

# Simultaneous Inhibition of Ornithine Decarboxylase 1 and Pyruvate Kinase M2 Exerts Synergistic Effects Against Hepatocellular Carcinoma Cells

This article was published in the following Dove Press journal:  
*OncoTargets and Therapy*

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**Purpose:** Previously, we showed that lactate promoted the proliferation and mobility of hepatocellular carcinoma (HCC) cells by increasing the expression of ornithine decarboxylase 1 (ODC1). In this study, we determined the relationship between ODC1 and pyruvate kinase M2 (PKM2, a key lactate metabolism enzyme), and determined the combined effects of difluoromethylornithine (DFMO; an ODC1 inhibitor) and compound 3k (a PKM2 inhibitor) on HCC cells.

**Methods:** First, the relationship between PKM2 and ODC1 was analyzed using Western blotting, Cell Counting Kit (CCK)-8 assays, transwell assays, bioinformatics, quantitative real-time fluorescent PCR (qRT-PCR), and immunohistochemical staining. Thereafter, the ODC1 inhibitor DFMO and the PKM2 inhibitor compound 3k were employed. Their combined effects on HCC cell proliferation and mobility were evaluated via CCK-8 assay, flow cytometry, a subcutaneous xenograft tumor model in mice, wound healing assays, and transwell assays. Additionally, the effects of DFMO and compound 3k on the epithelial-mesenchymal transition phenotype and the AKT/GSK-3 $\beta$ / $\beta$ -catenin pathway were explored using Western blotting and immunofluorescence.

**Results:** PKM2 knockdown significantly decreased the ODC1 expression, and the proliferation and invasion of HCC cells, while ODC1 overexpression reversed the inhibitory effects of PKM2 knockdown. Similarly, inhibition of ODC1 also decreased the expression of PKM2 via reducing the c-myc-induced transcription. PKM2 was co-expressed with ODC1 in HCC samples, while simultaneously upregulated PKM2 and ODC1 led to the poorest survival outcome. DFMO and compound 3k synergistically inhibited HCC cell proliferation, induced apoptosis, and suppressed cell mobility, as well as the EMT phenotype and the AKT/GSK-3 $\beta$ / $\beta$ -catenin pathway. The AKT activator SC79 reversed the inhibitory effects.

**Conclusion:** PKM2/ODC1 are involved in a positive feedback loop. The simultaneous inhibition of ODC1 and PKM2 using DFMO and compound 3k exerts synergistic effects against HCC cells via the AKT/GSK-3 $\beta$ / $\beta$ -catenin pathway. Thus, DFMO combined with compound 3k may be a novel effective strategy for treating HCC.

**Keywords:** hepatocellular carcinoma, ornithine decarboxylase 1, pyruvate kinase M2, DFMO, compound 3k, synergistic effect

## Introduction

Hepatocellular carcinoma (HCC) is a common malignancy of the digestive system, and it is particularly prevalent in China. There are more than 60,000 new cases of liver cancer each year, with >40% of cases occurring in China.<sup>1</sup> As most HCC

patients are diagnosed late, <20% of HCC cases can be successfully treated by surgical excision alone, and chemotherapy is still the main treatment strategy for HCC.<sup>2</sup> However, it is widely reported that HCC cells are highly resistant to various chemotherapeutic drugs. Therefore, identifying novel drugs for HCC is important.

Compared to normal cells, HCC cells tend to use glycolysis to produce ATP even in the presence of abundant oxygen. Therefore, lactate is generated and transferred to the extracellular matrix, leading to acidosis.<sup>3</sup> Several key enzymes involved in glycolysis are upregulated in HCC, including pyruvate kinase M2 (PKM2).<sup>4</sup> High expression of *PKM2* in HCC tissues predicts poor outcomes.<sup>5</sup> Targeting PKM2 inhibits the development of HCC by suppressing glycolysis.<sup>6</sup> Several drugs that target PKM2 have shown potential antitumor activity, such as benserazide<sup>7</sup> and compound 3k.<sup>8</sup> Compound 3k showed anti-proliferative activity against colorectal and cervical cancer cells.<sup>9</sup> However, evidence on the effect of compound 3k on HCC cells remains limited.

Ornithine decarboxylase 1 (ODC1) is a rate-limiting enzyme involved in the first step of the polyamine metabolism pathway. It catalyzes the conversion of ornithine to putrescine, which leads to increases in the higher-order polyamines spermidine and spermine.<sup>10</sup> Dysregulated *ODC1* has been demonstrated in various cancers.<sup>11,12</sup> Inhibition of *ODC1* via gene silencing and pharmacological methods led to anti-proliferation effects on cancer cells, including HCC cells.<sup>13,14</sup> Difluoromethylornithine (DFMO) is an irreversible inhibitor of ODC1 that binds to ODC1 and induces its degradation, decreasing intracellular polyamine levels. Clinical trials have revealed that DFMO benefits patients with cervical and colon cancer.<sup>15,16</sup> However, DFMO only exerts anti-tumor effects when the dosage reaches millimole levels, and high-dose DFMO results in gastrointestinal and hematologic toxicities.<sup>17</sup>

Our previous study showed that an acidic microenvironment (simulated by lactate) promoted proliferation and migration of HCC cells by increasing ODC1 expression.<sup>18</sup> In the current study, we aimed to reveal the relationship between the key lactate metabolism enzyme PKM2 and the polyamine metabolism enzyme ODC1, and to determine whether simultaneous inhibition of ODC1 and PKM2 by DFMO and compound 3k, respectively, exerts synergistic effects against HCC cells. The results suggested that this approach may represent a novel treatment for HCC.

## Materials and Methods

### Cell Culture and Drugs

The HCC cell line HepG2 was purchased from the American Type Culture Collection (ATCC; Virginia, USA). The HCC cell line SMMC-7721 was purchased from Shanghai Institute of Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C in 5% CO<sub>2</sub>. The ODC1 inhibitor DFMO and the AKT activator SC79 were purchased from MCE (Wuhan, China). The PKM2 inhibitor compound 3k was bought from Selleck (Wuhan, China). Spermidine and spermine were purchased from Sigma (New Jersey, USA).

### Cell Transfection

Small interfering RNAs (siRNAs) targeting *PKM2*, *ODC1*, and *c-myc* and scramble siRNAs were purchased from GeneCopoeia (Wuhan, China). An *ODC1* overexpression plasmid and a negative control (NC) plasmid were obtained from Vigenebio (Wuhan, China). Transfection of the siRNAs and plasmids was performed using Lipo2000 (GeneCopoeia; Wuhan, China) based on the manufacturer's instructions. The sequences of siRNAs targeting *PKM2*, *ODC1*, and *c-myc* and scramble siRNA were as follows:

*PKM2* siRNA1 sense: GCCAUCUACCACUUGCA AUTT,

*PKM2* siRNA1 anti-sense: AUUGCAAGUGGUA GAUGGCTT;

*PKM2* siRNA2 sense: GAUAUGGUGUUUGCGU CAUTT,

*PKM2* siRNA2 anti-sense: AUGACGCAAACACCAU AUCTT;

*ODC1* siRNA sense: GGUUGGUUUCAGCAU GUAUTT,

*ODC1* siRNA antisense: AUACAUGCUGAAACCAA CCTT;

*c-myc* siRNA sense: AAGCCACAGCATAATC CTGT,

*c-myc* siRNA antisense: AAACAGGATGTATGC TGTGGC;

scramble siRNA sense: UUCUCCGAACGUGUCA CGUTT,

scramble siRNA anti-sense: ACGUGACACGUUCGG AGAATT.

