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Metformin attenuates hepatoma cell proliferation by decreasing glycolytic flux through the HIF-1α/PFKFB3/PFK1 pathway

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ABSTRACT

Aims: Enhanced aerobic glycolysis is an essential hallmark of malignant cancer. Blocking the glycolytic pathway has been suggested as a therapeutic strategy to impair the proliferation of tumor cells. Metformin, a widely used anti-diabetes drug, exhibits anti-tumor properties. However, the underlying molecular mechanism of its action linking glucose metabolism with the suppression of proliferation has not been fully clarified.

Main methods: Stable isotope tracing technology and gas chromatography–mass spectrometry method were utilized to analyze the effect of metformin on glycolytic flux in HCC cells. Western blot and immunohistochemistry were utilized to analyze the expression of phosphofructokinase-1 (PFK1) and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) in HCC cells or xenograft tumor tissues. Lactate measurement and glucose uptake assay were used to analyze the level of lactate and glucose in the presence of frucose-2,6-diphosphate (F2,6BP) in HCC cells treated with metformin.

Key findings: We found that metformin significantly impaired hepatoma cell proliferation by inhibiting the glycolytic flux via PFK1 blockade. Interestingly, activation of PFK1 by F2,6BP reverses the inhibitory effect of metformin on hepatoma cell proliferation and glycolysis. Mechanistically, PFKFB3 , a potent allosteric activator of PFK1, was markedly suppressed through inhibiting hypoxia-induced factor 1 (HIF-1α) accumulation mediated by metformin.

Significance: Taken together these data indicate that HIF-1α/PFKFB3/PFK1 regulatory axis is a vital determinant of glucose metabolic reprogramming in hepatocellular carcinoma, which gives new insights into the action of metformin in combatting liver cancer.

1. Introduction

Hepatocellular carcinoma (HCC) is a common malignancy with high mortality rates and few effective treatment options [1,2]. HCC is typically asymptomatic until the later stages when surgical resection is often no longer applicable [3]. Sorafenib has been approved by the FDA for HCC treatment, but it reportedly has limited survival benefits and very low response rates [4]. Consequently, more effective therapeutic strategies are urgently required.

Normally differentiated hepatocytes in the liver maintain glucose homeostasis by regulating the catabolic glycolysis and anabolic gluconeogenesis pathway [5]. However, in hepatoma cells, this process is disrupted because of changes in various metabolic enzymes [6]. Anaerobic glycolysis is important for cell survival [7–9]. However, malignant hepatocytes sustain an abnormally high rate of glycolysis, even in the presence of ample oxygen [10,11]. The glycolytic flux is regulated mainly through the key rate-limiting step controlled by phosphofructokinase-1 (PFK1), which catalyzes the transformation of fructose-6-phosphate (F6P) to fructose-1,6-bisphosphate (F1,6BP). The activity of PFK1 is controlled by the intracellular ratio of ATP to AMP and inhibited by an increased level of ATP [12]. Homodimeric bifunctional 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3

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(PFKFB3) phosphorylates F6P to fructose-2,6-bisphosphate (F2,6BP), which subsequently allosterically activates PFK1 and stimulates high glycolytic flux in human cancers [13]. Moreover, PFKFB3 is overexpressed in human cancers [14–17] and is regulated by several genes, including HIF-1α, Akt, and PTEN [17–20]. These proteins may contribute to the transformation or proliferation of cancer cells.

Metformin, a first-line therapy for type II diabetes, has shown potential in *in vitro* and *in vivo* studies as an anticancer drug or an adjuvant chemotherapy sensitizer [21–23]. Several clinical trials using metformin as a treatment for non-diabetic cancer patients are ongoing [24]. Despite the current interest in using metformin as a cancer therapeutic drug, it is not known whether the predominant mechanism of its antitumor effect is alteration of the host metabolism or direct action on tumor cells [25,26]. Metformin reportedly affects mitochondrial metabolism [27] by the liver kinase B1/adenosine mono-phosphate-activated protein kinase (AMPK) pathway [28,29] or AMPK-independent signaling [30,31]. Several recent studies have focused on the emerging action of metformin on glycolysis in tumor cells [32]. Metformin also reportedly inhibits proliferation and induces apoptosis of cancer cells as a result of decreased levels of glycolytic enzymes, including hexokinase II and pyruvate kinase M2 (PKM2) [33,34]. However, to the best of our knowledge, no study has directly analyzed the action of metformin on PFK1, a pacemaker of glycolysis, during the metabolic transformation of hepatoma cells. Therefore, we investigated the ability of metformin to modulate an important glycolytic rate-limiting enzyme and hypothesized that this is a new mechanism promoting anti-neoplastic effects.

