

rAAV-Delivered PTEN Therapeutics for Prostate Cancer

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Abstract

Background: Prostate cancer (PCa) is the second most popular diagnosed cancer and the fifth leading cause of cancer-related mortality for males globally. At present, effective treatments for PCa need to be further developed. To further understand the molecular mechanism and develop novel therapeutics for PCa, the role of phosphatase and tensin homolog (PTEN) signaling in PCa progression was investigated. Previous studies have reported that PTEN and its downstream target cyclin-dependent kinase inhibitor 1B (CDKN1B) are significantly downregulated in PCa cells compared to normal controls; therefore, modulation of PTEN and CDKN1B expression might be a promising therapeutic approach for PCa treatment.

Methods: The expression of PTEN and CDKN1B was first verified in specimens from PCa patients or transgenic adenocarcinoma mouse prostate (TRAMP) mice. The effect of PTEN on PCa cell migration, apoptosis and cell cycle was analyzed by wound healing assay and flow cytometry *in vitro*. Next, we tested the concept of intraprostatic and intratumoral injection of recombinant adeno-associated virus (rAAV) 9 expressing Pten or Cdkn1b (4×10^{11} genome copies (GCs)/prostate) into 8-week-old TRAMP mice and a subcutaneous tumor xenograft mouse model (5×10^{11} GCs/tumor), respectively, to inhibit PCa progression.

Results: PTEN and CDKN1B were significantly downregulated in human and mouse PCa samples, and CDKN1B expression was positively correlated with PTEN expression. Further, PTEN overexpression significantly inhibited the cell migration and cell cycle progression and promoted the apoptosis of PCa cells by decreasing Ccnd1 expression and increasing Cdkn1b expression. Importantly, rAAV9.Pten or rAAV9.Cdkn1b treatment significantly extended the lifespan of TRAMP mice and inhibited the growth rate of tumor xenografts by regulating downstream gene expression. Moreover, we confirmed that neoplasia in the treated prostates was significantly diminished compared to that in the control prostates and that apoptosis was markedly observed in xenografts treated with Pten or Cdkn1b, highlighting changes in two crucial factors for PCa progression.

Conclusions: Taken together, these data indicate that rAAV-based PTEN/CDKN1B delivery holds promise for the development of novel therapeutics for PCa.

1. Background

Prostate cancer (PCa) is the second most commonly diagnosed cancer and the second leading cause of cancer-related death among men in 46 countries[1]. To date, drugs for different targets have been developed for the treatment of PCa, including sipuleucel-T[2], abiraterone[3], enzalutamide[4] and docetaxel[5]. However, tumor cell growth and metastasis is not completely inhibited by these therapeutics, and relapse of PCa is a predominant challenge for the effective treatment of PCa. In recent decades, a large number of studies have investigated the underlying mechanism involved in the occurrence and development of PCa, and many candidates have been provided for drug innovation[6, 7]. However, their

efficacies still need to be further improved. As reported previously, androgen receptor (AR) signaling plays a crucial role in PCa progression, and drugs targeting this pathway show significant PCa suppression[8]; nevertheless, off-target effects are induced by mutation, alternative splicing or ligand mismatching of these targets. Under this condition, novel therapeutics should be developed for effective PCa treatment using different perspectives.

Phosphatase and tensin homolog (PTEN) is a well-known tumor suppressor gene in many kinds of carcinomas, including prostate cancer[9], liver cancer[10], breast cancer[11], lung cancer[12] and colorectal cancer[13]. In particular, a PTEN gene copy is commonly lost in prostate cancer patients with androgen-dependent PCa (ADPC) and castration-resistant PCa (CRPC)[14]. PTEN loss causes an increase in PIP3 cellular content and ultimately promotes AKT activity and its downstream function[9]. Therefore, PTEN was deemed an important negative regulator of P13K/AKT signaling, which plays important roles in cancer. Inhibitors of PI3K have been developed to inhibit tumor progression; however, their clinical applications have been hampered by the serious side effects and toxicity[9]. As a critical modulator of P13K/AKT signaling, it is worth studying the availability of therapeutics targeting PTEN.

Interestingly, Jing Li *et al.* reported that CDKN1B and CCND1, two downstream genes of PTEN, present copy number alteration (CNA) deletion and amplification in prostate cancer, respectively^[15]. Furthermore, as a cyclin-dependent kinase inhibitor, CDKN1B has been shown to be a reliable prognostic marker for prostate cancer; hence, low expression of CDKN1B correlates with poor prognosis^[16]. PTEN enhances CDKN1B expression levels by activating its gene transcription and reducing its degradation[17]. Moreover, the expression and nuclear localization of CCND1 is also negatively regulated by PTEN, and PTEN orchestrates cell proliferation by synergistically regulating CCND1 and CDKN1B[17]. These findings imply that PTEN signaling has a pivotal effect on tumor cell proliferation by regulating cell cycle arrest; thus, it could be an ideal therapeutic target for inhibiting PCa progression. However, it is still a great challenge to deliver therapeutic genes into normal prostate or PCa cells.

Our previous studies showed that recombinant adeno-associated virus (rAAV) can effectively and safely transduce normal prostate tissue and PCa cells, and this provides a powerful tool for developing novel therapeutics for PCa[18, 19]. Compared to other viral vectors, rAAVs possess many advantages for gene delivery, i.e., long-term gene expression, high efficacy of transduction, wide tissue tropism and low immunogenicity[18, 20]. Masami Watanabe *et al.* utilized AAV serotype 2 to deliver Maspin into PCa xenografts, and tumor growth was significantly suppressed[21].

In this study, we first investigated the role of PTEN in inhibiting PCa progression, and second, we explored the therapeutic effects of rAAV-based gene delivery *in vivo* using a transgenic mouse model and a xenograft model. The findings can provide an important foundation for the clinical application of rAAV-based gene therapy for PCa, which would be greatly beneficial to PCa patients.

2. Methods

2.1. TCGA data analysis

The alteration frequency of PTEN among different studies was obtained from cBioPortal for Cancer Genomics (<http://www.cbioportal.org/>)[22, 23]. The gene expression profile and promoter methylation data were obtained from UALCAN (<http://ualcan.path.uab.edu/index.html>)[24] and TCGA datasets (<https://portal.gdc.cancer.gov/>). Further, the gene expression data were normalized by an algorithm of transcripts per kilobase of exon model per million mapped reads (TPM).

2.2. Cell culture and TRAMP model

PC3 and TRAMP-C2 cells, human and mouse PCa cell lines, respectively, were obtained from ATCC (CRL-1435 and CRL-2731, Manassas, VA, USA), and they were cultured with F-12K and DMEM supplemented with 10% fetal bovine serum (FBS) and 1% ampicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO₂. In addition, the mouse model of transgenic adenocarcinoma of the mouse prostate (TRAMP) was purchased from JAX lab, and mice were housed in a cycle of 12-hour light and 12-hour dark as per the requirements of Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts Medical School.

2.3. Production and titration of rAAV vectors.

rAAV vectors in this study were produced, purified and titrated by the vector core of the University of Massachusetts Medical School as described previously[25].

2.4. Animal studies

Eight-week-old TRAMP male mice were enrolled in this study, and 4×10^{11} GC of rAAV9.Pten or rAAV9.Cdkn1b vectors in 40 μ L of PBS were intraprostatically injected into two lobes of the anterior prostate and dorsal lateral prostate. The body weight of the mice was recorded every other week, and the survival status was observed each day. TRAMP-C2 cells were cultured as described above. In total, 3×10^6 cells were injected into the right flank of a male C57/B6 mouse. The tumor volume reached approximately 100 mm³ after 7–8 weeks of subcutaneous injection. Subsequently, 5×10^{11} GC rAAV particles were injected into the tumor at a single site. The length and width of the tumor were measured every 3 days. The tumor volume was calculated using the following formula: $L \times W^2 \times 0.5236$, where L is tumor length and W is width[21]. Fifteen days later, mice bearing TRAMP-C2 xenografts were sacrificed, and tumor tissues were harvested and subjected to H&E staining and TUNEL assay.

