Knockdown of the Sonic Hedgehog (*SHH*) Gene Inhibits Proliferation of Hep3B and SMMC-7721 Hepatocellular Carcinoma Cells via the PI3K/Akt/PCK1 Signaling Pathway

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDE **Youping Chen**
ABEF **Weifang Zhu**

Department of Gastroenterology, The First Peoples' Hospital of Tianmen City, Tianmen, Hubei, P.R. China

Corresponding Author: Weifang Zhu, e-mail: zhu_wf1786@outlook.com
Source of support: Departmental sources

Background: The *PCK1* gene encodes phosphoenolpyruvate carboxykinase (PEPCK), which has been shown have a role in metabolic events in hepatocellular carcinoma (HCC). This study aimed to investigate the role of the *SHH* gene and its encoded protein, sonic hedgehog (SHH), in two human hepatocellular carcinoma (HCC) cell lines.

Material/Methods: The human HCC cell lines Hep3B and SMMC-7721 were cultured. Cells were transfected with plasmids carrying specific *SHH* gene short-hairpin RNA (shRNA) and negative control (NC) shRNA. The effects of knockdown of expression levels of the *SHH* gene were studied on cell survival, cell apoptosis, the cell cycle, gluconeogenesis, and the expression of *PCK1*. Anchorage-independent growth, a characteristic of transformed cells, was detected by the colony formation assay. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) and Western blot were performed 24 h after transfection.

Results: Knockdown of expression levels of the *SHH* gene reduced cell proliferation and growth of HCC cells and induced cell apoptosis and G1 cell cycle arrest in both HCC cell lines. Knockdown of the *SHH* gene decreased the levels of glycolysis products and increased the production of glucose and reduced the phosphorylation of PI3K and Akt but induced the expression of *PCK1*.

Conclusions: Knockdown of the *SHH* gene reduced cell survival of HCC cells by increasing apoptosis, reducing cell proliferation, inducing G1 cell cycle arrest, and restoring gluconeogenesis, and was associated with the inhibition of the PI3K/Akt axis and induced the expression of *PCK1*.

MeSH Keywords: Hedgehog Proteins • Liver Neoplasms • Phosphatidylinositol 3-Kinases • Phosphoenolpyruvate Carboxykinase (ATP)

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/914768

📄 3005   📊 3   📈 7   📚 34

Background

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer [1] and is the second leading cause of cancer-related death, worldwide [2,3]. Chronic inflammatory diseases of the liver are known risk factors for the development of HCC [4–7], which might explain the high incidence of HCC during the past decades [8]. However, with the rapid development of treatment strategies, the incidence, morbidity, and mortality associated with HCC continue to increase. In 2017, the overall 5-year survival for patients with HCC was reported to be between 10–20% [9], which supports the need for the identification of novel therapeutic targets for HCC.

Metabolic reprogramming has been shown to be involved in oncogenesis [10]. The liver is the primary organ involved in endogenous glucose production and energy turnover, the normal function of the liver is a prerequisite for gluconeogenesis. However, during the progression of HCC, changed metabolic profiles associated with the tumor growth increase glucose uptake, glycolysis, and lactic acid production [11,12], which results in the Warburg effect, and supports the growth and survival of tumor cells [13,14]. There is increasing interest in the pathways involved in the regulation of the metabolic changes that occur during tumor progression [15]. Phosphoenolpyruvate carboxykinase (PEPCK/PCK) encoded by the *PCK* genes are the key enzymes regulating the process of gluconeogenesis process in the liver and govern the rate-limiting step in gluconeogenesis [15]. The activity of PEPCK is identified in the cytosol and mitochondria and two distinct isozymes exist that are encoded by different genes (*PCK1* and *PCK2*) [16–18]. Previous studies have shown that *PCK1* is a candidate target for developing treatments for HCC that act by restoring the metabolic properties of liver cells [19–21]. Khan et al. reported that the inhibition of mTOR in HCC initiated glycolytic flux in the gluconeogenesis pathway by upregulating the expression of *PCK1*, which suppressed the proliferation and survival of cancer cells [20]. Therefore, regulating the expression of *PCK1* has been considered as a possible future targeted treatment strategy in HCC.

The function of the *PCK1* gene is influenced by multiple upstream regulators and the identification of these regulators would be important to understand before considering the applications of *PCK1* in the treatment of HCC. The sonic hedgehog (SHH) and PI3K/Akt axis is a well-established signaling transduction axis that has been identified in multiple cancer types, including HCC [22,23]. Therefore, the inhibition of *SHH* gene signaling has now been considered as a promising method to inhibit the progression of multiple cancers [24]. The PI3K/Akt pathway has been shown to promote phosphorylation of forkhead box O (*FOXO*) transcription factors, including FOXO1, which will result in the suppression of *PCK1* gene transcription [25]. Also, the PI3K/Akt pathway is closely associated with gluconeogenesis in the liver. For example, activation of the PI3K/Akt pathway can suppress gluconeogenesis, as shown in several previous studies [26–28]. Therefore, it can be hypothesized that knockdown of the expression of the *SHH* gene may have a potential role in suppressing tumor cell growth in HCC associated with downstream activation of *PCK1*-mediated gluconeogenesis following inhibition of the PI3K/Akt pathway.

Therefore, this study aimed to investigate the role of the *SHH* gene and its encoded protein, SHH, in two human HCC cell lines, with the assessment of cell viability, cell apoptosis, and production of gluconeogenesis-related enzymes and PI3K/Akt and *PCK1* signaling activity following *SHH* gene knockdown.

Material and Methods

Agents and antibodies

The following primary antibodies were used in this study: SHH (bs-1544R) and p-PI3K (bs-5538R) (Beijing Biosynthesis Biotechnology Co., Ltd., China); PCK1 (PAA936Hu01) (USCN Life Science Inc., China); cleaved caspase-3 (ab2302) and cleaved poly ADP-ribose polymerase (PARP) (ab32561) (Abcam, Cambridge, MA, USA); Bcl-2 (BA0412), Bax (BA0315), and PI3K (BA1352) (Boster Bio, Beijing, China); p-Akt (Ser 473) (sc-8312), Akt (sc-135651) and β-actin (sc-47778) (Santa Cruz Biotechnology Inc., Dallas, TX, USA). The following secondary antibodies were used: goat anti-rabbit horseradish peroxidase (HRP)-conjugated IgG (A0216) and goat anti-mouse HRP-conjugated IgG (A0208) (Beyotime, Shanghai, China).

The transfection kit (c1507) was purchased from Applygen Technologies Inc. (Shanghai, China) and the RNA extraction kit (RP1201) and reverse transcription-polymerase chain reaction (RT-PCR) kit (PR6502) were purchased from BioTeke (Beijing, China). The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay solution (M-2128) was purchased from Sigma-Aldrich, St. Louis MO, USA. Detection kits for pyruvic acid (A081), glucose (F006), citrate (A128), and lactate (A019-2) were purchased from Nanjing Jiancheng Bioengineering Institute, China. PicoProbe™ fructose-6-phosphate fluorometric assay kit (K689) and the malate colorimetric assay kit (K637) were obtained from Biovision Inc., (Milpitas, CA, USA). RIPA lysis buffer (P0013B) and the BCA protein concentration kit (P0009) were provided by Beyotime, Shanghai, China.

