PCP4 promotes the growth and castration-resistant development of prostate cancer

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Abstract

Purkinje cell protein 4 (PCP4), which shows homology to the calcium-binding β-chain, regulates the calmodulin (CaM)-dependent signaling pathway by modulating CaM-dependent protein kinase 2 (CaMKK2) activity in Purkinje cells. In this work, we demonstrate that PCP4, which plays a role in tumorigenesis, induces prostate cancer (PCa) cell proliferation and migration *in vitro* and *in vivo*. Moreover, androgen receptor (AR) regulates the expression and phosphorylation activity of CaMKK2 by inducing the transcription of PCP4 in castration-resistant PCa (CRPC). Thus, exogenous overexpression of PCP4 blocks the biological function that EPI, the inhibitor of AR, suppressed the expression and phosphorylation of CaMKK2 in hormone-sensitive LNCap cells. A clinicopathological study of PCP4 was subsequently conducted on a cohort of 51 human PCa patients, and protein and mRNA expression levels of PCP4 and CaMKK2 were positively correlated with the sensitivity of androgen-deprivation treatment ($p < 0.05$). Moreover, PCP4 and CaMKK2 were significantly correlated with PCa relapse. This study reveals the oncogenic activity of PCP4 *in vitro* and provides insights into relevant mechanisms that may lead to novel treatments for CRPC.

Introduction

Prostate cancer (PCa) is one of the most common malignancies among males worldwide [1]. Because patients with localized PCa are usually at an advanced age upon diagnosis, radical prostatectomy is not recommended as an initial treatment because of the high risk of postsurgical complications. The first-line treatment for metastatic PCa is androgen deprivation therapy (ADT) [2]. However, after 18–24 months of ADT, the second stage of treatment poses great challenges because of the development of castration-resistant PCa (CRPC) [3]. While CRPC is unresponsive to ADT, androgen receptor (AR)-regulated signaling pathways remain active and are necessary for cancer progression [4]. Consequently, AR and its downstream factors and processes are a primary target of therapeutic interventions for PCa [5,6].

Purkinje cell protein (PCP) 4, also known as peptide (PEP) 19, shows homology to the calcium binding β-chain of S100 protein. PCP4 has been detected in prostate cancer [7], thyroid cancer [8], and breast cancer [9] tissues. A previous study showed that PCP4 maintains antiapoptic functions via the Akt signaling pathway [10]. Saulo *et al.* demonstrated that PCP4 is a regulator of aldosterone production in normal, hyperplastic, and neoplastic human adrenocortical cells [11].

In Purkinje cells and stellate neurons, PCP4 regulates calmodulin (CaM) dependent signaling by modulating CaM-dependent protein kinase 2 (CaMKK2) activity to influence a variety of neuronal processes. In PCa, CaMKK2 induces cell migration by upregulating AMPK phosphorylation [12]. Mark *et al.* showed that CaMKK2 signaling promotes prostate cancer cell growth by regulating GLUT12 [13].

In this article, we show that PCP4, which plays an important role in tumorigenesis, promotes cell proliferation, invasion, and migration by regulating CaMKK2 in PCa. The results of this work indicate that PCP4-CaMKK2 may be a novel therapeutic target.

Results

PCP4 promotes prostate cancer proliferation, migration, and invasion *in vitro* and *in vivo*

The PCP4 wild-type plasmid and control vector were transfected into the PCa cell lines LNCap and C4-2b, respectively, to establish PCP4-OE) cell lines (Fig. 1A). MTT, tumor sphere formation, and transwell assays revealed that PCP4 induces the proliferation, migration, and invasion of LNCap and C4-2b *in vitro* (Figs. 1B–1D). Moreover, the xenograft tumor results showed that the volumes and weights of PCP4-OE tumors are significantly higher than those of the control group (Figs. 1E, 1F). These findings indicate that PCP4 promotes prostate cancer proliferation, migration, and invasion *in vitro* and *in vivo.*

PCP4 expression increases in CRPC xenograft tumors and androgen deprivation treatment

IHC of PCP4 revealed that cells in the lesions of androgen-resistant PCa have higher nuclear immunoreactivity compared with those in androgen-sensitive PCa tissues (Fig. 4A). We investigated the tendencies of hormone-resistant and -sensitive PCa tissues and found that the PCP4 mRNA levels of the former are sharply increased compared with those of the latter (Fig. 4B). These findings indicate that the expression of PCP4 in PCa cells is closely correlated with the androgen level. To confirm this hypothesis, we built a CRPC tumor xenograft model. The graph in Fig. 2A shows a continuous decrease tendency in the volume of PCa xenograft tumors over 12 days after castration surgery, and then shrinkage from the 12th day to the 27th day postsurgery. PCP4 and the AR protein of PCa xenograft tumors were explored in different periods postcastration by Western blotting. Interestingly, the levels of these two proteins decreased over the first 6 days after surgery and then increased in subsequent days as the tumors recurred. Androgen levels in the blood dropped dramatically after testectomy, but AR levels were gradually increased by endogenic transcriptors, thereby promoting PCa tumor growth and androgen resistance [14]. In Fig. 2B, levels of AR and PCP4 in PCa tumors decreased within the first 3 days postcastration and then increased and showed higher expression in CRPC than in normal PCa tumors. These results indicate that the expression of PCP4 is regulated by the AR standard from normal PCa to CRPC in the mice xenograft model. To confirm this supposition, we explored the mRNA expression of PCP4 in different postcastration days. The mRNA level of PCP4 decreased over the first 6 days postcastration and was overexpressed in CRPC tumors (Fig. 2C). These results indicate that AR may positively regulate the transcription and translation of PCP4 mRNA. Charcoal medium was modified by charcoal-stripped FBS, which is commonly used to study androgen responsiveness and metabolism in cultured PCa cells. To explore the mechanism through which AR positively influences PCP4, we used the LNCap cell line, an androgen-sensitive line, for experiments *in vitro*. When LNCap cells were treated with charcoal medium for 1 day, the protein and mRNA levels of PCP4 obviously decreased compared with those of cells cultured with normal medium (Figs. 2D, 2E). Cells continuously treated with charcoal medium for 15 days showed higher protein and mRNA expression of PCP4 compared with cells cultured with charcoal medium for 1 day