2. Materials and methods

2.1. Cell culture and treatment

Human HCC cell lines $HepG_2$ and Huh7 were purchased from the Cell Bank of Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences. $HepG_2$ and Huh7 cells were cultured in Dulbecco's modified Eagle's medium and RPMI 1640 (Gibco, Grand Island, NY, USA), respectively, supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA). Isotopic labelling was performed in Dulbecco's modified Eagle's medium (without sodium pyruvate) with 10% fetal bovine serum supplemented with 25 mM D-[U–^{13}C] glucose. Cell lines were maintained in a humidified atmosphere with 95% air and 5% CO_2 at 37 °C. Metformin (Sigma, St. Louis, MO, USA), Compound C (Abcam, Cambridge, MA, USA), and CoCl2 (Abcam) were added to the medium at the indicated times and concentrations.

2.2. Reagents and antibodies

D-Glucose (U- [13]C, 99%) was purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA). Norvaline and fructose-2,6-biphosphate were acquired from Sigma Chemical. Lactate Assay Kits and Glucose Uptake Colorimetric Assay Kits were purchased from Biovision (Milpitas, CA, USA). The primary antibodies against AMPKα and phospho-AMPK (Thr172) were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-PFK1 antibody was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). PFKFB3, HIF-1α, and GAPDH antibodies were from Abcam.

2.3. MTS assays

The MTS assays were used to test cell viability. Cells were plated in 96-well plates at a density of 3000 cells/well overnight. Various reagents were then added to treat the cells at the indicated times. To explore cell proliferation, 20 μl/well MTS reagent was added to each well and incubated for 2–4 h. Absorbance was measured at a wavelength of 490 nm.

2.4. Real-time cell analysis

The RTCA S16 System (ACEA Biosciences, San Diego, CA, USA) was utilized to monitor the process of cell proliferation. Cells were plated in 16-well electronic plates at a density of 5000 cells/well. Plates were then placed in the special locating groove of the system in the normal cell incubator. The cell index was recorded every 15 min for 72 h to measure cell proliferation. The changes in cell index with time reflect the number of cells in the wells of the plates.

2.5. Metabolite extraction in cell culture

$HepG_2$ and Huh7 cells were respectively seeded at a density of 1×10^6 cells per well in a six-well plate overnight and incubated in full medium. The old medium was discarded and the cells were washed twice with 1 ml PBS before the addition of 2 ml indicated medium (in the absence or presence of [U–^{13}C] glucose) with or without the incubation of 2 mM metformin for 24 h. The medium was then quickly removed and the plates were placed on ice. Cells were washed three times with precooled PBS. The content of each well was added to 0.4 ml precooled (–80 °C) methanol that contained 1 μg/ml tridecanoic acid (Sigma-Aldrich, 91988-5G) for high performance liquid chromatography. Next, the cells were put in a freezer at –80 °C for 30 min and then placed on ice to raise the temperature. The cells in each well were submerged in 0.4 ml water containing 1 μg/ml norvaline (Sigma-Aldrich, 53721-100 mg) and gently blended. The cells were then scraped and collected with all the solvent and put into the specific centrifuge tube.

2.6. Gas chromatography–mass spectrometry

Instrumental analysis was implemented with an Agilent 7890A gas chromatography system and an Agilent 5975C inert MSD system (Agilent Technologies Inc., Palo Alto, CA, USA). An Optima 5 MS Accent fused-silica capillary column (30 m × 0.25 mm × 0.25 μm; Macherey-Nagel, Düren, Germany) was used to separate the derivatives. Helium (> 99.999%) was utilized as the carrier gas at a constant flow rate of 1 ml/min through the column. The injection volume was 1 μl in splitless mode, and the solvent delay time was 4.4 min. The initial oven temperature was maintained at 100 °C for 1 min, ramped to 280 °C at a rate of 12 °C/min, to 320 °C at a rate of 40 °C/min, and finally held for 3 min. The temperatures of the injector, the transfer line, and the electron impact ion source were set to 250 °C, 250 °C, and 230 °C, respectively. The electron energy was 70 eV, and data were collected in full scan mode (m/z 50–600).

2.7. Animal experiments

Athymic male nude mice (BALB/C, 4–6 weeks old) were purchased from the Laboratory Animal Service Center of Guangdong Province. According to the study from Morris et al. [35], $HepG_2$ is more suitable to be used for HCC cell metabolic research than Huh7 cells. Besides, in consideration of the results from Huh7 were consistent with those from $HepG_2$ in our *in vitro* experiments, thus $HepG_2$ cells were utilized for the construction of nude mice model of HCC. Briefly, $HepG_2$ cells were grown in culture medium before collection for subcutaneous injection. Cells were injected subcutaneously into the right flank of each mouse (5×10^6 in 100 μl per site). After tumor formation, mice from each group were randomly allocated to one of two groups (six mice/group) for treatment with a metformin diet (250 mg/kg/day) or a regular chow diet. The body weight of each mouse was measured every 5 days. All animal experiments were performed with protocols approved by the Animal Experimentation Ethics Committee of Guangzhou Medical University.

