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FULL LENGTH ARTICLE

PCK1 attenuates tumor stemness via activating the Hippo signaling pathway in hepatocellular carcinoma

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Abstract Liver cancer stem cells were found to rely on glycolysis as the preferred metabolic program. Phosphoenolpyruvate carboxylase 1 (PCK1), a gluconeogenic metabolic enzyme, is down-regulated in hepatocellular carcinoma and is closely related to poor prognosis. The oncogenesis and progression of tumors are closely related to cancer stem cells. It is not completely clear whether the PCK1 deficiency increases the stemness of hepatoma cells and promotes the oncogenesis of hepatocellular carcinoma. Herein, the results showed that PCK1 inhibited the self-renewal property of hepatoma cells, reduced the mRNA level of cancer stem cell markers, and inhibited tumorigenesis. Moreover, PCK1 increased the sensitivity of hepatocellular carcinoma cells to sorafenib. Furthermore, we found that PCK1 activated the Hippo pathway by enhancing the phosphorylation of YAP and inhibiting its nuclear translocation. Verteporfin reduced the stemness of hepatoma cells and promoted the pro-apoptotic effect of sorafenib. Thus, combined treatment with verteporfin and sorafenib may be a potential anti-tumor strategy in hepatocellular carcinoma.

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Introduction

The incidence and mortality of primary liver cancer are among the top 10.[1] The high recurrence rate and chemotherapy resistance of hepatocellular carcinoma (HCC) are the main reasons for its poor prognosis. Increasing evidence supported cancer stem cells (CSCs) as a major driver of chemoresistance.[2] CSCs are a subpopulation of tumor cells with self-renewal and differentiation abilities, which contribute to poor prognosis and high mortality in patients with HCC. Several liver CSC markers have been found, including CD90, CD13, CD133, EpCAM, CD24, and CD44.[3] Metabolic remodeling is one of the hallmarks of cancer. Tumor cells generally prefer to obtain cellular energy and biomacromolecules through glycolysis.[4] Unlike tumor cells, CSCs utilize oxidative phosphorylation (OXPHOS) or glycolysis as their primary metabolic source, depending on the various cancer types.[5] Liver CSCs are glycolysis-dependent and are characterized as an up-regulated expression of glycolysis-related genes including hexokinase 2 (HK2), phosphoglycerate mutant enzymes 1 (PGAM1), glucose transporter 1 (GLUT1), and pyruvate dehydrogenase kinase 4 (PDK4).[6] Gluconeogenesis is the reverse reaction of glycolysis. As a key enzyme of gluconeogenesis, phosphoenolpyruvate carboxykinase 1 (PCK1) is down-regulated in HCC and is associated with poor prognosis. PCK1 plays a suppressive role in HCC, including inhibiting aerobic glycolysis, promoting oxidative stress and apoptosis, blocking cell cycle progression, inhibiting cell proliferation and metastasis, and restraining tumor angiogenesis.[7–10] A previous study has reported that PCK1 is up-regulated in melanoma tumor-repopulating cells and promotes the tumorigenicity of tumor-repopulating cells.[11] However, whether PCK1 is involved in the regulation of stemness of hepatoma cells is currently unclear.

The Hippo pathway has vital biological functions in organ growth and tumorigenesis. In the Hippo pathway, mammalian STE20-like protein kinase 1 and 2 and large tumor suppressors 1 and 2 (LATS1/2), as tumor suppressors restrain the oncogenic effects of YAP/TAZ-TEAD.[12] In addition to participating in cell proliferation, organ growth, and cancer development, recent evidence indicates that the Hippo pathway has important effects on the biological functions of CSCs, including drug resistance, self-renewal, and epithelial-to-mesenchymal transition.[13]

In this study, we found that PCK1 attenuated the stemness of hepatoma cells and inhibited its tumorigenicity. Furthermore, PCK1 increased the sensitivity of hepatoma cells to sorafenib. Further, we found that PCK1 deletion contributed to the stemness of hepatoma cells by up-regulating YAP. Verteporfin, a YAP/TAZ-selective inhibitor, sensitized hepatoma cells to sorafenib-induced apoptosis. Therefore, verteporfin combined with sorafenib could be a potential anti-tumor strategy for HCC.

Materials and methods

Full details are available in Supplementary Materials.

Cell lines and clinical specimens

Human hepatoma cell line PLC/PRF/5 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), and Huh7 and MHCC-97H were from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in DMEM supplemented with 10% FBS, 100 mg/mL penicillin, and 100 U/mL streptomycin (all were purchased from Gibco, Grand Island, NY, USA) in 5% CO_2 at 37 °C. HCC tumor specimens were obtained from the Second Affiliated Hospital of Chongqing Medical University with the informed consent of the patients according to the Institutional Review Board approval (reference number: 2022052).

Cells were cultured in medium supplemented with Sorafenib (HY-10201; MedChemExpress, New Jersey, USA) or Verteporfin (YAPi; HY-B0146; MedChemExpress), OSMI-1 (T16409; TargetMol, MA, USA), Thiamet G (TG; T6056; TargetMol, MA, USA) and then collected for analysis.

Western blot

Total protein was extracted by cell lysis buffer (Beyotime Biotechnology, Jiangsu, China) with 1 mM phenylmethanesulfonyl fluoride (Beyotime). The extraction and isolation of nuclear and cytoplasmic protein were performed using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime) according to the manufacturer's instructions. Protein samples were separated by SDS/PAGE and electro-transferred to PVDF membranes (Millipore, Billerica, MA, USA). The immunoblots were probed with the indicated antibodies against PCK1 (1:1000; BS6870; Bioworld, Atlanta, GA, USA), YAP (1:1000; 14074; Cell Signaling Technology, Danvers, MA, USA), p-YAP (1:10,000; ab76252; Abcam, Cambridge, UK), O-GlcNAc (1:1000; ab2739; Abcam), Survivin (1:1000; 10508-1-AP; Proteintech Group Inc., Rosemont, IL, USA), H3 (1:5000; H0164; Millipore), β-Tubulin (1:50,000; 66240-1-Ig; Proteintech Group Inc.), and β-actin (1:4000, TA-09; ZSGB-BIO, Bejing, China). Protein bands were visualized with Clarity™ Western ECL Substrate (Bio-Rad).

Immunofluorescence staining

Cell slides were fixed with 4% paraformaldehyde for 20 min, and penetrated with 0.5% Triton X-100 (Sigma-Aldrich, St Louis, MO, USA) for 15 min. Then, the cells were blocked with normal goat serum and incubated with anti-YAP (1:100; Cell Signaling Technology) at 4 °C overnight, and

specific signals were visualized with Alexa Fluor 552 secondary antibody (ZF0316; ZSGB-BIO). The cells were counterstained with 1 μg/mL DAPI (10236276001, Roche Diagnostics GmbH, Mannheim, Germany) and visualized by a laser-scanning confocal microscope (Leica TCS SP8, Solms, Germany).

Sphere formation assay

Tumor cells were cultured in DMEM/F12 (DMEM/F12 basic, Gibco) supplemented with 1X B27 (B-27 Supplement (50X), serum-free, Gibco), 20 ng/mL human recombinant epidermal growth factor (Animal-Free Recombinant Human EGF, PeproTech, Cranbury, NJ, USA) and 20 ng/mL human recombinant basic fibroblast growth factor (Recombinant Human FGF-basic, PeproTech). Cells were cultured in 6-well ultra-low adhesion plates (Corning, Tewksbury, MA, USA) for 10—14 days.

