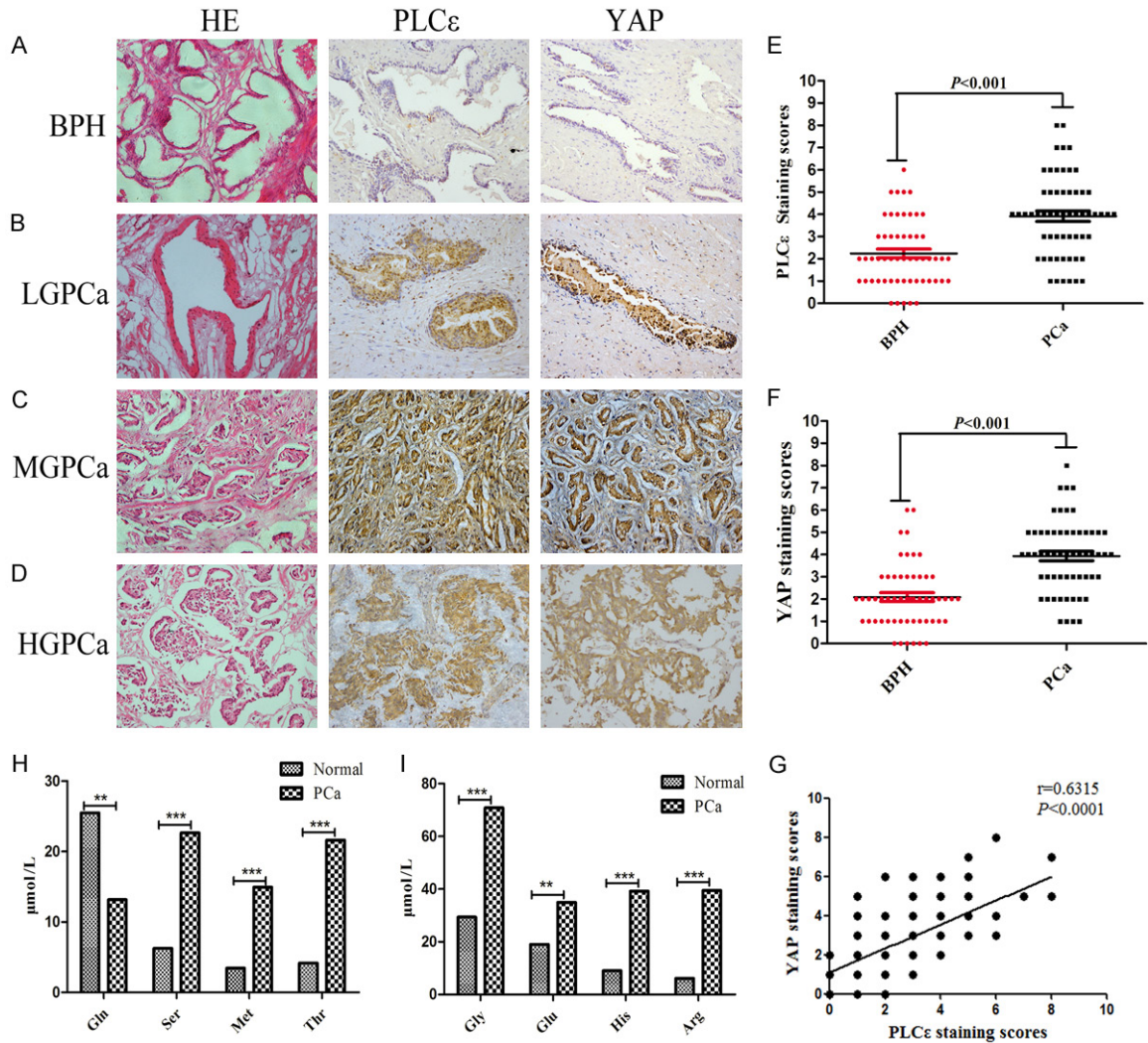
#### High correlation between PLC $\epsilon$ and YAP in PCa tissues

The expression levels of PLC $\epsilon$  and YAP in 55 BPH and 58 PCa samples were determined by IHC assay. Results showed the positive rate of PLC $\epsilon$  reached 74.14% (43/58) in PCa samples, vs 16.36% (9/55) in BPH samples. In addition, the positive rate of YAP was 82.76% (48/58) in PCa, vs 23.64% (13/55) in BPH (**Table 1**). Semi-quantitative staining scores showed an obviously increased PLC $\epsilon$  (**Figure 1E**,  $P < 0.001$ ) and YAP (**Figure 1F**,  $P < 0.001$ ) in PCa compared with BPH tissues. Moreover, the higher the tumor grade, the stronger the staining (**Figure 1A-D**). Pearson's linear correlation analysis results revealed that increased expression of PLC $\epsilon$  was closely related to the high expression of YAP (**Figure 1G**,  $P < 0.0001$ ). More clearly, PLC $\epsilon$  and YAP were significantly correlated with the Gleason grade among the clinical pathological parameters (**Table 1**).