(Figs. 2D, 2E). These results demonstrate that the expression of AR decreases during short-term androgen deprivation but recovers over long-term androgen absence. Moreover, PCP4 was regulated by AR in PC.

Androgen regulates CamKK2 by promoting PCP4 expression in PC

CamKK2, androgen receptor (AR)-regulated signaling axis in prostate cancer cells by pharmacological inhibitors, blocks androgen-mediated cell migration and invasion [12]. PCP4 promotes the migration and adhesion of human breast cancer via the CaMKK2 and Akt signaling pathways [9]. Co-immunoprecipitation experiments confirmed that endogenous CaMKK2 could decrease endogenous PCP4 levels in the three PCa cell lines examined (Fig. 3A), thus suggesting a possible connection between PCP4 and CaMKK2. To examine whether the activation of PCP4 affects the expression of CaMKK2, we detected levels of CaMKK2 and pCaMKK2 in PCP4-OE and control PCa cells by Western blotting. We found that the levels of the two proteins increased in PCP4-OE LNCap and C4-2b cells (Fig. 3B). EPI-001 (EPI), an AR N-terminal domain antagonist, blocks the transactivation of the AR NTD, interacts with the AF-1 region, inhibits protein–protein interactions with AR, and reduces AR interaction with the androgen-response elements of target genes [15]. To examine whether PCP4 and CaMKK2 are regulated by androgens, we explored LNCap and androgen-sensitive PCa cells treated with EPI. The expression of PCP4 and CaMKK2 decreased as the EPI concentration increased (Fig. 3C). We also found that the expression of PCP4, CaMKK2, and pCaMKK2 CRPC xenograft tumors is significantly higher than that in normal PCa tumors (Fig. 3D). We used LNCap cells stably overexpressing PCP4 and control cells cultured with EPI to confirm that androgen regulates CaMKK2 via PCP4. The protein level of CaMKK2 revealed stable expression in PCP-OE PCa cells treated with EPI but dramatically decreased in control cells as PCP4 recession (Fig. 3E). MTT and tumor sphere formation assays revealed that EPI, an AR inhibitor, could not suppress the proliferation of PCP4-OE cells in PCa; however, the proliferation of control cells was inhibited by EPI (Figs. 3F, 3G). These results demonstrate that CamKK2 is regulated via androgen-promoting PCP4.

Expression and correlation of PCP4 and CaMKK2 in clinical PCa tissues

We analyzed 51 cases of human PCa samples by IHC to detect the expression of PCP4 and CaMKK2 (Fig. 4A). The clinical characteristics of the cases were analyzed and are summarized in Table 1. The difference of mRNA between androgen-sensitive and androgen-resistant PCa about PCP4 (Fig. 4B) and CaMKK2 (Fig. 4C) were analyzed. The correlation between PCP4 and CaMKK2 was also analyzed (Fig. 4D). The expression of PCP4 and CaMKK2 was significantly correlated with PCa relapse over a 150-month follow-up period ($P < 0.05$; Figs. 4E, 4F https://www.kmplot.com/). These results suggest that the correlation of PCP4 and CaMKK2 is manifested in human PCa and plays important roles in PCa invasion, metastasis, recurrence, and androgen resistance.

Table 1
Correlation between the staining of PCP4, CaMKK2 and clinicopathologic characteristics in 51 cases of prostate cancer tissues

	n	PCP4			CaMKK2		
		-	+	P	-	+	P
Age(years)	51						
≤ 60		14	24	> 0.99	16	22	> 0.99
> 60		5	8		6	7	
Depth of tumor invasion							
T1-T2		10	22	0.0407	12	20	0.5631
T3-T4		12	7		9	10	
Histologic type							
Poor and undifferentiated		8	14	0.0090	7	15	0.0245
Well and moderate		22	7		19	10	
Metastasis							
No		13	15	0.5681	18	10	0.0952
Yes		8	15		9	14	

Discussion

PCP4, which was initially detected in Purkinje cells and stellate neurons, plays a critical role in ventricular arrhythmias associated with heritable and acquired syndromes [16]. Increasing research shows that PCP4, as an anti-apoptotic factor in neural cells, enhances cell migration, proliferation, and invasion and inhibits cell apoptosis in a number of human carcinoma cell lines [10,17]. In this study, we found that the PCP4 OE induces PCa cell growth and metastasis *in vitro* and *in vivo*. Moreover, in clinicopathological patients, the expression of PCP4 is higher in androgen-resistant samples than in androgen-sensitive samples. In the CRPC tumor xenograft model, AR levels decreased in the first few days following castration and then returned to OE levels thereafter. PCP4 levels showed a trend similar to that of AR levels in CRPC tumors. Interestingly, the variation trend of xenograft tumor size was similar to the trend of AR and PCP4 expression in CRPC development. Accumulated evidence suggests that interconnected molecular mechanisms are related to dysregulated persistent AR signaling and alter androgen biosynthesis and metabolism [18]. Intraprostatic conversion of adrenal androgens into testosterone and intratumoral androgen biosynthesis resulting in AR reactivation in CRPC has received special attention, and persistent AR-axis signaling is regarded a critical therapeutic target. In Fig. 3C, PCP4 and CaMKK2 decreased after treatment with EPI, an AR inhibitor. We also found that PCP4 expression is clearly regulated by AR. The

clinicopathological data further showed that PCP4 is positively correlated with AR expression (Fig. 4D). These results demonstrate that PCP4, as the downstream target of AR, may contribute to promote CRPC growth and PCa hormone resistance.