Flow cytometry

Cells were collected and resuspended in 100 μL PBS containing PE anti-CD44 antibody (103007, BioLegend, San Diego, CA, USA) or APC anti-CD133 antibody (566596, BD Biosciences, San Jose, CA, USA). After 30-min incubation at 4 °C in the dark, cells were washed with PBS and resuspended with 300 μL PBS. Samples were detected by FACSCanto™ II flow cytometer (BD Biosciences) and the data were analyzed by FlowJo.

Animal models

BALB/c male nude mice were randomly grouped ($n = 7$/group). Amount of 50 μL of MHCC-97H cell suspension was mixed with 50 μL of the Matrigel (356234, Corning, NY, USA). The cell mixtures were subcutaneously injected into the mice. Tumor incidence was monitored 4 weeks after tumor cells inoculation. Tumor-initiating frequency was calculated by limiting dilution according to the protocol provided on the web (http://bioinf.wehi.edu.au/software/elda/).[14] In addition, BALB/c nude mice were randomly divided into five groups ($n = 6$/group). Parental and PCK1-KO PLC/PRF/5 cells were collected and implanted into the livers of nude mice (1×10^5 cells per injection). The mice implanted with PCK1-KO cells were randomly divided into four groups including (i) vehicle, (ii) sorafenib (30 mg/kg) intraperitoneally every other day, (iii) verteporfin (100 mg/kg) intraperitoneally every other day, and (iv) the combination of sorafenib and verteporfin for 2 weeks, respectively. Those mice were sacrificed after implantation for 5 weeks. All animal procedures were approved by the Research Ethics Committee of Chongqing Medical University (reference number: 2022052).

Data mining

Gene expression data and corresponding clinical data for patients with HCC were obtained from The Cancer Genome Atlas Liver Hepatocellular Carcinoma (TCGA-LIHC) dataset.[15,16] The Kaplan-Meier survival curves were generated by the "survminer" package of R (Version 3.6.3). Raw data from the GSE14897 and GSE25417 datasets were downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/).

Statistical analysis

Data were shown as mean ± standard deviation (SD). Statistical analyses were performed using Student's *t*-test for two-group comparisons, and one-way ANOVA for multi-group comparisons. Pearson correlation coefficient (r) was used to test the linear correlation. P-values <0.05 were considered statistically significant ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$), and "ns" indicates no significance. Statistical analyses were conducted using GraphPad Prism 6.0 software (La Jolla, CA, USA).

Results

PCK1 attenuates the stemness of hepatoma cells and suppresses tumorigenicity *in vitro* and *in vivo*

CSCs are a crucial tumor cell population with high tumorigenicity in HCC.[17] To investigate the effect of PCK1 on the stemness of hepatoma cells, we detected the mRNA expression of CSC markers. The results indicated that PCK1 deficiency significantly increases the mRNA expression of well-characterized stemness markers including *CD133*, *EPCAM*, *CD44*, and *NANOG*, as compared with parental cells (Fig. 1A). Conversely, PCK1 overexpression obtained the opposite effects (Fig. 1B). Moreover, *in vitro* sphere formation assays showed that PCK1 deletion enhanced the self-renewal ability of PLC/PRF/5 cells (Fig. 1C), but PCK1 overexpression inhibited the self-renewal ability of Huh7 and MHCC-97H cells (Fig. 1D). It has been reported that $CD133^{+}$ $CD44^{+}$ hepatoma cells showed CSCs' properties, including proliferation and self-renewal.[18] In our study, flow cytometry analysis showed that the proportion of $CD133^{+}$ or $CD44^{+}$ HCC cells was increased in PLC/PRF/5 cells with PCK1 deficiency (Fig. 1E, F, upper panel) and was decreased in Huh7 and MHCC-97H cells overexpressing PCK1 (Fig. 1E, F, middle and lower panels). These results indicated that PCK1 reduces mRNA expression of CSC markers and the self-renewal property of hepatoma cells.

To further clarify the above findings, the tumorigenicity of HCC cells on PCK1 overexpression *in vivo* was examined in a nude mouse model, with subcutaneous inoculation and a limiting dilution approach with 1×10^3, 1×10^4, and 1×10^5 cells. Consistent with the *in vitro* results, PCK1 attenuates the tumorigenicity of hepatoma cells *in vivo* (from 7/7 to 4/7 with 1×10^5 cells; from 4/7 to 2/7 with 1×10^4 cells; from 3/7 to 0/7 with 1×10^3 cells). In addition, tumor size and mass were smaller in the AdPCK1 group when compared with the AdGFP group. Estimated tumor-initiating frequency was reduced from 1/7290 to 1/88308 after overexpression of PCK1 (Fig. 2A). These findings indicated that PCK1 attenuates the stemness properties of hepatoma cells and tumorigenicity *in vitro* and *in vivo*.