Cell cultures and transfection

The human hepatocellular carcinoma (HCC) cell line, Hep3B (TCHu106) was provided by the Shanghai Cell Bank of the

Table 1. Sequence data for the short-hairpin RNA (shRNA).

	Sequence (5'-3')
SHH shRNA-1	CCCGACATCATATTTAAGGAT
SHH shRNA-2	GTTTCTTGTGAGATCTTCCC
Control shRNA	GCTGTTGGACAGCGAGACCAT

Chinese Science Academy. The human HCC cell line, SMMC-7721 (ZQ0029) was purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd (Shanghai, China). Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37°C in an atmosphere consisting of 5% CO_2 and 95% air. The cell lines were cultured to a density that allowed sufficient cells for further experiments.

Plasmids carrying specific *SHH* gene short-hairpin RNA (shRNA) and negative control (NC) shRNA (Table 1) were constructed by Sangon Biotech, Shanghai, China, with the sequences as previously published [29]. Each HCC cell line was divided into three groups: the parental group, the NC group, HCC cells transfected with NC plasmid, and the sh*SHH* group, HCC cells transfected shRNA plasmid. Transfection was conducted using transfection agents according to the manufacturer's instruction and stable knockdown of the *SHH* gene was screened with G418 (geneticin) (0.5 µg/µl) and was further verified using reverse transcription quantitative polymerase chain reaction (RT-qPCR) and Western blot assays, 24 h after transfection. The off-target effect of gene knockdown was eliminated with experiments using another shRNA (Table 1, Supplemenatry Figure 1).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Whole RNAs in cells under different treatments were extracted and reverse transcribed into cDNA template using specific kits, following the manufacturer's instruction. The final qPCR reaction mixture volume of 20 µl contained 10 µL of SYBR Green Mastermix and 0.5 µl of each primer (10 µM) (Table 2), 1 µl of the cDNA template, and 8 µl of RNase-free H_2O. The amplification parameters were set as follows: denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 10 s, 60°C for 20 s, and

72°C for 30 s, and the reaction was stopped at 4°C for 5 min. The relative expression level of the *SHH* gene was calculated with Data Assist Software version 3.0 (Applied Biosystems, Foster City, CA, USA) according to the expression of $2^{-\Delta\Delta ct}$.

MTT assay

HCC cell proliferation after knockdown of the *SHH* gene was detected with the MTT assay. Briefly, 1×10^3 cells/ml were inoculated into a 95-well plate and cultured for 96 h. Every 24 h, 5 mg/ml of MTT solution was added into three randomly selected wells for each group and incubated for another 4 h. The cell viability at each time point was represented by the optical density (OD) value at 490 nm, detected using an ELX-800 microplate reader (Biotek, Winooski, VT, USA).

Colony formation assay

The effect of knockdown of the *SHH* gene on HCC cell anchorage-independent growth, a characteristic of transformed cells, was detected by the colony formation assay. Briefly, the cells were seed into RPMI-1640 medium containing 3% methylcellulose at a concentration of 500 cells per plate. After two weeks of culture, the colonies were stained histochemically on the plates with Wright-Giemsa for 5 min. The numbers of colonies were recorded to compare the anchorage-independent growth capability between the groups. The colony formation rate was calculated as the ratio of colony number/inoculated cell number per plate ×100%

Flow cytometry for cell apoptosis and evaluation of the cell cycle

Twenty-four hours after transfection, HCC cells were collected by centrifugation at 2000 rpm for 10 min. Flow cytometry was performed to determine the effect of knockdown of the *SHH* gene on the cell cycle distribution and cell apoptosis. For detection of cell cycle distribution, cells were incubated with 500 µl propidium iodide (PI)- fluorescein isothiocyanate (FITC) at 4°C for 30 min to determine the DNA content using an Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). For detection of cell apoptosis, 5 µl of Annexin-V was added to the culture wells and incubated for 10 min at room

Table 2. Primer sequences.

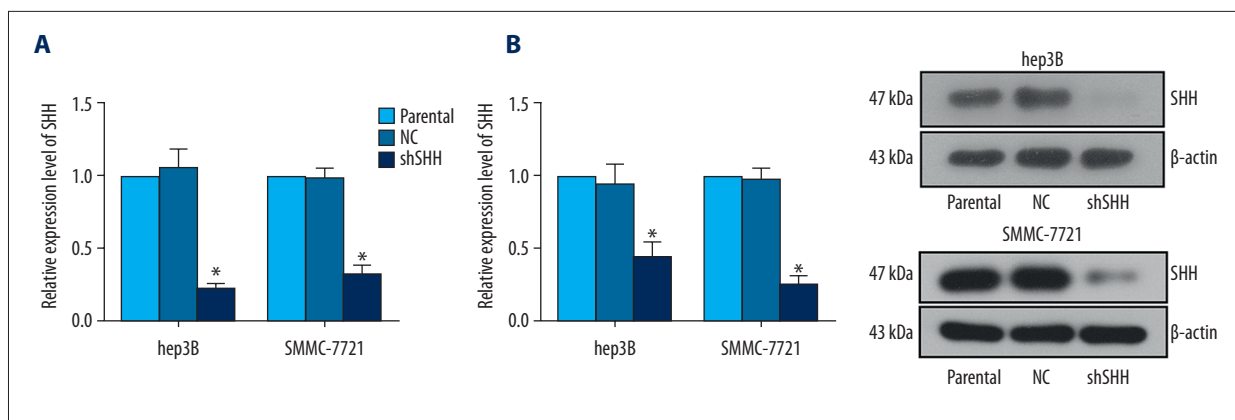| Gene	Direction	Sequence (5'-3')	Tm	Product length (bp)
SHH	Forward	AGCAACAGCAACGCAAAG	55.3	219
	Reverse	AATAGCCAGGAGAGGAGGA	53.5	
β-actin	Forward	CTTAGTTGCGTTACACCCTTTCTTG	62	156
	Reverse	CTGTCACCTTCACCGTTCCAGTTT	64.4	

Figure 1. Transfection of specific short-hairpin RNA (shRNA) reduced the expression of sonic hedgehog (SHH) at the mRNA and protein level. (**A**) Reverse transcription quantitative polymerase chain reaction (RT-qPCR) detection of the *SHH* gene in Hep3B and SMMC-7721 human hepatocellular carcinoma (HCC) cells. (**B**) Western blot detection of sonic hedgehog (SHH) protein in Hep3B and SMMC-7721 cells. * P<0.05 *vs.* the normal control (NC) group.

temperature before the cells were incubated with 5 μl of PI. The apoptosis rate was measured using an Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Gluconeogenesis activity

The activity of the gluconeogenesis pathway was determined by quantifying the levels of glycolysis products, including pyruvic acid, lactate, fructose-6-phosphate, and malate, as well as the level of glucose using different detection kits according to the manufacturer's instructions.