In the CRPC xenograft tumor model, the tumor size decreased with hormone deprivation shortly after castration. As AR expression recovered, however, the tumor volume and PCP4 and CaMKK2 levels increased once more. In castration-resistant patients, AR signaling is often reactivated in the absence of androgens [19,20]. Previous articles demonstrated that targeting CaMKK, the downstream target of AR, provides an attractive strategy to combat advanced PCa [21,22]. Inhibition of AMPK, the target pathway of CaMKK, decreases tumor hormone resistance [12,23].

In Purkinje cells, CaMKK2, which binds to calmodulin and phosphorylates CaMKI, is activated by PCP4 [5,24]. We initially confirmed the interaction of CaMKK2 and PCP4 to induce the proliferation of PCa cells. Although various upstream signaling pathways regulate the phosphorylation of CaMKK2, the AR's regulation is the first exhibited one of CaMKK2 expression by any signaling pathway. Our results show that the OE of mRNA and protein levels of CaMKK2 in CRPC tumors could be decreased by AR. This finding demonstrates the critical role of genomic androgens in cellular migration and proliferation. Other researchers have suggested that androgens alter cytoskeletal reorganization [25,26]. Moreover, we found that the exogenous overexpression of PCP4 blocks the signaling pathway of AR-CaMKK2 and that AR inhibition is invalid in hormone-sensitive cells. In clinical patients, the expression of PCP4 and CaMKK2 is positively correlated with the sensibility of androgen-deprivation treatment.

In conclusion, we found that PCP4-CaMKK2, a molecule necessary for tumorigenesis, plays an important role of transmitting signaling in the mechanism of AR regulation CaMKK2. In future research, we will focus on developing a PCP4 inhibitor and illustrate the complete regulatory mechanism of AR in CRPC.

Materials And Methods

Cells and reagents

The PCa cell lines LNCap and C4-2b were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic–antimycotic and incubated at 37°C in a moist atmosphere containing 5% CO_2. Charcoal-stripped FBS was purchased from Hyclone (Waltham, MA, USA). Anti-AR antibody (D6F11) and anti-CaMKK2 (D8D4D) were purchased from Cell Signaling Technology. Anti-PCP4 antibody was purchased from Abcam (ab197377; USA). The PCP4 wild-type plasmid (HG21760-UT) was purchased from Sino Biological (China).

Cell transfection

PCa cells were cultured to 80–90% confluence, and polyethylenimine (PEI) was used to transfect plasmids (i.e., PCP4-overexpressing [OE] and empty vector control plasmids) for 24 h. The cells were then dispersed in a 10 cm culture dish with an antibiotic (puromycin). After 2–3 weeks of culture, the cell

colonies were collected, and PCP4 expression was determined via Western blotting and real-time PCR. Cells were cultured to 75–90% confluence, and the lentiviral PCP4-shRNA (Sigma) was transduced into the cells with PEI. After 24 h, PCP4 levels were determined via Western blotting.

Western blotting

Exactly 20 μg of protein was loaded into each well of 24-well plate. The transferred membranes were incubated overnight (over 16 h) at 4°C with the primary antibody (1:1000) followed by the secondary antibody (1:3000) for 1 h.

Cell proliferation assay

PCa cells were inoculated in 96-well plates at a density of 4×10^3 cells per well and cultured for 8–12 h. Then, 10 μL of MTT dye was added to the wells at different time points, and the plate was incubated for another 3 h at 37°C. The original medium was removed from the wells, 100 μL of DMSO was added to each well, and the plates were gently shaken on a shaker for 10 min. A microboard reader (Tecan, USA) was used to measure the spectrometric absorbance of the wells at 570 and 630 nm.

Tumor sphere formation assay

Cells (1×10^2 cells/mL) were inoculated into each well of a 24-well ultra-low attachment plate and treated with serum-free DMEM/F12 medium supplemented with 15–20 ng/mL basic fibroblast growth factor, 15–20 ng/mL epidermal growth factor, 3–5 μg/mL insulin, and B-27 supplement. Approximately 50% of the medium was changed every 3–5 days. Images of the cells were obtained using an inverted bright-field microscope.

Transwell migration assay

Transwell invasion assays were performed in 24-well 8 μm pore-sized Transwell plates. The bottom of the plates was coated with BD Matrigel™ Basement Membrane Matrix, the upper chamber was filled with 5×10^4 cells in medium with 1% FBS, and the lower chamber was filled with medium with 10% FBS as a chemoattractant. The number of cells invading through the Matrigel was counted from four randomly selected microscopic fields of each filter. The test was repeated thrice.