## Cell Counting Kit-8 (CCK-8) Assay

The HepG2 and SMMC-7721 cells were plated on 96-well plates at a density of 3000 cells/well and the cells adhered to the bottom. Dimethyl sulfoxide (DMSO; the control), DFMO (500  $\mu$ M), compound 3k (2.5  $\mu$ M), SC79 (10  $\mu$ M), or treatment combinations were then added to the wells. After 24 and 48 h, 10  $\mu$ L CCK-8 was added to each well and optical density (OD) at 450 nm was assessed. The proliferation rate in each group was calculated according to the following formula: proliferation rate (%) =  $(OD_{\text{experimental group}} - OD_{\text{CCK-8}}) / (OD_{\text{control group}} - OD_{\text{CCK-8}})$ . The combination index (CI) of compound 3k and DFMO was calculated according to the following formula: CI =  $\text{combination inhibition rate} / (\text{compound 3k inhibition rate} + \text{DFMO inhibition rate} - \text{compound 3k inhibition rate} \times \text{DFMO inhibition rate})$ . CI >1.15 was set as the cutoff that represents a synergistic effect between the two drugs.

## Apoptosis Assay

A total of  $1 \times 10^5$  HepG2 and SMMC-7721 cells were seeded in six-well plates and treated with DMSO (the control), 500  $\mu$ M DFMO, 2.5  $\mu$ M compound 3K, and the combination (DFMO and compound 3K) for 24 and 48 h. After washing three times with phosphate-buffered saline (PBS), the cells were stained using 5  $\mu$ L Annexin V (Beyotime, Wuhan, China) and 10  $\mu$ L propidium iodide (PI; Beyotime, Wuhan, China) for 30 min at room temperature. The apoptotic rate was evaluated using a Becton-Dickinson FACScan cyto-fluorometer (New Jersey, USA). Total apoptosis rate (%) = Q2+Q3.

## Wound Healing Assay

A total of  $5 \times 10^5$  HepG2 and SMMC-7721 cells was seeded in six-well plates. When the cells reached >95% confluence, a 200- $\mu$ L sterile pipette tip was used to create a wound. After washing away the floating cells using PBS and replacing the medium, DMSO (the control), 500  $\mu$ M DFMO, 2.5  $\mu$ M compound 3K, and the combination were added to the well. The wound healing results, indicating migration ability, were recorded up to 24 h using an optical microscope.

## Transwell Assay

A total of  $5 \times 10^4$  HepG2 and  $2 \times 10^4$  SMMC-7721 cells were resuspended in serum-free DMEM and placed in the upper chamber of a Transwell system (Corning, USA) with a membrane coated with Matrigel (Corning,

USA). Concurrently, DMSO (the control), DFMO (500  $\mu$ M), compound 3k (2.5  $\mu$ M), SC79 (10  $\mu$ M), or treatment combinations were also added to the top chamber. 10% FBS was used as a chemoattractant and placed in the lower chamber. After culture for 24 h, the chambers were fixed with paraformaldehyde and stained with crystal violet for 30 min at room temperature. After removing the non-invasive cells, five fields in the chamber were photographed using an optical microscope ( $\times 100$  magnification) and the numbers of cells were counted.

## Quantitative Real-Time Fluorescence PCR (qRT-PCR)

Total RNA was extracted from HCC tissues and adjacent normal tissues using TRIzol reagent (Takara, Japan). The RNAs were reverse transcribed into cDNA using a 1st Strand cDNA Synthesis Kit (Yeasen, Shanghai, China). The qRT-PCR was performed using SYBR Green Master Mix (Yeasen, Shanghai, China).  $\beta$ -actin was used as the loading control. The primers were as follows:

*ODC1* forward primer: TTTACTGCCAAGGACA TTCTGG;

Reverse primer: GGAGAGCTTTTAACCACCTCAG;

*PKM2* forward primer: ATGTCGAAGCCCCATA GTGAA;

Reverse primer: TGGGTGGTGAATCAATGTCCA;

$\beta$ -actin forward primer: CATGTACGTTGCTATCC AGGC;

Reverse primer: CTCCTTAATGTACGCACGAT.

## Western Blotting

Protein samples of HepG2 and SMMC-7721 cells were homogenized in radioimmunoprecipitation assay (RIPA) buffer containing phenylmethylsulfonyl fluoride (PMSF) for total protein extraction according to the manufacturer's instructions. Equal amounts of proteins (30  $\mu$ g per lane) were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After transferring to Polyvinylidene Fluoride (PVDF) membranes, the membranes were blocked using 5% non-fat milk in Tris-buffered saline with Tween 20 (TBST) at room temperature for 2 h and then incubated overnight at 4°C with primary antibodies against PKM2, ODC1, c-myc, E-cadherin, vimentin,  $\beta$ -catenin, and  $\beta$ -actin (Proteintech, Wuhan, China) and p-AKT (Ser473) and p-GSK-3 $\beta$  (Ser9) (CST, USA). The membranes were washed with TBST and incubated with horseradish

peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG secondary antibody. Protein bands were visualized using Bio-Rad Chemidoc imaging software (California, USA).

### Immunofluorescence Assay

After fixation with 4% paraformaldehyde for 30 min, HepG2 cells were washed, permeabilized using PBS containing 1% Triton X-100, and blocked with 5% BSA. The cells were then incubated with anti- $\beta$ -catenin and anti-c-myc antibody for 12 h at 4°C. After washing with PBST, the cells were incubated with CY3 488-conjugated secondary antibody. Next, 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nuclei. Finally, images were acquired by microscopy.

### Analysis of the Cancer Genome Atlas (TCGA) HCC Data

Gene expression profiles of 377 HCC specimens and 50 adjacent tissues were downloaded from the University of California, Santa Cruz (UCSC) Genome Browser (<https://xenabrowser.net/datapages/>). The expression of *ODC1* and *PKM2* was compared between HCC and adjacent tissues, with  $\log(\text{fold change}) > 1$  and  $P < 0.05$  being set as the cutoffs representing a significant fold change. The co-expression relationship was analyzed using Pearson correlation analysis, with  $R > 0.3$  and  $P < 0.05$  being set as the cutoffs representing co-expression. HCC patients (aged  $\leq 80$  years) in the TCGA database were divided into four groups according to the expression of *ODC1* and *PKM2*. The 5-year survival rate was analyzed using the Kaplan–Meier method, with  $P < 0.05$  being set as the cutoff representing significance.

### Tissue Samples and Ethics Approval

From November 2018 to November 2019, 32 pairs of HCC and normal adjacent tissues were obtained from patients at the Affiliated Hospital of Guizhou Medical University. The collection and use of the tissue specimens were approved by the Human Research Ethics Review Committee of Guizhou Medical University and the analyses were performed according to the tenets of the Declaration of Helsinki. All patients provided written informed consent.

### Immunohistochemistry Assay

The HCC and adjacent tissues were fixed, dehydrated, embedded in paraffin (Boster, Wuhan, China), and then

were cut into 4- $\mu\text{m}$  specimens. The specimens were stained with hematoxylin and eosin (Sigma, USA) following the manufacturer's protocol. Specimens were deparaffinized using a graded series of xylene and rehydrated with a graded series of ethanol. Next, antigen retrieval was performed using sodium citrate and blocking was performed using  $\text{H}_2\text{O}_2$  and BSA (Boster, Wuhan, China). The sections were then incubated with a primary anti-*ODC1*, anti-*PKM2*, anti-*KI67*, and anti-*PCNA* antibody for 16 h at 4°C and then with horseradish peroxidase-conjugated secondary antibody (Boster, Wuhan, China) for 2 h at room temperature. After using a Cell and Tissue Staining Horse Radish Peroxidase (HRP) - Diaminobenzidine (DAB) Kit (Zhongshan Jinqiao Biotechnology Co. LTD; Beijing, China) to detect the presence of the secondary antibody, orthophoto microscopy was used to obtain images.

### Pyruvate Kinase Activity Assay to Detect PKM2

The HepG2 and SMMC-7721 cells were seeded in six-well plates and treated with DMSO (the control), compound 3k, DFMO, and the combination. After 12 h, the cells were harvested and lysed. After centrifugation at  $8000 \times g$  for 20 min at 4°C and detection of the protein concentration using the bicinchoninic acid (BCA) method, the relative PKM2 activity of 2  $\mu\text{g}$  protein was measured using a Pyruvate Kinase Assay Kit (cat no. BC0540; Solarbio; Wuhan China) according to the manufacturer's instructions.