2.5. Quantitative polymerase chain reaction (qPCR)

First, total RNA was extracted using the AllPrep DNA/RNA Mini Kit from QIAGEN (80204, Germantown, MD), and cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit from Thermo Fisher (4368814, Thermo Fisher Scientific, Waltham, MA). Second, qPCR was performed using the following profiles: a cycle of 95 °C, 2 min, 40 cycles of 95 °C, 5 s and 60 °C, 10 s. The relative mRNA expression was obtained by calculating the $2^{-\Delta\Delta t}$ value. Beta-actin was used as an internal control, and all experiments were performed in triplicate.

2.6. Western blotting

The total proteins were harvested using RIPA lysis buffer. Briefly, proteins were separated using 10% SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane at 220 mA for 90 min. After blocking with 5% skimmed milk, the membranes were incubated with the primary antibodies at 4 °C overnight. Next, the membranes were incubated with the secondary antibodies at room temperature (RT) for 1 hour (h). Finally, the protein bands were developed using reagents from an ECL chemiluminescence kit. Additionally, primary antibodies against Pten (Cat No: ab267787), Ccnd1 (Cat No: ab134175), Cdkn1b (Cat No: ab193379), ACTB (Cat No: ab6276), and p-Ctnnb (Cat No: ab27798) and secondary antibodies against goat anti-rabbit IgG H&L (HRP) (Cat No: ab6721) and goat anti-mouse IgG H&L (HRP) (Cat No: ab6789) were purchased from Abcam (Cambridge, MA). The intensities of the protein bands were evaluated using ImageJ software.

2.7. Wound healing assay

Cell migration *in vitro* was assessed by wound healing assay. A total of 3×10^5 cells were seeded into a 6-well plate, and the cells were transfected with PTEN overexpression vector for 48 h. Then, the monolayer cells were scratched by a 10 μ L tip, and the wound widths were recorded after 0, 24, 48 and 72 h of transfection. The wound width was measured using ImageJ software.

2.8. Cell apoptosis analysis

Cell apoptosis was analyzed by a kit from KeyGEN BioTECH (KGA108-2, Nanjing, China). After 48 h of transfection, PC3 cells in a 6-well plate were digested with trypsin without EDTA, and cells were washed with phosphate buffered saline (PBS) twice for 5 min. Next, cells were suspended in 500 μ L of binding buffer and incubated with 5 μ L of annexin V-FITC and 5 μ L of propidium iodide for 15 min. The apoptotic rates were obtained using a CytoFLEX flow cytometer from Beckman Coulter (Brea, CA).

2.9. Cell cycle detection

The cell cycle was analyzed by a kit from KeyGEN BioTECH (KGA512, Nanjing, China). Cells transfected with the PTEN vector for 48 h were harvested by digestion and centrifugation at 400 g for 5 min, and cells were further fixed with 70% precooled ethanol overnight. Fixed cells were centrifuged at 400 g for 3 min and incubated with 500 μ L of propidium iodide (PI)/RNase A mixture (9:1, v/v). Then, the cell cycle was detected using a CytoFLEX flow cytometer from Beckman Coulter (Brea, CA). The proliferation index was calculated as follows: Proliferation index (PI) = $(S + G_2/M) / (G_{0/1} + S + G_2/M) \times 100\%$.

2.10. Cell viability assay

The Cell Counting Kit-8 (CCK-8) from Dojindo was used to evaluate cell proliferation. After transfection with the PTEN vector for 48 h, 10 μ L of the CCK-8 solution was added into each well of a 96-well plate, and the plate was further incubated at 37 °C for 2 h. Further, the optical density at 450 nm was measured using a microplate reader.

2.11. H&E Staining

The histological analysis was performed as described previously[18]. Briefly, tissues were fixed in 4% paraformaldehyde overnight at RT, embedded in paraffin and sectioned to 4-micron thickness. The sections were stained with hematoxylin and eosin (HE) and imaged using a microscope (Leica, Buffalo Grove, IL, USA).

2.12. TUNEL assay

Prostate cancer cell apoptosis was assessed by the DeadEnd™ Colorimetric terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) System from Promega (G7360, Madison, WI). In brief, the paraffin was removed by washing with xylene twice for 5 min, and the slides were rehydrated in decreasing concentrations of ethanol (100%, 95%, 85%, 70%, 50%), 3 min each time. After fixing slides with 4% paraformaldehyde in PBS for 15 min, tissues were treated with 100 µL of a 20 µg/mL Proteinase K solution at RT for 30 min and refixed with 4% paraformaldehyde in PBS for 5 min. Next, slides were equilibrated using 100 µL of equilibration buffer at RT for 10 min and were incubated with 100 µL of TdT reaction mixture for 1 h at 37 °C in a humidified chamber. Then, the reaction was stopped using 2 × SSC for 15 min, and the slides were blocked with 0.3% hydrogen peroxide for 5 min. Further, slides were incubated with 100 µL of streptavidin horseradish peroxidase (HRP) (diluted 1:500 in PBS) for 30 min at RT and stained with 100 µL of diaminobenzidine (DAB) solution until a light brown background appeared. Finally, slides can be visualized using a light microscope after washing several times.

2.13. Statistical analysis

All data are presented as the means ± standard error of the mean (SEM) or the standard deviation (SD). Statistical significance for comparisons among multiple groups (>3) and between groups was determined using analysis of variance (ANOVA) and Student's paired *t*-test, respectively, in GraphPad Prism 7.0. *P* < 0.05 was considered significant.

3. Results

3.1. Downregulation of PTEN in human and mouse PCa specimens

To determine the expression pattern of PTEN in PCa tissues, we first analyzed its alteration frequency using The Cancer Genome Atlas (TCGA) data from cBioPortal. As shown in Fig. 1A, deep deletion and mutation of PTEN commonly occurred in PCa samples. Thus, PTEN expression was significantly downregulated in PCa samples, and its expression was unrelated to Gleason score (Fig B and C). Interestingly, PTEN expression was negatively correlated with lymph node metastasis (Fig D). In the TRAMP model mice, PTEN expression was downregulated accompanied by the progression of PCa (Fig. 1E).

3.2. Decreased expression of CDKN1B in PCa samples

By mining the TCGA data, we found that CDKN1B expression was markedly reduced in PCa samples (Fig. 2A), and its expression was positively correlated with PTEN expression ($r = 0.4776$) (Fig. 2B). Intriguingly, an obvious high methylation level was detected at the promoter region of CDKN1B in PCa tissue, and this level was not affected by the status of lymph node metastasis (Fig. 2C and D), which could be one reason for the reduction of CDKN1B expression since a high methylation level can inhibit gene transcription.

3.3. PTEN overexpression significantly inhibits PCa cell migration and cell cycle progression and promotes cell apoptosis

To further study the role of PTEN in regulating PCa progression, PTEN was overexpressed in PC3 cells, a PCa cell line, and cell migration was assessed using a wound healing assay. As shown in Fig. 3A-C, the mRNA and protein levels of PTEN were dramatically upregulated in PC3 cells. Moreover, the wound width in the PTEN overexpression group was significantly greater than that in the mock group (Fig. 3D and E). Next, the apoptosis and cell cycle of PC3 cells were evaluated with flow cytometry. As shown in Fig. 4A and B, PTEN overexpression markedly promoted PC3 cell apoptosis; nevertheless, the cell cycle was inhibited significantly by PTEN (Fig. 4C and D). Moreover, cell proliferation was assessed by CCK-8 assay, and the data indicated that PTEN obviously decreased PC3 cell proliferation (Fig. 4E). To unravel the underlying mechanism, the expression of two important downstream genes of PTEN, CCND1 and CDKN1B, was assayed, and the results showed that PTEN overexpression markedly decreased CCND1 and increased CDKN1B expression (Fig. 4F). These findings indicated that PTEN can inhibit PCa cell migration and the cell cycle and promote cell apoptosis at least partially by regulating CCND1 and CDKN1B expression.