Quantitative real-time PCR

cDNA was synthesized using 1 μg of the total RNA extracted using Trizol (Invitrogen, USA) and TaqMan reverse transcription reagent. The primers of PCP4 were 5'-GCTGGGCCAACCAATGGAA-3' and 5'-CACGTTCTGTCTCTGGTGCAT-3', while the primers of CaMKK2 were 5'-CGGTCGCAAGCTGTCTCTG-3' and 5'-GCGTCCGTTCATGTCCAGG-3'. Relative levels of the target gene mRNAs were expressed as a ratio of target β-actin and calculated from the standard curve as directed.

Animal studies

The backside of 4-week-old female NOD SCID mice was injected subcutaneously with PCP4-OE or control LNCap cells (1×10^6 cells in 200 μL of culture medium). After 30 days, the mice were killed by drowning in

CO_2, and the tumors were collected and weighed.

CRPC tumor xenograft model was established. Twenty 4-week-old male NOD SCID mice that had been injected with wild-type LNCap cells (1×10^6 cells) for 16 days were subjected to testectomy. The tumor size was measured, and a random mouse was euthanatized (drowning into pured CO_2) to collect tumors every 3 days after castration.

Patient selection and tissue microarray preparation

A total of 51 patients with PCa (age, 68–89 years) and admitted at the Department of Urinary Surgery of Xiangya Second Hospital, Central South University (Changsha, China) from July 2018 to December 2019 were enrolled in this study. Only ADT was performed on these patients during the observation period. Cancer relapse was confirmed by the detection of increased serum tumor markers (prostate-specific antigen, PSA) and imagological examination. **Statement**: This study was approved by the Research Ethics Committee of Xiangya Second Hospital. All participants signed the informed consent which were performed in accordance with the Declaration of Helsinki. We confirmed that informed consent was obtained from all subjects and their legal guardians.

Immunohistochemistry

Immunohistochemical (IHC) staining for PCP4 (1:200) and CaMKK2 (1:400) was performed on tissue slides. Four areas on each slide were randomly chosen for IHC scoring. The staining results were simultaneously evaluated by two independent pathologists (double-blinded). Samples in which staining intensity was absent or weak and less than half of the cells were stained were considered negative (–), whereas samples with moderate or strong staining in more than half of the cells were considered positive (+).

Declarations

ACKNOWLEDGMENTS

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CONFLICTS OF INTEREST

The authors declare no conflicts of interests.

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STATEMENT

We all authors confirmed the study is reported in accordance with ARRIVE guidelines.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

References

1. Bray, F. *et al.* Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin **68**, 394–424, doi:10.3322/caac.21492 (2018).
2. Valenca, L. B., Sweeney, C. J. & Pomerantz, M. M. Sequencing current therapies in the treatment of metastatic prostate cancer. Cancer Treat Rev **41**, 332–340, doi:10.1016/j.ctrv.2015.02.010 (2015).
3. Harris, W. P., Mostaghel, E. A., Nelson, P. S. & Montgomery, B. Androgen deprivation therapy: progress in understanding mechanisms of resistance and optimizing androgen depletion. Nat Clin Pract Urol **6**, 76–85, doi:10.1038/ncpuro1296 (2009).
4. Chen, C. D. *et al.* Molecular determinants of resistance to antiandrogen therapy. Nat Med **10**, 33–39, doi:10.1038/nm972 (2004).
5. Racioppi, L. CaMKK2: a novel target for shaping the androgen-regulated tumor ecosystem. Trends Mol Med **19**, 83–88, doi:10.1016/j.molmed.2012.12.004 (2013).
6. Racioppi, L. *et al.* CaMKK2 in myeloid cells is a key regulator of the immune-suppressive microenvironment in breast cancer. Nat Commun **10**, 2450, doi:10.1038/s41467-019-10424-5 (2019).
7. Han, Y. *et al.* Microarray analysis of copy-number variations and gene expression profiles in prostate cancer. Medicine (Baltimore) **96**, e7264, doi:10.1097/md.0000000000007264 (2017).
8. Schulten, H. J. *et al.* Comparison of microarray expression profiles between follicular variant of papillary thyroid carcinomas and follicular adenomas of the thyroid. BMC Genomics **16 Suppl 1**, S7, doi:10.1186/1471-2164-16-s1-s7 (2015).
9. Yoshimura, T. *et al.* PCP4/PEP19 promotes migration, invasion and adhesion in human breast cancer MCF-7 and T47D cells. Oncotarget **7**, 49065–49074, doi:10.18632/oncotarget.7529 (2016).
10. Hamada, T. *et al.* Anti-apoptotic effects of PCP4/PEP19 in human breast cancer cell lines: a novel oncotarget. Oncotarget **5**, 6076–6086, doi:10.18632/oncotarget.2161 (2014).
11. Felizola, S. J. *et al.* PCP4: a regulator of aldosterone synthesis in human adrenocortical tissues. J Mol Endocrinol **52**, 159–167, doi:10.1530/jme-13-0248 (2014).
12. Frigo, D. E. *et al.* CaM kinase kinase beta-mediated activation of the growth regulatory kinase AMPK is required for androgen-dependent migration of prostate cancer cells. Cancer Res **71**, 528–537, doi:10.1158/0008-5472.Can-10-2581 (2011).
13. White, M. A. *et al.* GLUT12 promotes prostate cancer cell growth and is regulated by androgens and CaMKK2 signaling. Endocr Relat Cancer **25**, 453–469, doi:10.1530/erc-17-0051 (2018).