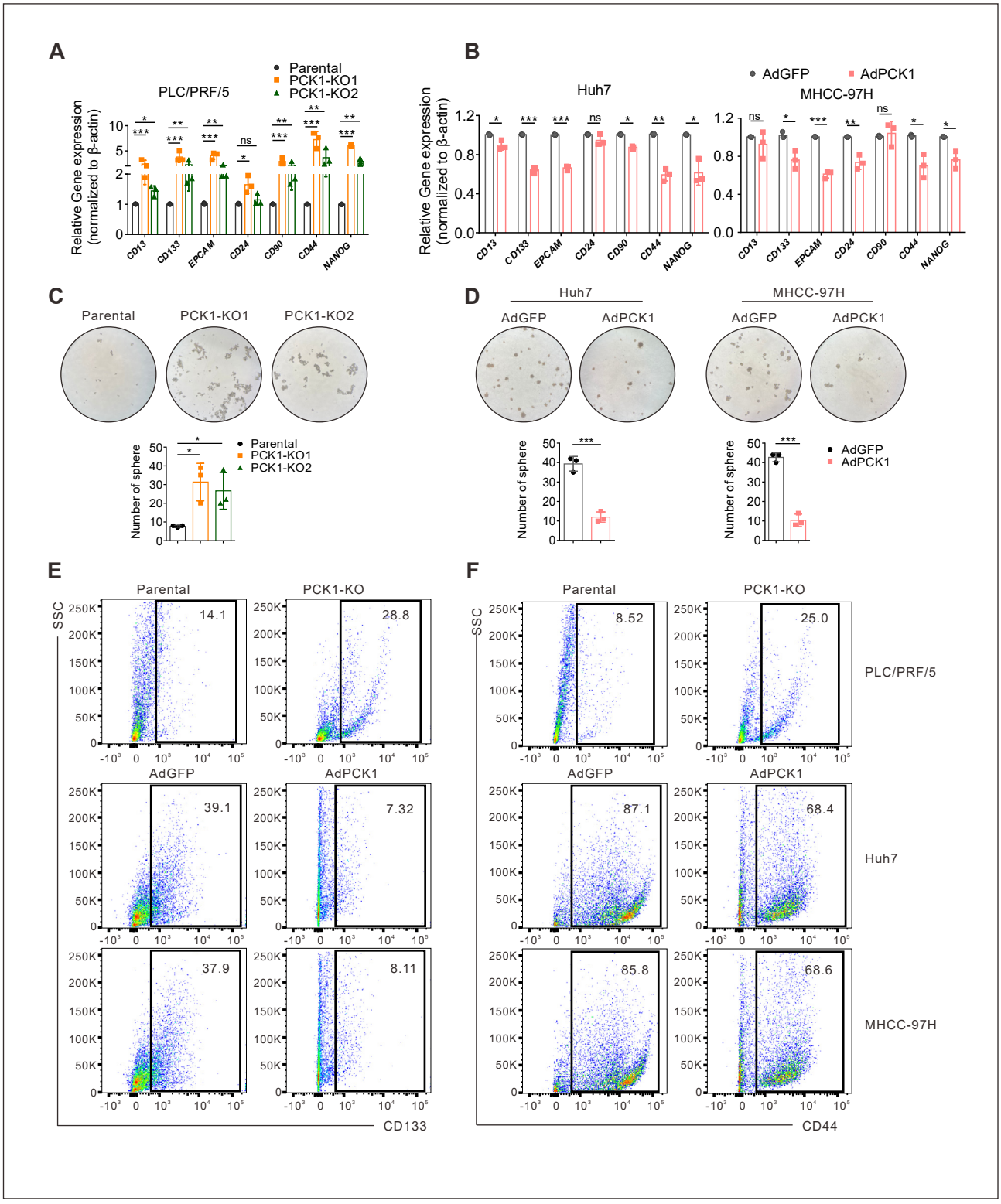

Figure 1 PCK1 attenuates the stemness properties of hepatoma cells. **(A—F)** The effect of PCK1-KO (parental and PCK1-KO PLC/PRF/5 cells) and PCK1-OE (Huh7 or MHCC-97H cells infected with AdGFP, AdPCK1) on the stemness of HCC cells showed by mRNA level of CSC markers (A, B), abilities of sphere formation (C, D), and $CD133^{+}$ cell population (E) and $CD44^{+}$ cell population (F). Statistical analysis was shown as mean ± SD (*$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$; ns, no significance). Three samples one-way ANOVA was performed for (A, C) and two sample *t*-test for (B, D). Magnification: 40 × .

A

	Tumor incidence			Tumor latency (Days, mean ± SD)			Estimated tumor initiating frequency
	10^3 cells	10^4 cells	10^5 cells	10^3 cells	10^4 cells	10^5 cells	
AdGFP	3/7	4/7	7/7	62.00±6.00	38.75±1.71	33.43±2.23	1/7290
AdPCK1	0/7	2/7	4/7		42.00±1.41	36.50±1.29	1/88308

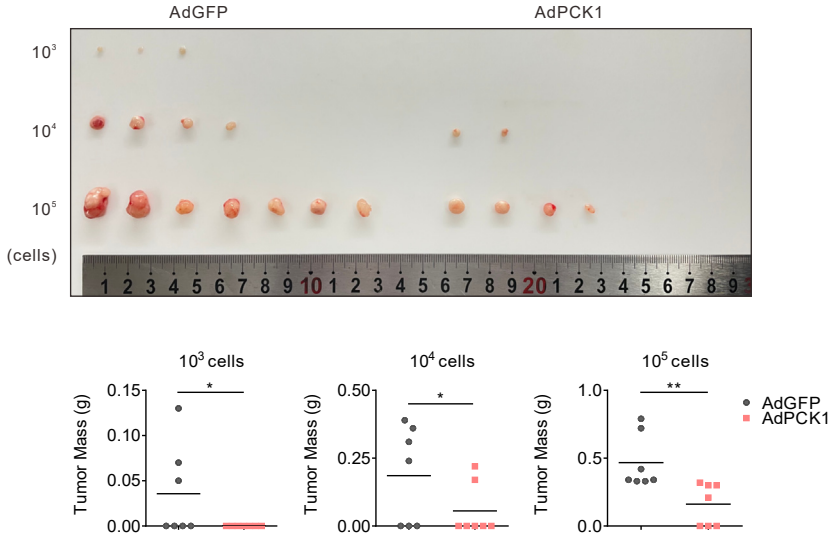

Figure 2 PCK1 suppresses *in vivo* tumorigenicity. MHCC-97H cells infected with AdGFP or AdPCK1 were subcutaneously inoculated with 1×10^3, 10^4, or 10^5 cells in nude mice. Tumor growth, tumor incidence, and latency period were evaluated. Tumor latency data were shown as mean ± SD. Two sample *t*-test; $n = 7$; $^*P < 0.05$, $^{**}P < 0.001$.

PCK1 enhances the sensitivity of hepatoma cells to sorafenib treatment

Liver CSCs contribute to initiating tumor development, inducing tumor progression, and correlating with chemotherapy resistance.[19] Sorafenib-resistant hepatoma cells exhibited significant stemness features.[20] To explore the role of PCK1 in the sensitivity of hepatoma cells to sorafenib treatment, we examined the cell proliferation ability in PCK1 knockout (PCK1-KO) and PCK1 overexpression (PCK1-OE) cells treated with sorafenib. The results of cell growth curves and colony formation assays indicated that PCK1-OE significantly restricted cell growth and sensitized hepatoma cells to sorafenib (Fig. 3A–D; Fig. S1A, B). To observe the effects of sorafenib on apoptosis, we examined the apoptosis rate of cells after sorafenib treatment. Flow-cytometric analysis showed that sorafenib induced cell apoptosis. More importantly, PCK1-KO attenuated the pro-apoptotic effect of sorafenib on hepatoma cells (Fig. 3E). Conversely, PCK1-OE obtained the opposite effects (Fig. 3F; Fig. S1C). Together, these results implied that PCK1 enhances the sensitivity of hepatoma cells to sorafenib via promoting apoptosis.

PCK1 inhibits YAP nuclear translocation

The Hippo pathway has important effects on CSCs' self-renewal and drug resistance.[13] YAP/TAZ-TEAD is a tumor promoter in the Hippo pathway.[21] Thus, we examined the effect of PCK1 on YAP activation. Immunoblotting revealed YAP and downstream Survivin were significantly increased and p-YAP decreased in PCK1-KO cells (Fig. 4A). Conversely, PCK1 overexpression decreased protein levels of YAP and Survivin but increased the expression of p-YAP (Fig. 4B; Fig. S2A). Previous studies have found that YAP is modified by O-GlcNAc and its phosphorylation is inhibited.[22–25] Our previous study found that PCK1 deletion up-regulated global cellular O-GlcNAcylation.[9] Therefore, we hypothesized that PCK1 deletion could up-regulate YAP O-GlcNAcylation and inhibit its phosphorylation. To verify this hypothesis, we examined whether PCK1 regulates YAP O-GlcNAcylation in PCK1-KO or PCK1-OE cells. The results