Western blot

The expression levels of components of the PI3K/Akt/PCK1 axis were detected with Western blot. Total protein was extracted using RIPA lysis buffer, and the protein concentrations were determined using a BCA protein measurement kit. Primary antibodies to SHH (1: 500), cleaved caspase-3 (1: 1000), cleaved PARP (1: 1000), Bcl-2 (1: 400), Bax (1: 400), p-PI3K (1: 500), PI3K (1: 400), p-Akt (1: 200), Akt (1: 200), PCK1 (1: 400), and β-actin (1: 1000) were incubated with polyvinylidene difluoride (PVDF) membranes at 4°C overnight. The secondary HRP IgG antibodies (1: 5000) were incubated with the membranes for 45 min at 37°C. The integrated optical density (IOD) of developed blots was detected using the gel Image-Pro system and the relative expression levels of proteins were calculated with Image-Pro analyzer (Media Cybernetics, Rockville, MD, USA).

Statistical analysis

Each assay was performed in triplicate and the data were expressed the mean ± standard deviation (SD). Statistical analysis and graphical data presentation were performed using GraphPad Prism version 6.0 (GraphPad Software, Inc.,

San Diego, CA). Statistical significance was considered to be a two-tailed P-value <0.05.

Results

Knockdown of the *SHH* gene inhibited cell proliferation of Hep3B and SMMC-7721 hepatocellular carcinoma (HCC) cells

The transfection of the *SHH* gene specific short-hairpin RNA (shRNA) suppressed the expression levels of *SHH* both at mRNA and protein levels (Figure 1). Knockdown of the *SHH* gene reduced Hep3B cell viability and proliferation at each time point when compared with cells in Parental and NC groups (P<0.05) (Figure 2A). Similar effects following *SHH* gene knockdown were also found for the SMMC-7721 cells (Figure 2B). These findings supported the role of *SHH* in HCC cell proliferation *in vitro*.

Knockdown of the *SHH* gene reduced anchorage-independent growth of Hep3B and SMMC-7721 HCC cells

Anchorage-independent growth, a characteristic of transformed cells, was detected by the colony formation assay for HCC cells after knockdown of the *SHH* gene. Following gene knockdown, significantly fewer colonies were detected for the Hep3B cells when compared with cells in Parental and NC groups (P<0.05) (Figure 3A) (Table 3). Inhibition of anchorage-independent growth by knockdown of the *SHH* gene was also found in the SMMC-7721 cells (Figure 3B) (Table 3). These findings supported the role of *SHH* in the maintenance of the HCC cell transformed state *in vitro*.
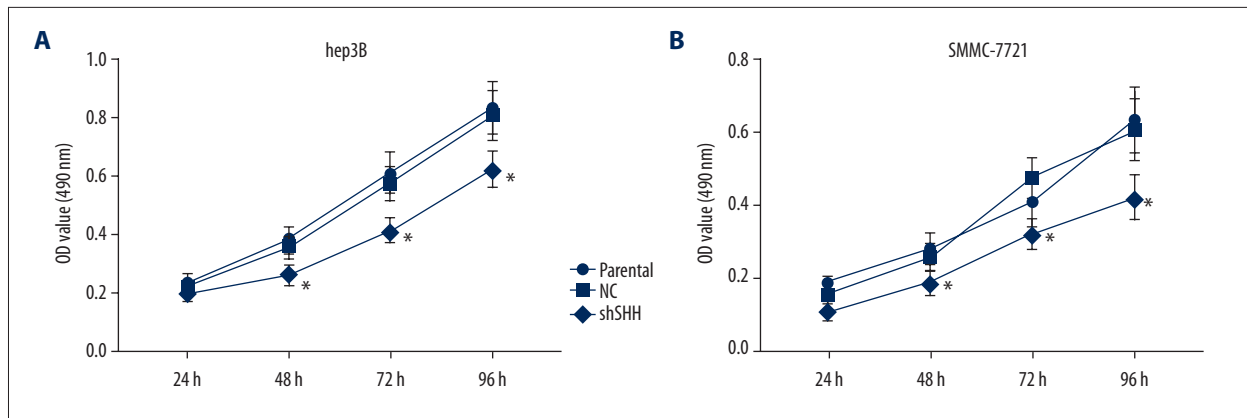
Figure 2. Inhibition of the *SHH* gene suppressed cell proliferation of the hepatocellular carcinoma (HCC) cell lines, Hep3B and SMMC-7721. (**A**) Quantitative analysis results of the MTT assay of cell proliferation in Hep3B cells. (**B**) Quantitative analysis results of the MTT assay of cell proliferation in SMMC-7721 cells. * P<0.05 *vs.* the normal control (NC) group.
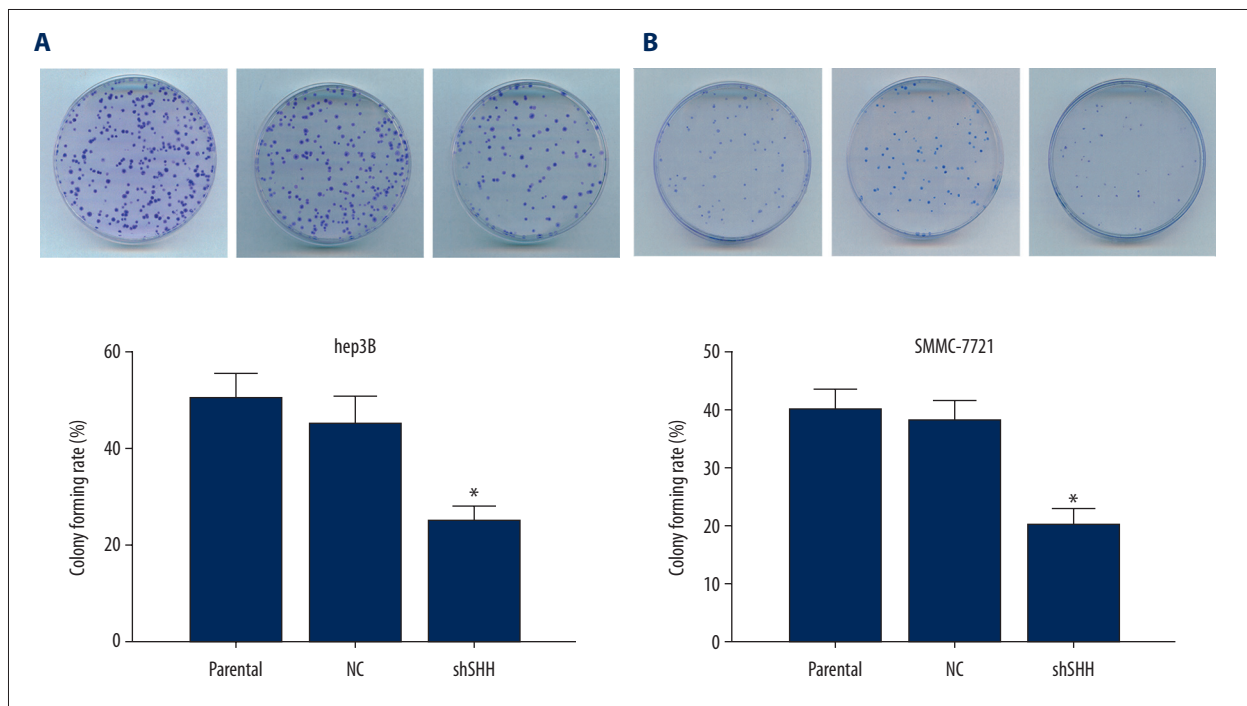

Figure 3. Knockdown of the *SHH* gene suppressed anchorage-independent growth of the HCC cell lines, Hep3B and SMMC-7721. (**A**) Quantitative analysis result of colony formation detection of anchorage-independent growth in Hep3B cells. (**B**) Quantitative analysis result of colony formation detection of anchorage-independent growth in SMMC-7721 cells. * P<0.05 *vs.* the normal control (NC) group.
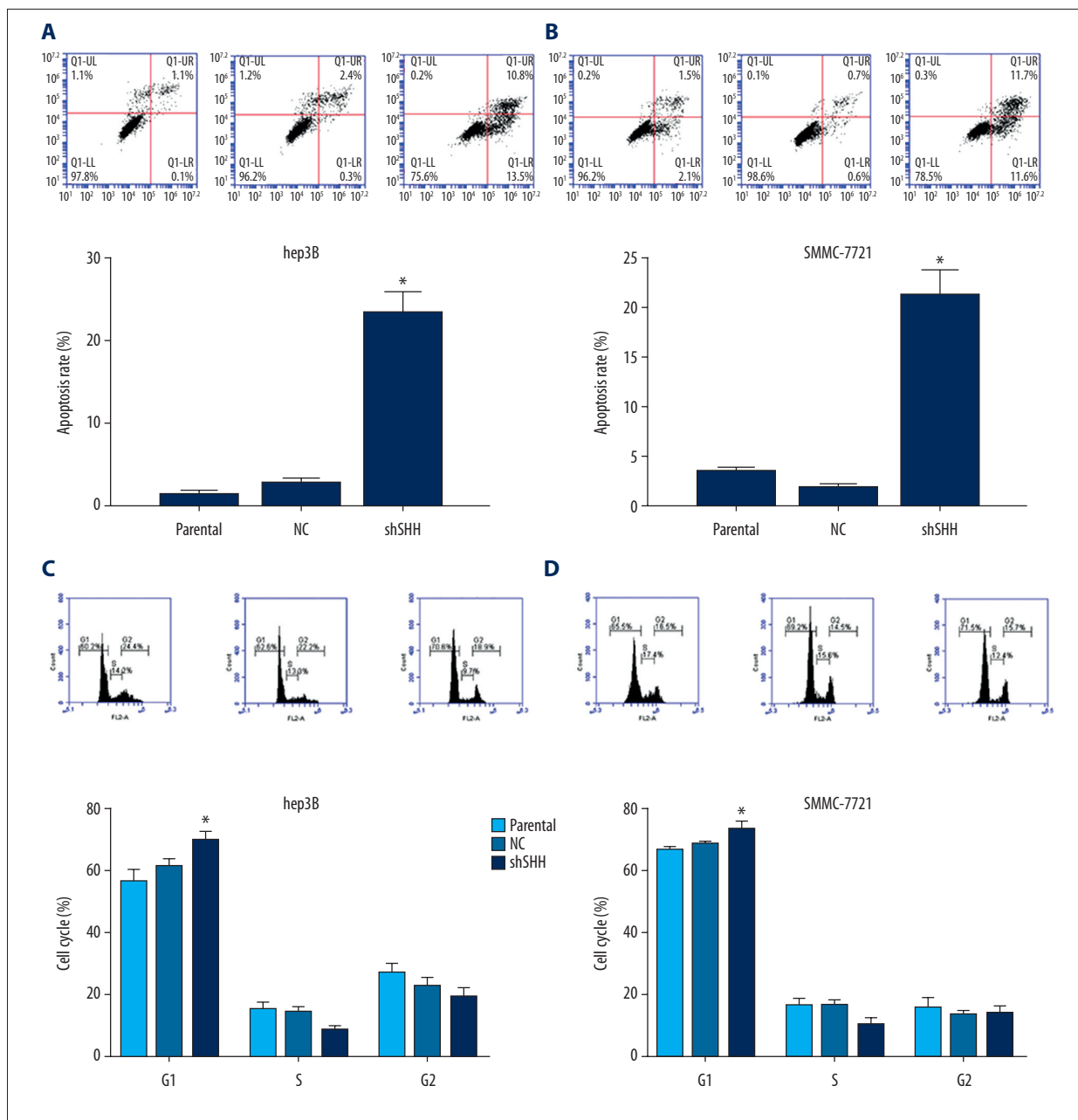
Knockdown of the *SHH* gene induced apoptosis and cell cycle arrest in Hep3B and SMMC-7721 HCC cells

The effect of knockdown of the *SHH* gene on the migration and invasion potential of HCC cells has previously been reported [22], but its effect on cell apoptosis and the cell cycle was not previously studied. In the present study, apoptosis and the cell cycle in HCC cells were studied after *SHH*

gene knockdown, which increased the rate of cell apoptosis rate and the proportion of cell distributed in the G1 phase in both Hep3B and SMMC-7721 cells (Figure 4). The apoptosis rate was associated with increased expression of cleaved caspase-3, cleaved PARP, Bax, and reduced expression of Bcl-2 (Figure 4E, 4F). These results supported that inhibition of *SHH* gene expression reduced cell survival and increased cell apoptosis in HCC cells *in vitro*.

Table 3. Data of the colony formation assays.

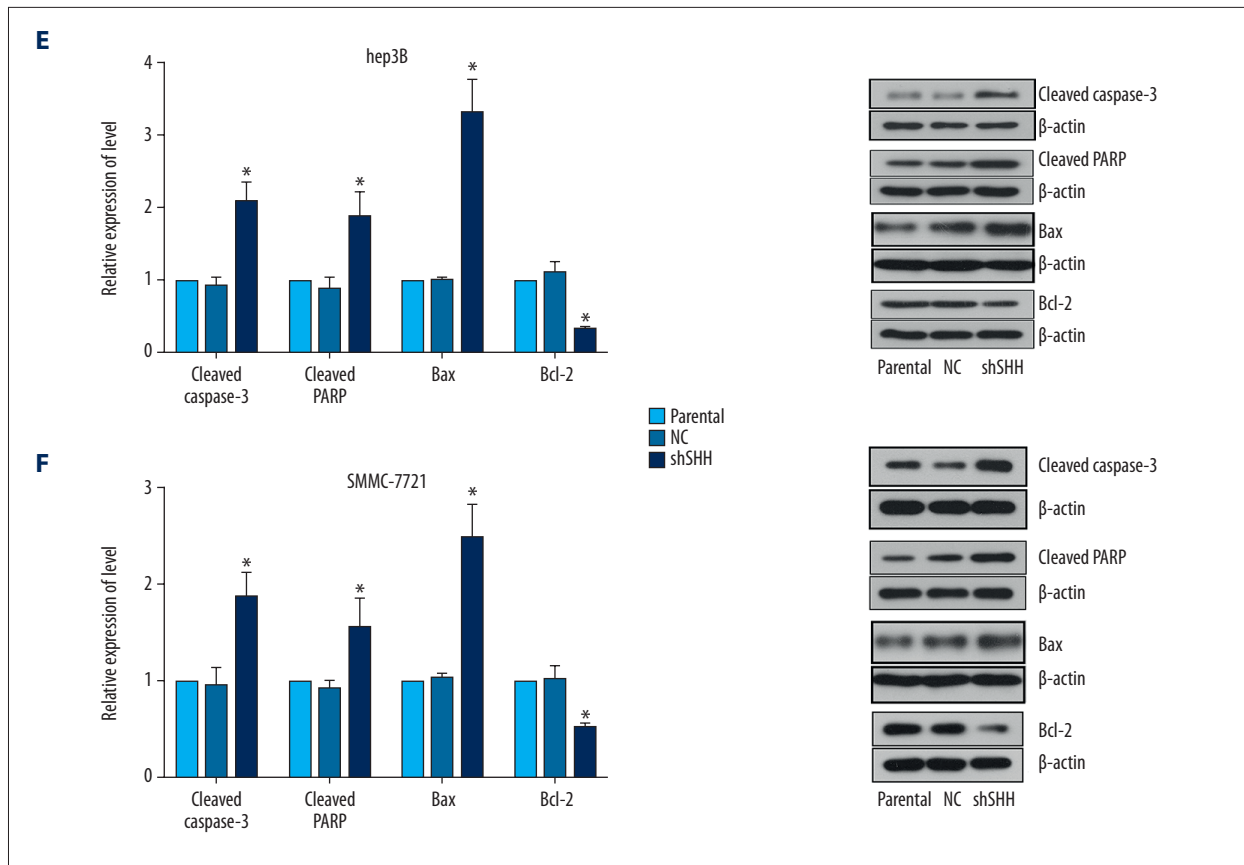| Cell line	Group	Data (%)		
		Rep 1	Rep 2	Rep 3
hep3B	Parental	55.67	45.83	49.00
	NC	49.17	43.97	42.67
	shSHH	25.50	27.33	22.00
SMMC-7721	Parental	40.40	39.80	40.20
	NC	39.40	37.20	42.80
	shSHH	20.40	23.00	18.60

This work is licensed under Creative Common Attribution-
NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0)

6028

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System]
[ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica]
[Chemical Abstracts/CAS]

Figure 4. Knockdown of the *SHH* gene induced cell apoptosis and G1 cell cycle arrest in the HCC cell lines, Hep3B and SMMC-7721. (**A**) Representative images and quantitative analysis results of the flow cytometry detection of cell apoptosis in Hep3B cells. (**B**) Representative images and quantitative analysis results of the flow cytometry detection of cell apoptosis in SMMC-7721 cells. (**C**) Representative images and quantitative analysis results of flow cytometry detection of the cell cycle distribution in Hep3B cells. (**D**) Representative images and quantitative analysis results of flow cytometry detection of the cell cycle distribution in SMMC-7721 cells. (**E**) Quantitative analysis results and representative images of the Western blot results for caspase-3, PARP, Bax, and Bcl-2 in Hep3B cells. (**F**) Quantitative analysis results and representative images of the Western blot results for caspase-3, PARP, Bax, and Bcl-2 in SMMC-7721 cells. * $P<0.05$ *vs.* the normal control (NC) group.

Knockdown of the *SHH* gene activated gluconeogenesis in Hep3B and SMMC-7721 HCC cells

A hypothesis that drove this study was that the expression of the *SHH* gene might be associated with gluconeogenesis in HCC cells, and so the levels of products involved in glycolysis were measured. Knockdown of the *SHH* gene suppressed the production of products involved in glycolysis, including pyruvic acid, lactate, citrate, and malate in Hep3B and SMMC-7721 cells, and increased the production of glucose associated with gluconeogenesis (Figure 5) ($P<0.05$). However, the production of fructose-6-phosphate was not significantly affected by knockdown of the *SHH* gene (Figure 5), which might be attributed to the increased production of fructose-6-phosphate in gluconeogenesis.

Knockdown of the *SHH* gene induced the expression of PCK1 and inhibited the PI3K/Akt pathway in Hep3B and SMMC-7721 HCC cells

This preliminary study attempted to investigate the downstream pathway that mediated the effect of knockdown of the *SHH* gene. Based on the findings from previous studies [22,23], the activity of the PI3K/Akt/PCK1 axis was investigated. As shown by Western blot, knockdown of the *SHH* gene suppressed the phosphorylation levels of PI3K and Akt in both Hep3B and SMMC-7721 cells (Figure 6). Also, inhibition of the PI3K/Akt pathway was associated with increased levels of PCK1, partially explaining the regulating sequence driving the activation of gluconeogenesis following *SHH* gene knockdown.
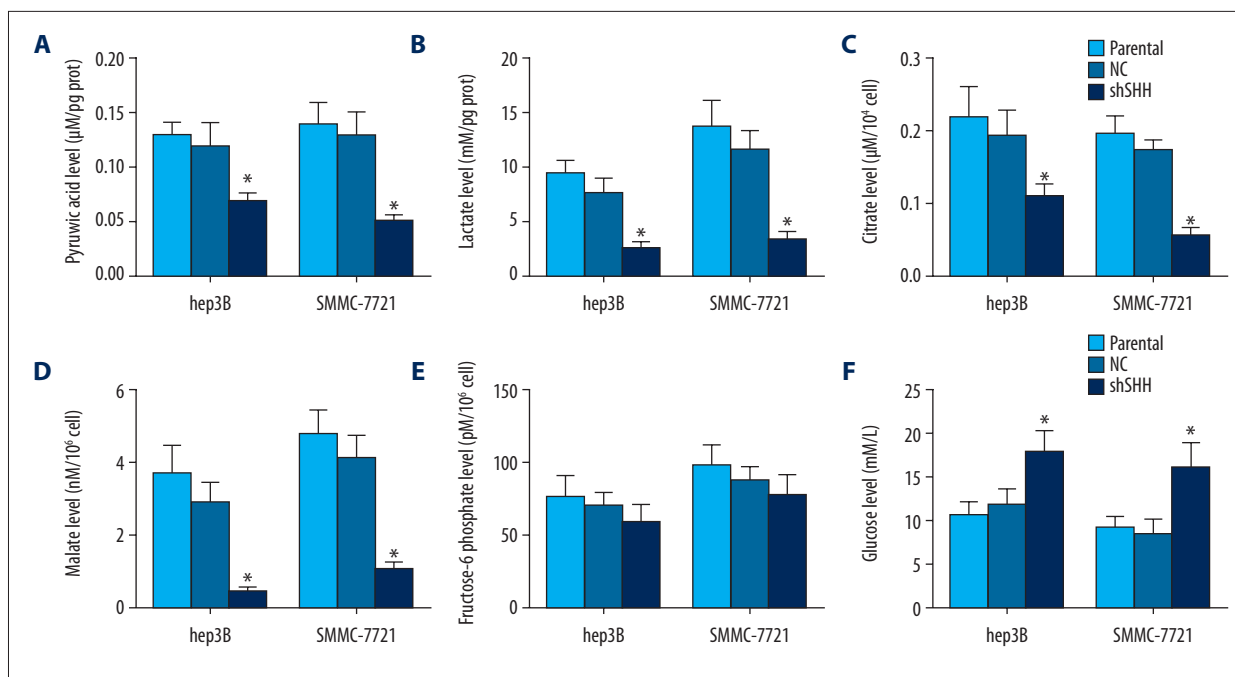
Figure 5. Knockdown the *SHH* gene suppressed glycolysis and activated gluconeogenesis in the HCC cell lines, Hep3B and SMMC-7721. Quantitative analysis results for pyruvic acid (**A**), lactate (**B**), citrate (**C**), malate (**D**), fructose-6 phosphate (**E**), and glucose (**F**) levels in the two cell lines, Hep3B and SMMC-7721. * P<0.05 *vs.* the normal control (NC) group.

Discussion

Sonic hedgehog (SHH) signaling transduction was first identified in *Drosophila melanogaster* has been shown to be involved in the development and maintenance of homeostasis of normal organs. In oncogenesis, the abnormal activity of SHH signaling has been reported in several tumor types. For example, Kasperczyk et al. showed that *SHH* signaling promoted resistance to apoptosis and that this was mediated by NF-κB in pancreatic carcinoma and rhabdomyosarcoma [30]. Similar results were also found by Feldman et al., who showed that inhibition of *SHH* gene signaling reduced the metastatic potential of pancreatic cancer [31]. The role of the *SHH* gene in the metastatic potential of HCC was also previously reported by Sun et al., who showed the association with reduced activity of the PI3K/Akt pathway, which inhibited migration ability of HCC cells by inhibition of SHH-Gli1 signaling [22]. Based on the findings from these previous studies, the main aim of the present study was to further explore the downstream pathway mediating the function of the *SHH* gene in the progression of HCC. The findings showed that the *SHH* gene was also involved in maintaining the survival of HCC cells *in vitro* and that inhibition of expression of the *SHH* gene might activate gluconeogenesis by inducing the expression of PCK1, mediated through inhibition of the PI3K/Akt pathway.

In previous studies, the role of SHH in the liver cell oncogenesis has been studied in terms of its effects on HCC cell migration and

metastasis [22,23]. In this study, the effects of *SHH* gene knockdown on HCC cells were investigated in terms of its effects on cell proliferation and anchorage-independent growth, a characteristic of transformed cells. Gene knockdown significantly suppressed the viability of HCC cells at each sampling time point as well as inhibiting anchorage-independent growth. The finding from the present study supplement those from previous studies on the role of *SHH* signaling and support its role not only in metastasis but also in survival and cell proliferation in HCC.

Also, the findings of the present study identified the levels of glycolysis products following knockdown of the *SHH* gene. The results showed that, apart from fructose-6-phosphate, the products of glycolysis were suppressed while the production of glucose was increased, indicating inhibition of glycolysis and the initiation of gluconeogenesis. The liver is the main organ involved in the maintenance of blood glucose levels by both supplying glucose to the circulation and removing glucose from the circulation [33]. One of the most important processes during the turnover of glucose is gluconeogenesis, and when this process is disturbed due to the concomitant activation or suppression of glycolysis [15], metabolic reprogramming enhances the metabolic flexibility of cancer cells [34]. Therefore, normal gluconeogenesis in the liver might represent a strategy for the control of HCC, which is a theory that was partially supported in the current study in that induced gluconeogenesis was associated with reduced survival of HCC cells. At the molecular level, the expression levels of p-PI3K, p-Akt, and anti-apoptosis
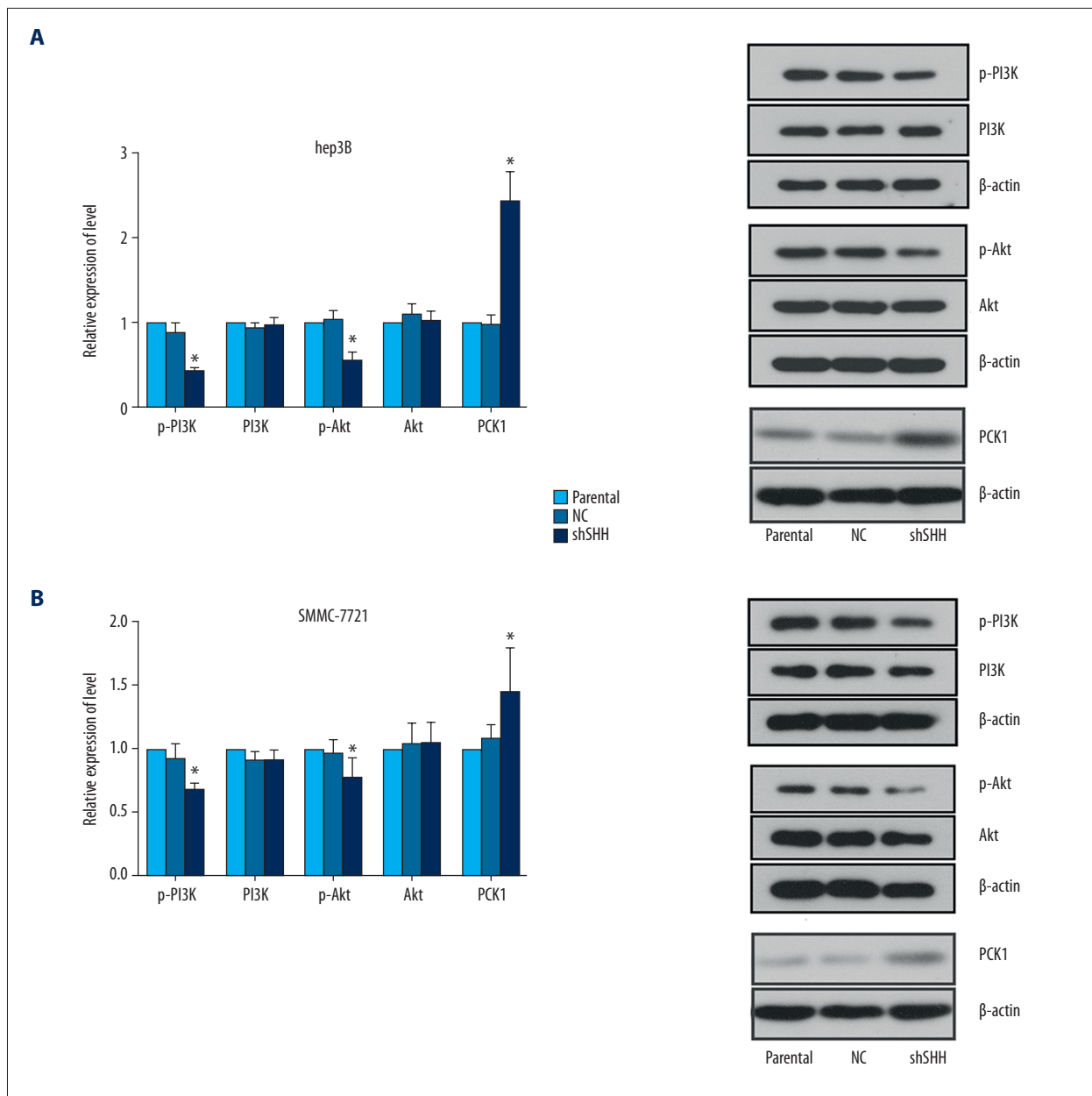
Figure 6. Knockdown of the *SHH* gene inhibited PI3K/Akt activity and increased *PCK1* expression in the HCC cell lines, Hep3B and SMMC-7721. (**A**) Quantitative analysis results and representative images of the Western blot results for PI3K, Akt, and PCK1 in Hep3B cells. (**B**) Quantitative analysis results and representative images of the Western blot results for PI3K, Akt, and PCK1 in SMMC-7721 cells. * P<0.05 *vs.* the normal control (NC) group.