14. Takayama, K. I., Suzuki, T., Fujimura, T., Takahashi, S. & Inoue, S. COBLL1 modulates cell morphology and facilitates androgen receptor genomic binding in advanced prostate cancer. Proc Natl Acad Sci U S A **115**, 4975–4980, doi:10.1073/pnas.1721957115 (2018).
15. Andersen, R. J. *et al.* Regression of castrate-recurrent prostate cancer by a small-molecule inhibitor of the amino-terminus domain of the androgen receptor. Cancer Cell **17**, 535–546, doi:10.1016/j.ccr.2010.04.027 (2010).
16. Kim, E. E. *et al.* PCP4 regulates Purkinje cell excitability and cardiac rhythmicity. J Clin Invest **124**, 5027–5036, doi:10.1172/jci77495 (2014).
17. Honjo, K. *et al.* PCP4/PEP19 upregulates aromatase gene expression via CYP19A1 promoter I.1 in human breast cancer SK-BR-3 cells. Oncotarget **9**, 29619–29633, doi:10.18632/oncotarget.25651 (2018).
18. Attar, R. M., Takimoto, C. H. & Gottardis, M. M. Castration-resistant prostate cancer: locking up the molecular escape routes. Clin Cancer Res **15**, 3251–3255, doi:10.1158/1078-0432.Ccr-08-1171 (2009).
19. Yuan, X. *et al.* Androgen receptor functions in castration-resistant prostate cancer and mechanisms of resistance to new agents targeting the androgen axis. Oncogene **33**, 2815–2825, doi:10.1038/onc.2013.235 (2014).
20. Karantanos, T. *et al.* Understanding the mechanisms of androgen deprivation resistance in prostate cancer at the molecular level. Eur Urol **67**, 470–479, doi:10.1016/j.eururo.2014.09.049 (2015).
21. Flores-Morales, A. *et al.* Proteogenomic Characterization of Patient-Derived Xenografts Highlights the Role of REST in Neuroendocrine Differentiation of Castration-Resistant Prostate Cancer. Clin Cancer Res **25**, 595–608, doi:10.1158/1078-0432.Ccr-18-0729 (2019).
22. Penfold, L. *et al.* CAMKK2 Promotes Prostate Cancer Independently of AMPK via Increased Lipogenesis. Cancer Res **78**, 6747–6761, doi:10.1158/0008-5472.Can-18-0585 (2018).
23. Massie, C. E. *et al.* The androgen receptor fuels prostate cancer by regulating central metabolism and biosynthesis. Embo j **30**, 2719–2733, doi:10.1038/emboj.2011.158 (2011).
24. Kobuke, K. *et al.* Purkinje Cell Protein 4 Expression Is Associated With DNA Methylation Status in Aldosterone-Producing Adenoma. J Clin Endocrinol Metab **103**, 965–971, doi:10.1210/jc.2017-01996 (2018).
25. Kampa, M. *et al.* The opioid agonist ethylketocyclazocine reverts the rapid, non-genomic effects of membrane testosterone receptors in the human prostate LNCaP cell line. Exp Cell Res **294**, 434–445, doi:10.1016/j.yexcr.2003.11.027 (2004).
26. Papakonstanti, E. A., Kampa, M., Castanas, E. & Stournaras, C. A rapid, nongenomic, signaling pathway regulates the actin reorganization induced by activation of membrane testosterone receptors. Mol Endocrinol **17**, 870–881, doi:10.1210/me.2002-0253 (2003).