3.4. Pten and Cdkn1b suppress PCa progression in a TRAMP model

To elucidate the therapeutic roles of Pten and Cdkn1b, rAAV9.Pten or rAAV9.Cdkn1b was intraprostatically injected into the mouse prostate, and the DNA and mRNA levels were detected in the anterior prostate (AP) and dorsal lateral prostate (DLP). As shown in Fig. 5, the DNA and mRNA levels of Pten and Cdkn1b were significantly elevated in AP and DLP. Additionally, the body weights of the mice in the three groups did not show marked differences (Fig. 6A). Critically, the survival status of mice in the Pten and Cdkn1b groups was obviously improved, and the median survival of mice in the Mock, Pten and Cdkn1b groups was 220, 301 and 365 days, respectively (Fig. 6B). Furthermore, H&E staining showed that the neoplasia of AP and DLP in Cdkn1b and Pten diminished when compared to the mock group (Fig. 6C). Importantly, Pten overexpression markedly promoted the expression of its downstream gene Cdkn1b at both the mRNA and protein levels (Fig. 7A, B and C) and altered the phosphorylation level of Ctnnb1, another

target of Pten (Fig. 7B and C). However, the mRNA and protein expression of Ccnd1 was dramatically decreased by Pten (Fig. 7A, B and C). On the one hand, the results were consistent with previous studies, and on the other hand, the studies showed that the regulation of gene expression by Pten can explain its role in the inhibition of PCa progression. These findings suggest that Pten and Cdkn1b can effectively inhibit PCa development by modulating the expression of their downstream genes.

3.5. Pten and Cdkn1b repress tumor growth in an immunocompetent xenograft mouse model by boosting apoptosis

To confirm the inhibitory role of Pten and Cdkn1b in PCa progression, a xenograft model was first established using TRAMP-C2 cells in C57/B6 mice, and the rAAV vectors were intratumorally injected when the tumor volume reached approximately 100 mm³ (Fig. 8A). As illustrated in Fig. 8B, the tumor growth rates of mice in the Pten and Cdkn1b groups were significantly lower than those of the mock group, and the expression of Pten and Cdkn1b in tumor tissues was also markedly increased (Fig. 8C and D). H&E staining of tumor tissue did not show obvious pathological differences among these three groups; however, the apoptosis rate of tumor cells in the Pten and Cdkn1b groups was significantly increased compared to that in the mock group (Fig. 8E). These findings indicated that Pten and Cdkn1b can effectively inhibit tumor growth and could be ideal targets for PCa treatment.

4. Discussion

Currently, PCa is challenging the survival and quality of life of patients; its mortality rate has increased stably even with early surveillance using prostate-specific antigen (PSA)[26]. Androgen-dependent PCa (ADPC) can be effectively treated with androgen deprivation therapy (ADT). However, tumor cells can become resistant to this kind of treatment due to genomic reprogramming or other causes; in this case, ADPC evolves toward castration-resistant PCa (CRPC). In CRPC, AR mutation, overexpression and alternative splicing occurs in tumor cells, and the effect of AR signaling on tumor cell growth is significantly diminished[27]. Furthermore, androgen can be released from tumor cells themselves or the adrenal gland[28]. Based on this understanding, novel endocrine therapeutics have been developed that decrease androgen synthesis and the binding between androgen and AR. Abiraterone is an inhibitor of CYP17, which is crucial for androgen synthesis in testis, adrenal gland and tumor cells[29]. After ADT treatment, tumor cells are more sensitive to low levels of androgen; hence, it is an alternative choice for effective PCa treatment that completely blocks androgen synthesis. Moreover, AR activity in tumor cells is promoted by binding androgen; therefore, agents blocking the binding of AR and androgen could show an inhibitory effect on PCa progression. Enzalutamide was designed to competitively bind AR, and tumor cell growth can be inhibited by repressing the activity of AR signaling[30]. Generally, both abiraterone and enzalutamide were designed to target the AR signaling axis, and their efficacies for PCa inhibition were promising at the early stage. However, these inhibitory roles can be alleviated or even disappear after several months of treatment[31].

Therefore, the underlying mechanism for resistance or relapse needs to be further explored, and a breakthrough for effective treatment of PCa could be achieved from this point of view. PTEN and CDKN1B have been extensively demonstrated to be deleted or mutated in PCa using cohorts from Western countries and Asian populations. These findings suggest that PCa progression can be significantly inhibited by PTEN and CDKN1B overexpression. By combination with the rAAV vector, the genes of interest can be effectively delivered into normal prostate tissue and PCa cells as described previously. Furthermore, our data showed that PTEN and CDKN1B indeed inhibited tumor cell growth *in vitro* and *in vivo*, revealing an opportunity for the development of alternative treatments for PCa.

In terms of the suppressive role of PTEN, the TCGA data suggested that PTEN deletion or mutation dramatically decreased the survival of PCa patients (data not shown), and its low expression was stably detected in samples with different Gleason scores and lymph nodal metastatic status, suggesting that PTEN could be an ideal target for PCa treatment. Interestingly, the synergistic expression of PTEN and CDKN1B further hinted at the potential role of PTEN gene delivery in inhibiting PCa. Through regulating CDKN1B and CCND1 expression, PTEN can significantly inhibit the cell cycle at G1 phase and promote both early-stage and late-stage apoptosis of PCa cells *in vitro*.

Notably, intraprostatic injection of rAAV9.Pten and rAAV9.Cdkn1b markedly increased the survival rates of TRAMP mice. The median survival time increased from 220 days in the mock group to 301 and 365 days in the Pten and Cdkn1b groups, respectively. Single deletion of CDKN1B did not lead to tumorigenesis; however, the reduction of Cdkn1b gene expression accelerated tumorigenesis in Pten^{+/-} mice[16]. Conversely, the combined delivery of PTEN and CDKN1B would probably show a greater therapeutic effect on PCa than single gene delivery. Consistent with the *in vitro* results, PTEN promoted CDKN1B expression and CTNNB1 phosphorylation and decreased CCND1 expression *in vivo*, again verifying the mechanism of PTEN in regulating PCa progression. Importantly, the phosphorylation of CTNNB1 is directly regulated by PTEN/AKT/GSK3 β signaling pathway, namely, PTEN overexpression activates GSK3 β function and increases CTNNB1 phosphorylation, and then the phosphorylation of CTNNB1 was degraded by proteasome[32, 33]. Therefore, the expression of its downstream genes promoting cell cycle and survival was decreased, and the cancer progression was further inhibited.

In our study, 4×10^{11} GC rAAVs were administered intraperitoneally amounting to 1.74×10^{13} GC/kg, based on an average weight of 8-weeks old mice of 23 g. Although, this dose appears to be high, a preclinical study in dogs using semisystemic intraportal administration of rAAV8 reported the safe administration of 4.95×10^{13} GC/kg, supporting the safety and feasibility of our dose[34]. In particular, our study employed two *in vivo* mouse models to investigate the therapeutic role of PTEN and CDKN1B in PCa. The TRAMP mouse model simulated the PCa condition by exhibiting various forms of disease from mild intraepithelial hyperplasia to large multinodular malignant neoplasia[35]. The TRAMP-C2 xenograft model was obtained by injecting TRAMP-C2 cells into wild-type C57/B6 mice. These two models were established with a normal immune environment; hence, the therapeutic effect of rAAV-based gene delivery, demonstrated by its inhibition of PCa progression, provides strong evidence for its clinical transformation and application.

5. Conclusions

In summary, PTEN and its downstream gene, CDKN1B, are significantly downregulated in human and mouse PCa samples compared to control samples; these genes can effectively inhibit PCa progression *in vitro* and *in vivo* by promoting apoptosis and arresting the cell cycle. By utilizing the rAAV-based gene delivery system, rAAV.PTEN and rAAV.CDKN1B showed promising curative effects on PCa. The findings of our study provide an important alternative choice for clinicians and patients.

Abbreviations

p>PCa, prostate cancer; TRAMP, transgenic adenocarcinoma of mouse prostate; ADPC, androgen-dependent PCa; ADT, androgen deprivation therapy; PSA, prostate specific antigen; CRPC, castration resistant PCa; AAV, adeno-associated virus; AR, androgen receptor.

Declarations

Ethics approval and consent to participate

The animal experiments were performed as per the requirements of Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts Medical School.

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

G.G. is a co-founder of Voyager, Adrenas and AspA Therapeutics specialized in rAAV based gene therapy, and holds equity in the companies. G.G. is an inventor on patents with potential royalties licensed to Voyager, AspA and other biopharmaceutical companies.

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Authors' contributions

J. Ai and G. Gao conceived the project and drafted the manuscript, J. Ai and J. Li, Qin. Su, H. Ma, R. He conducted experiments and analyzed data, Q. Wei and H. Li revised the manuscript.