#### The concentration of serine/glycine in the blood of PCa is abnormally elevated

Plasma samples from 66 patients with PCa and 43 specimens from normal subjects were col-

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**Figure 1.** High expression of PLCε and YAP in tissues and high levels of serine/glycine in blood of PCa. (A-D) Representative hematoxylin and eosin (H&E) staining and IHC staining in 55 PCa and 58 BPH samples. Magnification × 200. Representative IHC staining of different staining intensities was used as a criterion for staining scores: BPH (A): with no staining; Low-grade (LG) PCa (B): with light staining; Middle-grade (MG) PCa (C): with moderate staining; High-grade (HG) PCa (D): with strong staining. (E, F) Staining scores of PLCε (E) and YAP (F) expression in BPH and PCa tissues. Data were represented as the means ± SD. (G) Correlation analysis between PLCε and YAP in tissues analyzed by Pearson analysis. (H, I) Multiple common amino acids concentration levels in different blood samples tested by mass spectrometry and analyzed by Mann-Whitney test. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  vs. controls.

lected for detection of many amino acid concentrations by LC-MS/MS and GC-MS testing. As shown in **Figure 1H, 1I**, with the exception of glutamine, the concentrations of serine (**Figure 1H**), glycine (**Figure 1I**) and some of its downstream metabolites such as threonine and methionine in their blood of PCa patients were higher than that in normal samples. It was also found that high levels of serine and glycine in PCa were significantly associated with Gleason score (**Table 2**). The experiment therefore

confirmed that the abnormal metabolism of serine/glycine in PCa patients.

*Knockdown of PLCε can inhibit the expression of YAP in PCa cells*

At its most basic, expression of YAP in normal prostate epithelial cell (RWPE-1) with PCa cell lines (LNCaP, PC3, DU145) were compared. As **Figure 2A-C** illustrated both the mRNA (**Figure 2A**) and protein (**Figure 2B, 2C**) of YAP in cancer

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**Table 2.** Serine/glycine concentrations and clinical pathological parameters in PCa

Characteristics	No. specimens (%)	Serine (μmol/L)			Glycine (μmol/L)		
		Median = 17.639			Median = 54.484		
		<17.639	≥17.639	P value	<54.484	≥54.484	P value
<b>Histology</b>							
Normal	43	42	1	<b>0.000***</b>	34	9	<b>0.000***</b>
PCa	66	12	54		20	46	
<b>Age (year) of PCa</b>							
<60	4 (6.1)	1 (1.5)	3 (4.5)	0.561	2 (3.05)	2 (3.05)	0.352
≥60	62 (93.9)	11 (16.7)	51 (77.3)		18 (27.3)	44 (66.7)	
<b>PSA (μg/L) of PCa</b>							
Median = 20.67							
<20.67	26 (39.4)	5 (7.6)	21 (31.8)	0.553	9 (13.6)	17 (25.8)	0.364
≥20.67	40 (60.6)	7 (10.6)	33 (50.0)		11 (16.7)	29 (43.9)	
<b>Gleason score of PCa</b>							
<7	13 (19.7)	5 (7.6)	8 (12.1)	<b>0.049*</b>	1 (1.5)	12 (18.2)	<b>0.043*</b>
≥7	53 (80.3)	7 (10.6)	46 (69.7)		19 (28.8)	34 (51.5)	

Note. PSA: prostate specific antigen; PCa: prostate cancer. Statistical method:  $\chi^2$  test. The bold entries represent statistically significant differences. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .

cells were apparently higher than RWPE-1. Three plasmids short hairpin(sh)RNAs (vector-sh-YAP#1, vector-sh-YAP#2, and vector-sh-YAP#3) were constructed to knockdown YAP of PCa cells, whose effect were validated immediately. The results displayed sh-YAP#3 had the most significant knockdown effect both in mRNA (**Figure 2D**) and protein level (**Figure 2E, 2F**) which was used in next experiments. Then the expression of YAP was detected when depletion of PLC $\epsilon$ , found that down-regulation expression of YAP in sh-PLC $\epsilon$  group compared with sh-NC and blank group no mater in mRNA (**Figure 2G**) and protein level (**Figure 2H, 2I**).