### Subcutaneous Xenotransplant Tumor Model

Four-week-old female BALB/c nude mice were purchased from the Animal Center of Guizhou Medical University. The mice were housed in a specific-pathogen-free environment with a 12 h light/dark cycle. HepG2 cells were subcutaneously injected ( $1 \times 10^7$  cells in 150  $\mu\text{L}$  PBS) into the nude mice. At day 6 after injection, the size of the tumors reached 40–60  $\text{mm}^3$  (mice with tumor size  $< 40 \text{ mm}^3$  or  $> 60 \text{ mm}^3$  were excluded). The mice were randomly divided into four groups: control, compound 3k, DFMO, and combination. The mice in the compound 3k and combination groups were intraperitoneally injected with 40 mg/kg compound 3k every 3 days, while the mice in the control and DFMO groups were intraperitoneally injected with an equal volume of DMSO (the control) in water every 3 days. The mice in the

DFMO and combination groups drank autoclaved water containing 0.05% DFMO every day, while the mice in the control and compound 3k groups drank normal autoclaved water every day. The tumor volume was measured every 3 days, and the health condition of the mice was monitored once a day. All mice were euthanized after 15 days of treatment, and tumor, liver, kidney, and heart tissues were extracted for assessment. This animal experiment was approved and administered by the Animal Ethics Committee of Guizhou Medical University.

## Statistical Analyses

The data were analyzed using one-way analysis of variance (ANOVA) combined with least significant difference (LSD) tests using SPSS 19.0 (IBM SPSS Statistics, USA).  $P < 0.05$  was considered statistically significant.

## Results

### PKM2 Affects the Proliferation and Mobility of HCC Cells via Regulating ODC1

Our previous research revealed that ODC1 in HCC cells was increased in an acidic microenvironment (simulated by 10 mM lactate), so we assessed whether inhibiting endogenous lactate metabolism (also known as glycolysis) affects ODC1. The results showed that suppression of the endogenous lactate metabolism enzyme PKM2 (using either of two siRNAs) significantly decreased the expression of ODC1 in HepG2 and SMMC-7721 cells (Figure 1A,  $P < 0.01$ ), accompanied by reduced proliferation rate and invasion ability (Figure 1B and C,  $P < 0.01$ ). We then overexpressed ODC1 in PKM2-downregulated HCC cells (Figure 1D), which relieved the inhibitory effects of PKM2 inhibition on HCC cell proliferation and invasion (Figure 1E and F,  $P < 0.05$ ). Taking the results together, there was a regulated relationship between PKM2 and ODC1, with PKM2 affecting proliferation and invasion via regulating ODC1.

### ODC1 Regulates the c-myc-Induced PKM2 Transcription Modulation via Upregulating Polyamines in HCC Cells

Interestingly, we found that overexpression of ODC1 increased the protein and mRNA levels of PKM2, while suppression of ODC1 reduced both (Figure 2A and B,  $P < 0.01$ ). Previous studies demonstrated that ODC1

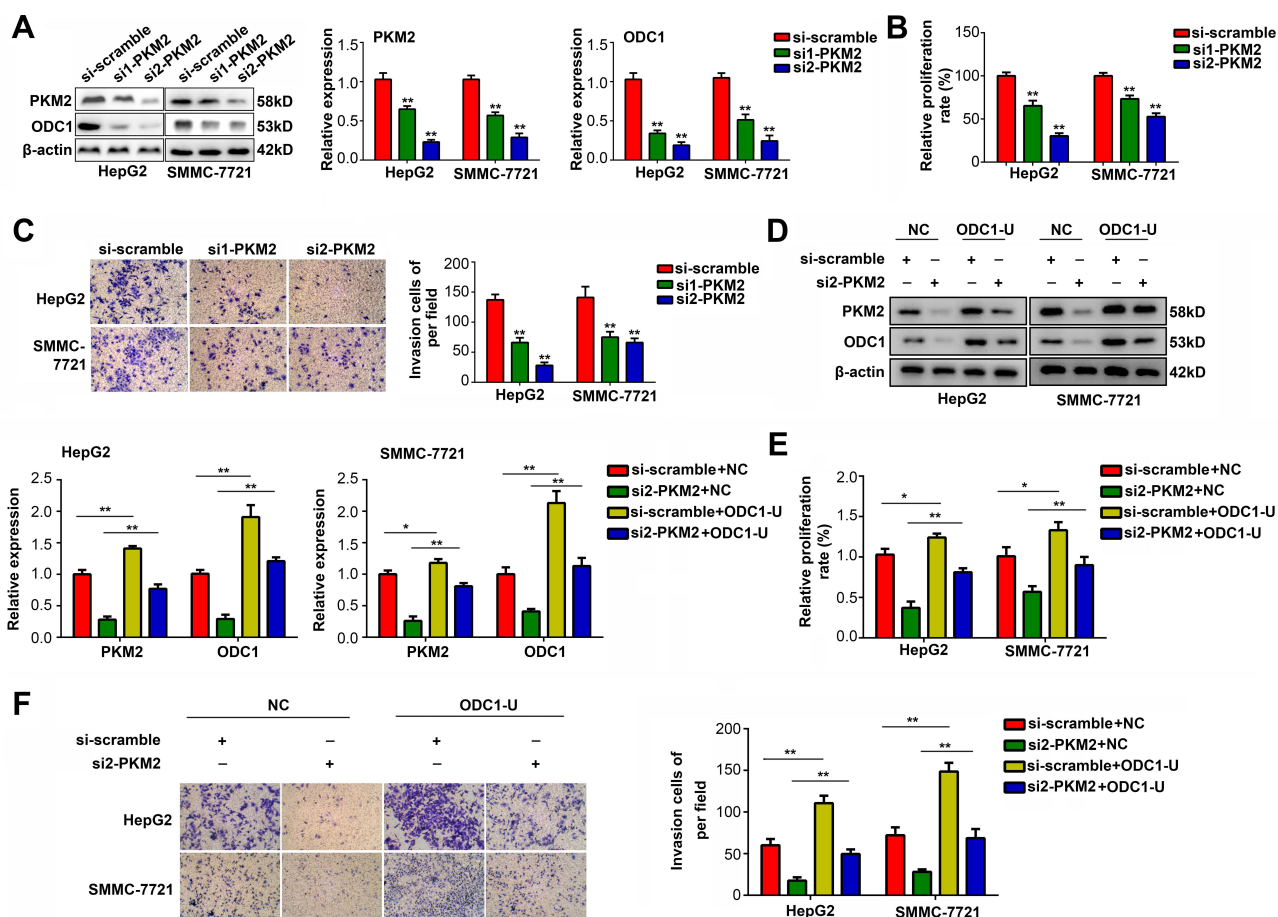
increases the synthesis of polyamines, which could lead to upregulated c-myc expression, maintenance of c-myc protein stability, and an increase in its transcriptional regulation ability.<sup>19,20</sup> As PKM2 is a target gene of c-myc, we hypothesized that ODC1 may regulate PKM2 expression via affecting c-myc-induced PKM2 transcription. Western blotting was performed, and the results showed that ODC1 overexpression increased the expression of c-myc, while ODC1 inhibition reduced the expression of c-myc (Figure 2C,  $P < 0.01$ ). Inhibition of c-myc reversed the PKM2 upregulation induced by ODC1 overexpression (Figure 2D,  $P < 0.05$ ). Furthermore, we found that treatment with spermidine and spermine in ODC1-downregulated cells increased c-myc and PKM2 expression (Figure 2E,  $P < 0.05$ ), as well as the nuclear translocation of c-myc (Figure 2F). Taken together, these results indicate that ODC1 regulated the c-myc-induced PKM2 transcription modulation via upregulating polyamines.

### PKM2 and ODC1 are Co-Expressed in HCC Tissues

We then investigated the relationship between PKM2 and ODC1 in HCC tissues in the TCGA database. We found that both PKM2 and ODC1 were upregulated in HCC tissues (Figure 3A and B,  $P < 0.05$ ), and they were clearly co-expressed (Figure 3C,  $R = 0.422$ ,  $P < 0.05$ ). Similarly, we detected the mRNA level of PKM2 and ODC1 in 32 pairs of HCC and adjacent tissues, and we showed that both were upregulated in HCC tissues compared to adjacent tissues (Figure 3D and E,  $P < 0.01$ ). PKM2 and ODC1 were co-expressed in the HCC tissues at both the mRNA and protein levels (Figure 3F-G). Furthermore, we found that simultaneously high expression of PKM2 and ODC1 in the HCC tissues predicted the poorest survival outcome (Figure 3H). These results indicated that simultaneous inhibition of ODC1 and PKM2 may be beneficial for HCC patients.