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References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2018;68:394–424.
2. Olson BM, McNeel DG. Sipuleucel-T: immunotherapy for advanced prostate cancer. *Open Access J Urol.* 2011;3:49–60.
3. de Bono JS, Logothetis CJ, Molina A, Fizazi K, North S, Chu L, et al. Abiraterone and increased survival in metastatic prostate cancer. *N Engl J Med.* 2011;364:1995–2005.
4. Scher HI, Fizazi K, Saad F, Taplin ME, Sternberg CN, Miller K, et al. Increased survival with enzalutamide in prostate cancer after chemotherapy. *N Engl J Med.* 2012;367:1187–97.
5. Barata PC, Sartor AO. Metastatic castration-sensitive prostate cancer: Abiraterone, docetaxel, or. *Cancer.* 2019;125:1777–88.
6. Muhammad LA, Saad F. The role of clusterin in prostate cancer: treatment resistance and potential as a therapeutic target. *Expert Rev Anticancer Ther.* 2015;15:1049–61.
7. Vaillant O, El Cheikh K, Warther D, Brevet D, Maynadier M, Bouffard E, et al. Mannose-6-phosphate receptor: a target for theranostics of prostate cancer. *Angew Chem Int Ed Engl.* 2015;54:5952–6.
8. Wu H, You L, Li Y, Zhao Z, Shi G, Chen Z, et al. **Loss of a negative feedback loop between IRF8 and AR promotes prostate cancer growth and enzalutamide resistance.** *Cancer Res.* 2020.
9. Braglia L, Zavatti M, Vinceti M, Martelli AM, Marmiroli S. Deregulated PTEN/PI3K/AKT/mTOR signaling in prostate cancer: Still a potential druggable target? *Biochim Biophys Acta Mol Cell Res.* 2020;1867:118731.
10. Akula SM, Abrams SL, Steelman LS, Emma MR, Augello G, Cusimano A, et al. RAS/RAF/MEK/ERK, PI3K/PTEN/AKT/mTORC1 and TP53 pathways and regulatory miRs as therapeutic targets in hepatocellular carcinoma. *Expert Opin Ther Targets.* 2019;23:915–29.
11. Carbognin L, Miglietta F, Paris I, Dieci MV. **Prognostic and Predictive Implications of PTEN in Breast Cancer: Unfulfilled Promises but Intriguing Perspectives.** *Cancers (Basel).* 2019; 11.
12. Gkoutakos A, Sartori G, Falcone I, Piro G, Ciuffreda L, Carbone C, et al. **PTEN in Lung Cancer: Dealing with the Problem, Building on New Knowledge and Turning the Game Around.** *Cancers*

- (Basel). 2019; 11.
13. Salvatore L, Calegari MA, Loupakis F, Fassan M, Di Stefano B, Bensi M, et al. **PTEN in Colorectal Cancer: Shedding Light on Its Role as Predictor and Target**. *Cancers (Basel)*. 2019; 11.
 14. Zhao D, Cai L, Lu X, Liang X, Li J, Chen P, et al. **Chromatin Regulator, CHD1, Remodels the Immunosuppressive Tumor Microenvironment in PTEN-Deficient Prostate Cancer**. *Cancer Discov*. 2020.
 15. Li J, Xu C, Lee HJ, Ren S, Zi X, Zhang Z, et al. A genomic and epigenomic atlas of prostate cancer in Asian populations. *Nature*. 2020;580:93–9.
 16. Di Cristofano A, De Acetis M, Koff A, Cordon-Cardo C, Pandolfi PP. Pten and p27KIP1 cooperate in prostate cancer tumor suppression in the mouse. *Nat Genet*. 2001;27:222–4.
 17. Radu A, Neubauer V, Akagi T, Hanafusa H, Georgescu MM. PTEN induces cell cycle arrest by decreasing the level and nuclear localization of cyclin D1. *Mol Cell Biol*. 2003;23:6139–49.
 18. Ai J, Li J, Gessler DJ, Su Q, Wei Q, Li H, et al. Adeno-associated virus serotype rh.10 displays strong muscle tropism following intraperitoneal delivery. *Sci Rep*. 2017;7:40336.
 19. Ai J, Wang D, Wei Q, Li H, Gao G. Adeno-associated Virus Serotype Vectors Efficiently Transduce Normal Prostate Tissue and Prostate Cancer Cells. *Eur Urol*. 2016;69:179–81.
 20. Ai J, Tai PWL, Lu Y, Li J, Ma H, Su Q, et al. Characterization of adenoviral transduction profile in prostate cancer cells and normal prostate tissue. *Prostate*. 2017;77:1265–70.
 21. Watanabe M, Nasu Y, Kashiwakura Y, Kusumi N, Tamayose K, Nagai A, et al. Adeno-associated virus 2-mediated intratumoral prostate cancer gene therapy: long-term maspin expression efficiently suppresses tumor growth. *Hum Gene Ther*. 2005;16:699–710.
 22. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov*. 2012;2:401–4.
 23. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal*. 2013;6:pl1.
 24. Chandrashekar DS, Bashel B, Balasubramanya SAH, Creighton CJ, Ponce-Rodriguez I, Chakravarthi B, et al. UALCAN: A Portal for Facilitating Tumor Subgroup Gene Expression and Survival Analyses. *Neoplasia*. 2017;19:649–58.
 25. Su Q, Sena-Esteves M, Gao G. **Production of Recombinant Adeno-Associated Viruses (rAAVs) by Transient Transfection**. *Cold Spring Harb Protoc*. 2020; 2020:pdb prot095596.
 26. Moradi A, Srinivasan S, Clements J, Batra J. Beyond the biomarker role: prostate-specific antigen (PSA) in the prostate cancer microenvironment. *Cancer Metastasis Rev*. 2019;38:333–46.
 27. Tan MH, Li J, Xu HE, Melcher K, Yong EL. Androgen receptor: structure, role in prostate cancer and drug discovery. *Acta Pharmacol Sin*. 2015;36:3–23.
 28. Penning TM. Mechanisms of drug resistance that target the androgen axis in castration resistant prostate cancer (CRPC). *J Steroid Biochem Mol Biol*. 2015;153:105–13.

29. Thoma C. Prostate cancer: Interfering with abiraterone metabolism to optimize therapy. *Nat Rev Urol.* 2016;13:370.
30. Scott LJ. Enzalutamide: A Review in Castration-Resistant Prostate Cancer. *Drugs.* 2018;78:1913–24.
31. Attard G, Antonarakis ES. Prostate cancer: AR aberrations and resistance to abiraterone or enzalutamide. *Nat Rev Urol.* 2016;13:697–8.
32. Zhang R, Li G, Zhang Q, Tang Q, Huang J, Hu C, et al. Hirsutine induces mPTP-dependent apoptosis through ROCK1/PTEN/PI3K/GSK3beta pathway in human lung cancer cells. *Cell Death Dis.* 2018;9:598.
33. Shen H, Li L, Yang S, Wang D, Zhong S, Zhao J, et al. MicroRNA-29a contributes to drug-resistance of breast cancer cells to adriamycin through PTEN/AKT/GSK3beta signaling pathway. *Gene.* 2016;593:84–90.
34. Marcos-Contreras OA, Smith SM, Bellinger DA, Raymer RA, Merricks E, Faella A, et al. Sustained correction of FVII deficiency in dogs using AAV-mediated expression of zymogen FVII. *Blood.* 2016;127:565–71.
35. Kido LA, de Almeida Lamas C, Marostica MR Jr, Cagnon VHA. Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) model: A good alternative to study PCa progression and chemoprevention approaches. *Life Sci.* 2019;217:141–7.