### *PLC $\epsilon$ -depletion prevents serine/glycine metabolism and proliferation of PCa cells*

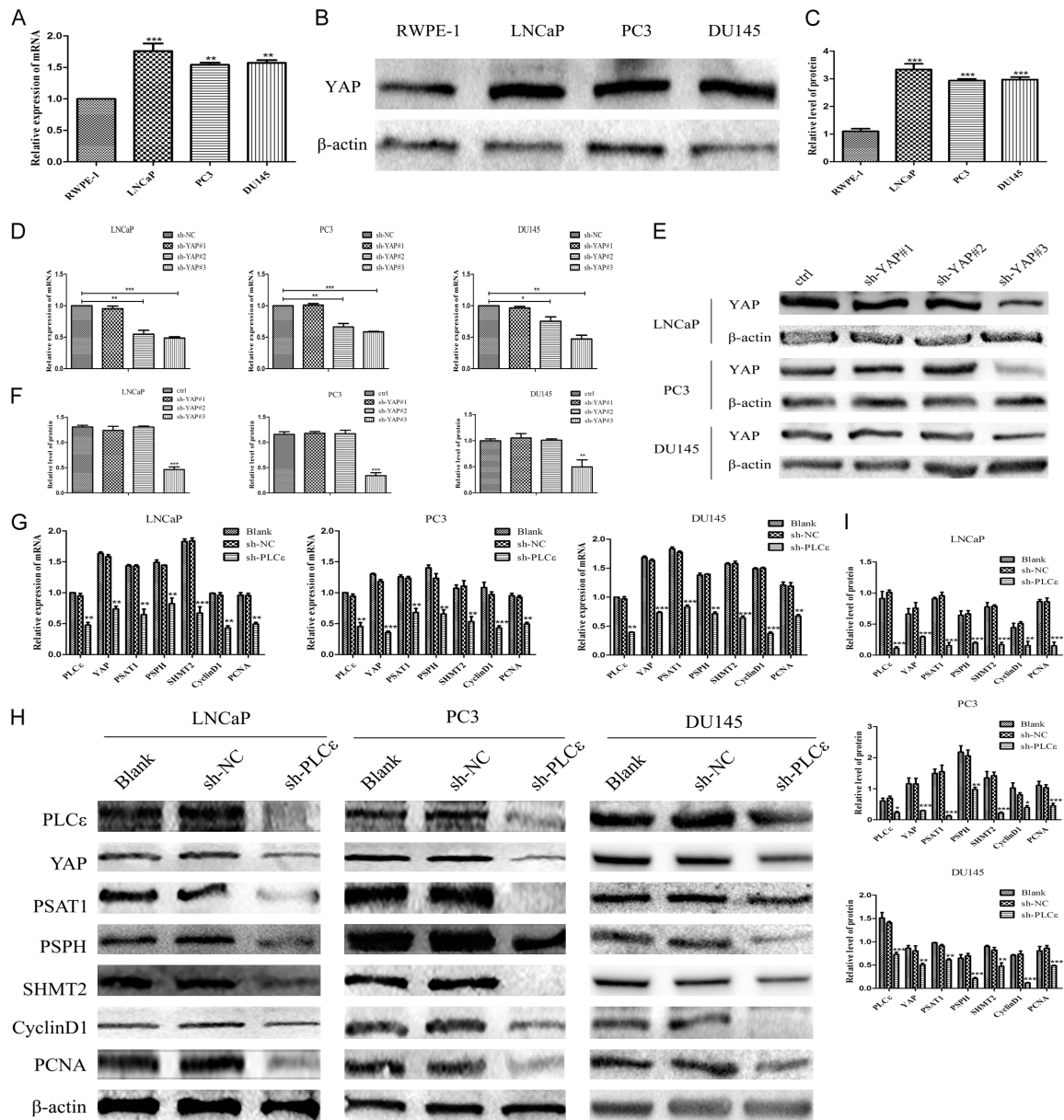
We were very curious whether PLC $\epsilon$  knockdown will have an influence on serine/glycine production and proliferation of PCa cells. So mRNA and protein were examined by q-PCR and western blot. The results obtained that compared with the control group, the expression of serine/glycine producing enzyme (PSAT1, PSPH, SHMT2) and proliferation-related gene (CyclinD1, PCNA) were decline in sh-PLC $\epsilon$  group (**Figure 2G-I**). As with the above results, the mass spectrometry results showed that both serine (**Figure 5I**) and glycine (**Figure 5J**) concentrations of cells in PLC $\epsilon$ -depletion group were lower than control group. As expected, clone

formation assay revealed the number of clones in sh-PLC $\epsilon$  group was also much less than that of the control group (**Figure 3G, 3H**). The above results demonstrated that reducing PLC $\epsilon$  can inhibit the serine/glycine production and proliferation of PCa cells.

### *PLC $\epsilon$ regulates serine/glycine production and cell proliferation of PCa by modulating YAP*

In order to explore whether PLC $\epsilon$  affects the serine/glycine metabolism of PCa by affecting YAP. The effect of over-expressing YAP plasmid was verified firstly. q-PCR (**Figure 3C**) and western blot (**Figure 3A, 3B**) indicated vector-YAP can successfully raise YAP. Same methods to test the expression of three key enzymes of serine/glycine and proliferating genes. The results prompted that the serine/glycine-producing enzymes (PSAT1, PSPH, SHMT2) and proliferating genes (CyclinD1, PCNA) showed a certain degree of up-regulation in sh-PLC $\epsilon$ +YAP group compared with sh-PLC $\epsilon$  group at mRNA (**Figure 3D**) and protein (**Figure 3E, 3F**) level. In contrast, the serine/glycine production enzymes and proliferation genes were further decline in sh-PLC $\epsilon$ +sh-YAP group. Supplementary, the join of the vector-YAP plasmid had no influence on PLC $\epsilon$  (**Figure 3D-F**). These results suggested that PLC $\epsilon$  may affect the serine/glycine production and cell proliferation of PCa by regulating YAP.