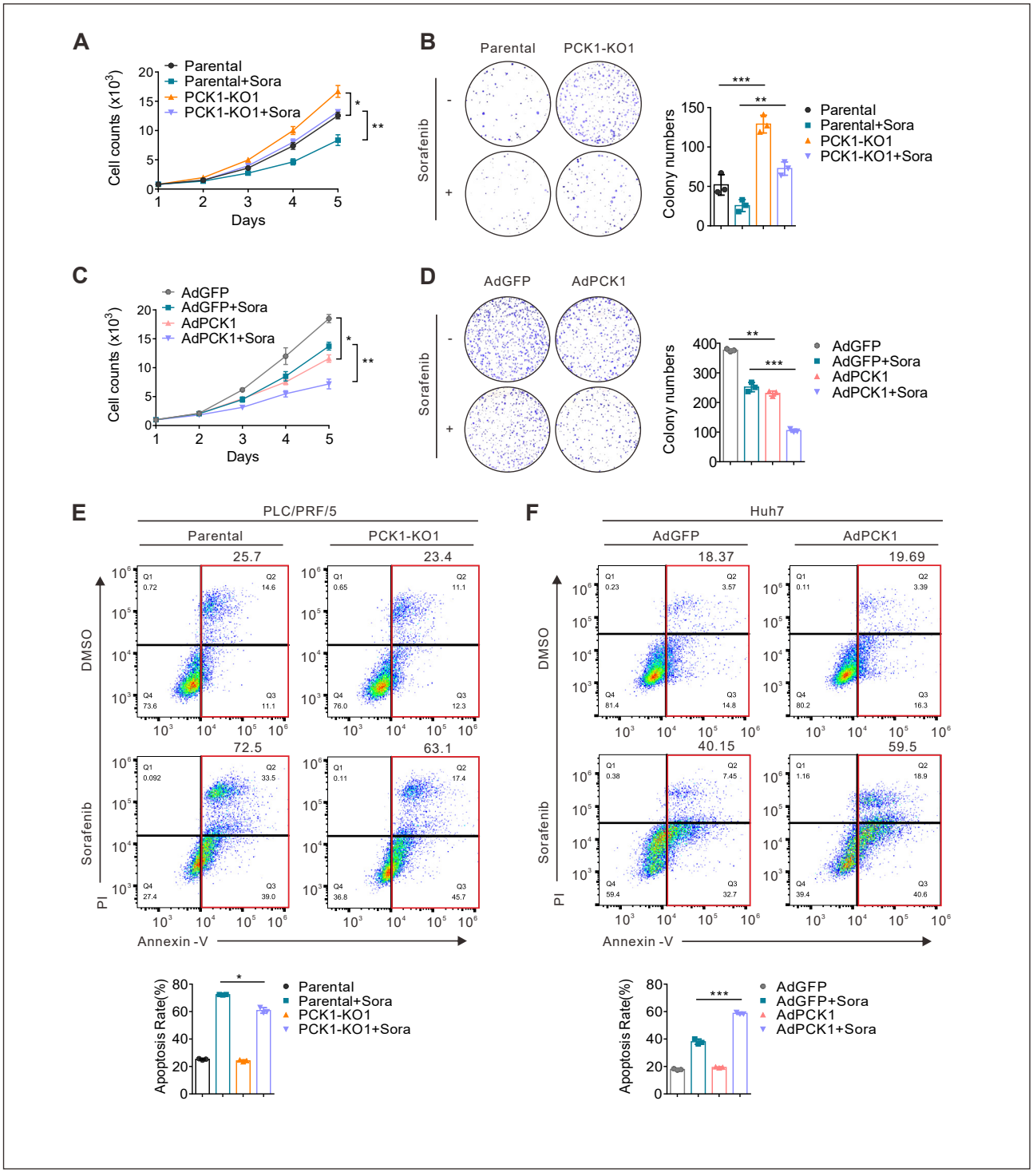

Figure 3 PCK1 enhances the sensitivity of hepatoma cells to sorafenib. **(A—D)** The effect of PCK1-KO or PCK1-OE (Huh7 cells) on cell growth curves and colony formation assays. **(E, F)** Flow-cytometric analysis of apoptosis. PLC/PRF/5 cells were treated with 5 μM sorafenib, and Huh7 cells were treated with 3 μM sorafenib. Statistical data were shown as mean ± SD. Two sample *t*-test; $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$).

showed that PCK1-KO strengthened YAP O-GlcNAcylation and decreased p-YAP, while OSMI-1 (the OGT inhibitor) partially alleviated YAP O-GlcNAcylation and increased p-YAP (Fig. 4C, D). In contrast, PCK1-OE decreased YAP O-GlcNAcylation and increased p-YAP, while TG (the OGA inhibitor) partially reversed YAP O-GlcNAcylation and weakened p-YAP (Fig. S2B—E), suggesting PCK1 deletion up-regulates YAP O-GlcNAcylation, inhibits its phosphorylation, and inhibits the activation of the Hippo/YAP signaling pathway. Next, we detected YAP expression in cytoplasm

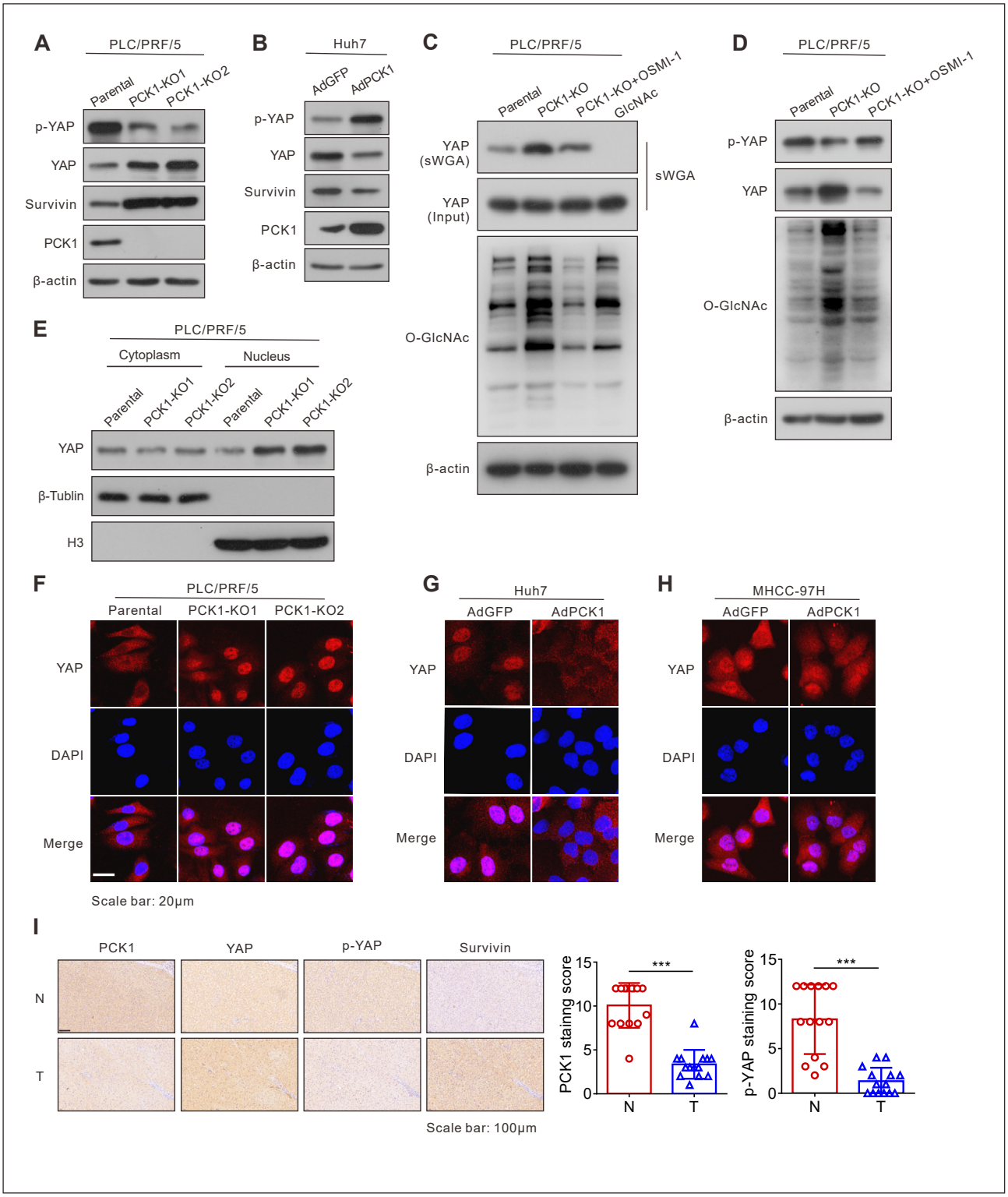

Figure 4 PCK1 inhibits YAP nuclear translocation and promotes its phosphorylation. **(A, B)** The protein level of YAP, p-YAP, and Survivin in PCK1-KO cells (A) and PCK1-OE cells (Huh7) (B). **(C, D)** PCK1-KO cells were treated with 50 μM OSMI-1 for 12 h, followed by sWGA pull-down assay (C) or Western blot (D). The presented input YAP was adjusted to a similar level (C). **(E—H)** YAP expression in cytoplasm and nucleus extraction in hepatoma cells. Cell samples were detected by Western blotting (E) or immunofluorescence (F—H). Scale bar, 20 μm. Nuclei were counterstained with DAPI. **(I)** PCK1 and p-YAP expression in non-tumor tissues (N) and tumor tissues (T) from 14 patients with HCC and semi-quantitative analyses of immunohistochemistry (IHC) data. Scale bars, 100 μm. Statistical data were shown as mean ± SD. Two sample *t*-test; ***$P < 0.0001$.