Figures

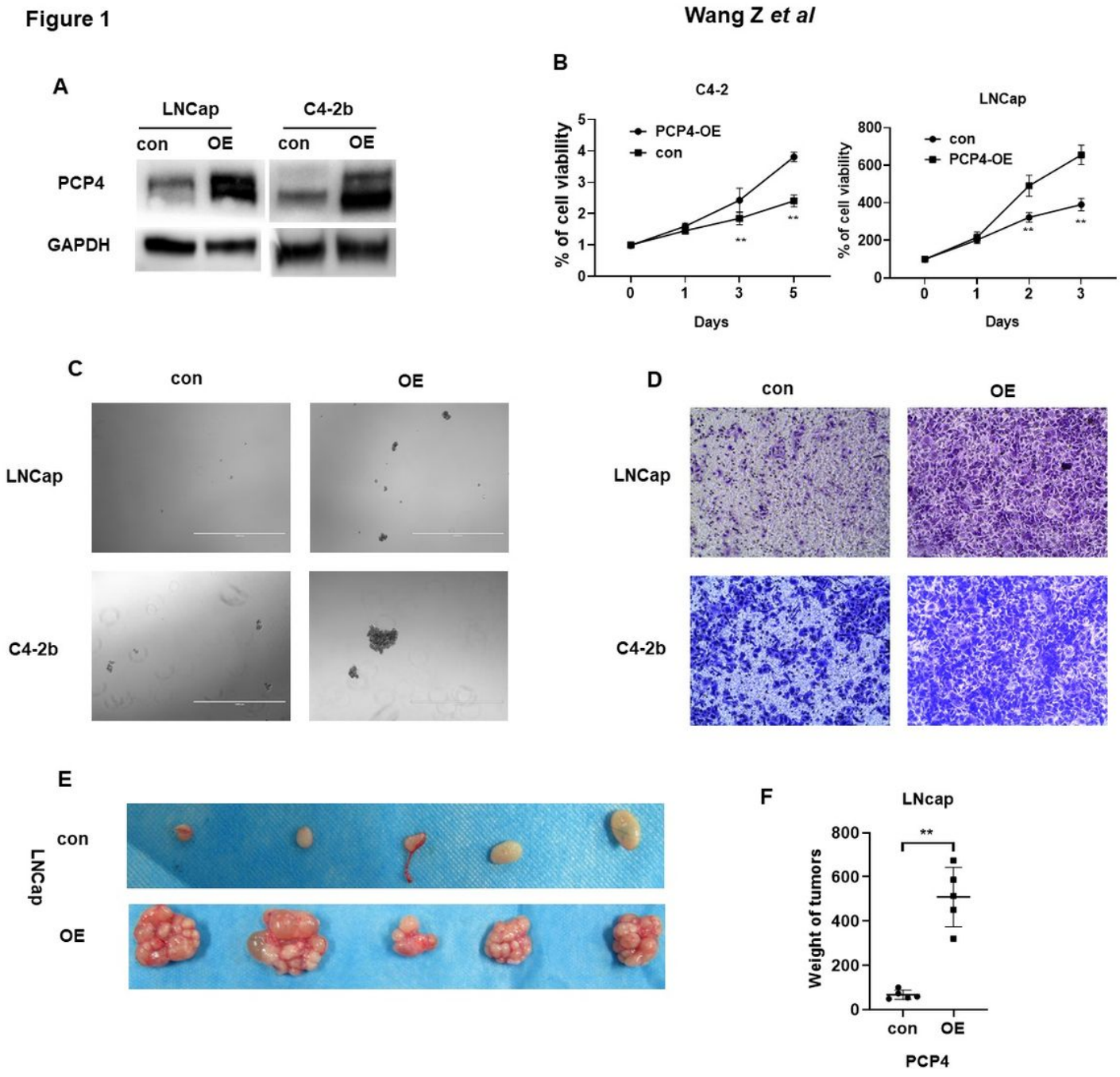

Figure 1

Overexpression PCP4 upregulated PC proliferation, migration and invasion *in vitro* and *in vivo*.

A. Western blot analysis of PCP4 protein expression in LNCap and C4-2b stably transfected with PCP4 plasmid (OE) and control vector (con).

B. MTT analysis for cell proliferation of LNCap (left) and C4-2b (right) cells stably transfected with PCP4 plasmid (OE) and control vector (con).

C. Representative images of colony formation assay of LNCap and C4-2b cells transfected with PCP4 plasmid (OE) and control vector (con).

D. Representative images of Transwell proliferation assay of LNCap and C4-2b cells transfected with PCP4 plasmid (OE) and control vector (con).

E, F. Xenograft tumor development in NOD SCID mice inoculated with LNCap cells transfected with PCP4 plasmid (OE) and control vector (con) (n = 5). Six weeks later, mice were sacrificed, and tumors were collected (**E**) and weighted (**F**) (mean and standard deviation).

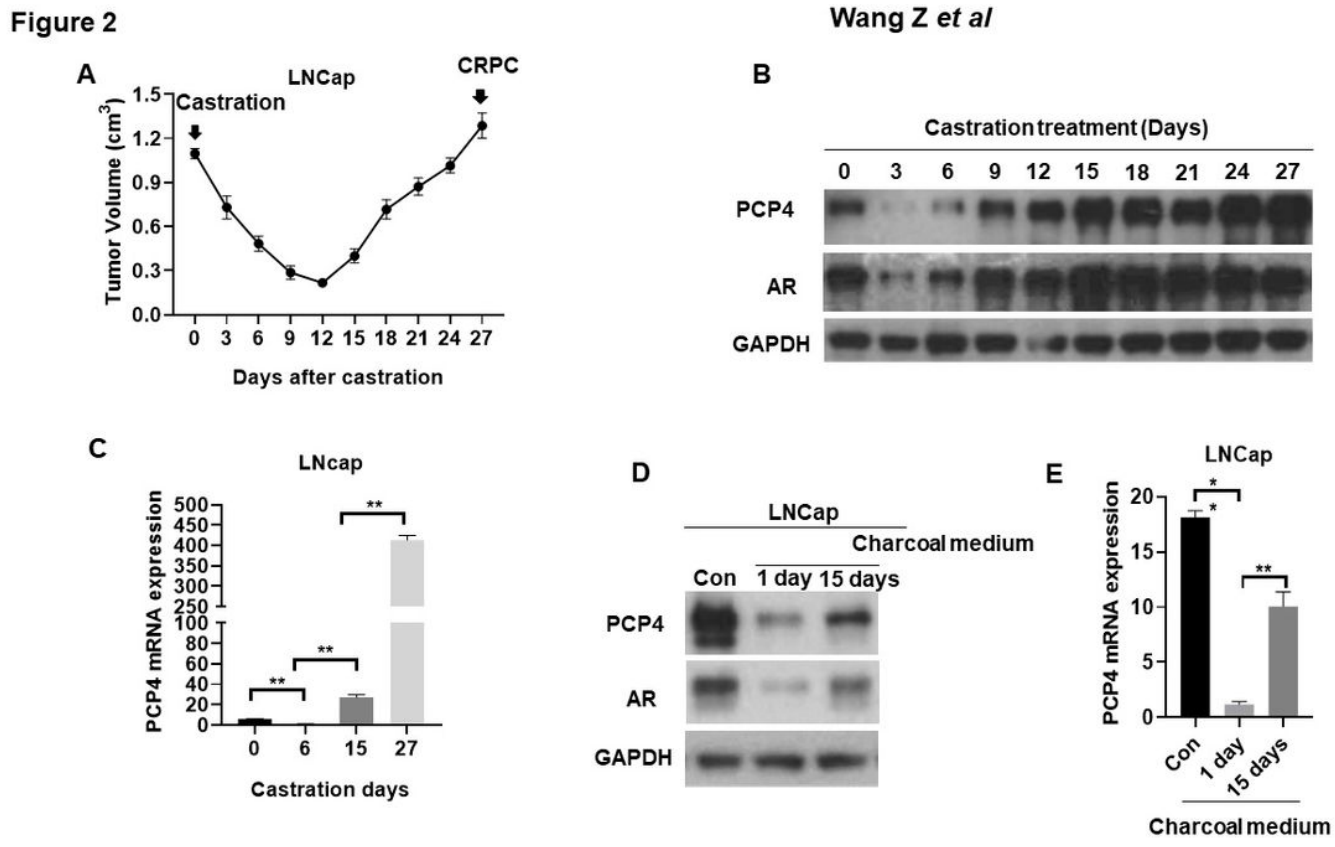

Figure 2

PCP4 expression was increased in CRPC xenograft tumors and androgen deprivation treatment.