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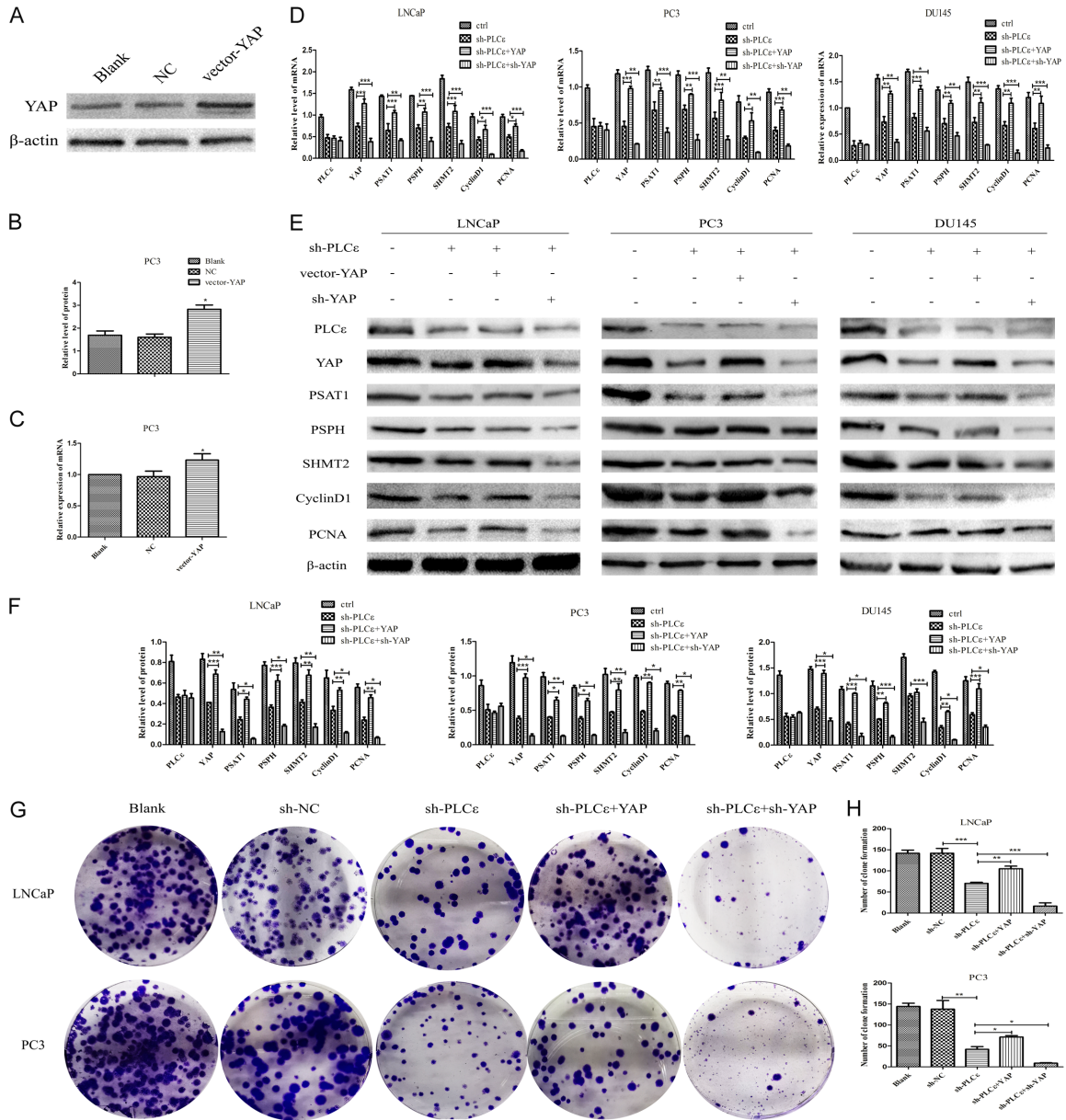
**Figure 2.** PLCε knockdown inhibits YAP mRNA and protein expression in PCa cell lines. (A-C) The messenger RNA mRNA (A) by quantitative polymerase chain reaction (q-PCR) and protein (B, C) levels by Western blot of YAP in different cell lines. (D-F) Knockdown of YAP plasmid on mRNA (D) and protein (E, F) levels of cell lines. (G-I) mRNA and protein levels of PLCε, YAP, PSAT1, PSPH, SHMT2, CyclinD1 and PCNA in cells were detected by qPCR (G) and Western blot analysis (H, I) after infected with lentiviral sh-PLCε. β-actin were used as internal controls. Data were represented as mean ± SD of three individual experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  vs. controls.

### *PLCε modulates phosphorylation/dephosphorylation and translocation of YAP*

In order to further explore the specific mechanism in which PLCε regulates YAP, following experiments were conducted. First, nuclear and cytoplasmic proteins of PCa cells were extracted. Then western blot were used to exam the expression of YAP and p-YAP in nucleus

and cytoplasm, respectively. The results suggested that when PLCε depletion, the expression of YAP in the nucleus was obviously down-regulated, corresponding to a certain degree of up-regulation of p-YAP in the cytoplasm (Figure 4A), and the gray value analysis of three cells were also significant (Figure 4B-D). To confirm the distribution of YAP in cells. Afterwards, IF was used to locate it. Most of YAP of PCa cells

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**Figure 3.** PLC $\epsilon$  mediates serine/glycine metabolism and proliferation by modulating YAP. (A, B) Protein level verification of vector-YAP by western blot. (C) mRNA of vector-YAP by q-PCR. (D) q-PCR detection of mRNA levels of PLC $\epsilon$ , YAP, PSAT1, PSPH, SHMT2, CyclinD1, and PCNA in cells after infected with vector-YAP or vector-sh-YAP. (E, F) Western blot detected and analyzed the protein expression of these gene mentioned in (D). (G, H) Clonal formation assay (G) and statistical analysis (H) of the numbers of colonies in cells after addition of vector-YAP or sh-YAP plasmid.  $\beta$ -actin were used as internal controls. Data were represented as mean  $\pm$  SD of three individual experiments. \* $P$ <0.05, \*\* $P$ <0.01, and \*\*\* $P$ <0.001 vs. controls.