Figures

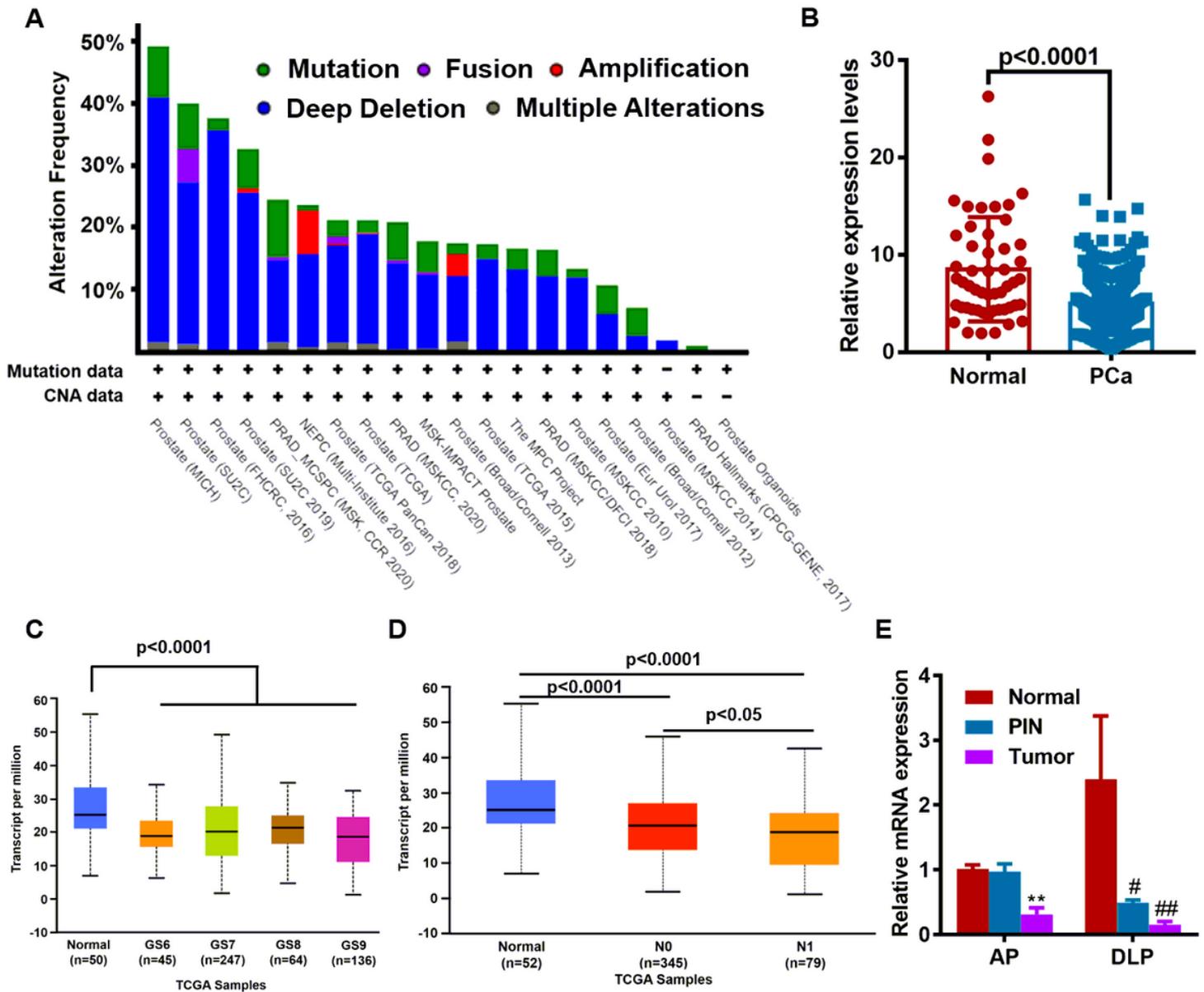


Figure 1

PTEN expression pattern in human and mouse PCa. A. The panels of PTEN gene alteration frequency showed that PTEN gene deletion and mutation are two common events among different studies. B. The data from TCGA database indicated significant downregulation. C. PTEN expression in the normal group and PCa groups with different Gleason scores. D. PTEN expression in the normal group and PCa groups with different lymph nodal metastases. E. PTEN expression was obviously decreased with PCa progression. CNA, copy number alteration; GS, Gleason score; AP, anterior prostate; DLP, dorsal lateral prostate. **, $p < 0.01$ compared to normal AP; #, $p < 0.05$ and ##, $p < 0.01$ compared to normal DLP.

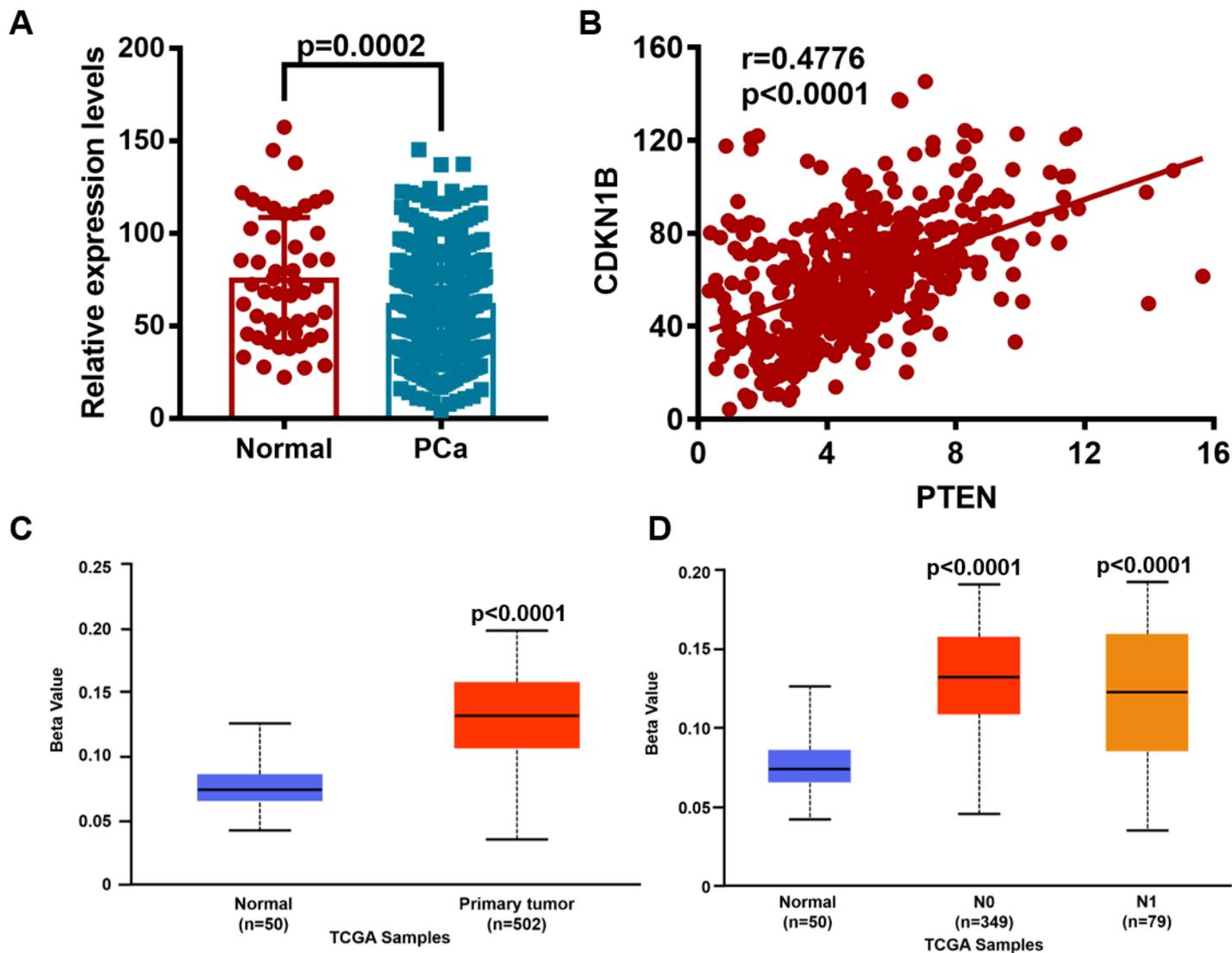


Figure 2

CDKN1B expression in human PCa. A. TCGA data analysis showed a marked reduction in CDKN1B in PCa samples compared to control samples. B. Correlation analysis of the expression of PTEN and CDKN1B. C and D. The methylation levels of the CDKN1B gene promoter were evaluated between the normal and PCa groups.