A. Growth curve of LNCap xenograft in 0-27 days postcastrated host mice. Significant relapse growth of tumors occurred at 12 days postcastration.

B. Western blot analysis of PCP4 and Androgen receptor (AR) protein expression in various tumors postcastration.

C. qRT-PCR analysis of PCP4 protein expression in various tumors postcastration.

D. Western blot analysis of PCP4 and AR protein expression in LNCap cell treated by normal medium or charcoal medium.

E. qRT-PCR analysis of PCP4 protein expression in LNCap and C4-2b cell treated by normal medium or charcoal medium.

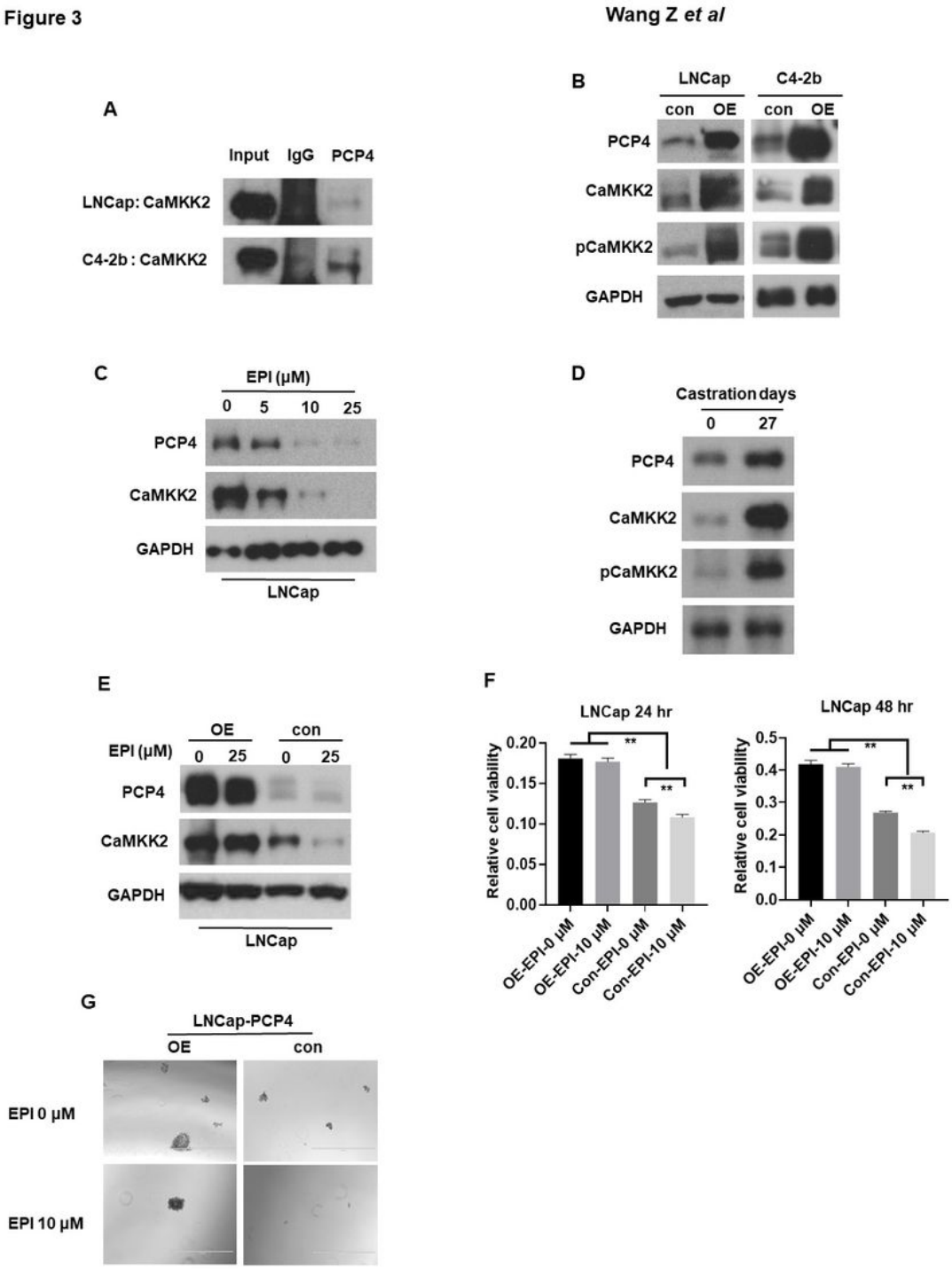

Figure 3

Androgen regulated CaMKK2 via promoting PCP4 expression in PC.

A. Western blot analysis of PCP4 and CaMKK2 protein in LNCap cells cultured with different concentration androgen inhibitor EPI.