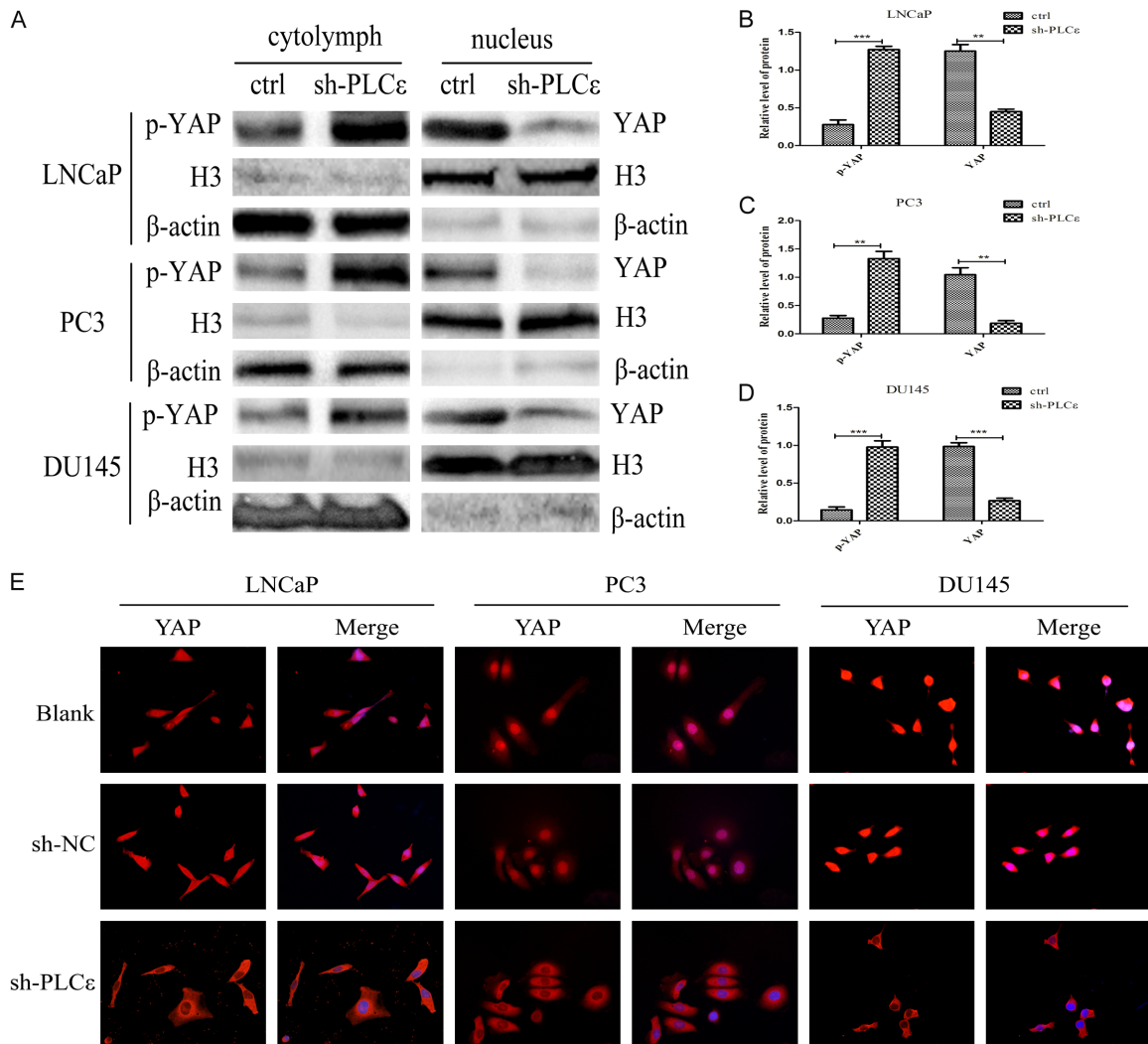
are distributed in the nucleus, a small part in cytoplasm in general. Experiment displayed that the YAP signal in nucleus were significantly reduced in the sh-PLC $\epsilon$  group compared with control group (Figure 4E). Therefore, we suspected that knocking out PLC $\epsilon$  can suppress the transfer of YAP from cytoplasm to the nucleus, thereby phosphorylation and degradation.