### ODC1 Inhibitor DFMO and PKM2 Inhibitor Compound 3k Exert Synergistic Effects on Cell Proliferation and Apoptosis in vitro

The above results indicated the advantages of simultaneous inhibition of ODC1 and PKM2, so we then investigated the feasibility of this treatment. The ODC1 inhibitor DFMO and the PKM2 inhibitor compound 3k were employed (Figure 4A). We found that both DFMO and compound 3k exhibited inhibitory effects on PKM2 and ODC1 expression, as well



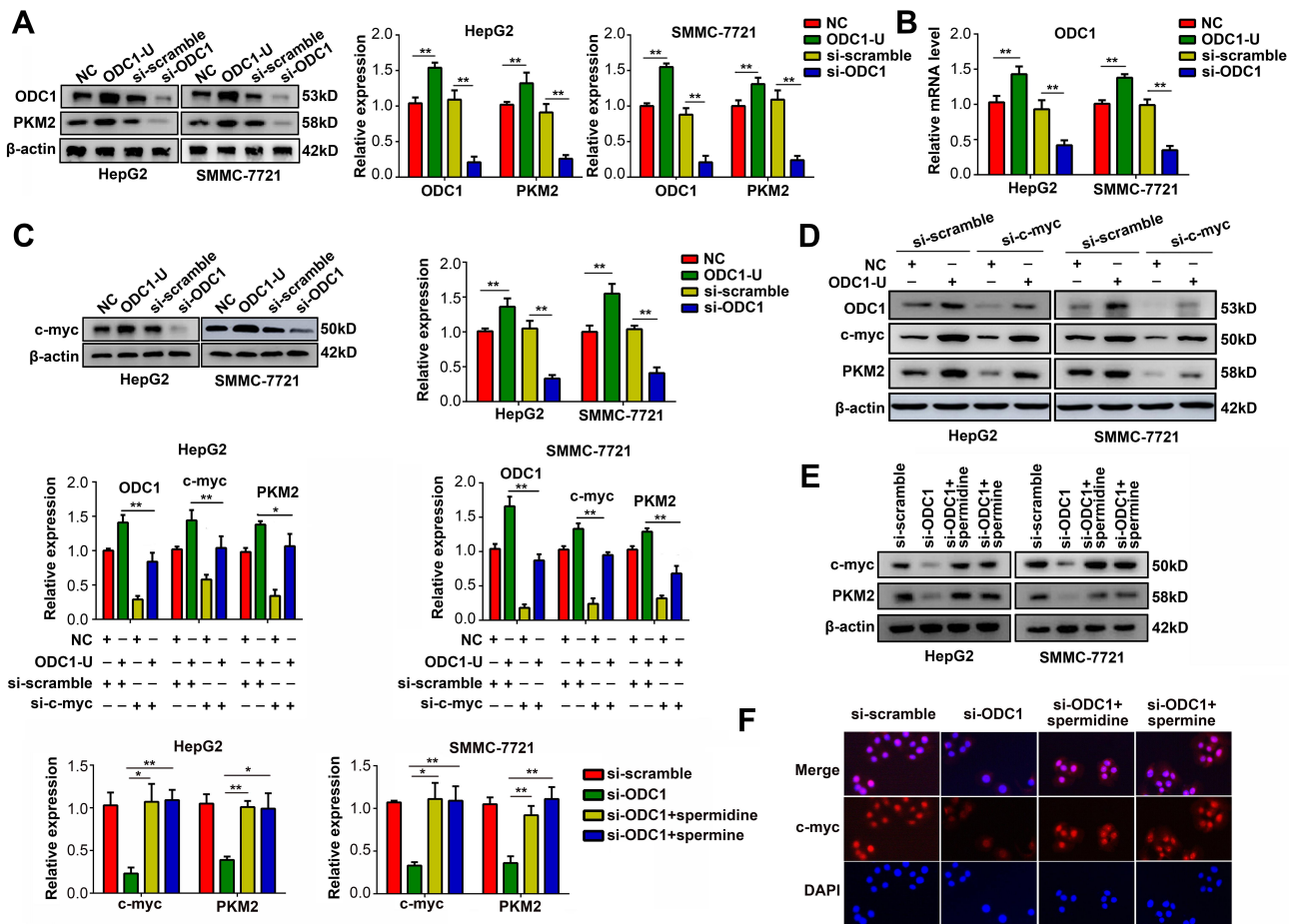
**Figure 1** PKM2 affected the proliferation and mobility of HCC cells via regulating ODC1. **(A)** Western blotting was used to detect the expression of PKM2 and ODC1 in HepG2 and SMMC-7721 cells after downregulating PKM2 using two siRNAs (si1-PKM2 and si2-PKM2). **(B)** CCK-8 assays were performed to detect the proliferation of the cells with downregulated PKM2. **(C)** Transwell assays were performed to detect the invasion of the cells with downregulated PKM2. **(D)** Western blotting was used to detect the expression of PKM2 and ODC1 in four groups of cells transfected with: 1. NC plasmid plus scramble siRNA; 2. NC plasmid plus siRNA targeting PKM2; 3. ODC1 overexpression plasmid plus scramble siRNA; 4. ODC1 overexpression plasmid plus siRNA targeting PKM2. **(E)** CCK-8 assays were used to detect the effects of ODC1 overexpression on the proliferation of the cells with downregulated PKM2. **(F)** Transwell assays were used to detect the effects of ODC1 overexpression on the invasion of the cells with downregulated PKM2. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

**Abbreviations:** HCC, hepatocellular carcinoma; PKM2, pyruvate kinase M2; ODC1, ornithine decarboxylase 1; siRNA, small interfering RNA; si, small interfering; CCK-8, cell counting kit-8; NC, negative control.

as PKM2 activity, while the combination exhibited stronger inhibitory effects compared to the single drugs (Figure 4B and C,  $P < 0.05$ ). The CCK-8 results showed that 500  $\mu$ M DFMO combined with 2.5  $\mu$ M compound 3k significantly inhibited the proliferation of HepG2 and SMMC-7721 cells compared to the single drugs and DMSO treatment at 24 and 48 h. The CI in HepG2 cells was 1.30 (24 h) and 1.45 (48 h), while the CI in SMMC-7721 cells was 1.21 (24 h) and 1.46 (48 h) (Figure 4D and E). Furthermore, compared to the single drugs and DMSO treatment, the apoptosis rate was significantly increased in HepG2 and SMMC-7721 cells in the combination group at 24 and 48 h (Figure 4F and G,  $P < 0.05$ ). Taking the results together, the ODC1 inhibitor DFMO and the PKM2 inhibitor compound 3k exert synergistic effects on cell proliferation and apoptosis in vitro.