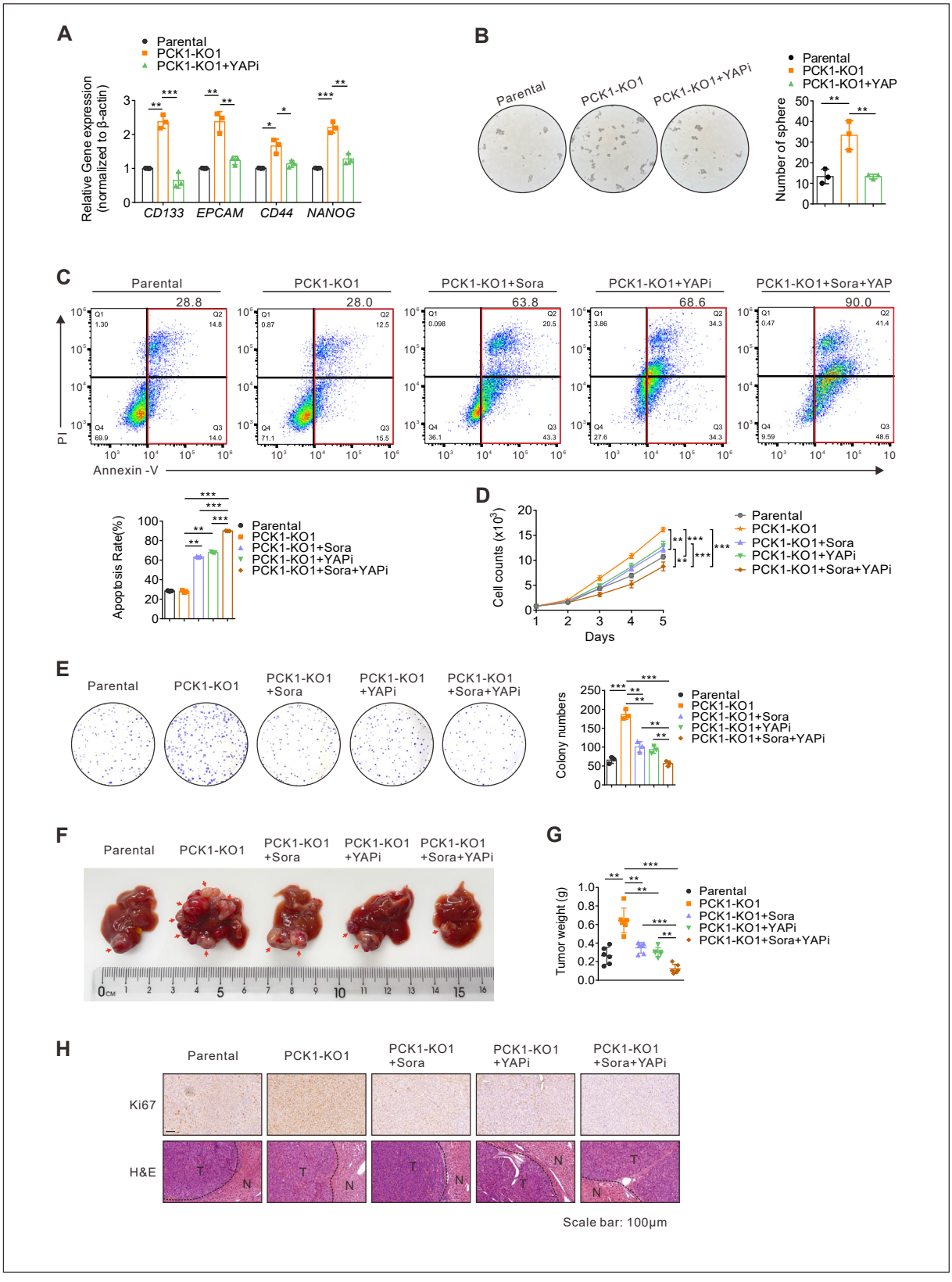

Figure 5 Verteporfin weakens CSCs' properties and potentiates the anti-tumor effect of sorafenib. **(A, B)** The mRNA expression of stemness-related genes (A) and abilities of sphere formation (B) in PCK1-KO cells treated with 20 μM YAP inhibitor (verteporfin). **(C—E)** Flow-cytometric analysis of apoptosis (C), cell growth curves (D), and colony formation assays (E) were performed after treatments with sorafenib and verteporfin as indicated. PCK1-KO cells were treated with 5 μM sorafenib and 20 μM verteporfin. YAPi, YAP inhibitor, verteporfin. **(F)** Representative images of orthotopic liver implantation of PCK1-KO cells in nude mice. **(G)** Liver