*Verteporfin enhances the role of knockdown PLC $\epsilon$  in inhibiting the biological behavior of PCa*

For further exploration, VP was applied which is the specific inhibitor of YAP. Then found its minimum inhibitory concentration (5  $\mu$ M) through CCK-8 assay (Figure 5A), and carried out the



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**Figure 4.** PLCε regulates dephosphorylation and nuclear translocation of YAP. (A-D) Western blot (A) and protein quantification analyses (B-D) detected the expression of YAP in nucleus and p-YAP in cytoplasm. β-actin and Histone (H) 3 were used as an internal control. (E) Immunofluorescence staining demonstrated YAP intracellular distribution in PCa cell lines. Magnification × 400. Data were represented as mean ± SD of three individual experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  vs. controls.

next experiments. Results firstly demonstrated the inhibitory effect of VP on cells at mRNA and protein levels. The results of q-PCR (Figure 5B) and Western blot (Figure 5C-E) indicated that the expressions of YAP, PSAT1, PSPH, SHMT2, CyclinD1 and PCNA in sh-PLCε or VP group were significantly lower than those in control group, but the effect of sh-PLCε+VP group was the best. More importantly, the addition of VP had almost no significant impact on PLCε.

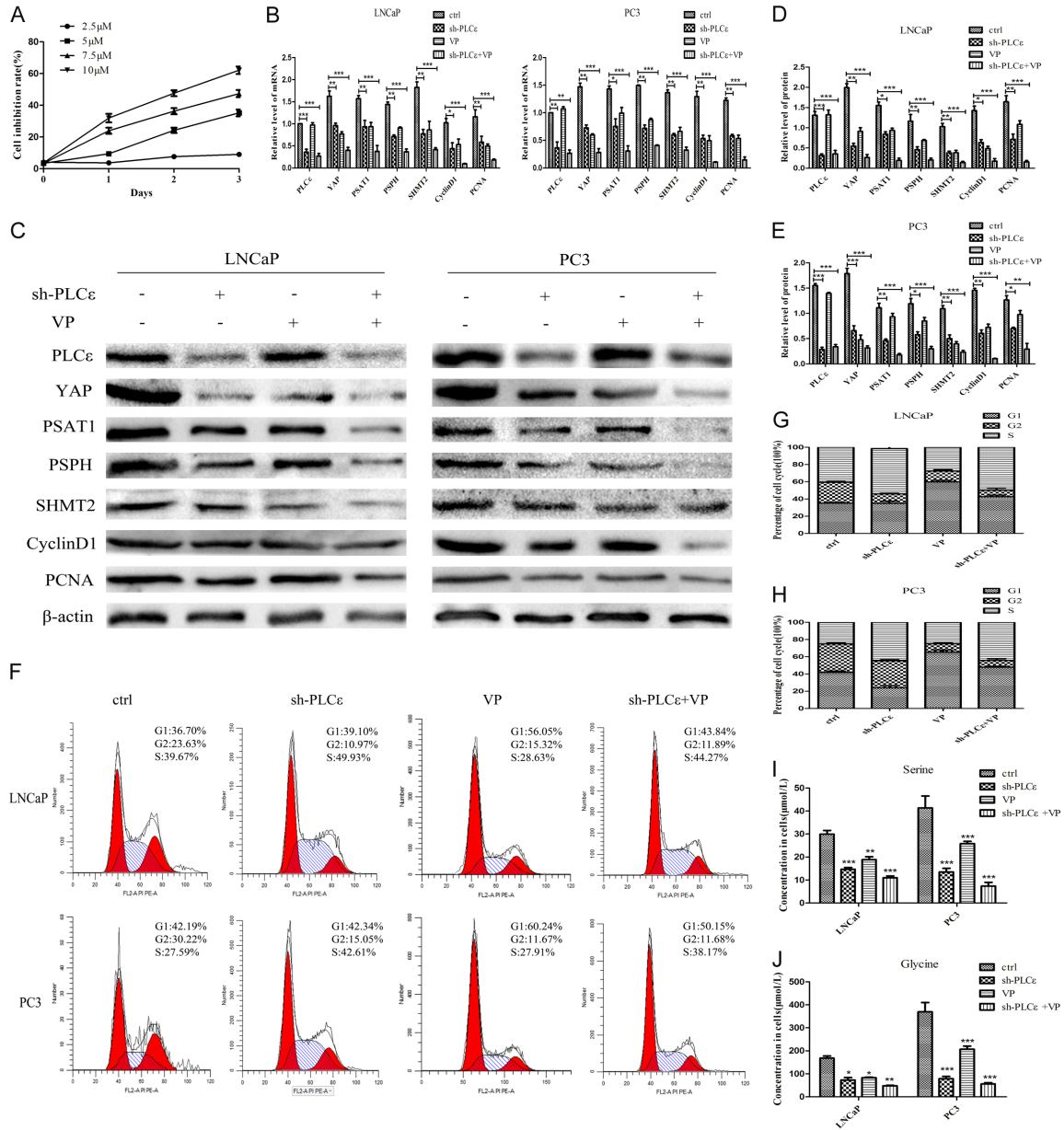
Followed by, VP was added to the cell culture medium for 48 hours. As shown in Figure 5F-H, flow cytometry confirmed that the cells in the S phase of the sh-PLCε group increased significantly, while the cells in the G1 phase of the VP

group increased significantly. More meaningful was the mass spectrum results displayed that compared with the control group, the concentration of serine and glycine of the cells decreased obviously in the sh-PLCε group or adding VP group. However, the concentration decrease of sh-PLCε+VP group is the most obvious (Figure 5I, 5J).