## ODC1 Inhibitor DFMO and PKM2 Inhibitor Compound 3k Exert Synergistic Effects on Cell Mobility and Epithelial–Mesenchymal Transition (EMT) Phenotype in vitro

The results of the wound healing assays showed that DFMO combined with compound 3k significantly inhibited the migration rate of HepG2 and SMMC-7721 cells compared to the single drugs and DMSO treatment (Figure 5A and B,  $P < 0.01$ ). Similarly, transwell assays showed that the number of invaded cells per field was significantly decreased by the combination treatment compared to single drugs and DMSO treatment (Figure 5C and D,  $P < 0.01$ ). As EMT plays a key role in cell migration and

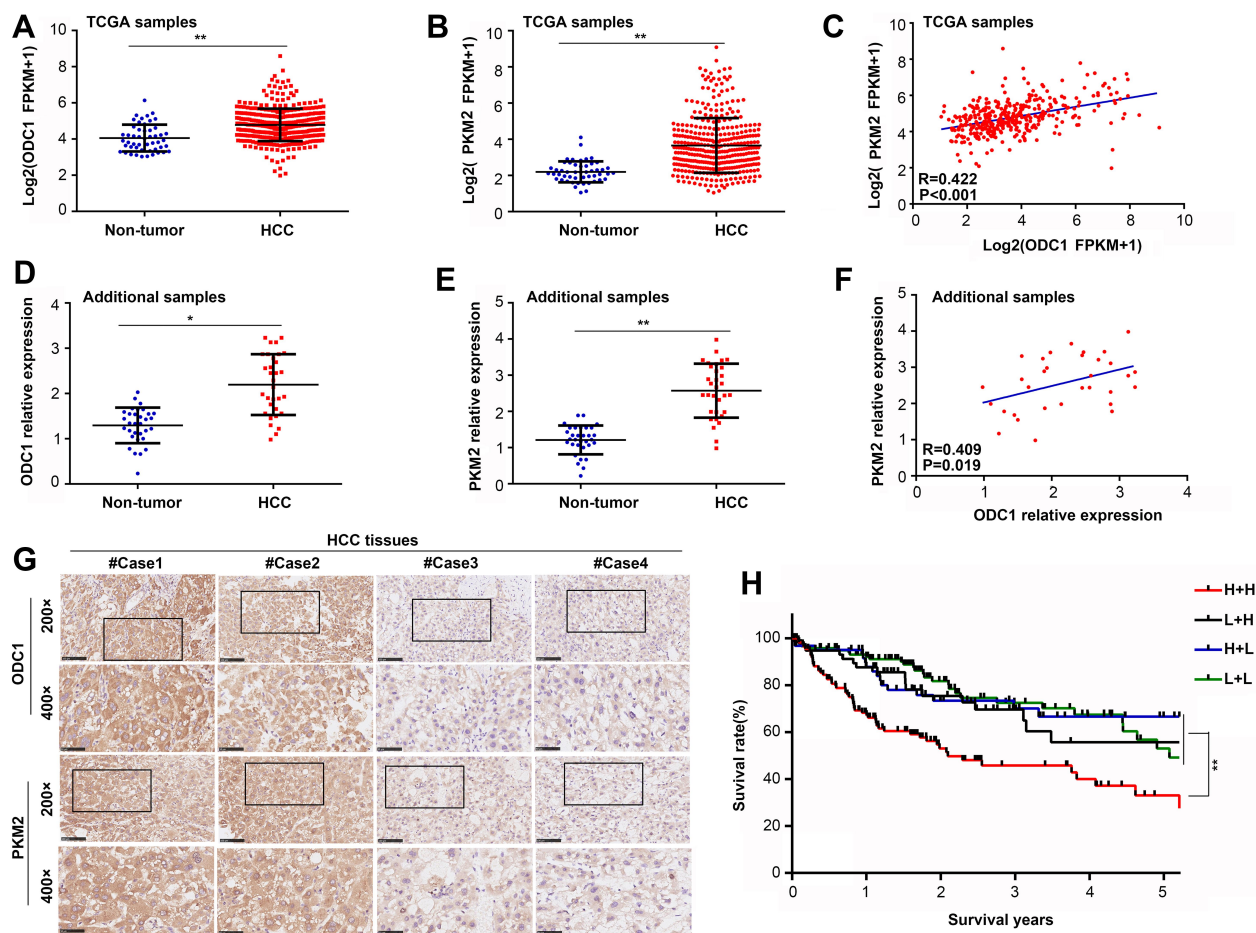


invasion,<sup>21</sup> we then assessed two markers of EMT, E-cadherin and vimentin. The results showed that treatment with DFMO and compound 3k significantly increased the expression of E-cadherin, as well as decreasing the expression of vimentin, compared to single drugs and DMSO treatment (Figure 5E-G,  $P < 0.01$ ).

## ODC1 Inhibitor DFMO and PKM2 Inhibitor Compound 3k Exert Synergistic Effects on HepG2 Cell Proliferation in vivo

To determine the combined effects of DFMO and compound 3k on HepG2 cell proliferation in vivo, a xenograft

tumor model in nude mice was used. The combination treatment significantly decreased the proliferation of HepG2 cells in vivo compared to single drugs and DMSO treatment (Figure 6A,  $P < 0.05$ ), as well as the weight of tumor tissues (Figure 6B and C,  $P < 0.05$ ). Similarly, the combination treatment significantly decreased the expression of PKM2, ODC1, KI67, and PCNA in the tumor tissues compared to single drugs and DMSO treatment (Figure 6D). Furthermore, we found that the use of DFMO (0.05% in water each day), compound 3k (40 mg/kg intraperitoneal injection every 3 days), and the combination treatment did not injure the liver, kidney, and heart in mice during the treatment process (Figure 6E). In conclusion, the ODC1 inhibitor DFMO and the PKM2



**Figure 3** PKM2 was co-expressed with ODC1 in HCC tissues. (A) ODC1 and (B) PKM2 expression in HCC and adjacent tissues and (C) co-expression relationship between ODC1 and PKM2 in TCGA samples. (D) ODC1 and (E) PKM2 expression detected using qRT-PCR in our samples (n=32) and (F) co-expression relationship between ODC1 and PKM2 based on qRT-PCR (G) and immunohistochemical staining. (H) Kaplan–Meier survival analysis of four groups in the TCGA database according to PKM2 and ODC1 expression (H+H: both high ODC1 and PKM2 expression; L+H: only high PKM2 expression; H+L: only high ODC1 expression; L+L: both low ODC1 and PKM2 expression). \*,  $P<0.05$ ; \*\*,  $P<0.01$ .

**Abbreviations:** HCC, hepatocellular carcinoma; PKM2, pyruvate kinase M2; ODC1, ornithine decarboxylase 1; TCGA, The Cancer Genome Atlas; qRT-PCR, quantitative real-time fluorescent PCR; H, high; L, low.

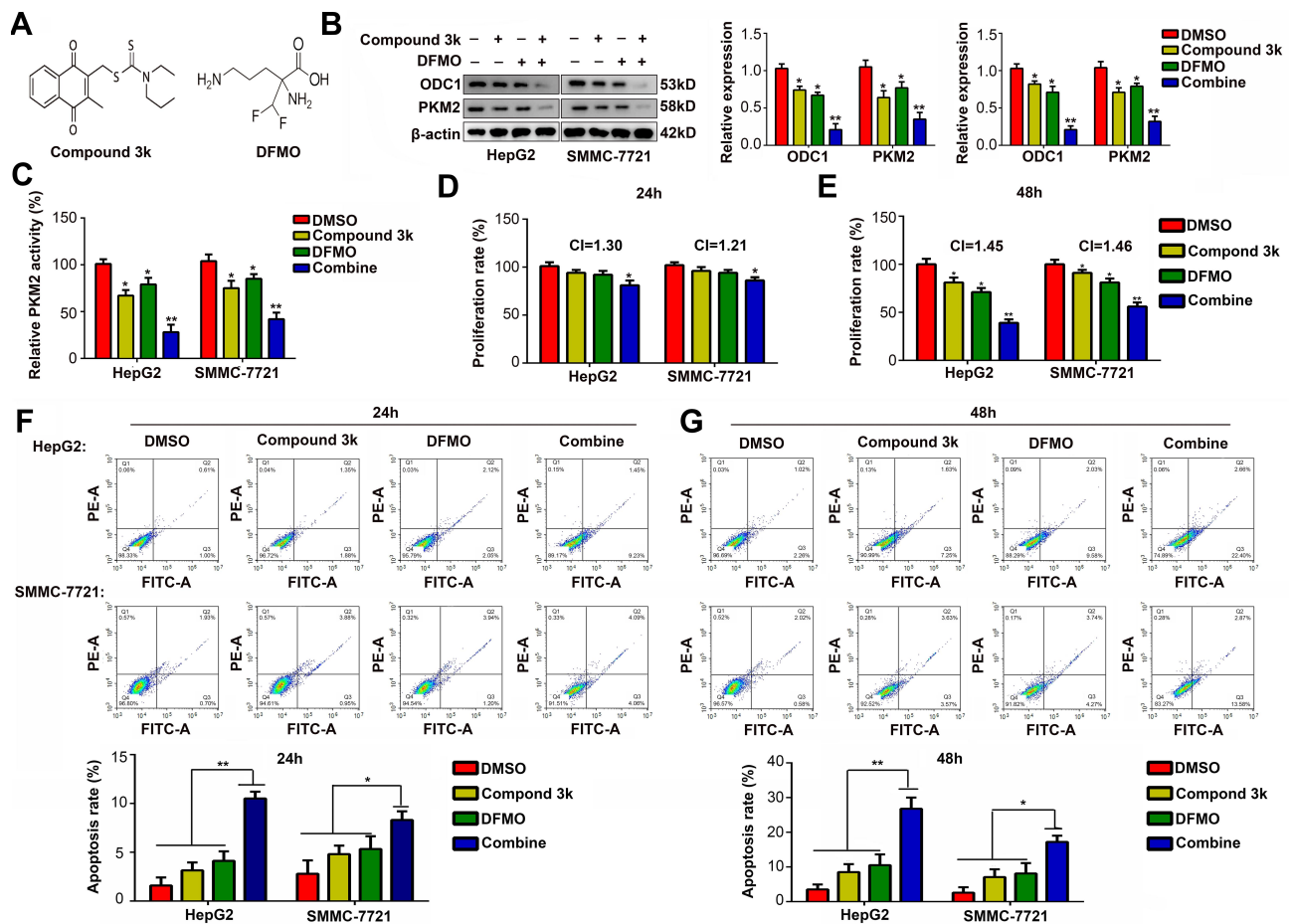
inhibitor compound 3k exert synergistic effects on HepG2 cell proliferation in vivo.