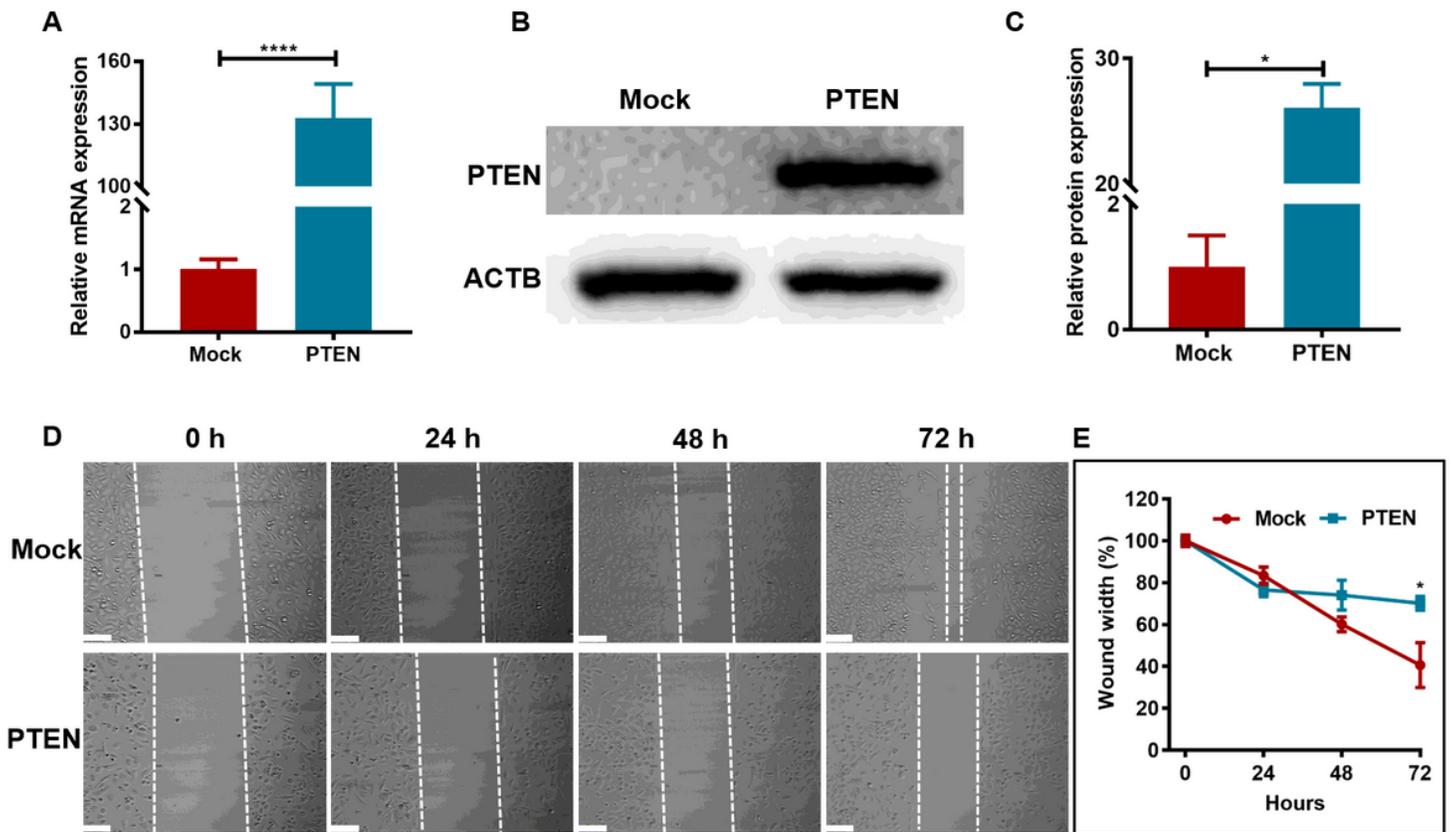


Figure 3

PTEN overexpression significantly inhibits PCa migration in vitro. A. qPCR detection of PTEN mRNA levels. B. The protein level of PTEN was detected in PC3 cells. C. The intensities of protein bands were calculated by ImageJ software. D. A wound healing assay was used to assess the migration ability of PC3 cells. E. The real-time measurement of the width of the wound showed that PTEN overexpression markedly decreased PC3 cell migration. *, $p < 0.05$; ****, $p < 0.0001$.

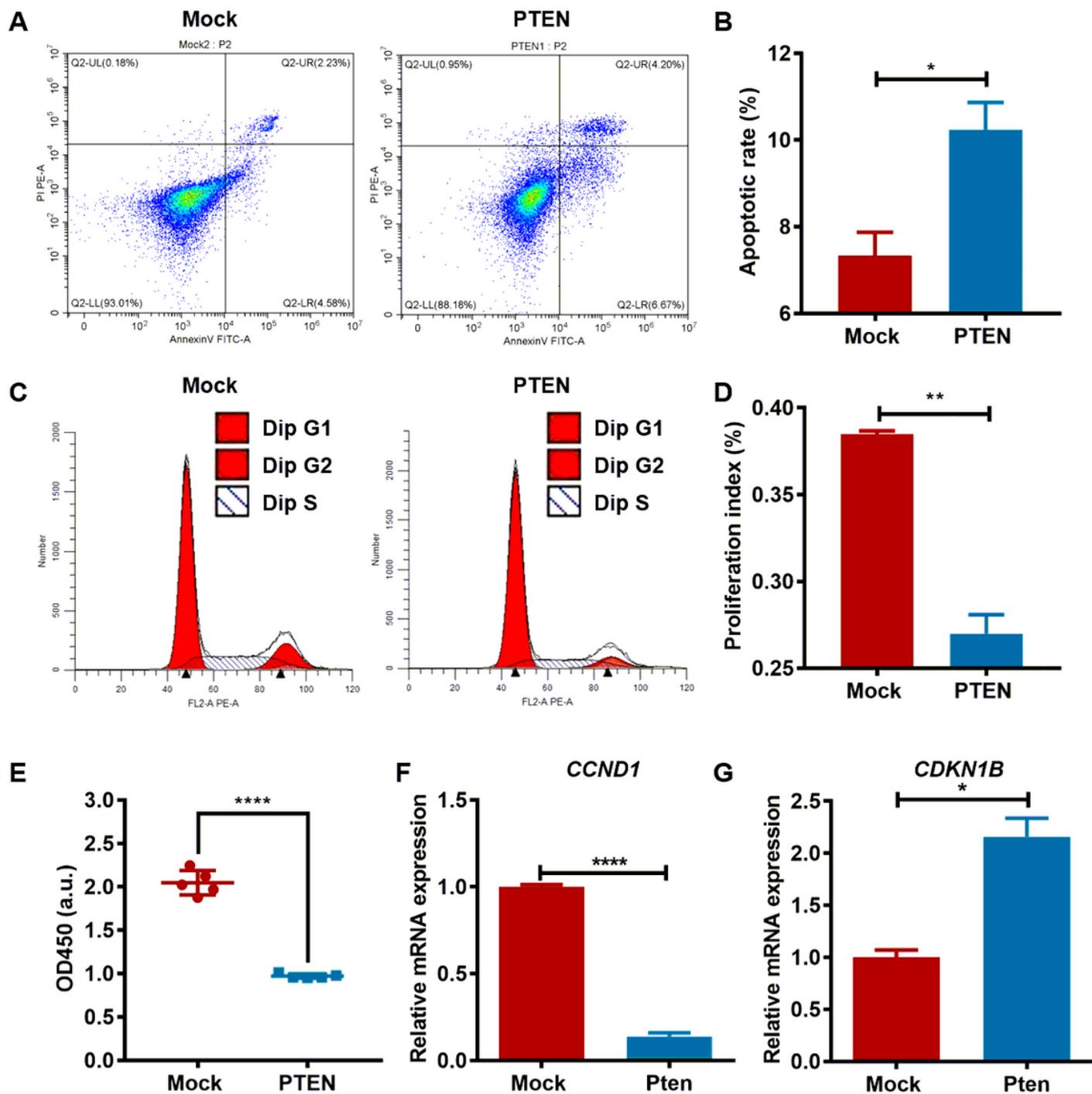


Figure 4

PTEN overexpression significantly promotes cell apoptosis and inhibits the cell cycle by regulating *CCND1* and *CDKN1B* expression. A and B. PC3 cell apoptosis was detected using flow cytometry. Q2-UR represents (double positive) late stage apoptosis, and Q2-LR represents (FITC positive/PI negative) early stage apoptosis. C and D. The cell cycle was analyzed by flow cytometry. The number of cells in the G2 and S phases in the PTEN group was significantly lower than that in the mock group. E. The effect of PTEN on PC3 cell proliferation was measured using the CCK-8 assay. F and G. The expression of *CCND1* and *CDKN1B* was detected using qPCR. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$.