*Verteporfin can effectively inhibit serine/glycine production and tumorigenesis of PCa in vivo*

Previously results confirmed that PLCε-depletion can inhibit the serine/glycine production and proliferation of PCa by regulating YAP

## Serine/glycine metabolism of prostate cancer

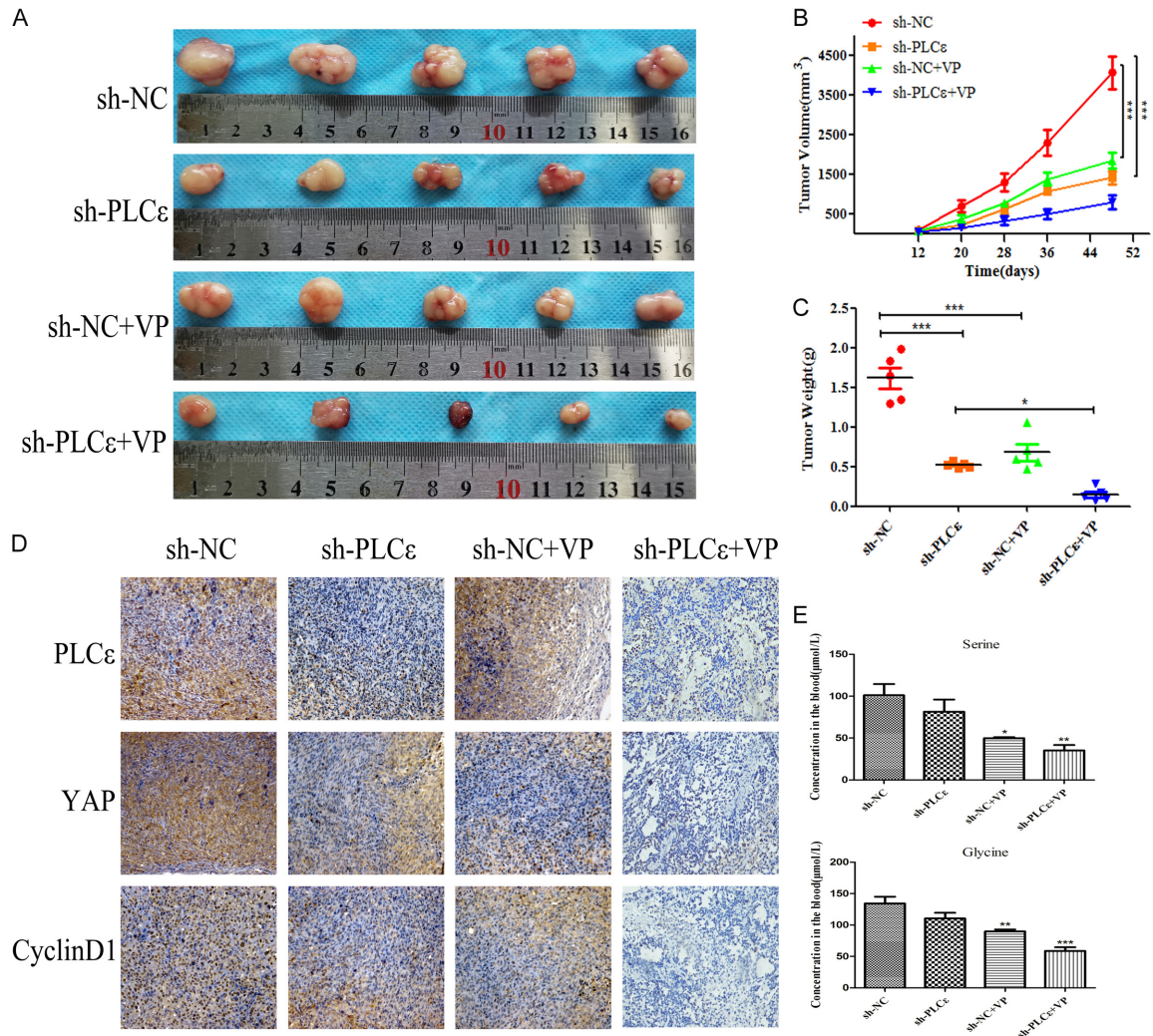


**Figure 5.** VP enhances the inhibitory effect of PLCε depletion on PCa in vitro. (A) CCK-8 assay to detect the toxicity of different concentrations of VP (2.5 μM, 5.0 μM, 7.5 μM, 10 μM) on PC3 cells. (B-E) mRNA (B) and protein expression (C-E) of PLCε, YAP, PSAT1, PSPH, SHMT2, CyclinD1, and PCNA in the cell lines after treated with VP (5 μM, 48 h). (F-H) Flow cytometry detected cell cycle after cultured with VP (F), and statistical analysis (G, H). (I, J) Mass spectrometry tested the effect of VP on the concentration of serine (I) and glycine (J) in cells. Data were represented as mean ± SD of three individual experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  vs. controls.