## ODC1 Inhibitor DFMO and PKM2 Inhibitor Compound 3k Exert Synergistic Effects on the AKT/GSK-3 $\beta$ / $\beta$ -Catenin Pathway

Previous studies have shown that both ODC1 and PKM2 regulate the AKT/GSK-3 $\beta$ / $\beta$ -catenin pathway and promote the development of various cancers.<sup>18,22</sup> Therefore, we determined whether DFMO and compound 3k exert synergistic effects on HCC cells via the AKT/GSK-3 $\beta$ / $\beta$ -catenin pathway. The Western blotting results showed that the combination treatment significantly decreased

the expression of p-AKT (Ser473), p-GSK-3 $\beta$  (Ser9), and  $\beta$ -catenin in HepG2 and SMMC-7721 cells compared to single drugs and DMSO treatment (Figure 7A,  $P<0.05$ ). Moreover, the immunofluorescence results showed that both nuclear and cytoplasmic  $\beta$ -catenin were decreased significantly by combination treatment compared to single drugs and DMSO treatment (Figure 7B). Thereafter, the AKT activator SC79 was used. The results indicated that SC79 activated the AKT/GSK-3 $\beta$ / $\beta$ -catenin pathway, and reversed the effects of compound 3k, DFMO, and the combination treatment on the AKT/GSK-3 $\beta$ / $\beta$ -catenin pathway (Figure 7C,  $P<0.05$ ). Similarly, SC79 increased HCC cell proliferation and invasion, reversing the effects of compound 3k, DFMO, and the combination treatment on cell





**Figure 4** ODC1 inhibitor DFMO and PKM2 inhibitor compound 3k exert synergistic effects on HCC cell proliferation and apoptosis in vitro. **(A)** Chemical formulae of DFMO and compound 3k. **(B)** ODC1 and PKM2 expression in HCC cells treated with control (DMSO), compound 3k (2.5  $\mu$ M), DFMO (500  $\mu$ M), and the combination was detected using Western blotting. **(C)** PKM2 activity in the four groups of HCC cells was determined using a Pyruvate Kinase Assay Kit. **(D-E)** CCK-8 assays were used to assess the proliferation rate in the four groups of HCC cells at 24 and 48 h. The CI index was also calculated. **(F-G)** Flow cytometry was used to determine the apoptosis rate at 24 and 48 h in the four groups of HCC cells. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

**Abbreviations:** CI, combination index; HCC, hepatocellular carcinoma; PKM2, pyruvate kinase M2; ODC1, ornithine decarboxylase 1; DFMO, difluoromethylornithine; DMSO, dimethyl sulfoxide; CCK-8, cell counting kit-8.

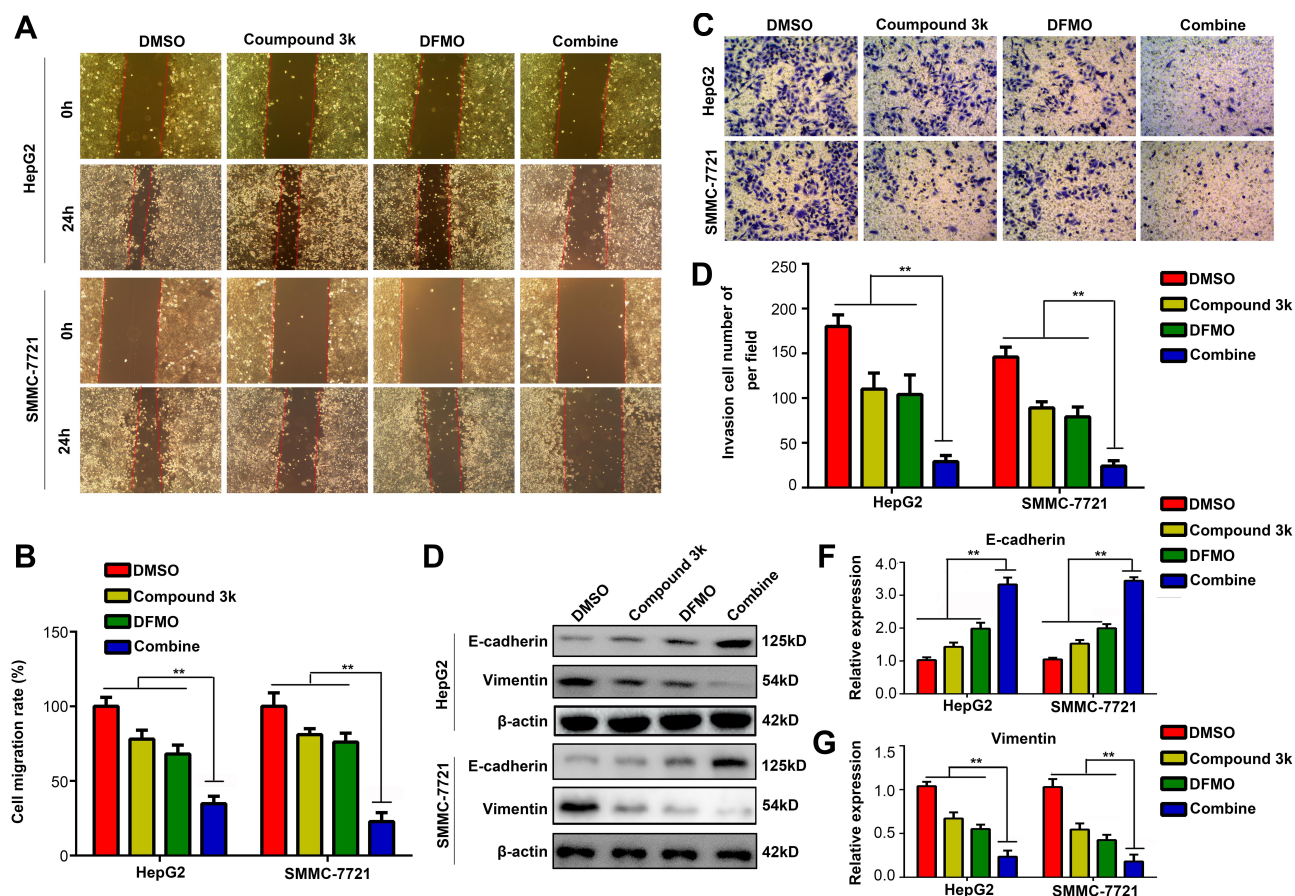
proliferation and invasion (Figure 7D and E,  $P < 0.05$ ). These results indicated that the ODC1 inhibitor DFMO and the PKM2 inhibitor compound 3k exert synergistic effects on the AKT/GSK-3 $\beta$ / $\beta$ -catenin pathway.