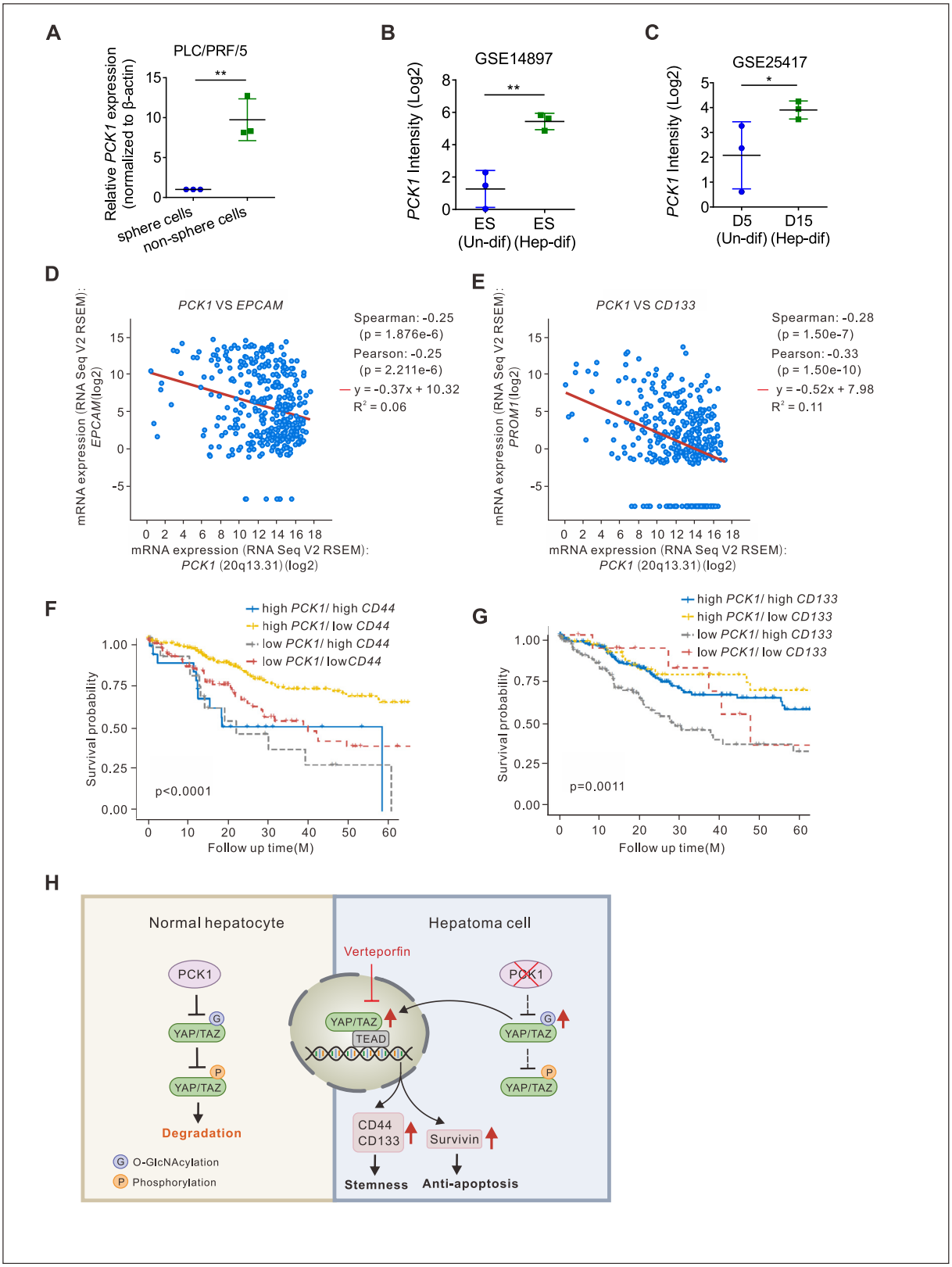

Figure 6 PCK1 is down-regulated in liver CSCs. **(A)** The mRNA level of *PCK1* in spheres derived from PLC/PRF/5 cells. **(B)** *PCK1* was analyzed in undifferentiated embryonic stem cells (ESCs) (GSE14897). Un-dif, undifferentiated; Hep-dif, hepatic-

tumor weight was assessed ($n = 6$ for each group). **(H)** Ki67 staining in liver tumor tissues. Scale bar, 100 μm. Statistical data were shown as mean ± SD. Multi samples one-way ANOVA; $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$. Magnification: 40 × .

and nucleus extraction in hepatoma cells. Surprisingly, endogenous YAP presents a strong nuclear localization in PCK1-KO cells. Whereas, PCK1 overexpression caused a significant down-regulation of nuclear YAP in Huh7 and MHCC-97H cells (Fig. 4E; Fig. S2F, G). The results were also confirmed by immunofluorescence (Fig. 4F—H). In addition, immunohistochemical staining showed that YAP and Survivin were highly expressed in human HCC tissues, while p-YAP was lowly expressed (Fig. 4I; Fig. S2H). Together, these results suggested that PCK1 down-regulates YAP O-GlcNAcylation, promotes YAP phosphorylation, and inhibits YAP nuclear translocation.

Verteporfin reduces hepatoma cell stemness and increases sensitivity to sorafenib

Previous studies indicate that Hippo pathway plays important roles in self-renewal and cell apoptosis.[26,27] We hypothesized that loss of PCK1 could promote stemness of CSCs via Hippo pathway. To verify this hypothesis, we examined the mRNA expression of *CD133*, *EPCAM*, *CD44*, and *NANOG*, and the self-renewal ability of PCK1-KO cells treated with verteporfin or of PCK1 and YAP combined knockout cells. The results showed that both YAP inhibitor and YAP knock-out reduced the mRNA expression level of CSC markers and sphere formation (Fig. 5A, B; Fig. S3A, B). Furthermore, PCK1-KO cells treated with verteporfin in combination with sorafenib showed significantly higher apoptosis (Fig. 5C). Verteporfin combined with sorafenib induced a synergistic effect on inhibiting proliferation (Fig. 5D, E). Similarly, YAP deficiency retarded the proliferation ability of PCK1-KO cells (Fig. S3C, D). To confirm the synergistic effect of verteporfin and sorafenib in inhibiting tumor growth *in vivo*, PCK1-KO cells were implanted into the nude mice livers. The synergistic inhibitory effect of verteporfin and sorafenib was confirmed in the orthotopic implantation model, consistent with the results of cell experiments *in vitro* (Fig. 5F—H). Collectively, these data indicated targeting YAP with verteporfin could attenuate stemness properties and increase sensitivity to sorafenib *in vitro* and *in vivo*.

PCK1 expression was negatively correlated with the stemness of hepatoma cells

Finally, we assessed the mRNA level of PCK1 in spheres derived from PLC/PRF/5 cells. The result showed PCK1 weakly expressed in spheres (Fig. 6A). In addition, PCK1 was also lowly expressed in embryonic stem cells by analysis of its expression profiles derived from GEO datasets (GSE14897[28] and GSE25417[29]) (Fig. 6B, C). Furthermore, *PCK1* was negatively correlated to *EPCAM* or *CD133* mRNA level (Fig. 6D, E) and survival analysis showed that patients with lower *PCK1* but higher *CD44* or *CD133* had poorer overall survival (Fig. 6F, G). In summary, these results supported that PCK1 suppresses the stemness properties in HCC.

Discussion

CSCs are a population of tumor cells with a self-renewal property. CSCs have distinct metabolic manners compared with tumor cells and primarily rely on glycolysis or OXPHOS in a tumor type-dependent manner. The CSCs of lung cancer, pancreatic cancer, glioblastoma, ovarian cancer, and AML relied on OXPHOS.[30—33] Nevertheless, the CSCs of breast cancer, nasopharyngeal cancer, and liver cancer mainly utilized glycolysis.[34—36] A previous study observed that the expression of glycolytic genes (*HK2*, *PDK4*, *LUT1*, *PGAM1*) was up-regulated and the expression of gluconeogenetic enzymes (*G6Pase*, *PCK*) was down-regulated in $CD133^+$ liver CSCs.[6] In this study, we found that PCK1 attenuates the stemness of hepatoma cells and tumorigenicity via Hippo pathway. Also, PCK1 enhances the sensitivity of hepatoma cells to sorafenib. Furthermore, verteporfin, a YAP/TAZ-selective inhibitor, could reduce the stemness of hepatoma cells and enhance the pro-apoptotic effect of sorafenib (Fig. 6H). This study uncovered the importance of the gluconeogenic rate-limiting enzyme PCK1 in the self-renewal and tumorigenic properties of liver CSCs.

Recent studies have shown that metabolic enzymes have essential roles in the maintenance and regulation of stemness in CSCs. Glutaminase 1 promoted CSCs' properties via ROS/Wnt/β-catenin pathway.[37] Fructose-1,6-bisphosphatase suppressed glycolysis and promoted apoptosis in cancer stem-like cells.[38] High levels of phosphoenolpyruvate carboxykinase 2 (PCK2) maintained tumor-initiating cells and promoted tumor aggressiveness in prostate cancer.[39] In another study on melanoma, down-regulation of PCK2 was essential for tumor-repopulating cell growth *in vitro* and tumorigenesis *in vivo* in melanoma.[40] Unexpectedly, up-regulation of PCK1 was necessary for the growth of tumorigenic melanoma tumor-repopulating cells.[11] Previous studies have shown that the role of PCK1 in cancers originating in different tissues may be inconsistent.[41,42] PCK1 promotes tumor cell proliferation and survival in cancers originating in non-gluconeogenic tissues, including melanoma, colon carcinoma, gastric cancer, and pancreatic cancer.[11,43—45] In contrast, PCK1 expression was decreased in cancers derived from gluconeogenic tissues, such as the liver and kidney. PCK1 inhibits tumor cell proliferation and survival in HCC and renal cell carcinoma.[7,46—48] It has been reported that acetylation-induced isoenzyme conversion resulted in PCK1 down-regulation and PCK2 up-regulation in

differentiation. Statistical data were shown as mean ± SD (*$P < 0.05$; **$P < 0.001$). **(C)** *PCK1* expression levels were analyzed on day 5 and day 15 during hepatic differentiation from ES cells (GSE25417). **(D, E)** Correlation analysis of *PCK1* and *EPCAM* **(D)** or *CD133* **(E)** mRNA levels in HCC using data from the TCGA database (Pearson's correlation coefficient). **(F, G)** Prognostic significance of the combination of *PCK1* with *CD44* **(F)** or *CD133* **(G)** with overall survival (OS). Two sample *t*-test was performed for (A—C) and Pearson correlation coefficient (r) for (D, E). **(H)** Schematic illustration of the role of PCK1 in liver CSCs. Loss of PCK1 increases the stemness of hepatoma cells by promoting YAP nuclear translocation. Inhibition of YAP can attenuate the stemness of hepatoma cells and promote cell apoptosis.