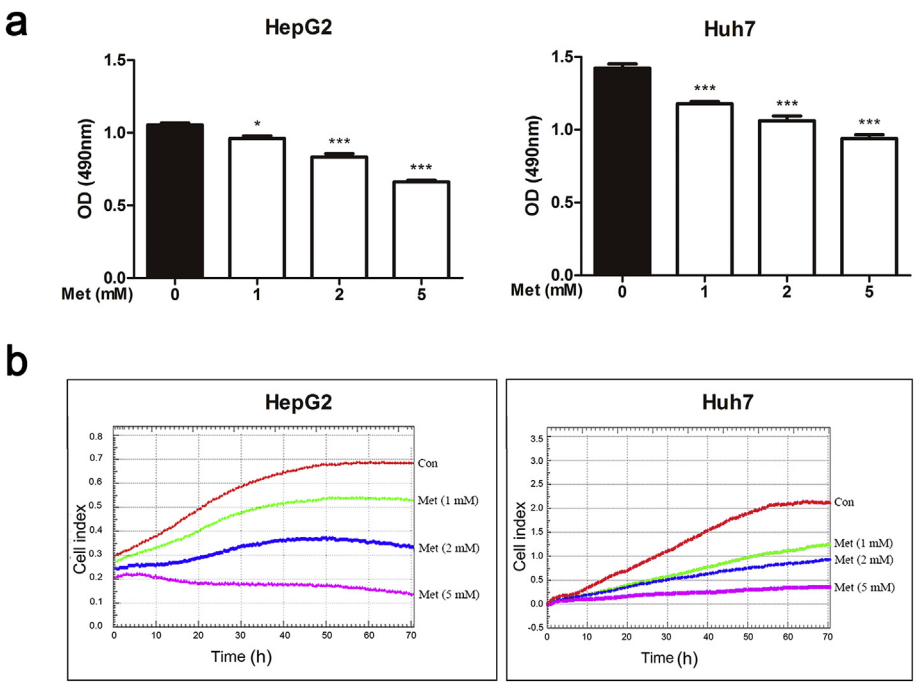

Fig. 1. Metformin suppresses hepatoma cell proliferation. (a) $HepG_2$ and Huh7 cells were treated with various concentrations of metformin (0, 1, 2 or 5 mM) for 24 h. Cell viability was determined by MTS assay. $^*P < 0.05$, $^{***}P < 0.001$ compared with the control group (0 mM). mM, mmol/L. Error bars obtained from SEM of n = 3 independent measurements. (b) Time-dependent cell response profiles of metformin (0, 1, 2 or 5 mM) treatment in liver cancer cells. The cell suspensions were transferred to E-plates and placed on the RTCA reader for real-time monitoring every 15 min for duration of the assay. The number of cells inside the well is displayed as cell index. min, minutes; h, hours; Con, control; Met, metformin.

2.8. Western blotting analysis

Cell lysates and tissue homogenates were extracted in RIPA buffer that contained protease and phosphatase inhibitors (Roche, Sandhofer Strasse 116, D-68305 Mannheim, Germany). The protein concentration was quantified using the bicinchoninic acid (BCA) assay. Total proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto PVDF membranes (Millipore Corporation, Billerica, MA, USA). The membranes were blocked in PBS containing 0.05% Tween-20 and 5% nonfat milk for 2 h at room temperature and probed with specific antibodies overnight at 4 °C. After being fully washed, membranes were incubated in their associated secondary antibodies. Visualization of proteins of interest was performed by enhanced chemiluminescence reagent (Pierce, Thermo Scientific, Waltham, MA, USA).

2.9. Immunohistochemistry

Paraffin-embedded, formalin-fixed tissues were immunostained for PFK1 protein using standard immunohistochemical procedures according to the manufacturers' instructions. The immunohistochemical score was independently assessed by two pathologists without knowledge of patient characteristics. Negative controls were prepared in the same way but without primary antibodies.

2.10. PFK1 activity assay

PFK1 activity was measured as previously described [10]. Briefly, PFK1 activity was assayed in a basic medium containing: 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$, 1 mM fructose-6-phosphate, 1 mM ATP, 0.5 mM NADH, 2 mU/ml aldolase, 2 mU/ml triosephosphate isomerase, 2 mU/ml α-glycerophosphate dehydrogenase, and 100 μg/ml of protein in a final volume of 200 μl. Reactions were started when the protein was added to the medium. NADH oxidation was detected by measuring the decrease in absorbance at 340 nm in a microplate reader.

2.11. Lactate measurement

Lactate levels in the culture medium of liver cancer cells were detected using a fluorometric assay according to the manufacturer's protocol.

2.12. Glucose uptake assay

Hepatoma cells ($HepG_2$ and Huh7) were seeded in six-well plates (2×10^5/well) and incubated at 37 °C for 24 h. Before the test, cells were deprived of glucose for 2–3 h by replacing the medium with glucose-free medium. Furthermore, cell medium was exchanged with fresh medium with or without fluorescent 2-NBDG (Thermo Scientific Technologies) and cells were incubated at 37 °C for an additional 45 min. