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Original Articles

Inhibition of GSK-3β activity suppresses HCC malignant phenotype by inhibiting glycolysis via activating AMPK/mTOR signaling

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ABSTRACT

Glycogen synthase kinase-3 beta (GSK-3β) has been shown to play a critical role in the development of many cancers, but its role in hepatocellular carcinoma (HCC) remains unclear. Deregulating cellular energetics is a signature hallmark of cancer, therefore modulating cancer metabolism has become an attractive anti-cancer approach in recent years. As a key enzyme in glucose metabolism, understanding the role of GSK-3β in cancer metabolic process may facilitate the development of effective therapeutic approach for HCC. In this study, we showed that inhibition of GSK-3β led to diminished viability, metastasis and tumorigenicity in HCC cells. Suppression of GSK-3β activity also reduced glucose consumption, lactate production and adenosine triphosphate (ATP) levels in HCC cells. The decreased extracellular acidification rate (ECAR) and down-regulated key enzymes on the glycolysis pathway by GSK3β inhibition demonstrated that GSK-3β was involved in glycolysis process of HCC. Mechanistically, the metabolic change and anti-cancer effect by GSK-3β inhibition was achieved mainly through activation of adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) signaling, which negatively affected glycolysis and cell proliferation. The results from primary HCC cells and from in vivo nude mice model confirmed our observations. Our study results indicated that GSK-3β may become a promising therapeutic target for HCC.

1. Introduction

Hepatocellular carcinoma (HCC), the most common primary liver cancer, ranks as the sixth most prevalent malignancy and the second leading cause in mortality worldwide [1]. Unfortunately, most patients with liver cancer have unresectable disease at the time of initial diagnosis, thus eliminating possibility of surgery. In the meantime, chemotherapy and radiation have achieved little benefit in halting disease progression or prolonging patients' lives. In the past decade, only molecular targeted therapy showed promising potential, as it is characterized by high specificity, manifest efficacy and limited side effect [2]. Searching for new drug targets hence became a paramount mission.

Glycogen synthase kinase-3 (GSK-3) is a multifunctional serine/threonine kinase, which plays an important role in a variety of biological processes, such as glycogen metabolism, cell proliferation, and oncogenesis [3]. Most studies focus on one of its two homologous mammalian GSK-3 isoforms: GSK-3β, which has two phosphorylation sites located at site 9 of serine (Ser9) and site 216 of tyrosine (Tyr216). It is believed that phosphorylation of the Tyr216 site can lead to activation of the kinase activity, and phosphorylation of the Ser9 site can deactivate it.

The influence of GSK-3β in tumorigenesis has been studied extensively, but conclusions remain controversial. In some studies, GSK-

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Abbreviations

HCC hepatocellular carcinoma
GSK-3β glycogen synthase kinase 3β
mTOR mammalian target of rapamycin
ATP adenosine triphosphate
AMPK adenosine 5′-monophosphate (AMP)-activated protein kinase
EMT epithelial-mesenchymal transition
DMEM dulbecco modified eagle medium
FBS fetal bovine serum
PBS phosphate buffer saline
shRNA small hairpin RNA
CCK-8 cell Counting Kit-8
ECAR extracellular acidification rate
qRT-PCR quantitative reverse transcription polymerase chain reaction
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBST tris-Buffered Saline and Tween-20
ITS insulin, transferrin, selenous acid
EGF epidermal growth factor
SD standard deviation
IC50 50% inhibited concentration
DMSO dimethyl sulfoxide
GLUT glucose transporter
HK hexokinase
PFKFB3 human 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
PKM pyruvate kinase isozyme type M
HIF hypoxia inducible factor
NADPH nicotinamide adenine dinucleotide phosphate

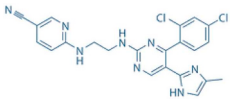
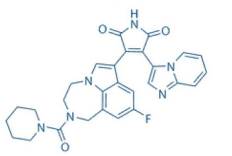
3β has been shown to inhibit androgen-receptor-stimulated prostate cancer cell growth [4], and suppression of its activity in the mammary epithelium of normal mice promotes the occurrence of breast cancer [5]. In addition, upregulated expression of phosphorylated GSK3β Ser9 (p-GSK3β Ser9), the inactivated form of GSK3β, was found in oral cancer (OSCC) [6] and esophageal cancer [7], suggesting that it plays a role of tumor suppressor. However, in other literature, GSK-3β was reported to have tumor promoting impact. Its inactivated form p-GSK3β Ser9 was found to be absent in multiple types of cancer, while the activated form, p-GSK3β Tyr216, was found increased [8–10]. Inhibition of GSK-3β has been shown to restrict the growth of various cancers such as neuroblastoma, glioma cells, colon cancer cells, pancreatic cancer cells, lymphoblastic leukemia cells and many others [11–27]. Regardless of the different influence, it is safe to claim that GSK-3β plays a critical role in the regulation of cancer development.

Like on other tumors, the influence of GSK3β on HCC is also highly disputed. While some studies claim that GSK-3β has anti-HCC effect [28,29], but more publications suggest that GSK-3β is a potential therapeutic target in HCC [30]. In-vitro experiments have indicated that inhibition of GSK-3β could positively affect HCC cell apoptosis and survival [31,32], and promote lysosome-dependent degradation of c-FLIPL in HCC [33].

One of the significant characteristics of cancer cells is the high level of glycolysis even in the presence of oxygen, known as the Warburg effect. This metabolic adaptation is believed to be critical for tumor cell growth and proliferation. Deregulating cellular energetics is recognized as a hallmark of human cancers [34]. Modulating cancer metabolism is currently an attractive anti-cancer approach. GSK-3β is a key enzyme in glucose metabolism, but how it regulates the metabolic process in HCC and the underlying mechanism remains elusive. A better understanding of the regulatory mechanisms of GSK-3β in the metabolic process of HCC has the potential to facilitate the development of effective therapeutic approach against liver cancer.

Our study results showed that GSK-3β inhibition could indeed suppress HCC cells malignant phenotype and glycolysis metabolic pattern. Through activating the AMPK/mTOR signaling pathway, GSK-3β inhibition could lead to down-regulation of many glycolysis-related key enzymes and several key factors involved in cell proliferation. Our results demonstrate that GSK-3β is a crucial metabolic controller in HCC, and proper regulation of its activity may be a promising strategy for HCC treatment.

Table 1

Full Name	Source	Molecula weight	Structure
CHIR-99021	Selleck, Cat. No. S1263	465.34	
BIO	Selleck, Cat. No. S7198	356.17	
LY2090314	Selleck, Cat. No. S7063	512.53	

2. Materials and methods

2.1. Patients

Specimens, including 123 primary HCC and Non-tumor-adjacent tissues from HCC patients who received curative surgery in the Eastern Hepatobiliary Surgery Hospital, Second Military Medical University (Shanghai, China) from January 2010 to December 2014, were used for tissue microarray. The study was approved by the Committee of Ethics of Biomedicine Research, Second Military Medical University. Informed consent was obtained from all patients before surgery for using their data in the research.

Table 2

CACCATTGGCAATGAGCGGTTC	β-Actin F
AGGTCTTTGCGGATGTCCACGT	β-Actin R
CCGACTAACACCACTGGAAGCT	GSK3β F
AGGATGGTAGCCAGAGGTGGAT	GSK3β R
GCCAGAAGGAGTCAGGTTCAA	GLUT1 F
TCCTCGGAAAGGAGTTAGATCC	GLUT1 R
GCCATGAGCTACGTCTCCATT	GLUT4 F
GGCCACGATGAACCAAGGAA	GLUT4 R
CTGCTGGTGAAAATCCGTAGTGG	HK1 F
GTCCAAGAAGTCAGAGATGCAGG	HK1 R
GAGCCACCACTCACCCTACT	HK2 F
CCAGGCATTCGGCAATGTG	HK2 R
CGAGCCTCAAGTCACTCCAC	PKM1 F
GTGAGCAGACCTGCCAGACT	PKM1 R
ATTATTTGAGGAACTCCGCCGCCT	PKM2 F
ATTCCGGGTCACAGCAATGATGG	PKM2 R
GGCAGGAGAATGTGCTGGTCAT	PFKFB3 F
CATAAGCGACAGGCGTCAGTTTC	PFKFB3 R
CTGTGATACGGATCAGAAACCG	PDK1 F
TCCACCAAACAATAAAGAGTGCT	PDK1 R
TTCCCGACTAGGCCCATTC	HIF1α F
CAGGTATTCAAGGTCCCATTTCA	HIF1α R

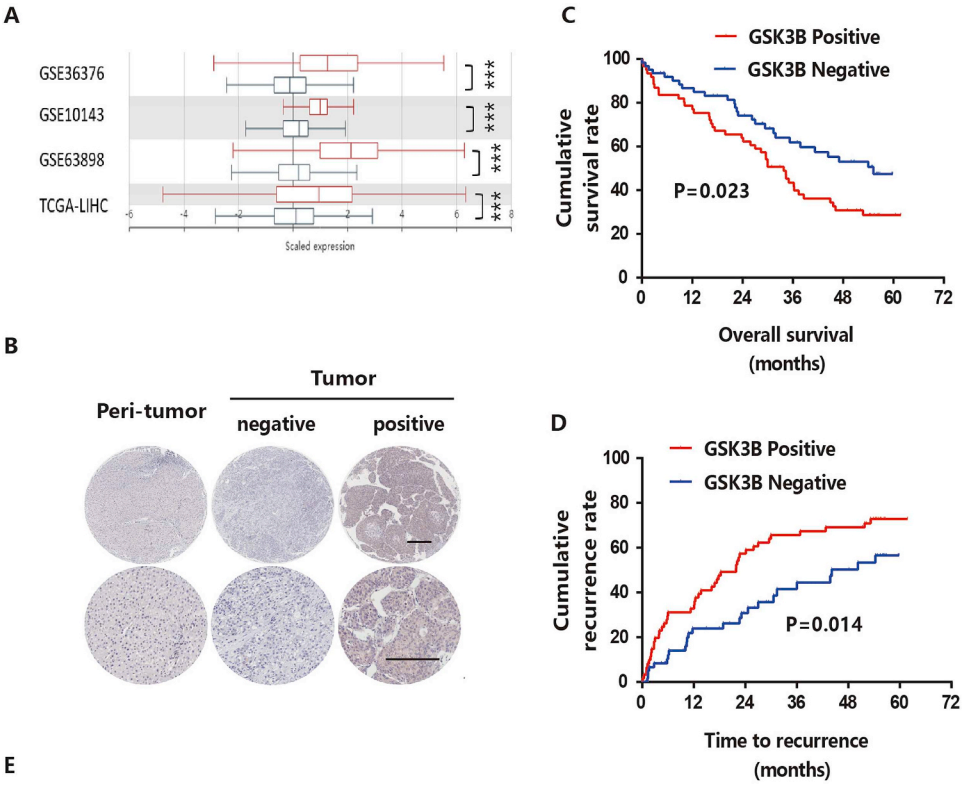


Fig. 1. GSK3β overexpression in HCC predicts a poor prognosis.
(A) The genechip data of HCC were obtained from TCGA and GEO databases, and the expression levels of GSK3β mRNA in HCC (red) and adjacent nontumor tissues (green) were statistically analyzed (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).
(B) Representative images of IHC staining of GSK3β in HCC and adjacent nontumor tissues from HCC tissue microarray (Scale bar = 100 μm).
(C and D) Cumulative survival rate and recurrence rate for the negative and positive GSK3β expression group in the 123 HCC patients, and their statistical difference (by the log-rank test).
(D) Analysis of relationship between intra-tumoral GSK3β expression and clinicopathologic features.

E

		GSK3B expression		
		negative (n = 62)	positive (n = 61)	P
Sex	female	7	4	0.358
	male	55	57	
Age	median	49.7	51.1	0.485
	range	22 – 74	30 – 71	
AFP level(mg/L)	>400	31	30	0.928
	<400	31	31	
HBsAg	positive	56	56	0.774
	negative	6	5	
HBVDNA	positive	40	40	0.902
	negative	22	21	
Liver cirrhosis	yes	31	34	0.524
	no	31	27	
Ascites	yes	11	8	0.479
	no	51	53	
Encapsulation	complete	27	28	0.793
	no	35	33	
TBL(umol/l)	median	18.5	18.2	0.838
	range	5.2-38.4	8.9-39.3	
Alb(g/dl)	median	42.2	41.1	0.274
	range	30.9-67.6	24.4-50.4	
ALT(U/L)	median	58.3	54	0.596
	range	5.8-206.3	6.3-251.5	
AST(U/L)	median	65.7	61	0.643
	range	15.5-270.2	16.5-263.7	
Diameter(cm)	>5	27	48	**P<0.001**
	<5	35	13	
Metastasis	yes	33	49	**P<0.001**
	no	29	12	
TNM	T1/T2	30	9	**P<0.001**
	T3	32	52	

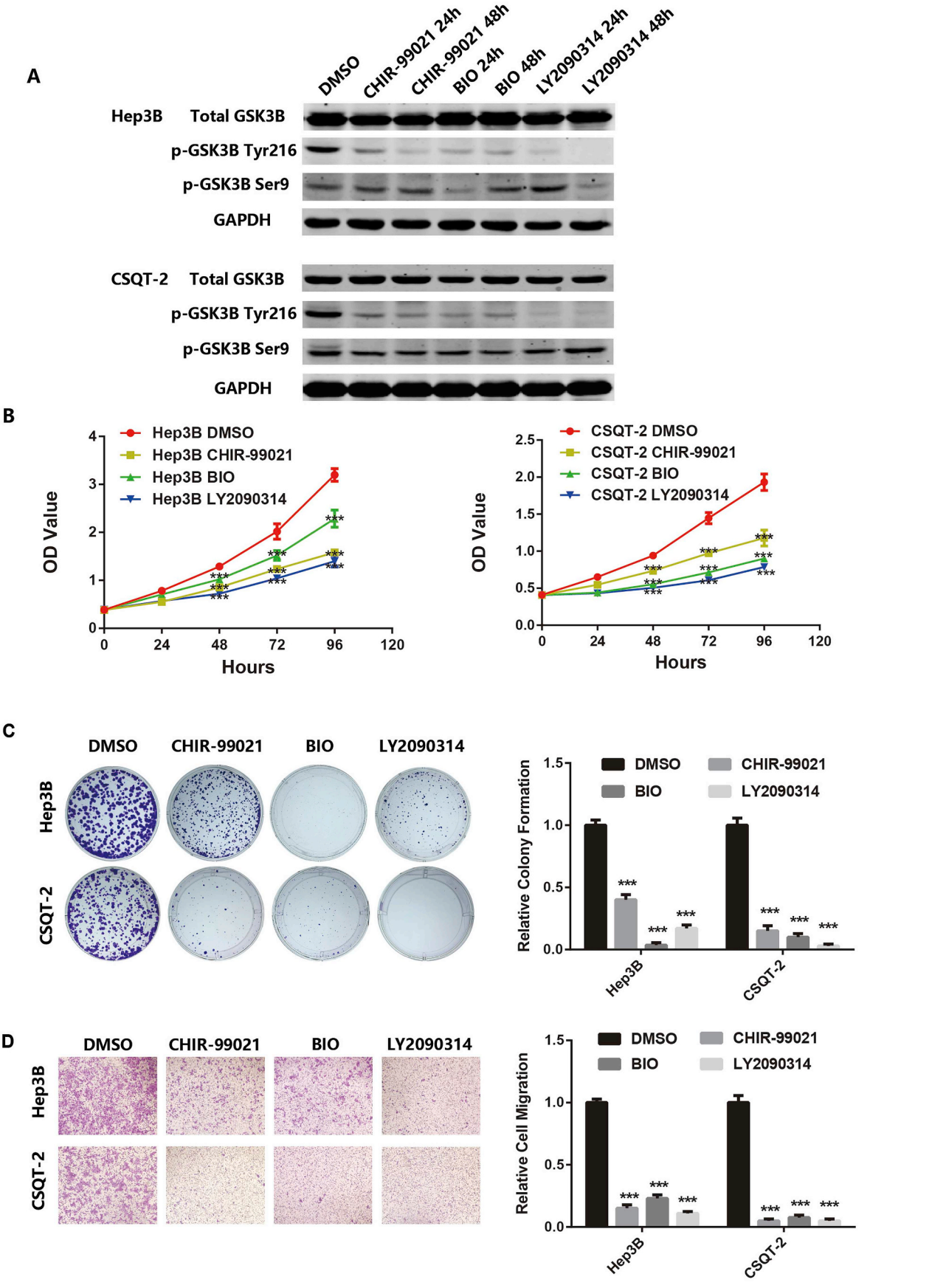


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Fig. 2. Impact of different GSK-3β inhibitors CHIR-99021, BIO and LY2090314 on biological behavior of HCC cells in vitro.
(A) GSK-3β inhibitors decreased the activity of GSK-3β by reducing Tyr216 phosphorylation site levels.
(B) Hep3B and CSQT-2 HCC cells were treated with DMSO (dimethyl sulfoxide) or GSK-3β inhibitors CHIR-99021, BIO and LY2090314 for designated times. Relative cell viability was measured by the CCK-8 assay at indicated time points as described under materials and methods.
(C) Hep3B and CSQT-2 HCC cells were treated with DMSO or CHIR-99021, BIO and LY2090314 for 14 days, and then fixed with formaldehyde solution and stained with crystal violet. Display of representative samples were photographed. Results were analyzed by Image J software and date was shown as mean ± SD.
(D) GSK-3β inhibitors suppressed cell migration in Hep3B and CSQT-2 HCC cells. Transwell assay was performed after cells treated with DMSO, CHIR-99021, BIO and LY2090314 for 48 h. Representative views were photographed. Results were analyzed by Image J software and date was shown as mean ± SD.
*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

2.2. GSK3β inhibitors

GSK3β inhibitors (CHIR-99021, BIO, LY2090314) were purchased from Selleck biotechnology. Please refer to Table 1 for details.

2.3. Cell culture

The HCC cell line Hep3B was purchased from Cell Bank of Chinese Science Academy (Shanghai, China) and the CSQT-2 cell line was provided by professor Shuqun Cheng, Eastern Hepatobiliary Surgery Hospital. All cell lines were maintained with dulbecco modified eagle medium (DMEM) (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (GIBCO BRL, Grand Island, NY, USA) and penicillin/streptomycin in a 5% CO_2 humidified incubator at 37 °C.

2.4. Cell viability

To evaluate cell proliferation, the cells were seeded in triplicates in 96-well plates at a density of 2×10^3 cells per well. Following one night incubation, cells were washed by phosphate buffer saline (PBS) and maintained in complete medium added with indicated reagents or not. At the indicated time, 10 μL of cell Counting Kit-8 (CCK-8) solution (Dojindo Laboratories, Kumamoto, Japan) was added per well and incubated for 60 min at 37 °C. OD values were detected at an absorbance of 450 nm using a microplate reader (BioTek, USA) at a range of time points.

2.5. Colony formation assay

For colony formation assay, cells were plated in 6-well plates in a density of 1000 per well. 14 days after plating, cells were washed by PBS, fixed with formaldehyde and stained with crystal violet for 15 min at room temperature. The visible colonies were counted manually and representative views were photographed.

2.6. Migration assay

For the migration assay, 5×10^4 cells were plated in 24-well transwell plates with chambers (Costar, Corning,USA) respectively. The medium in the chambers was 1% FBS, and the medium outside was supplemented with 20% FBS. After 48 h, the cell chambers were fixed with 10% formalin for 10 min. Next, the chambers were washed by PBS and stained with crystal violet for 10 min. After washing chambers, non-invading cells were removed using a cotton swab. Representative fields were photographed, and four random microscopic fields for each sample were analyzed using Image-J software (Media Cybernetics Inc, Bethesda, USA). These experiments were repeated at least 3 times independently.

2.7. Stable cell line construction

Plasmid containing short hairpin RNA (shRNA) targeting GSK-3β and the negative control (shCtrl) were purchased from OBiO Technology (Shanghai) Corp. GSK-3β shRNA expression vector was pLKD-EF1a-EGFP-LUC-F2A-Pruo-U6-shRNA. The sequence of shGSK-3β1: GCTAGATCACTGTAACATA. The sequence of shGSK-3β2: GAAA GCTAGATCACTGTAA. Overexpression GSK-3β vector was pLenti-CMV-EGFP-3FLAG-PGK-blasticidin. Stable GSK-3β expressed or knock down GSK-3β HCC cell lines were established by a lentiviral vector system.

2.8. RNA interfering

For RNA interfering, cells were transfected with 10 nM small interfering (si)RNAs by INTERFERin transfection reagent (409–10; Polyplus, New York, NY) according to manufacturer's instructions. siRNAs were purchased from the Biotend Company (Shanghai, China). The sequence of siAMPK is 5 ′-3 ′ GGCAUAAAGUAGCUGUGAAdTdT and UUCACAGCUACUUUAUGCCdTdT.

2.9. Quantification of glucose, lactate and ATP

The concentration of glucose, lactate in the culture medium and ATP levels of GSK-3β treatment groups and control groups were determined by means of spectrophotometric analysis using Assay kits (Glucose Assay kit, Lactate Assay Kit, Nanjing jiancheng bioengineering institute, China; ATP Assay kit, Beyotime, China). All measurements followed manufacturer's instructions and were normalized for the number of cells in each experiment.

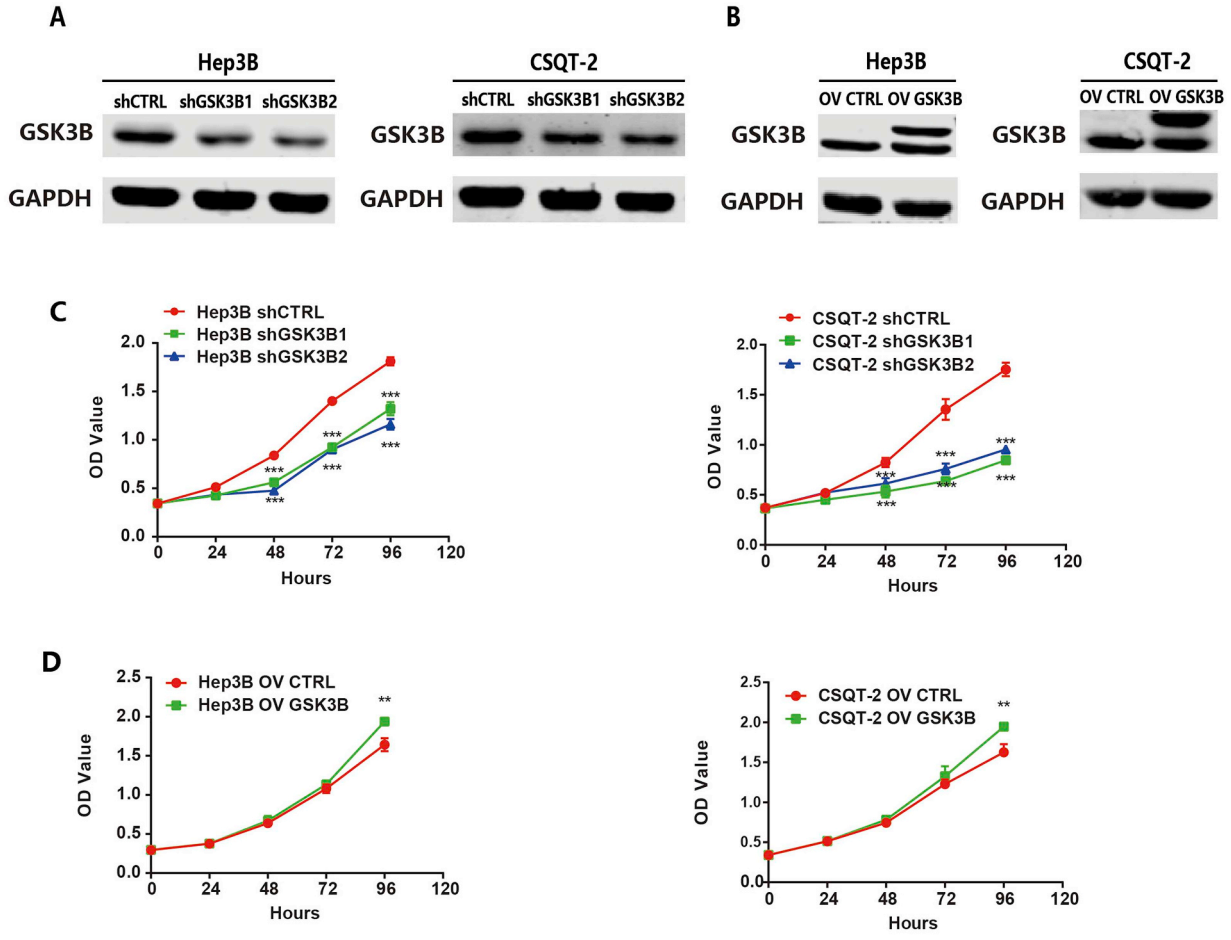

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Fig. 3. Impact of shGSK-3β and over-expression GSK-3β on proliferation, colony formation and migration ability of HCC cells.
(A and B) The expression level of GSK-3β protein in shGSK-3β (A) and over-expression GSK-3β (B) HCC cell samples were shown.
(C and D) shGSK-3β inhibited proliferation (C), while over-expression GSK-3β promoted proliferation (D) in HCC cells.
(E and F) shGSK-3β (E) and over-expression GSK-3β (F) HCC cells were seeded in 6-well plates for 14 days, and then fixed with formaldehyde solution and stained with crystal violet. Representative images are shown. Results were analyzed by Image J software and date was shown as mean ± SD.
(G and H) shGSK-3β (G) inhibited cell migration and over-expression GSK-3β (H) promoted cell migration in HCC cells. Representative images are shown. Results were analyzed by Image J software and date was shown as mean ± SD.
*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

2.10. Seahorse analysis

Extracellular acidification rate (ECAR) measurements were performed using the XF96 Extracellular Flux analyzer (Seahorse Bioscience, USA). Briefly, 1×10^4 cells were plated into XF96 polystyrene cell culture plates containing 10% FBS DMEM. The cells were incubated for 24 h in a humidified 37 °C incubator with 5% CO_2. The previous night, calibration solution was added in utility plate in a 37 °C/non-CO_2 incubator for 12 h. One hour before XF assay, 10% FBS DMEM was replaced by seahorse base medium (PH = 7.4) and take polystyrene cell culture plates incubated into a 37 °C/non-CO_2 incubator for 60 min. All experiments were performed at 37 °C. Analyses were performed at basal condition and after injection of glucose (10 mM), oligomycin (2 μM), 2-DG(50 mM) (XF Glycolysis Stress Test kit, Agilent, USA) at indicated time points.

2.11. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Cell total RNA was extracted using Trizol reagent (Invitrogen, USA) following the manufacturer's instructions. The quantity and quality of extracted RNA were assessed by the spectrophotometric (Dojindo Laboratories, Kumamoto, Japan) determination of absorbance ratio (A260/A280). Then, the prepared RNA was reversely transcribed into cDNA using reverse transcriptase (Invitrogen, USA) and random primers. One microliter of synthesized cDNA was used in each qPCR reaction. SYBR Green-based qRT-PCR was subsequently executed on ABI PRISM 7300HT Sequence Detection System (Applied Biosystems, USA). β-Actin was used as a control for normalization. Primers used in RT-PCR were as Table 2.

2.12. Western blot analysis

Protein samples extracted from HCC cell lines were subjected to sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) electrophoresis and transferred to nitrocellulose membranes. Then, the nitrocellulose membranes were blocked with 1 × TBST containing 5% BSA for 1 h and incubated overnight at 4 °C with primary antibodies against Phospho-GSK-3β Tyr216 (1:1000, ab75745, Abcam), GSK-3β (1:1000, 12456, Cell Signaling Technology), GAPDH (1:1000, 5174, Cell Signaling Technology), Phospho-mTOR (Ser2448) (1:1000, 5536, Cell Signaling Technology), Phospho-p70 S6 Kinase (Thr389) (1:1000, 9234, Cell Signaling Technology), Phospho-4E-BP1 (Thr37/46) (1:1000, 2855, Cell Signaling Technology), AMPKα (1:1000, 5831, Cell Signaling Technology), Phospho-AMPKα (Thr172) (1:1000, 2535, Cell Signaling Technology). After washing by 1 × tris-Buffered Saline and Tween-20 (TBST), the nitrocellulose membrane was incubated with goat anti-mouse or goat anti-rabbit luorescence-conjugated secondary antibody and scanned with an Odyssey scanner (Li-Cor, Lincoln, NE, USA).

2.13. Isolation of human primary hepatocellular carcinoma cells

Tumor tissue that derived from HCC patients were carefully dissected to remove necrotic tissue, connective tissue and blood vessels. After cutting tumor tissue into small pieces, about 10 ml collagenase solution (0.5 mg/ml) was added to the tumor for the digestion in 37 °C incubator for 20–30 min. By pipetting several times, the collagenase solution containing tumor cells was passed the sterile 70 μm nylon filters to collect single-cell solution. Cells were centrifuged and washed using culture medium for 3 times, cell yield and viability were assessed through trypan blue. Fresh isolated tumor cells were cultured in DMEM with 20% FBS, 1% insulin, transferrin, selenous acid (ITS) supplement, 20 ng/ml epidermal growth factor (EGF) addition. Medium was changed every two days.

2.14. Animal xenograft assays

6–8 weeks old male nude mice were purchased from Chinese Science Academy (Shanghai, China). Approximately 1×10^6 Hep3B cells were suspended in 50 μL DMEM and Matrigel (BD Bio-sciences) (1:1) and injected subcutaneously into nude mice. The size of subcutaneous tumors and mice weight were recorded at the indicated time point. All animal experiments were approved by the Ethical Committee of the SMMU and performed in accordance with relevant regulations and guidelines.

2.15. Data analysis and statistical procedures

The results were expressed as the mean ± standard deviation (SD) of independent experiments. The significance of differences between groups were determined by independent-samples T test. Differences were considered statistically significant for values of $P < 0.05$. All statistical analysis were performed with SPSS 22.0 and GraphPad Prism Version 5.0 softwares.

3. Results

3.1. GSK3β overexpression predicts poor prognosis in HCC patients

To identify the clinical relevance of aberrant GSK-3β expression with HCC, we analyzed TCGA-LIHC (The Cancer Genome Atlas) and Gene Expression Omnibus (GEO) database. We found that GSK-3β expression is upregulated in tumor tissues in comparison to para-tumor tissues (Fig. 1A). To confirm the data obtained from gene expression database, we conducted our own tissue microarray using 123 pairs of HCC and nontumor tissues. We found that nontumor tissues did not

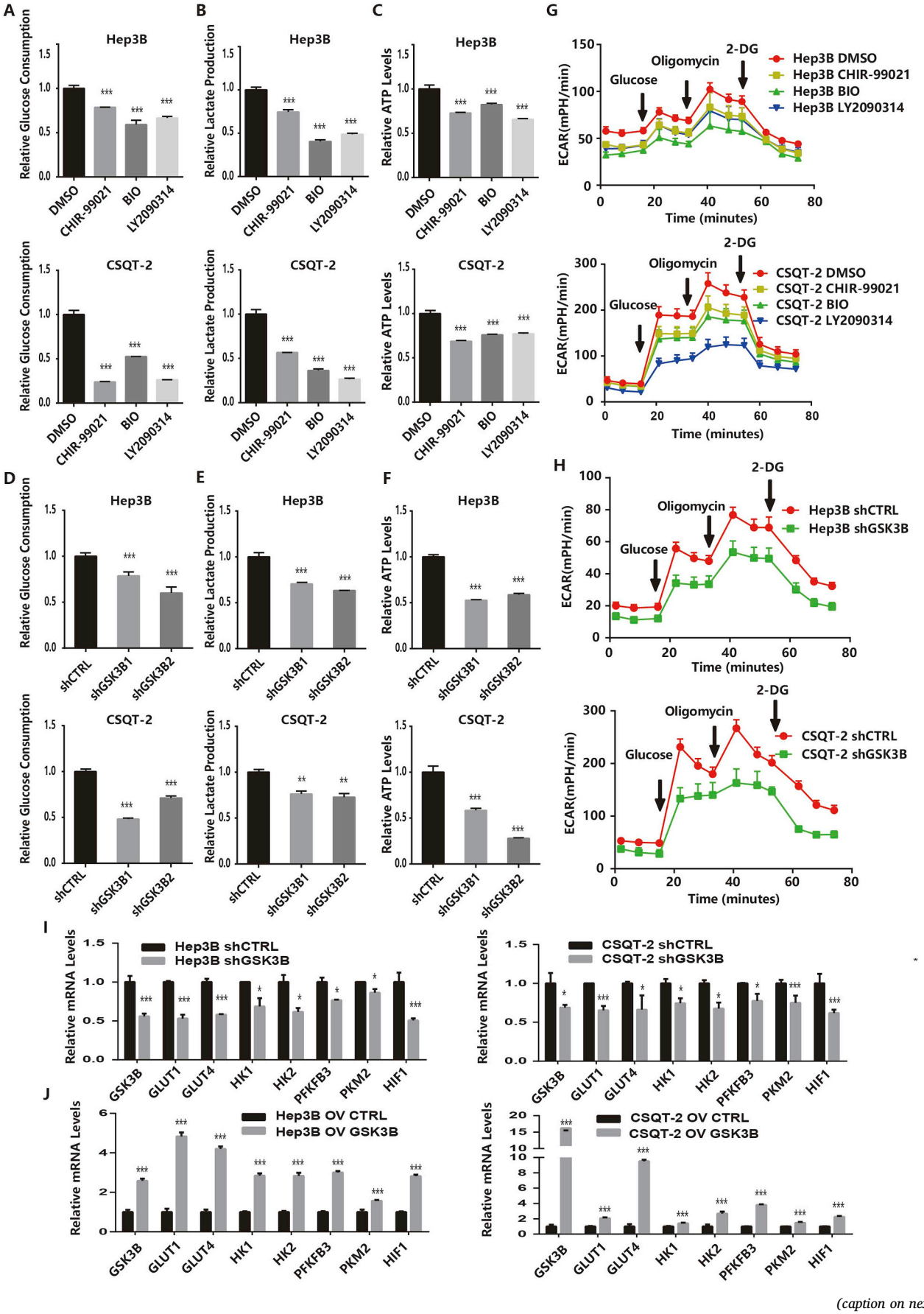

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Fig. 4. GSK-3β knock down by inhibitors or shRNA inhibits aerobic glycolysis of HCC cells.
(A and B and C) GSK-3β inhibitors reduced glucose consumption (A), lactate production (B) and ATP levels (C) of HCC cells. Three independent experiments were performed.
(D and E and F) Glucose consumption (D), lactate production (E) and cellular ATP level (F) were decreased in shGSK-3β HCC cells. Three independent experiments were performed.
(G and H) The effect of GSK-3β inhibitors (G) and shGSK-3β (H) on glycolysis were evaluated by monitoring extracellular acidification rate (ECAR) using XF96 Extracellular Flux Analyzer. Glucose (10 mM), the oxidative phosphorylation inhibitor (oligomycin 2 μM) and the glycolytic inhibitor 2-deoxyglucose (2-DG, 50 mM) were sequentially injected into each well at indicated time points. Five replicates for each group, and three independent experiments were performed.
(I and J) Glycolysis key enzymes gene expression levels of shGSK-3β (I) and over-expression GSK-3β (J) samples were detected by qRT-PCR.
All measurements were normalized to cell number calculated at the end of the experiment.
*P < 0.05,**P < 0.01, ***P < 0.001.

express GSK-3β. Tumor tissues could have either positive or negative GSK-3β expression (Fig. 1B). Based on this, we divided the 123 patients with HCC into two groups: a positive GSK-3β expression group (n = 61) and a negative GSK-3β expression group (n = 62). To evaluate the significant contribution of GSK-3β expression in the prognosis of patients with HCC, we analyzed the relevance of GSK-3β expression with overall survival and recurrence rate of patients. As shown in Fig. 1C and D, patients with GSK-3β expression exhibited worse overall survival (OS, median OS times were 29.97 vs. 53.97 months, respectively; difference 24 months, P < 0.05) and higher recurrence rate (P = 0.014) (Fig. 1C and D) than the GSK-3β negative expression group. The result analysis further indicated that GSK-3β expression levels was positively correlated with tumor size (P < 0.001), metastasis (P < 0.001), TNM staging (P < 0.001) (Fig. 1E). Thus, GSK-3β may be used as an independent factor for predicting the prognosis of HCC.

3.2. Inhibition of GSK-3β activity by inhibitors or shRNA suppresses tumor biological behavior of HCC cells

To investigate the effect of GSK-3β on HCC malignant phenotype, we tested chemical GSK-3β inhibitors (CHIR-99021, BIO, LY2090314) separately on two HCC cell lines: Hep3B and CSQT-2. The former is a commonly used classic HCC cell line, while the latter is derived from portal vein tumor thrombus of HCC and possesses high metastatic properties. We firstly determined the 50% inhibited concentration (IC50) of inhibitors with dosage-curve assay. The IC50 of CHIR-99021, BIO and LY2090314 in Hep3B cells were 22.85 μM, 2.19 μM and 6.89 μM respectively, and in CSQT-2 cells were 11.34 μM, 4.38 μM and 3.97 μM respectively (Supporting Fig. S1). These concentrations were then applied in the following GSK-3β inhibition assessments. We measured the level of phosphor-GSK3β Tyr216 (activated form) in HCC cells at different time points (24 h and 48 h) using Western blot assay (Fig. 2A), to make sure GSK-3β inhibition by small molecules was indeed successful. The results showed that phosphor-GSK3β Tyr216 expression was suppressed consistently by inhibitors under all conditions.

We then assessed the effect of GSK-3β inhibitors on viability of HCC cells in vitro. We observed significantly slowed proliferation in inhibitors-treated groups in a time-dependent manner (Fig. 2B). Next, we found that inhibition of GSK-3β led to the decrease of colony formation in HCC cells, which indicated reduced oncogenicity. Compared to dimethyl sulfoxide (DMSO)-treated control groups, CHIR-99021 can reduce colony formation of Hep3B cells by 55% and CSQT-2 cells by 85%, while BIO and LY2090314 can reduce colony formation of both HCC cells by over 85% (Fig. 2C). In the transwell assay which represents migration ability of tumor, we found that the migration of inhibitors-treated Hep3B and CSQT-2 HCC cells were significantly decreased compared to control group (Fig. 2D). The results indicated that inhibition of GSK-3β is directly translated into suppression of tumor biological behaviors. Results from other human HCC cell lines such as SMMC-7721 and LM3 further confirmed these observations (Supporting Figs. S2A–C).

To validate the effect of GSK-3β inhibitors on suppression of tumor biological behaviors and rule out any side effects by the small molecules, we constructed GSK-3β knockdown and over-expression HCC cell lines using lentiviral-based approaches and performed above experiments using the same strategies. The knockdown and over-expression efficiency of GSK-3β in HCC cells was confirmed by Western blot (Fig. 3A and B, Supporting Fig. S3A). In shGSK-3β groups, HCC cells' proliferation had a dramatic decrease (Fig. 3C, Supporting Fig. S3B). Similarly, HCC cells' colony formation was reduced by 60–95%, and migration ability decreased by 65–90% (Fig. 3E and G, Supporting Figs. S3D and S3F). The results indicated that shGSK-3β indeed had a strong inhibitory effect on HCC cells' viability, tumorigenicity and migration ability. In the meantime, over-expression of GSK-3β promoted HCC proliferation after 96 h (Fig. 3D, Supporting Fig. S3C). It also promoted HCC cells' tumorigenicity potential and migration ability in vitro (Fig. 3F and H, Supporting Figs. S3E and S3G). Together, these results demonstrated that GSK-3β plays a promotional role in the development of HCC, and its inhibition can effectively suppress tumor biological behavior of HCC cells.

Finally, we performed cell cycle and apoptosis analysis by flow cytometry, and senescence analysis of HCC cells by SA-β-Gal staining to determine the specific anti-growth effect of GSK3β inhibition on HCC cells. The results showed that inhibition of GSK3β increased the cell population in G0/G1 stage, but decreased population in S and G2/M stage (Fig. S4A) indicating cell growth arrest. It also promoted apoptosis (Fig. S4B) of HCC cells. The cell senescence analysis result, on the other hand, showed that GSK3β had no effect on the senescence of HCC cells(Fig. S4C). Taking together, the anti-growth effect by GSK3β inhibition was the synergistic effect of induced cell cycle arrest and apoptosis.

3.3. Inhibition of GSK-3β constrains HCC cells malignant phenotype primarily through glycolysis modulation

The results above showed that decreased GSK-3β activity by inhibitors or shRNA can suppress the malignant phenotype of HCC cells. Since GSK-3β is a key enzyme in glucose metabolism, we theorized that the inhibitory effect occurred was mainly through the regulation of glycolysis – the significant metabolic pattern of tumour cells. Glucose content assay revealed that GSK-3β inhibitors (CHIR-99021, BIO, LY2090314) led to reduction in glucose consumption in HCC cells

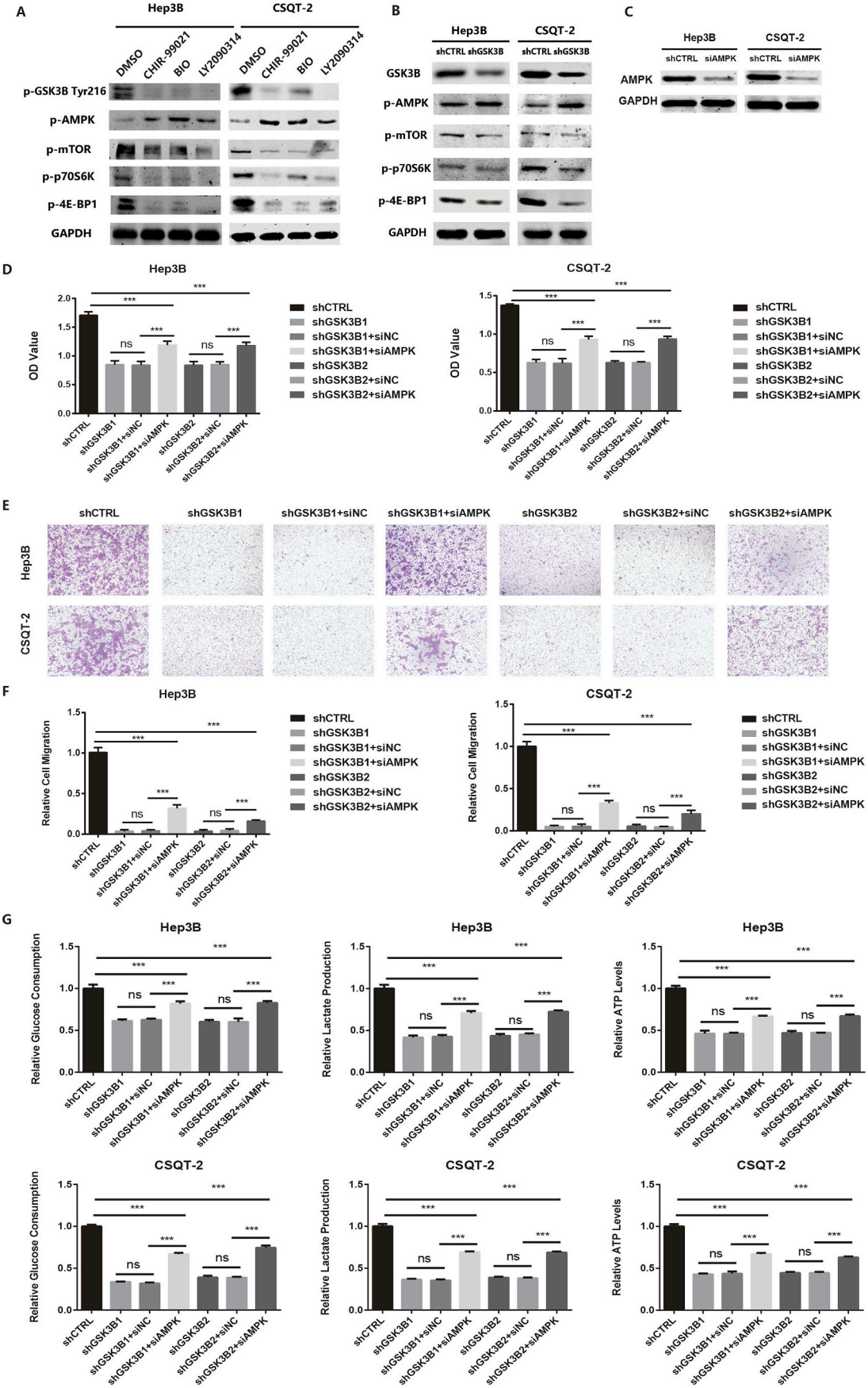

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Fig. 5. GSK-3β regulates glycolysis via AMPK/mTOR pathways in HCC cells.
(A) The levels of p-AMPK, p-mTOR, p-p70S6K and p-4E-BP1 were determined by Western blot in HCC cells treated with DMSO or GSK-3β inhibitors at the indicated time.
(B) The levels of p-AMPK, p-mTOR, p-p70S6K and p-4E-BP1 were determined by Western blot in shGSK-3β group and control group at the indicated time.
(C) Validation of interference efficiency of siAMPK in Hep3B and CSQT-2 cells.
(D) The proliferation of HCC cells in control group and treatment group at 72 h was detected by CCK8 assay.
(E) The metastasis ability of HCC cells in control group and treatment group was detected by Transwell assay.
(F) Quantitative analysis of the number of hepatocellular carcinoma cells crossing Transwell chamber in each group.
(G) Glucose consumption, lactate production and ATP levels of HCC cells in each control group and treatment group were detected by kits.
*P < 0.05,**P < 0.01, ***P < 0.001.

(Fig. 4A). Subsequently, lactate, the next stage product of glucose in the glycolysis process and cellular ATP levels also significantly decreased in inhibitors-treated HCC cells (Fig. 4B and C). We then used shRNA instead of chemical inhibitors, and observed similarly drastic reductions in glucose consumption, lactate production and eventually cellular ATP levels (Fig. 4D–F, Supporting Fig. S5A). Evidently, inhibition of GSK-3β had a strong and clear suppressive influence on HCC cells' metabolic process.

To further demonstrate GSK-3β's modulation on glucose metabolic process, we measured extracellular acidification rate (ECAR) using XF96 Extracellular Flux Analyzer. The results indicated that GSK-3β knockdown by inhibitors or shRNA suppressed the glycolytic capability of HCC cells with varied strengths (Fig. 4G and H, Supporting Figs. S5B and S5C). In addition, gene expressions of key enzymes involved in the glycolysis process including glucose transporter 1 and 4 (GLUT1 and GLUT4), hexokinase 1 and 2 (HK1 and HK2), human 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), pyruvate kinase isozyme typeM 2 (PKM2), Hypoxia inducible factor (HIF) were remarkable down-regulation in shGSK-3β HCC cells (Fig. 4I, Supporting Fig. S5D). Conversely, these genes were all up-regulated in over-expressed GSK-3β HCC cells (Fig. 4J, Supporting Fig. S5E).

3.4. GSK-3β regulates HCC cells' glycolysis and malignant phenotype via AMPK/mTOR pathway

To further determine the signaling pathway of GSK-3β regulating glycolysis in HCC cells, we investigated the activity of AMPK and mTOR, serine/threonine protein kinases that are highly sensitive to cellular energy status. AMPK is frequently down-regulated in cancer cells and its activation can often lead to inhibition of mTOR [35]. As shown in Fig. 5A and B, the p-AMPK levels were elevated and p-mTOR levels were decreased in both Hep3B and CSQT-2 cells after GSK-3β had been suppressed.

Aside from being a modulator of cell metabolism, mTOR also regulates cell proliferation, death, and survival [35]. We also analyzed the expression level of mTOR's downstream molecules related to cell survival and growth such as p-p70S6K, p-4E-BP1 in HCC cells, and found that their expression levels were also strongly decreased in GSK-3β suppressed HCC cells (Fig. 5A and B).

These findings combined with above metabolic alteration data suggested that GSK-3β inhibition resulted in the restriction of mTOR activity by activating AMPK, led to the inhibition of glycolytic abilities as well as the suppression of malignant phenotype in HCC cells. These results also indicated that AMPK holds a critical role in HCC cell metabolic regulation.

A rescue experiment was performed to confirm that AMPK up-regulation induced by the inhibition of GSK3β indeed caused the suppression of malignant phenotype and glycolytic ability of HCC cells. We constructed AMPK siRNA and verified the interference efficiency of siAMPK on Hep3B and CSQT-2 HCC cells (Fig. 5C). As showed in Fig. 5D and E and 5F, suppression of AMPK expression by siAMPK partially restored the proliferation and migration ability of HCC cells suppressed due to GSK3β inhibition (Fig. 5D and E and 5F). Also, siAMPK partially reversed the GSK-3β-inhibition-led downregulation of glucose consumption, lactate production and ATP levels of HCC cells (Fig. 5G). Thus, the results suggested that the regulation of GSK3β on biological behavior and glycolysis of HCC cells was dependent on AMPK.

3.5. Primary HCC cells confirm the anti-cancer effect by GSK-3β inhibition

To further test the anti-cancer effect of GSK-3β inhibition and confirm our observation from HCC cell lines, we applied GSK-3β inhibitors on primary HCC cells that isolated from cancer tissues derived from four HCC patients (Fig. 6A). After primary HCC cells from patient 1 and patient 2 were treated by GSK-3β inhibitors for four days, we found that primary HCC cells viability and migration ability were significantly decreased (Fig. 6B and C). Meanwhile, metabolic pattern of primary HCC cells had also changed. The glucose consumption (Fig. 6D), lactate production (Fig. 6E), ATP level (Fig. 6F) and glycolysis level (Fig. 6G) were considerably decreased in inhibitors-treated HCC cells. Western blot results showed that after primary HCC cells from patient 3 and patient 4 were treated by GSK-3β inhibitors, p-AMPK was notably up-regulated and p-mTOR, p-p70S6k and p-4E-BP1 were drastically down-regulated (Fig. 6H).

These results suggest that GSK-3β inhibitors can inhibit the aerobic glycolysis level of primary HCC cells by activating the AMPK signaling pathway, and ultimately affect tumor biological behavior of primary HCC cells, which is consistent with the experimental results of above HCC cell lines.

3.6. Inhibition of GSK-3β inhibits tumor growth in vivo

Furthermore, we used a xenograft nude mice model to investigate the effect of GSK-3β on tumor growth in vivo. Nude mice were subcutaneously injected with 1×10^6 shGSK-3β Hep3B cells, over-expression GSK-3β Hep3B cells, and their control Hep3B cells as comparison. About 4 weeks after tumor inoculation, the animals were euthanized to compare tumor burden between control groups and GSK-3β-treated groups. Tumors from mice injected with shGSK-3β Hep3B

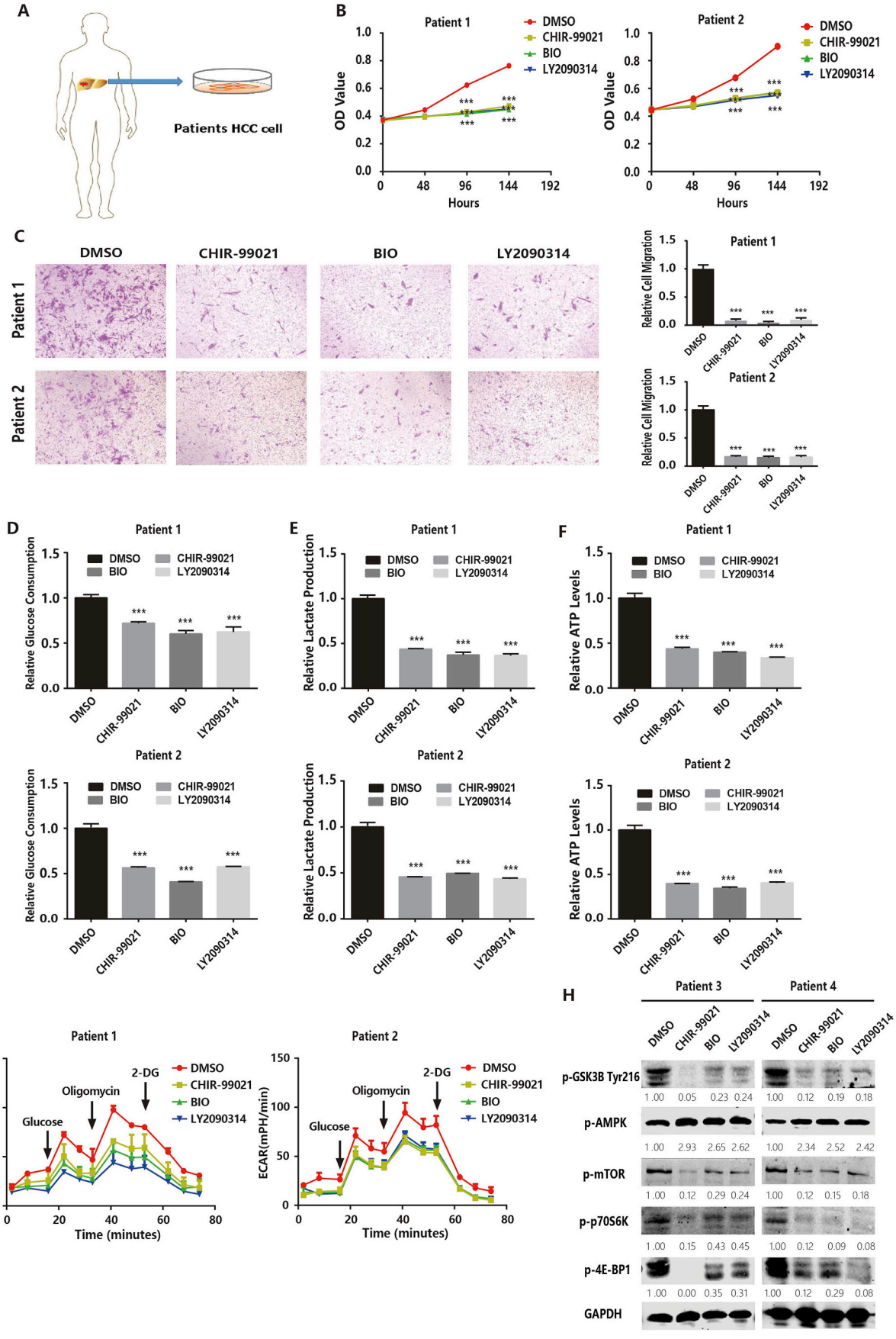

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Fig. 6. Primary HCC cells confirm the anti-cancer effect by GSK-3β inhibition.
(A) HCC tissues from four patients were obtained from the hospital, we isolated primary HCC cells by collagenase digestion.
(B) Primary HCC cells from patient 1 and patient 2 were treated with DMSO or GSK-3β inhibitors CHIR-99021, BIO, LY2090314 for designated times. Relative cell viability was measured by the CCK-8 assay at indicated time points as described under materials and methods.
(C) GSK-3β inhibitors suppressed cell migration in primary HCC cells from patient 1 and patient 2. Transwell assay was performed after primary HCC cells treated with DMSO, CHIR-99021, BIO, LY2090314 for 48 h. Representative images are shown.
(D–G) After primary HCC cells from patient 1 and patient 2 were treated by GSK-3β inhibitors, glucose consumption (D), lactate production (E), cellular ATP level (F) and glycolysis level (G) were significantly decreased. Three independent experiments were performed.
(H) After primary HCC cells from patient 3 and patient 4 were treated by GSK-3β inhibitors for 72 h, the levels of p-AMPK was upregulated and p-mTOR, p-p70S6K, and p-4E-BP1 were down-regulated.
*P < 0.05,**P < 0.01, ***P < 0.001.

cells had significantly slower growth as measured by tumor volume and weight (Fig. 7A). In contrast, tumors from mice injected with over-expression GSK-3β Hep3B cells were much bigger in volume and heavier in weight compared with tumors derived from control group (Fig. 7B), indicating that GSK-3β promote tumor growth in vivo. Similar results were obtained in another HCC cell line SMMC-7721 (Supporting Figs. S6A and S6B).

To further define the mechanism of tumor growth inhibition by modulating GSK-3β in vivo, we performed immunohistochemical assay on tumor tissues derived from mice models. As can be seen in Fig. 7C, GSK-3β, p-mTOR, p-p70S6K, p-4E-BP1 were lower expression and p-AMPK was higher expression in the shGSK-3β group compared with the control group, which is in line with the in-vitro results. Opposite results were obtained from measuring the protein levels in the OVGSK-3β group.

4. Discussion

The increased uptake of glucose and amino acids is recognized as one of the signature features of cancer metabolism [36]. In contrast to normal cells, glucose in cancer cells is predominantly metabolized by aerobic glycolysis rather than by oxidative metabolism. Upregulated glycolysis serves as a metabolic central hub that connects with other metabolic pathways. Among them, the critical ones are (1) the pentose phosphate pathway (PPP) for ribonucleotide synthesis and nicotinamide adenine dinucleotide phosphate (NADPH), (2) the hexosamine pathway to generate UDP-*N*-acetylglucosamine for protein glycosylation, and (3) the serine biosynthesis pathway [37,38]. As a result, increased glucose uptake by cancer cells also leads to upregulated levels of other intermediary metabolites. As a key regulator of glucose metabolism, GSK-3β essentially controls the entire metabolic process of cancer cells. Understanding the regulation mechanisms of GSK-3β on aerobic glycolysis may enable the development of therapeutic interventions for cancer. Our results indicated, as a promoter of HCC progression, GSK-3β inhibition by small molecules or shRNA led to suppression of tumor behavior such as proliferation, colony formation and migration in HCC cells. Decreased glucose consumption, lactate production and ATP levels were also observed in GSK-3β suppressed HCC cells, suggesting that the suppression of HCC cells' malignant phenotypes was likely through modulation on glycolysis. Assessment of extracellular acidification rate and down-regulated expression level of key enzymes (GLUT1, HK2, PFKFB3, PKM2) revealed that GSK-3β is indeed involved in glycolytic metabolic process.

Targeting the glycolysis key factors and enzymes to against cancer is currently an attractive approach. For example, GLUTs (glucose transporter) which are responsible for transporting glucose into the cancer cells, are popular drug targets. GLUT1 is highly expressed in many cancer types and inhibition of GLUT1 by small molecules has been shown to kill tumor cells in preclinical models [39]. HK2 is a key mediator of aerobic glycolysis and is overexpressed in many tumor cells [40]. Systemic targeting of HK2 blocks tumor growth without adverse physiologic consequences [41]. PFKFB3 isozyme is extensively expressed in tumor cells and required for the high glycolytic rate. A small molecule inhibitor of PFKFB3, 3- (3-pyridinyl)- 1-(4-pyridinyl) -2-propen -1- one (3PO), suppresses glycolytic flux and is cytostatic to tumor cells [42]. Muscle type of pyruvate kinase (PKM) is one of the key mediators of the Warburg effect and tumor metabolism. Specific knockdown of the PKM2 results in decreased viability and increased apoptosis in multiple cancer cells and causes substantial tumor regression of established xenografts [43]. Targeting the key enzymes of glycolysis pathway can effectively halt cancer progression, however, focusing on a single target tend to have limited impact due to the heterogeneity of cancer cells. Our results indicated that inhibition of GSK-3β activity could suppress several key enzymes activity throughout the glycolysis-related pathways, which makes it a far more attractive target than single enzyme.

AMPK is a conserved and ubiquitously expressed energy sensor. It is also a negative regulator of the Warburg Effect [44]. AMPK activation leads to reduction in phosphorylated mTOR, its direct downstream target [35], which plays a crucial role in glycolysis and protein synthesis. In our study, we observed that p-AMPK T172 protein level was greatly enhanced and p-mTOR level was visibly decreased in GSK-3β suppressed HCC cells. Changes in these two signaling pathways were in accordance with previous changes in metabolic processes of HCC cells, indicating that GSK-3β modulates glycolysis via these two pathways. Moreover, diminished expression of p-mTOR by inhibition of GSK-3β was capable of reducing its downstream targets expression level, such as p-p70S6K, p-4E-BP1. These proteins directly control proliferation and metastasis of tumor cells. This impact combined with suppression of tumor cell metabolic abilities, eventually translated into suppression of HCC malignant phenotype. Interestingly, since GSK-3β is a key mediator in the Wnt/β-catenin pathway, its inhibition led to increased expression of β-catenin in both RNA and protein level as expected, which however did not result in tumor promoting effect, implying that the impact of GSK-3β on HCC is likely independent of the Wnt/β-catenin signaling.

In this study, we demonstrated that GSK-3β could be a potent target for HCC eradication. Inhibition of GSK-3β resulted in decreased cell growth-related proteins and glycolysis-related enzymes levels through activating AMPK/mTOR signaling. Several questions remain to be clarified in future investigation. First, we still have not discovered why inhibition of GSK3β activity could promote malignant phenotypes in certain cancers, as reported by previous literature. Additionally, several reports have demonstrated that the GSK-3β Ser9 play critical role on HCC development. However, Ser9 site is only the regulation site of GSK-

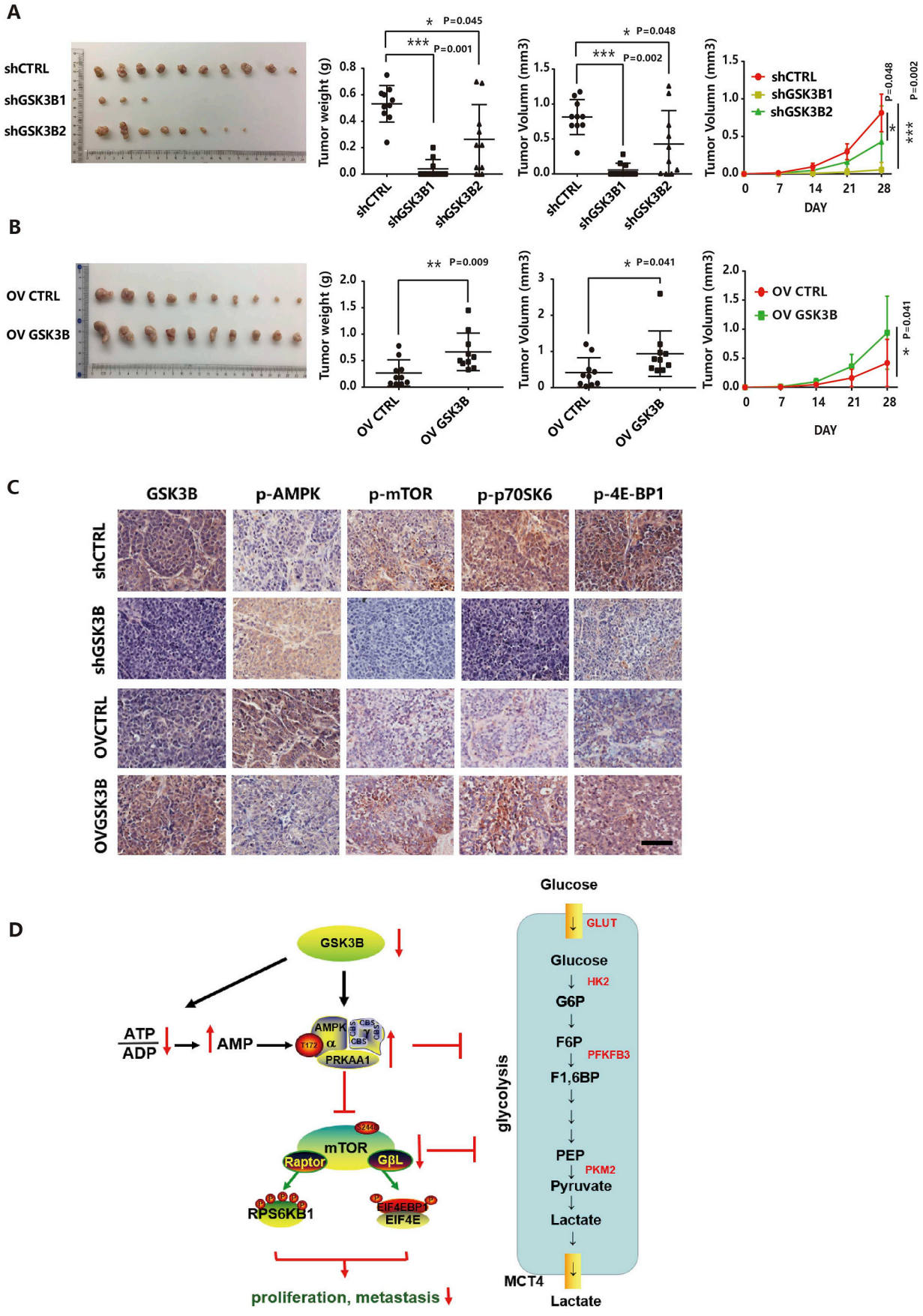

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Fig. 7. Effects of GSK-3β on tumor formation in vivo.
(A) Representative subcutaneous tumors from mice implanted with 1×10^6 control or shGSK-3β Hep3B cells were shown. Tumor weights were measured after the mice had been sacrificed. Tumor volumes from each group were evaluated at the indicated time points and data were shown as mean ± SD.
(B) Representative subcutaneous tumors from mice implanted with 1×10^6 control or GSK-3β over-expression Hep3B cells were shown.
(C) Immunohistochemistry assay were performed on paraffin embedded specimens of xenograft tumors.
(D) Schematic depiction of the mechanisms underlying inhibition of GSK-3β activity suppressed HCC glycolysis by activating AMPK/mTOR signaling pathway.
*P < 0.05,**P < 0.01, ***P < 0.001.

3β. The kinase site of GSK-3β is Tyr216. Whether the different activation of two phosphorylation sites of GSK-3β is responsible for HCC promotion or suppression needs to be elucidated. Finally, while inhibition of GSK-3β activity has the capability to suppress the glycolysis of HCC cells, many of the enzymes it affects also exist in normal tissues. How to specifically target HCC cells without incurring negative side effects need to be answered prior to clinical application. Nevertheless, our research proved that inhibition of GSK-3β can suppress not only glycolysis, but also cell growth related protein synthesis. Therefore, GSK-3β is a promising therapeutic target for HCC.

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Author contributions

G.F., P.Z., X.Z., and X.Z. performed the experiments and G.F., P.Z., and J.L. analyzed the data. P.Z. and H.W. designed the project. G.F., and P.Z. analyzed the data and wrote the manuscript. P.Z. and H.W. revised the manuscript.

Statement

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.08.003.

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