in vitro. Next, experiments further verified the conjecture by performing a nude mouse tumor xenograft test in vivo. Consistent with in vitro studies, both sh-PLCε and VP groups showed significant tumor suppression (Figure 6A). As expected, the above two groups showed a reduction in tumor volume (Figure 6B) and weight (Figure 6C) compared with control group. If sh-PLCε combined with injection of VP,

the effect was most significant. Simultaneously, IHC staining of mouse tumors to observe the expression of PLCε, YAP and CyclinD1. Same as the guess, the above three genes performed lower level both in sh-PLCε and VP group than control. While sh-PLCε+VP group was far lower than other three groups (Figure 6D). Finally, the tail vein blood of nude mice was obtained for serine/glycine concentration, mass spectrom-

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**Figure 6.** VP effectively inhibits serine/glycine production and tumor growth in vivo. (A) Photographs showed the tumors dissected from the nude mice after subcutaneous tumor formation. (B, C) Volumetric (B) and weight (C) changes of tumors after injection of PC3 cells with sh-PLCε and/or VP in nude mice. (D) IHC staining of PLCε, YAP and CyclinD1 in tumor tissues from mice. Magnification  $\times 200$ . (E) Serine/glycine concentration in venous blood of mice was detected by mass spectrometry. Data were represented as mean  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  vs. controls.

etry revealed that consumption of PLCε and VP separately can both suppress serine/glycine levels, the combination of the two still had better results (**Figure 6E**). Both experiments in vivo and in vitro have further proven VP can enhance the role of knockdown PLCε in inhibiting the biological behavior of PCa.

### Discussion

Abnormal metabolism of serine/glycine is closely related to the occurrence and development of tumors [29-31]. In addition, serine/glycine metabolites can also regulate other meta-

bolic pathways [32, 33]. Moreover, imbalance regulation of PLCε can lead to the development of urinary tumors [34, 35]. Results firstly observed aberrant levels of serine and glycine in the blood of PCa patients. And just as YAP can regulate certain key enzymes in the process of serine/glycine production. After that IHC found high expression of PLCε always accompanied with high YAP in tissues, and both were closely related to the patient's low survival and Gleason score. Coincidentally, PLCε and YAP were significantly related after correlation analysis. Therefore, we hypothesized there may be a potential association between PLCε and YAP.

As the most unique member of the PLC family, PLC $\epsilon$  not only participates in the occurrence and development of tumors [36, 37], it also plays a role in tumor suppression [38]. As an oncogene, in addition to the traditional hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>), producing inositol 1,4,5-triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG), it can also directly play an important role in the regulation of small molecular weight or small G proteins [13, 39, 40]. Therefore, the specific role of PLC $\epsilon$  still has doubts. This study has demonstrated that weakening of PLC $\epsilon$  with lentivirus can suppress the production of serine/glycine and the proliferation by cell and animal experiments, which suggested that PLC $\epsilon$  may be involved in serine/glycine metabolism of PCa.

Research then explored how PLC $\epsilon$  affects the serine/glycine metabolism of PCa. Recent reports suggested YAP is highly expressed in PCa, and YAP-AR axis may be related to the development of it [41]. The presence of YAP in breast [25] and rhabdomyosarcoma [42] may partake the serine biosynthesis pathway. That being the case, to prove that YAP participates in the production of serine/glycine in PCa. Then results displayed down-regulation of YAP by using vector-sh-YAP can successfully suppress the expression of key enzymes of serine/glycine production at the mRNA and protein level. Similarly, the consumption of YAP also reduced the level of serine/glycine concentration of PCa cells. Results verified that YAP induces the process of serine/glycine production in PCa.

Zhou et al. recently discovered that G protein-coupled estrogen receptor (GPER) can activate YAP through PLC $\beta$  signaling pathway [43] and is related to cell invasion and metastasis in breast cancer [44]. As an isozyme of PLC $\beta$ , we suspected that PLC $\epsilon$  can also regulate YAP. As expected, this study confirmed that the mRNA and protein levels of YAP were significantly down-regulated after knocking down PLC $\epsilon$ . Using plasmid to overexpress YAP can reverse this phenomenon. More clearly, experiments further proved that consume of PLC $\epsilon$  can transfer YAP from nucleus into cytoplasm, so that can be rephosphorylated and finally degraded. The above results verified that PLC $\epsilon$  does participate in the regulation of YAP-induced serine/glycine metabolism in PCa.

VP, currently used clinically for the treatment of age-related macular degeneration [45]. However, it has been found to be effective in the treatment of liver cancer [46], breast cancer [47], pancreatic cancer [48, 49] and other tumors [50] in recent years. The above description of VP as a potential tumor-targeted drug is increasingly important. Although our research strongly demonstrated that PLC $\epsilon$  can inhibit serine/glycine metabolism and proliferation of PCa. But the addition of VP could greatly improve the inhibition caused by knockdown of PLC $\epsilon$ , which will provide meaningful reference for clinical treatment of PCa.

### Conclusion

On the whole, our study displayed that abnormal serine and glycine concentrations, which may be a potentially important indicator for the diagnosis of PCa. This was also the first time to discover that PLC $\epsilon$ /YAP axis may be involved in serine/glycine metabolism and growth of PCa. More specifically, PLC $\epsilon$  works by modulating the dephosphorylation and nuclear translocation of YAP. Most critical, the combination of VP and knockout PLC $\epsilon$  could effectively inhibit the production of serine/glycine and tumor growth, which would provide a certain clinical value for the treatment of PCa.

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### Disclosure of conflict of interest

None.

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