## Discussion

Excision and chemotherapy are the main treatment measures for HCC, but few patients benefit due to side effects and recurrence.<sup>23</sup> Therefore, there is an urgent need for understanding the mechanisms underlying HCC carcinogenesis, as well as for developing new therapeutic strategies for patients with HCC.

PKM2 is a key metabolism enzyme involved in lactate metabolism as it catalyzes the transformation of phosphoenolpyruvate to pyruvate. Consistent with other metabolism enzymes (such as Glut1 and PFKP), PKM2

is also upregulated in a series of cancer types including HCC.<sup>24,25</sup> By regulating the AKT/mTOR pathway, PKM2 increases the development of bladder cancer.<sup>26</sup> Similarly, PKM2 promotes the mobility of gastric cancer cells via regulating the PI3K/AKT pathway.<sup>27</sup> High expression of PKM2 during hypoxia leads to resistance to an mTOR inhibitor in prostate cancer.<sup>28</sup> In HCC patients, high expression of PKM2 predicts a poor 5-year survival rate.<sup>5</sup> ODC1 is the rate-limiting enzyme in polyamine biosynthesis and it is dysregulated in various cancers such as endometrial, prostate, and colorectal cancer. ODC1 promotes tumor growth through the generation of polyamines including putrescine and the higher-order polyamines spermidine and spermine.<sup>29</sup> Our previous study revealed that ODC1 in HCC cells was increased in an acidic microenvironment (simulated



**Figure 5** ODC1 inhibitor DFMO and PKM2 inhibitor compound 3k exert synergistic effects on cell mobility and EMT phenotype in vitro. (A-B) Wound healing assays were used to assess the migration rate at 24 h of HCC cells treated with control (DMSO), compound 3k (2.5  $\mu$ M), DFMO (500  $\mu$ M), or the combination. (C-D) Transwell assays were used to detect the invasion rate at 24 h in the four groups of HCC cells. (E-G) Western blotting was used to detect the expression of E-cadherin and vimentin in the four groups of HCC cells. \*\*,  $P < 0.01$ .

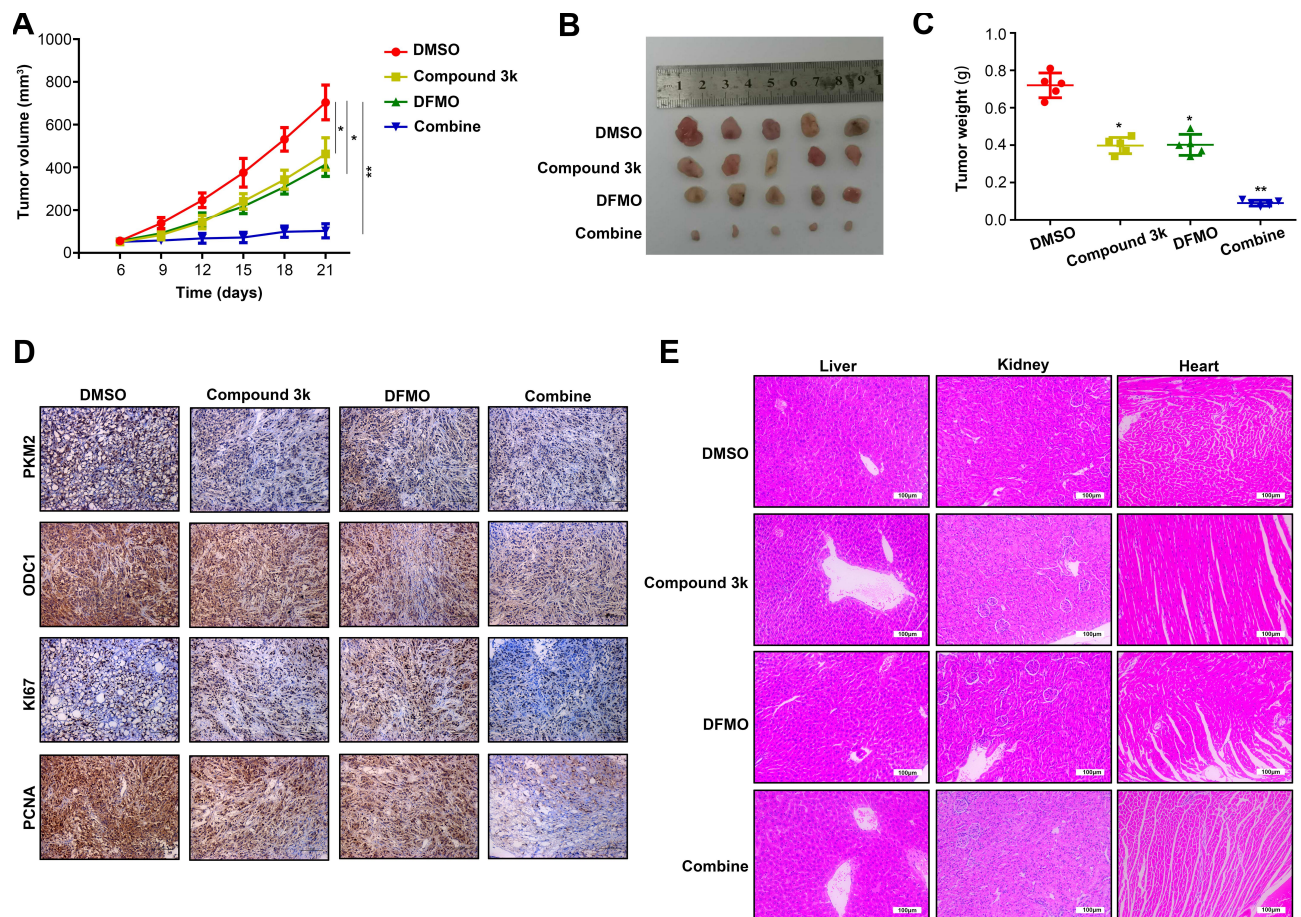
**Abbreviations:** HCC, hepatocellular carcinoma; PKM2, pyruvate kinase M2; ODC1, ornithine decarboxylase 1; DFMO, difluoromethylornithine; DMSO, dimethyl sulfoxide.

by lactate) and promoted the development of HCC. Therefore, in the present study, we determined the relationship between lactate metabolism enzyme PKM2 and ODC1 in HCC.

We found that inhibition of *PKM2* significantly decreased the expression of ODC1, as well as HCC cell proliferation and invasion. Overexpression of ODC1 reversed the inhibitory effects of PKM2 inhibition on cell proliferation and invasion. These findings were consistent with the opinion that ODC1 expression is sensitive to changes in glycolysis in cells.<sup>30</sup> Interestingly, we also found that ODC1 can affect the expression of PKM2. *PKM2* is a target gene of c-myc<sup>31</sup> and previous studies have shown that ODC1 has the potential to regulate the target genes of c-myc via producing polyamines. Therefore, we further studied the relationship between ODC1 and PKM2. We found that ODC1 can regulate the c-myc-induced *PKM2*

transcription modulation via upregulating polyamines. Moreover, we found that *PKM2* and *ODC1* are co-expressed in terms of both mRNA and protein levels in HCC tissues. Simultaneous upregulation of *PKM2* and *ODC1* in HCC tissues predicted the poorest survival outcome. Taken together, these were the first results showing that *PKM2/ODC1* form a positive feedback loop that promotes the development of HCC, and we believe that simultaneous inhibition of ODC1 and PKM2 may prove advantageous as a cancer treatment approach.