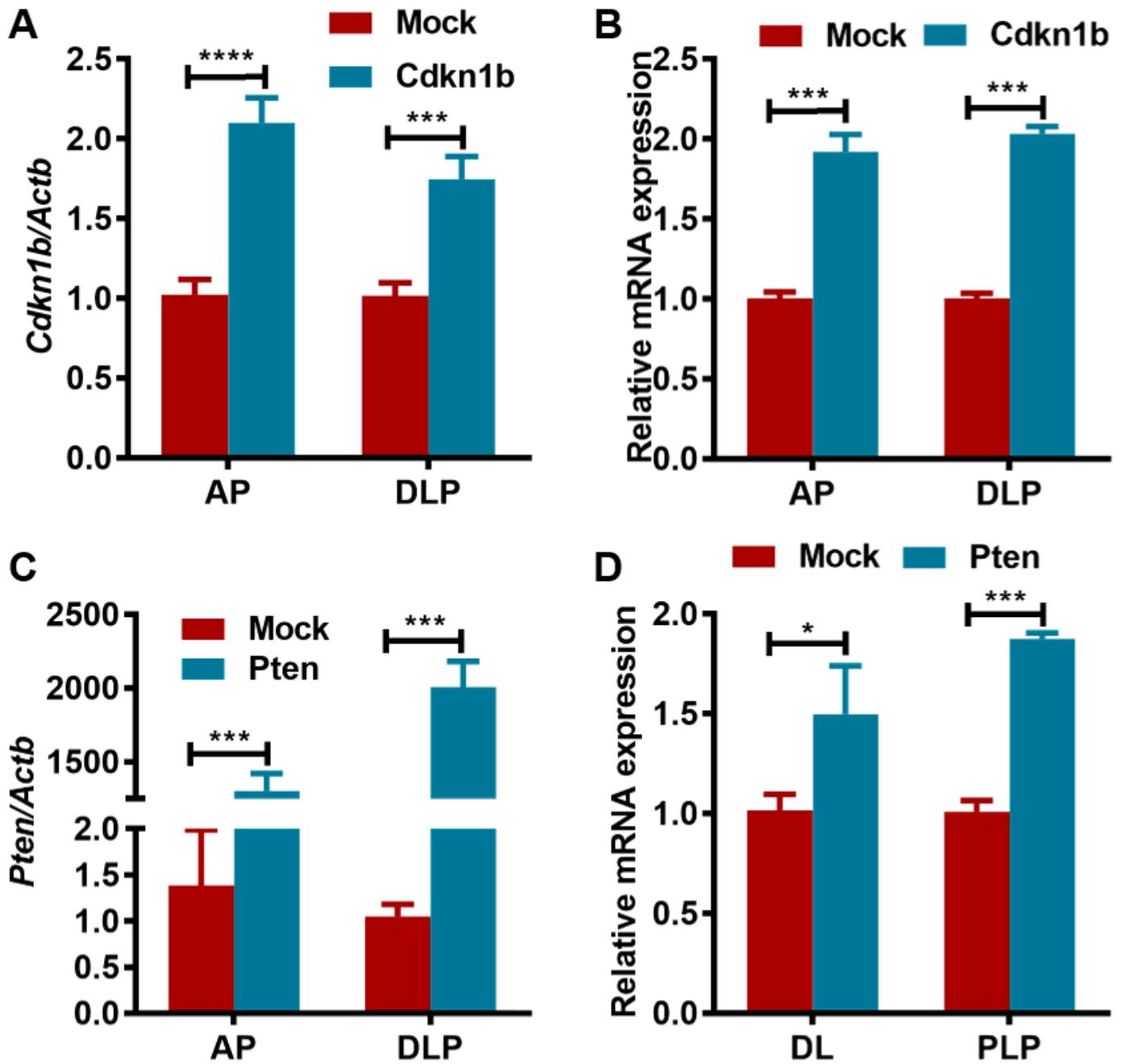


Figure 5

Cdkn1b and Pten DNA and mRNA levels in AP and DLP after intraprostatic injection. A and B. DNA and mRNA levels of Cdkn1b were detected using qPCR. C and D. DNA and mRNA levels of Pten were detected using qPCR. *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$.

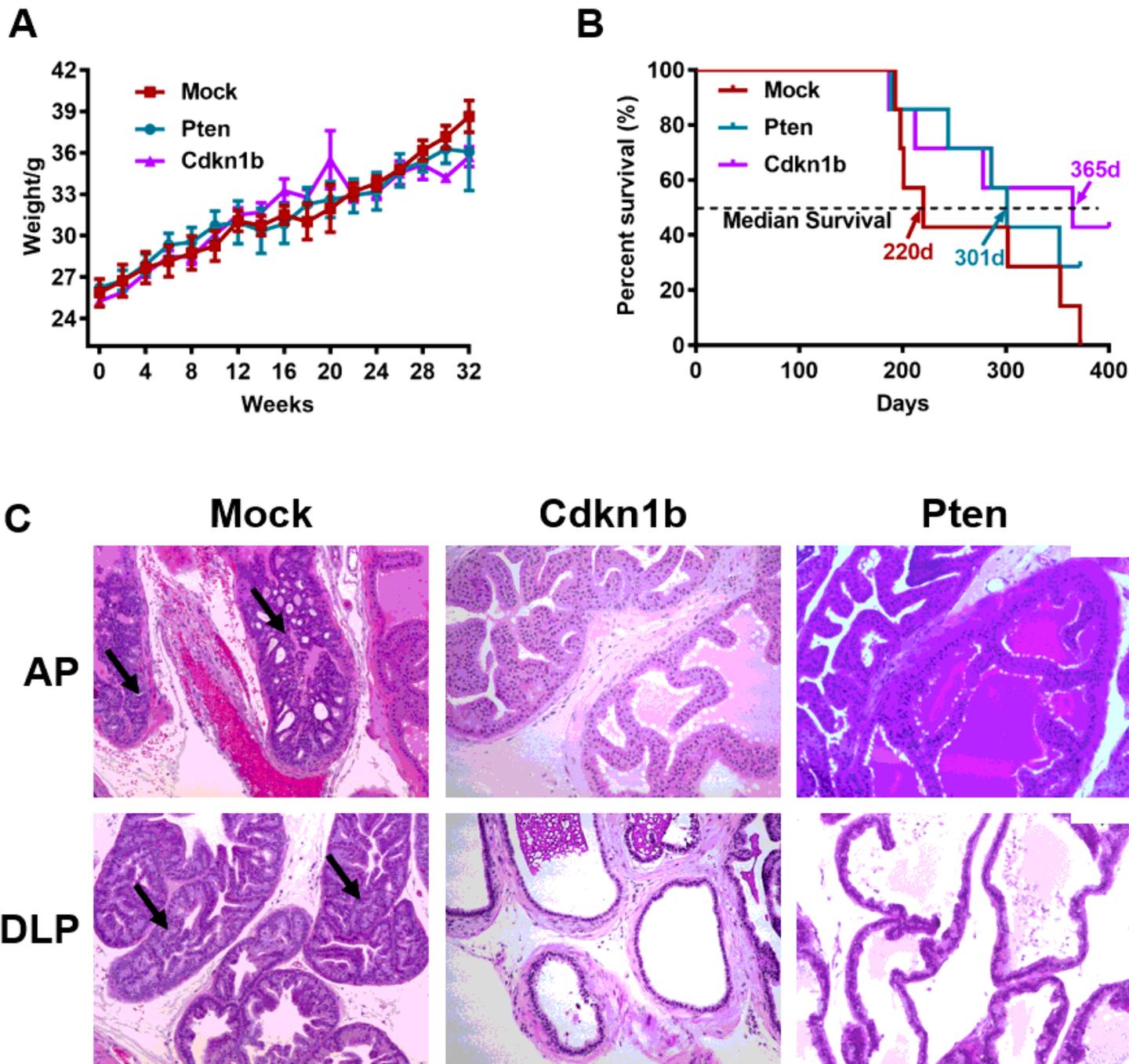


Figure 6

Pten and Cdkn1b improve survival by alleviating neoplasia. A. The body weights of the mice were recorded every two weeks. B. The survival of mice was monitored every day, and the median survival is shown by the arrows. C. H&E staining of AP and DLP to illustrate the pathological changes in mouse prostates treated with Cdkn1b and Pten. The neoplasia areas are indicated by black arrows.