HepG2-resistant sorafenib cells, and high expression of PCK2 reduced the sensitivity of HCC cells to sorafenib.[49] However, it did not completely deny the role of PCK1 in the sensitivity of hepatoma cells to sorafenib. The biological phenotype and gene expression of sorafenib-resistant HCC cells have been altered. In this study, PCK1 expression increased gradually with cell differentiation. PCK1 inhibits the expression of CSC markers, the stemness of hepatoma cells, and tumorigenicity. This study helps improve the understanding of the involvement of PCK1 in the tumorigenesis and development of different cancer types.

The Hippo pathway is involved in cell proliferation, organ growth, tumorigenesis, and stemness of CSCs.[50] YAP/TAZ is a tumor promoter in the Hippo pathway. LATS1/2 phosphorylates YAP/TAZ and inhibits nuclear translocation of YAP/TAZ, thereby inhibiting downstream gene transcription.[51] Our results showed that PCK1 promotes the activation of Hippo pathway, decreases YAP nuclear translocation, and inhibits the stemness of hepatoma cells. Previous studies have shown that YAP is modified by O-GlcNAc, and YAP O-GlcNAcylation disrupts its interaction with LATS1, preventing its phosphorylation and activating its activity.[22–25] We have found that PCK1 deficiency increases global O-GlcNAcylation in HCC.[9] In this study, we found that PCK1 deficiency suppresses the phosphorylation of YAP by increasing its O-GlcNAcylation. These results suggest that gluconeogenesis dysregulation and abnormal energy metabolism play an important role in maintaining the stemness and tumorigenesis of hepatoma cells.

Sorafenib is approved as a first-line treatment for patients with HCC, but its clinical efficacy is poor. Drug resistance was identified as a major barrier to the failure of sorafenib therapy in HCC.[52] Drug resistance was thought to be related to tumor heterogeneity. Tumors are considered a heterogeneous population of cells, and CSCs represent a small population of cancer cells.[53] In HCC treated with sorafenib, most cancer cells were apoptotic, but liver CSCs remained and showed resistance to sorafenib.[54] Stemness-related signaling pathways, including Hedgehog, Hippo, Notch, and Wnt pathways, play vital roles in the acquisition of CSC phenotypes. Previous studies have shown that blocking the Wnt, Hedgehog, and Notch pathways can sensitize sorafenib in HCC.[55–57] In this study, we found that PCK1 attenuates the stemness of CSCs via activating Hippo pathway and increases the sensitivity of HCC cells to sorafenib. Importantly, the combination of sorafenib and verteporfin, a YAP inhibitor, significantly suppressed hepatoma cell proliferation and promoted apoptosis.

In summary, our study showed that PCK1 suppresses the stemness of hepatoma cells and enhances the sensitivity of hepatoma cells to sorafenib by promoting YAP phosphorylation. Inhibition of YAP can attenuate the stemness of hepatoma cells and inhibit cell proliferation. Therefore, the combination of verteporfin with sorafenib may be a potential anti-tumor strategy for HCC.

Author contributions

RL performed most experiments, analyzed the data, and wrote the original draft. YL, WLZ, and GJZ performed most experiments and analyzed the data. ZRZ assisted with animal experiments. LYH provided technical assistance. NT and KW contributed to supervision, writing — review & editing, and funding acquisition.

Conflict of interests

The authors declare that there is no conflict of interests.

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Appendix A. Supplementary data

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References

1. Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA A Cancer J Clin*. 2021;71(3):209–249.
2. Wang T, Fahrmann JF, Lee H, et al. JAK/STAT3-regulated fatty acid β-oxidation is critical for breast cancer stem cell self-renewal and chemoresistance. *Cell Metabol*. 2018;27(1):136–150.e5.
3. Nio K, Yamashita T, Kaneko S. The evolving concept of liver cancer stem cells. *Mol Cancer*. 2017;16(1):1–12.
4. Hanahan D. Hallmarks of cancer: New dimensions. *Cancer Discov*. 2022;12(1):31–46.
5. Sancho P, Barneda D, Heeschen C. Hallmarks of cancer stem cell metabolism. *Br J Cancer*. 2016;114(12):1305–1312.
6. Le A. *The Heterogeneity of Cancer Metabolism*. 1311. Springer International Publishing; 2021.
7. Liu MX, Jin L, Sun SJ, et al. Metabolic reprogramming by PCK1 promotes TCA cataplerosis, oxidative stress and apoptosis in liver cancer cells and suppresses hepatocellular carcinoma. *Oncogene*. 2018;37(12):1637–1653.
8. Tuo L, Xiang J, Pan X, et al. PCK1 negatively regulates cell cycle progression and hepatoma cell proliferation via the AMPK/$p27^{Kip1}$ axis. *J Exp Clin Cancer Res*. 2019;38(1):50.
9. Xiang J, Chen C, Liu R, et al. Gluconeogenic enzyme PCK1 deficiency promotes CHK_2 O-GlcNAcylation and hepatocellular