B. Western blot analysis of PCP4, CaMKK2, and pCaMKK2 in LNCap and C4-2b cells stably transfected with PCP4 plasmid (OE) and control vector (con).

C. PCP4 immunoprecipitation with CaMKK2 in PC cell lines.

D. Western blot analysis of PCP4 and CaMKK2 in LNCap cells stably transfected with PCP4 plasmid (OE) and control vector (con) treated by EPI or not.

E. Western blot analysis of PCP4, CaMKK2, and pCaMKK2 in LNCap xenograft tumors postcastration 0 day and 27 days.

F. MTT analysis for cell proliferation of LNCap cells stably transfected with PCP4 plasmid (OE) and control vector (con) treated by EPI or not, 24 hr (left) or 48 hr (right).

G. Representative images of colony formation assay of LNCap cells stably transfected with PCP4 plasmid (OE) and control vector (con) treated by EPI or not.

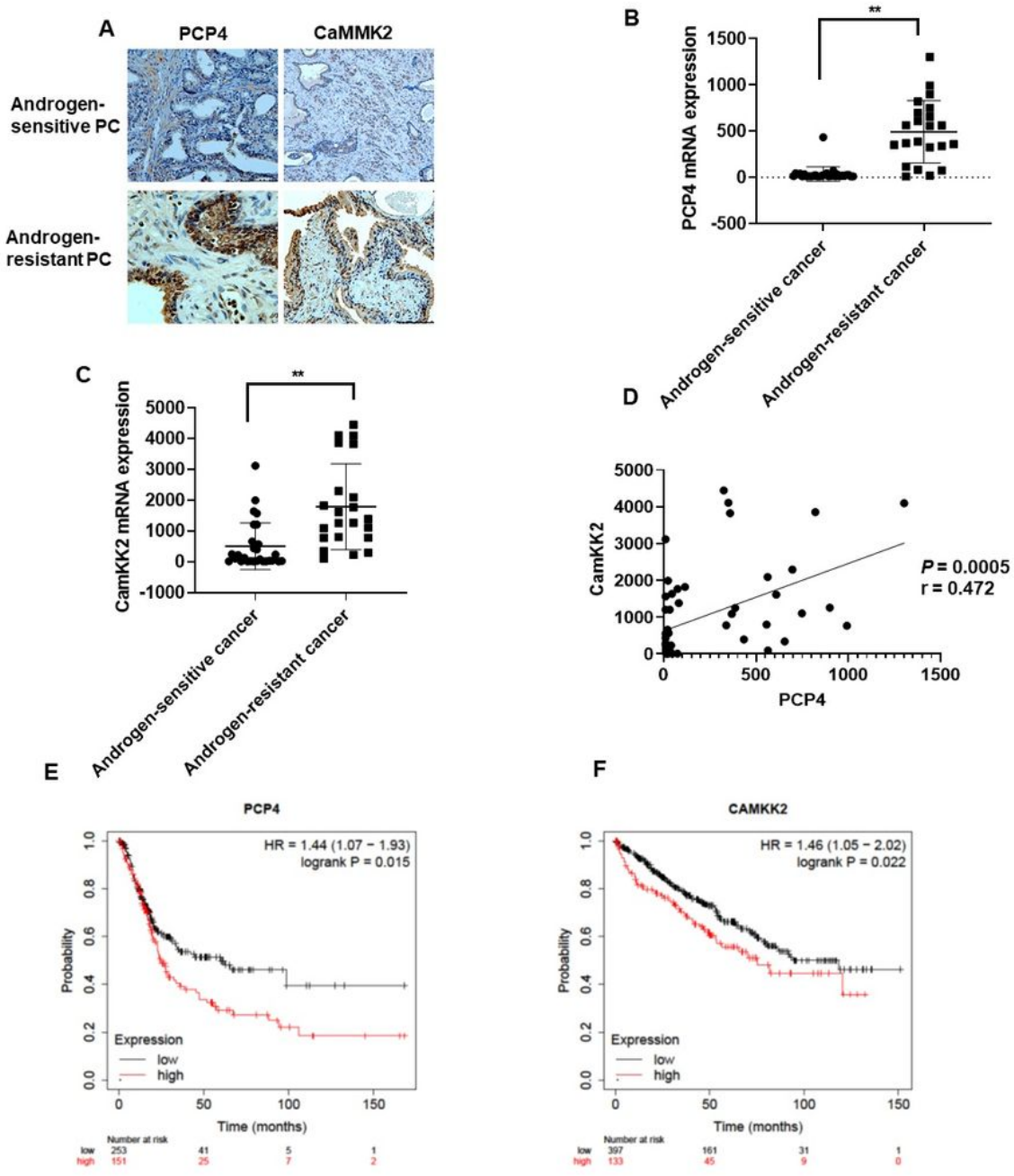

Figure 4

PCP4 and CaMKK2 expression and correlation in clinic PC tissues.

A. Immunohistochemical analysis (IHC) for the expression of PCP4 and CaMKK2 in hormone sensitive and resistant PC tissues.

B. qRT-PCR analysis of PCP4 protein expression in hormone sensitive and resistant PC tissues.

C. qRT-PCR analysis of CaMKK2 protein expression in hormone sensitive and resistant PC tissues.

D. The graph showed the correlation between PCP4 and CaMKK2 in PC tissues.

E, F. Kaplan-Meier survival curves comparing overall survival rates on the basis of high and low PCP4 (**E**) and CaMKK2 (**F**) expression of in prostate cancer patient cohort (https://www.kmplot.com/).