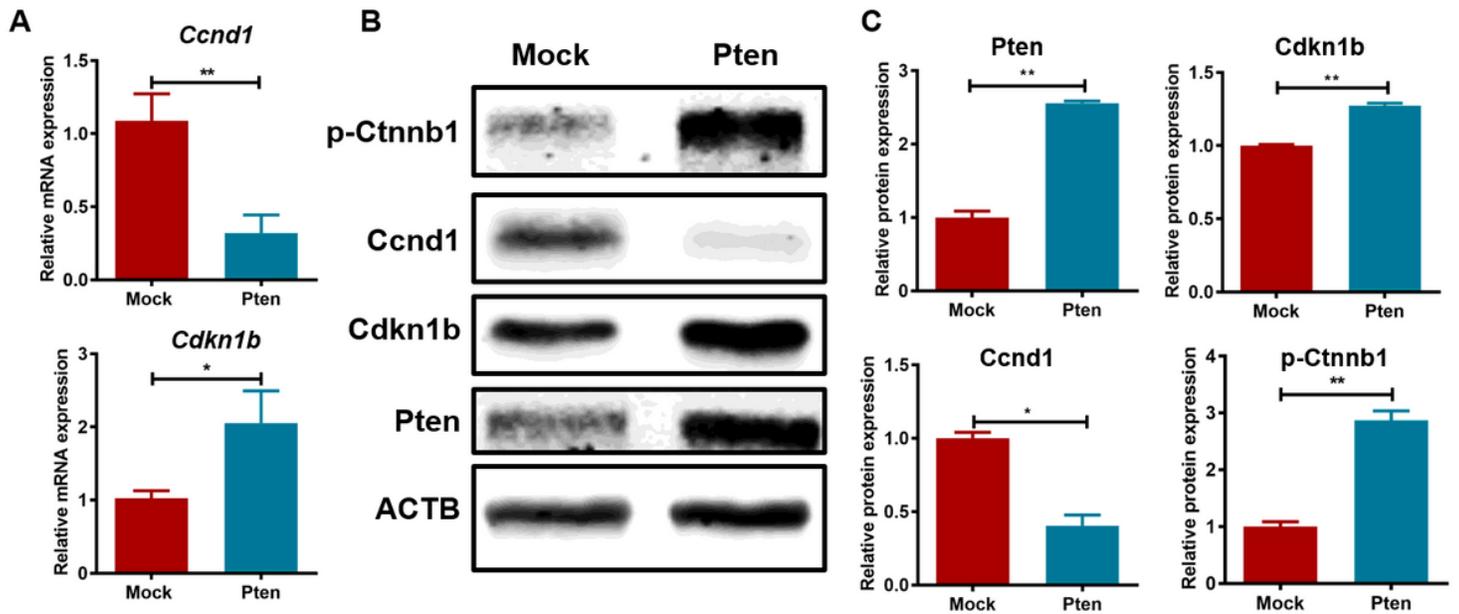


Figure 7

Pten regulates downstream gene expression. A. qPCR detection of *Ccnd1* and *Cdkn1b* in mouse prostates. B. The expression of *Ccnd1*, *Cdkn1b*, Pten and Ctnnb1 phosphorylation was detected by western blotting. C. The protein bands shown in B were quantitated using ImageJ software. *, $p < 0.05$; **, $p < 0.01$.

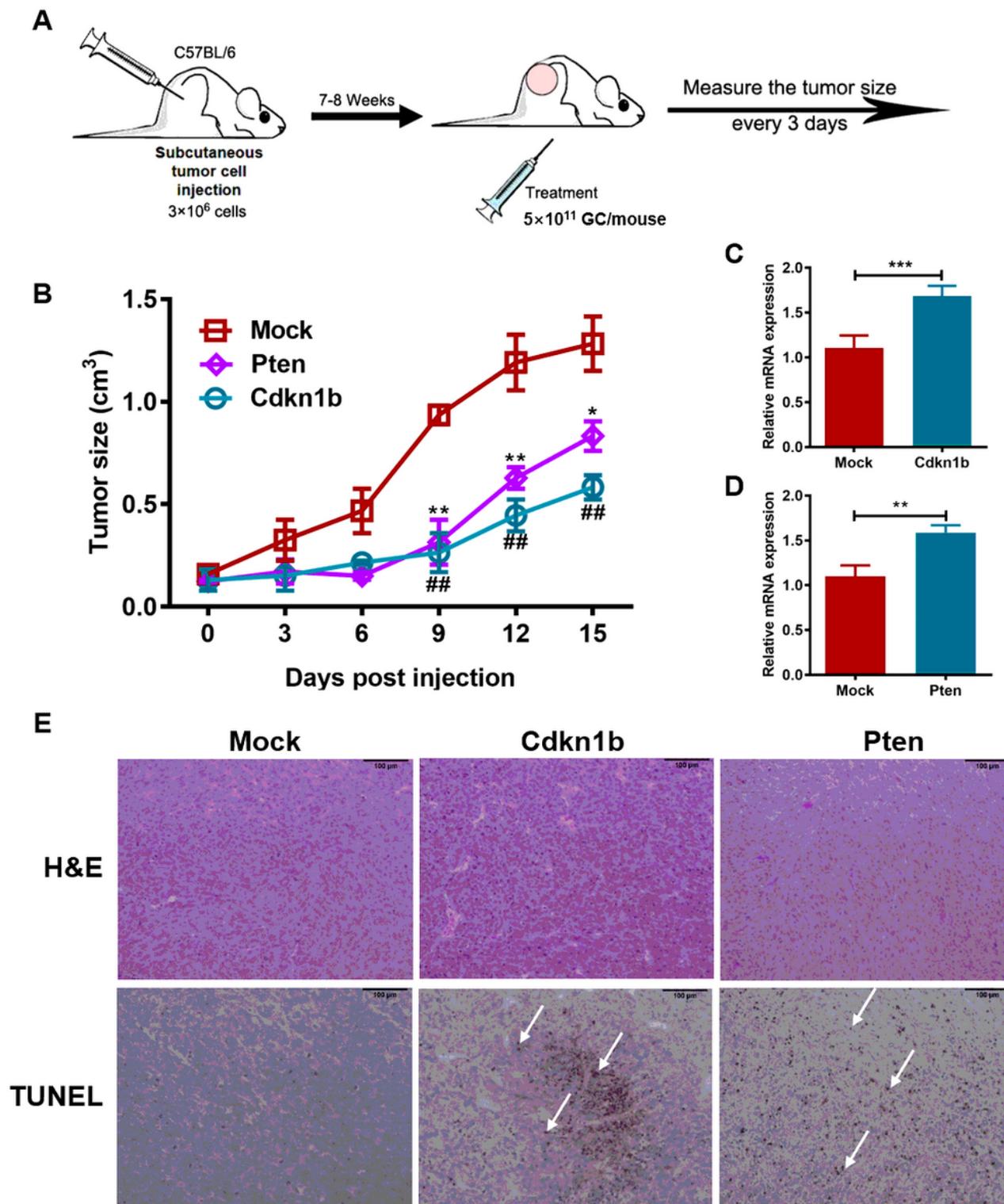


Figure 8

Pten and Cdkn1b significantly inhibit tumor growth in a subcutaneous xenograft model. A. Schematic diagram illustrating the establishment of the xenograft model and intratumoral injection of rAAV.Pten or rAAV.Cdkn1b. B. The tumor volume was measured every 3 days. Significant differences among these groups were observed after 9 days of injection. C. The expression of Cdkn1b and Pten in xenografts was

detected using qPCR. E. H&E staining showed the pathological status of xenograft tumors, and TUNEL assay was used to evaluate tumor cell apoptosis as indicated by the white arrow. **, $p < 0.01$; ***, $p < 0.001$.