carcinoma growth upon glucose deprivation. *J Clin Invest*. 2021;131(8):e144703.
10. Liu R, Gou D, Xiang J, et al. O-GlcNAc modified-TIP60/KAT5 is required for PCK1 deficiency-induced HCC metastasis. *Oncogene*. 2021;40(50):6707—6719.
11. Li Y, Luo S, Ma R, et al. Upregulation of cytosolic phosphoenolpyruvate carboxykinase is a critical metabolic event in melanoma cells that repopulate tumors. *Cancer Res*. 2015;75(7):1191—1196.
12. Ma S, Meng Z, Chen R, Guan KL. The Hippo pathway: biology and pathophysiology. *Annu Rev Biochem*. 2019;88:577—604.
13. Park JH, Shin JE, Park HW. The role of Hippo pathway in cancer stem cell biology. *Mol Cell*. 2018;41(2):83—92.
14. Lo RCL, Leung CON, Chan KKS, et al. Cripto-1 contributes to stemness in hepatocellular carcinoma by stabilizing Dishevelled-3 and activating Wnt/β-catenin pathway. *Cell Death Differ*. 2018;25(8):1426—1441.
15. Cerami E, Gao J, Dogrusoz U, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov*. 2012;2(5):401—404.
16. Gao J, Aksoy BA, Dogrusoz U, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal*. 2013;6(269):pl1.
17. Yamashita T, Wang XW. Cancer stem cells in the development of liver cancer. *J Clin Invest*. 2013;123(5):1911—1918.
18. Zhu Z, Hao X, Yan M, et al. Cancer stem/progenitor cells are highly enriched in $CD133^+$ $CD44^+$ population in hepatocellular carcinoma. *Int J Cancer*. 2010;126(9):2067—2078.
19. Ji J, Wang XW. Clinical implications of cancer stem cell biology in hepatocellular carcinoma. *Semin Oncol*. 2012;39(4):461—472.
20. Xia S, Pan Y, Liang Y, Xu J, Cai X. The microenvironmental and metabolic aspects of sorafenib resistance in hepatocellular carcinoma. *EBioMedicine*. 2020;51:102610.
21. Yang Y, Li X, Wang T, Guo Q, Xi T, Zheng L. Emerging agents that target signaling pathways in cancer stem cells. *J Hematol Oncol*. 2020;13(1):60.
22. Peng C, Zhu Y, Zhang W, et al. Regulation of the Hippo-YAP pathway by glucose sensor O-GlcNAcylation. *Mol Cell*. 2017;68(3):591—604.e5.
23. Zhang X, Qiao Y, Wu Q, et al. The essential role of YAP O-GlcNAcylation in high-glucose-stimulated liver tumorigenesis. *Nat Commun*. 2017;8:15280.
24. Li X, Wu Z, He J, et al. OGT regulated O-GlcNAcylation promotes papillary thyroid cancer malignancy via activating YAP. *Oncogene*. 2021;40(30):4859—4871.
25. Zhu G, Murshed A, Li H, et al. O-GlcNAcylation enhances sensitivity to RSL3-induced ferroptosis via the YAP/TFRC pathway in liver cancer. *Cell Death Dis*. 2021;7(1):83.
26. Shao DD, Xue W, Krall EB, et al. KRAS and YAP1 converge to regulate EMT and tumor survival. *Cell*. 2014;158(1):171—184.
27. Cao X, Wang C, Liu J, Zhao B. Regulation and functions of the Hippo pathway in stemness and differentiation. *Acta Biochim Biophys Sin*. 2020;52(7):736—748.
28. Si-Tayeb K, Noto FK, Nagaoka M, et al. Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. *Hepatology*. 2010;51(1):297—305.
29. DeLaForest A, Nagaoka M, Si-Tayeb K, et al. HNF4A is essential for specification of hepatic progenitors from human pluripotent stem cells. *Development*. 2011;138(19):4143—4153.
30. Ye XQ, Li Q, Wang GH, et al. Mitochondrial and energy metabolism-related properties as novel indicators of lung cancer stem cells. *Int J Cancer*. 2011;129(4):820—831.
31. Janiszewska M, Suvà ML, Riggi N, et al. Imp2 controls oxidative phosphorylation and is crucial for preserving glioblastoma cancer stem cells. *Genes Dev*. 2012;26(17):1926—1944.
32. Lagadinou ED, Sach A, Callahan K, et al. BCL-2 inhibition targets oxidative phosphorylation and selectively eradicates quiescent human leukemia stem cells. *Cell Stem Cell*. 2013;12(3):329—341.
33. Sancho P, Burgos-Ramos E, Tavera A, et al. MYC/PGC-1α balance determines the metabolic phenotype and plasticity of pancreatic cancer stem cells. *Cell Metabol*. 2015;22(4):590—605.
34. Dong C, Yuan T, Wu Y, et al. Loss of FBP_1 by Snail-mediated repression provides metabolic advantages in basal-like breast cancer. *Cancer Cell*. 2013;23(3):316—331.
35. Shen YA, Wang CY, Hsieh YT, Chen YJ, Wei YH. Metabolic reprogramming orchestrates cancer stem cell properties in nasopharyngeal carcinoma. *Cell Cycle*. 2015;14(1):86—98.
36. Chen CL, Uthaya Kumar DB, Punj V, et al. NANOG metabolically reprograms tumor-initiating stem-like cells through tumorigenic changes in oxidative phosphorylation and fatty acid metabolism. *Cell Metabol*. 2016;23(1):206—219.
37. Li B, Cao Y, Meng G, et al. Targeting glutaminase 1 attenuates stemness properties in hepatocellular carcinoma by increasing reactive oxygen species and suppressing Wnt/beta-catenin pathway. *EBioMedicine*. 2019;39:239—254.
38. Dai J, Ji Y, Wang W, et al. Loss of fructose-1, 6-bisphosphatase induces glycolysis and promotes apoptosis resistance of cancer stem-like cells: an important role in hexavalent chromium-induced carcinogenesis. *Toxicol Appl Pharmacol*. 2017;331:164—173.
39. Zhao J, Li J, Fan TWM, Hou SX. Glycolytic reprogramming through PCK2 regulates tumor initiation of prostate cancer cells. *Oncotarget*. 2017;8(48):83602—83618.
40. Luo S, Li Y, Ma R, et al. Downregulation of PCK2 remodels tricarboxylic acid cycle in tumor-repopulating cells of melanoma. *Oncogene*. 2017;36(25):3609—3617.
41. Grasmann G, Smolle E, Olschewski H, Leithner K. Gluconeogenesis in cancer cells — repurposing of a starvation-induced metabolic pathway? *Biochim Biophys Acta Rev Cancer*. 2019;1872(1):24—36.
42. Wang Z, Dong C. Gluconeogenesis in cancer: function and regulation of PEPCK, FBPase, and G6Pase. *Trends Cancer*. 2019;5(1):30—45.
43. Montal ED, Dewi R, Bhalla K, et al. PEPCK coordinates the regulation of central carbon metabolism to promote cancer cell growth. *Mol Cell*. 2015;60(4):571—583.
44. Li Y, Zhang M, Dorfman RG, et al. SIRT2 promotes the migration and invasion of gastric cancer through RAS/ERK/JNK/MMP-9 pathway by increasing PEPCK1-related metabolism. *Neoplasia*. 2018;20(7):745—756.
45. Chaika NV, Yu F, Purohit V, et al. Differential expression of metabolic genes in tumor and stromal components of primary and metastatic loci in pancreatic adenocarcinoma. *PLoS One*. 2012;7(3):e32996.
46. Ma R, Zhang W, Tang K, et al. Switch of glycolysis to gluconeogenesis by dexamethasone for treatment of hepatocarcinoma. *Nat Commun*. 2013;4:2508.
47. Bian XL, Chen HZ, Yang PB, et al. Nur77 suppresses hepatocellular carcinoma via switching glucose metabolism toward gluconeogenesis through attenuating phosphoenolpyruvate carboxykinase sumoylation. *Nat Commun*. 2017;8:14420.
48. Khan M, Biswas D, Ghosh M, Mandloi S, Chakrabarti S, Chakrabarti P. mTORC2 controls cancer cell survival by modulating gluconeogenesis. *Cell Death Dis*. 2015;1:15016.
49. Jing Z, Gao J, Li J, et al. Acetylation-induced PCK isoenzyme transition promotes metabolic adaption of liver cancer to systemic therapy. *Cancer Lett*. 2021;519:46—62.
50. Yu FX, Zhao B, Guan KL. Hippo pathway in organ size control, tissue homeostasis, and cancer. *Cell*. 2015;163(4):811—828.

51. Wu Z, Guan KL. Hippo signaling in embryogenesis and development. *Trends Biochem Sci*. 2021;46(1):51—63.
52. Llovet JM, Ricci S, Mazzaferro V, et al. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med*. 2008;359(4):378—390.
53. Saunders NA, Simpson F, Thompson EW, et al. Role of intratumoural heterogeneity in cancer drug resistance: molecular and clinical perspectives. *EMBO Mol Med*. 2012;4(8):675—684.
54. Oishi N, Yamashita T, Kaneko S. Molecular biology of liver cancer stem cells. *Liver Cancer*. 2014;3(2):71—84.
55. Lin HH, Feng WC, Lu LC, Shao YY, Hsu CH, Cheng AL. Inhibition of the Wnt/β-catenin signaling pathway improves the antitumor effects of sorafenib against hepatocellular carcinoma. *Cancer Lett*. 2016;381(1):58—66.
56. Wang S, Wang Y, Xun X, et al. Hedgehog signaling promotes sorafenib resistance in hepatocellular carcinoma patient-derived organoids. *J Exp Clin Cancer Res*. 2020;39(1):22.
57. Wang X, Wang R, Bai S, et al. Musashi2 contributes to the maintenance of $CD44^{v6+}$ liver cancer stem cells via Notch1 signaling pathway. *J Exp Clin Cancer Res*. 2019;38(1):505.