factors were inhibited, while the level of pro-apoptosis factors was increased after *SHH* gene knockdown.

As previously reported, several factors can suppress the function of *PCK1* via the activation of PI3K/Akt signaling [25]. The interaction between *PCK1* and PI3K/Akt was also supported by the findings of the current study, as inhibition of the PI3K/Akt axis was associated with increased expression of *PCK1*, which confirmed the regulatory sequence from PI3K/

Akt to PCK1. Although previous studies also showed that the function of *SHH* gene expression was modulated by the PI3K/Akt axis, the current study showed that knockdown of the *SHH* gene impaired the function of the PI3K/Akt pathway, representing the formation of a regulating loop between *SHH* and PI3K/Akt. These results demonstrated that the inhibition of *SHH* signaling inhibited oncogenesis in HCC cells studied *in vitro* by suppressing the PI3K/Akt pathway and inducing the PCK1 pathway.

Conclusions

This study aimed to investigate the role of the sonic hedgehog *(SHH)* gene and its encoded protein, SHH, in two human hepatocellular carcinoma (HCC) cell lines, Hep3B and SMMC-7721. Knockdown of the *SHH* gene reduced cell survival of HCC cells by increasing apoptosis, reducing cell proliferation, inducing G1 cell cycle arrest, and restoring gluconeogenesis, and was associated with the inhibition of the PI3K/Akt axis and induced the expression of *PCK1*. The findings from this preliminary study supplement the findings from previous studies that have demonstrated a role for SHH in invasion and metastasis of HCC and have shown that the effects of inhibition of the *SHH* gene activated gluconeogenesis, was associated with the activation of *PCK1*, which might depend on the inhibition of PI3K/Akt pathway. These findings require further study but indicate the possibility that the future metabolic treatment approaches for HCC might focus on modulating the expression and function of the *SHH* gene.

Supplementary Figure
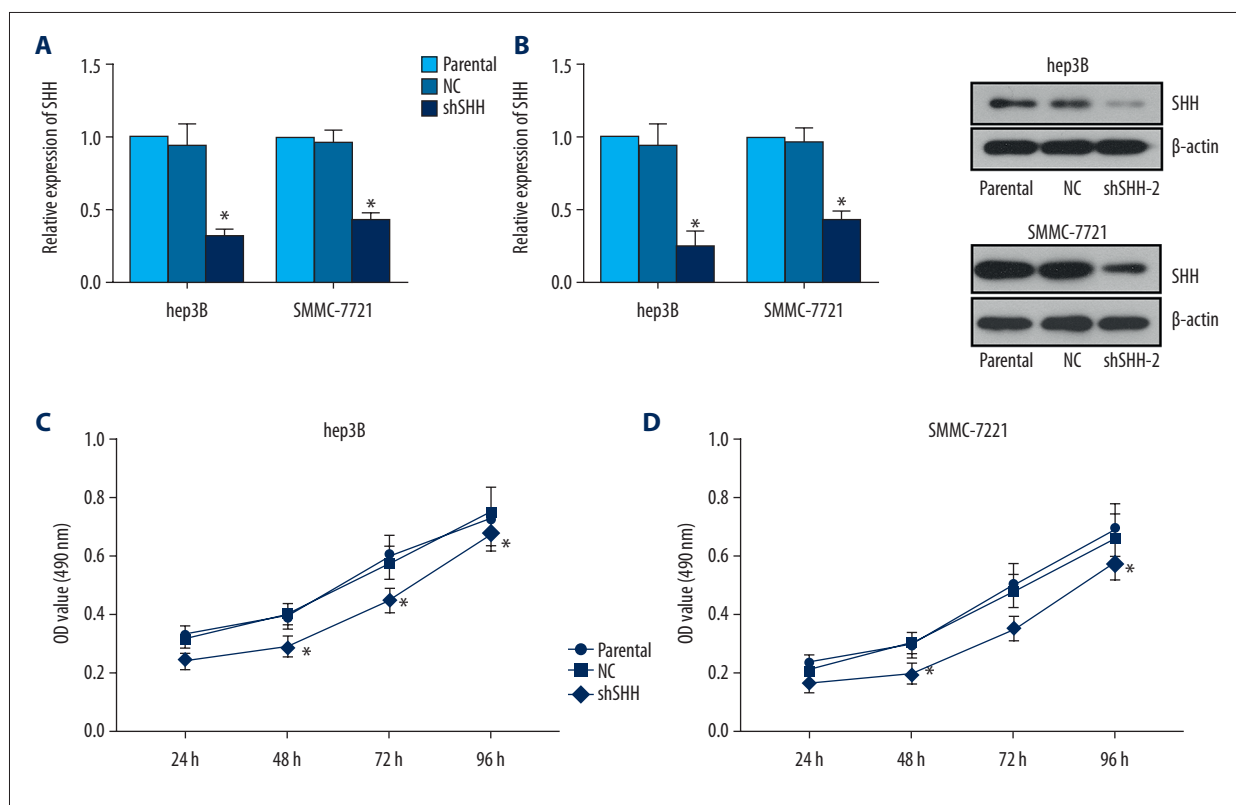
Supplementary Figure 1. Transfection of specific shRNA-2 suppressed the level of sonic hedgehog (SHH) at the mRNA and protein level and reduced proliferation of Hep3B and SMMC-7721 cells. (**A**) Reverse transcription quantitative polymerase chain reaction (RT-qPCR) detection of the *SHH* gene in Hep3B and SMMC-7721 cells. (**B**) Quantitative analysis results and representative image of Western blot detection of the SHH protein in Hep3B and SMMC-7721 cells. (**C**) Quantitative analysis results of the MTT assay for cell proliferation in Hep3B cells. (**D**) Quantitative analysis results of the MTT assay for cell proliferation in SMMC-7721 cells. * P<0.05 *vs.* the normal control (NC) group.