Targeting *PKM2* or *ODC1* using gene silencing or pharmacological methods has led to prominent anti-cancer effects. For example, shikonin, a PKM2 inhibitor, showed a significant effect in bladder cancer via inducing necroptosis.<sup>32</sup> Another PKM2 inhibitor, compound 3k, also exhibited anti-proliferation effects in breast and colon cancer. DFMO, an ODC1-specific



**Figure 6** ODC1 inhibitor DFMO and PKM2 inhibitor compound 3k exert synergistic effects on HCC proliferation in vivo. **(A)** Proliferation rate of HCC tumor treated with control (DMSO), compound 3k, DFMO, and the combination was detected. **(B)** Size of HCC tumor in the four groups. **(C)** Weight of HCC tumor in the four groups. **(D)** The expression of PKM2, ODC1, PCNA, and Ki67 in HCC tissues was detected using immunohistochemical staining (200 $\times$ ). **(E)** Effects of the four treatments on the liver, kidney, and heart in mice were detected using HE staining (200 $\times$ ). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

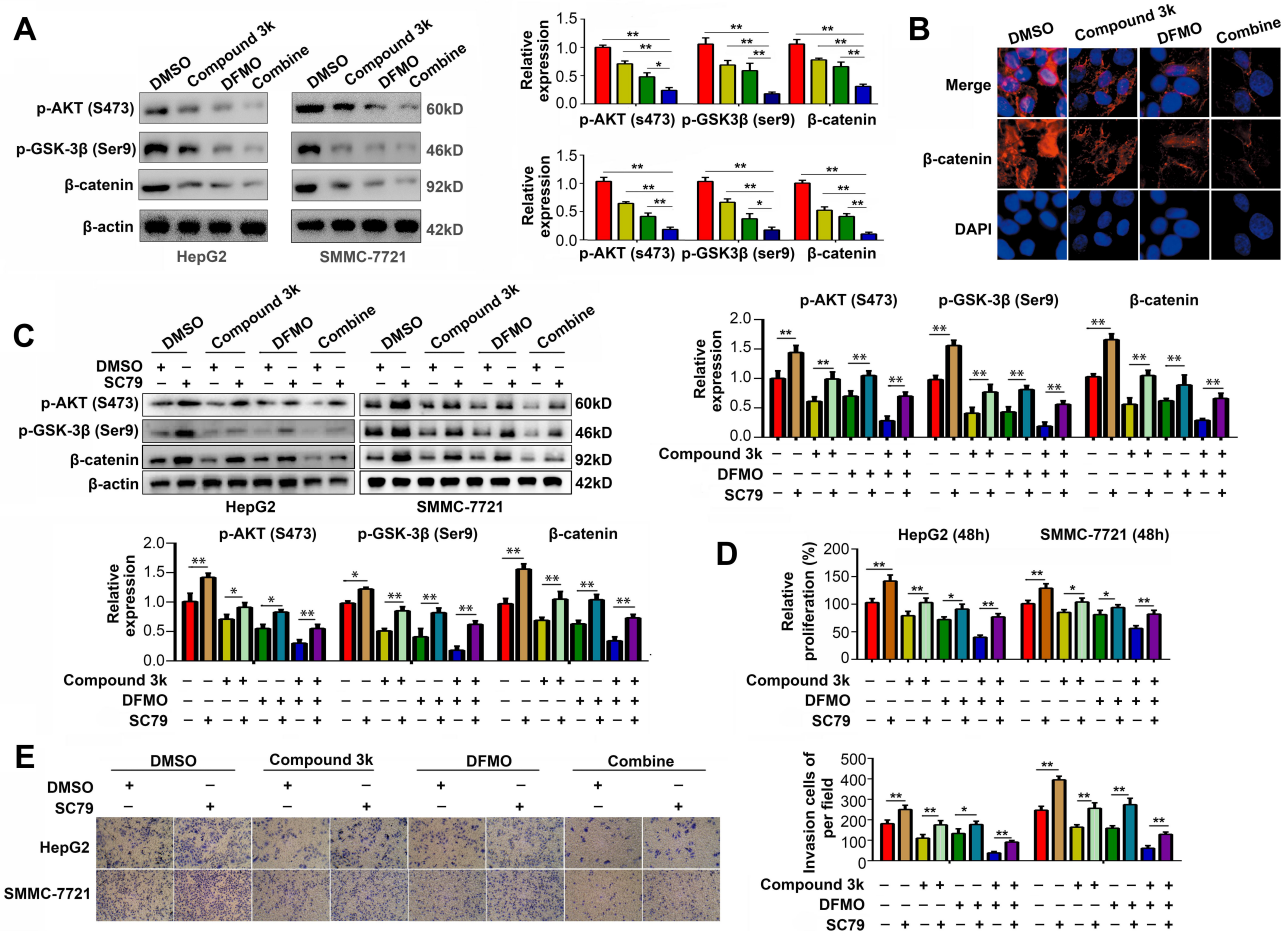
**Abbreviations:** HCC, hepatocellular carcinoma; PKM2, pyruvate kinase M2; ODC1, ornithine decarboxylase 1; PCNA, proliferating cell nuclear antigen; Ki67, marker of proliferation Ki-67; DFMO, difluoromethylornithine; DMSO, dimethyl sulfoxide; HE, hematoxylin and eosin.

inhibitor, has anti-cancer effects in various cancers, including cervical and colon cancer.<sup>9</sup> In the present study, the PKM2 inhibitor compound 3k and the ODC1 inhibitor DFMO were employed to determine the feasibility of simultaneous inhibition of PKM2 and ODC1 to treat HCC. We found that they exerted synergistic inhibitory effects on HCC cell proliferation, as well as inducing apoptosis. Similarly, we found that they exerted synergistic effects on HCC cell mobility and EMT, as well as the proliferation of HepG2 cells in vivo. Finally, we found that they exerted synergistic inhibitory effects on the AKT/GSK-3 $\beta$ / $\beta$ -catenin pathway, while the AKT activator SC79 reversed these inhibitory effects on the AKT/GSK-3 $\beta$ / $\beta$ -catenin pathway, cell proliferation, and invasion. This is the first

evidence revealing that it is feasible to combine compound 3k and DFMO to treat HCC, and that they exert synergistic inhibitory effects on HCC cell proliferation and invasion via the AKT/GSK-3 $\beta$ / $\beta$ -catenin pathway.

## Conclusion

Overall, PKM2/ODC1 form a positive feedback loop that promotes the development of HCC. The PKM2 inhibitor compound 3k and the ODC1 inhibitor DFMO showed synergistic effects against HCC cell proliferation, migration, invasion, and the EMT process by influencing the AKT/GSK-3 $\beta$ / $\beta$ -catenin pathway. Therefore, simultaneous inhibition of ODC1 and PKM2 using DFMO and compound 3k, respectively, maybe a novel and effective strategy for treating HCC.



**Figure 7** ODC1 inhibitor DFMO and PKM2 inhibitor compound 3k exert synergistic effects on the AKT/GSK-3β/β-catenin pathway. **(A)** Western blotting was used to detect the expression of p-AKT (Ser473), p-GSK-3β (Ser9), and β-catenin in HCC cells treated with control (DMSO), compound 3k (2.5 μM), DFMO (500 μM), and the combination. **(B)** Immunofluorescence was used to detect the location of β-catenin in the four groups of HepG2 cells. **(C)** Western blotting demonstrated that the AKT activator SC79 reversed the effects of the four treatments on the AKT/GSK-3β/β-catenin pathway. **(D)** CCK-8 assays demonstrated that the AKT activator SC79 reversed the effects of the four treatments on HCC cell proliferation. **(E)** Transwell assays demonstrated that the AKT activator SC79 reversed the effects of the four treatments on the HCC cell invasion. \* $P < 0.05$ ; \*\* $P < 0.01$ .

**Abbreviations:** HCC, hepatocellular carcinoma; PKM2, pyruvate kinase M2; ODC1, ornithine decarboxylase 1; AKT, AKT serine/threonine kinase; GSK-3β, glycogen synthase kinase 3 beta; CCK-8, cell counting kit-8; DMSO, dimethyl sulfoxide.

## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

## Disclosure

This work was supported by the High-level Scholarship Project for Returned Talents of Guizhou Province #(2018) 05 and Laboratory and Talent Project of Guizhou

Cooperational Science Foundation[2018]5779-76. The authors report no conflicts of interest in this work.

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