References:

1. Ferlay J, Soerjomataram I, Dikshit R et al: Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer, 2015; 136: E359–86

2. Song P, Tang Q, Feng X, Tang W: Biomarkers: Evaluation of clinical utility in surveillance and early diagnosis for hepatocellular carcinoma. Scand J Clin Lab Inv, 2016; 76: S70–76

3. Tsuchiya N, Yu S, Endo I et al: Biomarkers for the early diagnosis of hepatocellular carcinoma. World J Gastroenterol, 2015; 21: 10573–83

4. Mingqian F, Mitchell H: Glypican-3 antibodies: A new therapeutic target for liver cancer. FEBS Lett, 2014; 588: 377–82

5. Bruix J, Boix L, Sala M, Llovet JM: Focus on hepatocellular carcinoma. Cancer Cell, 2004; 5: 215–19

6. Sanyal A, Poklepovic A, Moyneur E, Barghout V: Population-based risk factors and resource utilization for HCC: US perspective. Curr Med Res Opin, 2010; 26: 2183–91

7. Ghouri YA, Mian I, Rowe JH: Review of hepatocellular carcinoma: Epidemiology, etiology, and carcinogenesis. J Carcinogen, 2017; 16: 1

8. Dhanasekaran R, Khanna V, Kooby DA et al: Chemoembolization combined with RFA for HCC: Survival benefits and tumor treatment response. J Cancer Ther, 2013; 4: 7

9. Yu R, Tan Z, Xiang X et al: Effectiveness of PIVKA-II in the detection of hepatocellular carcinoma based on real-world clinical data. BMC Cancer, 2017; 17: 608

10. Butler EB, Yuhua Z, Cristina MOP et al: Stalling the engine of resistance: Targeting cancer metabolism to overcome therapeutic resistance. Cancer Res, 2013; 73: 2709–17

11. Gutschner T, Diederichs S: The hallmarks of cancer. RNA Biol, 2012; 9: 703–19

12. Deberardinis RJ, Sayed N, Ditsworth D, Thompson CB: Brick by brick: Metabolism and tumor cell growth. Curr Opin Genet Dev, 2008; 18: 54–61

13. Leithner K, Hrzenjak A, Trötzmüller M et al: PCK2 activation mediates an adaptive response to glucose depletion in lung cancer. Oncogene, 2015; 34: 1044–50

14. Ward PS, Thompson CB: Metabolic reprogramming: A cancer hallmark even Warburg did not anticipate. Cancer Cell, 2012; 21: 297–308

15. Khan MW, Chakrabarti P: Gluconeogenesis combats cancer: Opening new doors in cancer biology. Cell Death Dis, 2015; 6: e1872

16. Chang HC, Lane MD: The enzymatic carboxylation of phosphoenolpyruvate. II. Purification and properties of liver mitochondrial phosphoenolpyruvate carboxykinase. J Biol Chem, 1966; 241: 2413–20

17. Nordlie RC, Lardy HA: Mammalian liver phosphoneolpyruvate carboxykinase activities. J Biol Chem, 1963; 238: 2259–63

18. Hanson RW, Garber AJ: Phosphoenolpyruvate carboxykinase. I. Its role in gluconeogenesis. Am J Clin Nutr, 1972; 25: 1010–21

19. Arai T, Kano F, Murata M: Translocation of forkhead box O1 to the nuclear periphery induces histone modifications that regulate transcriptional repression of PCK1 in HepG2 cells. Genes Cells, 2015; 20: 340–57

20. Khan MW, Biswas D, Ghosh M et al: mTORC2 controls cancer cell survival by modulating gluconeogenesis. Cell Death Discov, 2015; 1: 15016

21. Kim HJ, Jee HJ, Yun J: DNA damage induces down-regulation of PEPCK and G6P gene expression through degradation of PGC-1alpha. Acta Biochim Biophys Sin (Shanghai), 2011; 43(8): 589–94

22. Sun C, Zhang Z, Ping H et al: Involvement of PI3K/Akt pathway in the inhibition of hepatocarcinoma cell invasion and metastasis induced by SASH1 through downregulating Shh-Gli1 signaling. Int J Biochem Cell Biol, 2017; 89: 95–100

23. Zhang K, Sun C, Zhang Q, Wang X: Sonic hedgehog-Gli1 signals promote epithelial-mesenchymal transition in ovarian cancer by mediating PI3K/AKT pathway. Med Oncol, 2015; 32: 368

24. Ma J, Tian L, Cheng J et al: Sonic hedgehog signaling pathway supports cancer cell growth during cancer radiotherapy. Plos One, 2013; 8: e65032

25. Shi H, Fang R, Li Y et al: The oncoprotein HBXIP suppresses gluconeogenesis through modulating PCK1 to enhance the growth of hepatoma cells. Cancer Lett, 2016; 382: 147–56

26. Kuang JR, Zhang ZH, Leng WL et al: Dapper1 attenuates hepatic gluconeogenesis and lipogenesis by activating PI3K/Akt signaling. Mol Cell Endocrinol, 2017; 447: 106–15

27. Liu TY, Shi CX, Gao R et al: Irisin inhibits hepatic gluconeogenesis and increases glycogen synthesis via the PI3K/Akt pathway in type 2 diabetic mice and hepatocytes. Clin Sci (Lond), 2015; 129: 839–50

28. Chunjiong W, Yujing C, Jing L et al: FAM3A activates PI3K p110α/Akt signaling to ameliorate hepatic gluconeogenesis and lipogenesis. Hepatology, 2014; 59: 1779–90

29. Hung HC, Hsiao YH, Gean PW: Sonic hedgehog signaling regulates amygdalar neurogenesis and extinction of fear memory. Eur Neuropsychopharmacol, 2015; 25(10): 1723–32

30. Kasperczyk H, Baumann B, Debatin KM, Fulda S: Characterization of sonic hedgehog as a novel NF-kappaB target gene that promotes NF-kappaB-mediated apoptosis resistance and tumor growth in vivo. FASEB J, 2009; 23: 21–33

31. Georg F, Surajit D, Volker F et al: Blockade of hedgehog signaling inhibits pancreatic cancer invasion and metastases: A new paradigm for combination therapy in solid cancers. Cancer Res, 2007; 67: 2187–96

32. He P, Zhang HX, Sun CY et al: Overexpression of SASH1 inhibits the proliferation, invasion, and EMT in hepatocarcinoma Cells. Oncol Res, 2016; 24: 25–32

33. Jianhua S, Liping Q, Janssen RC et al: Chronic hyperglycemia enhances PEPCK gene expression and hepatocellular glucose production via elevated liver activating protein/liver inhibitory protein ratio. Diabetes, 2005; 54: 976–84

34. Cairns RA, Harris IS, Mak TW: Regulation of cancer cell metabolism. Nat Rev Cancer, 2011; 11: 85–95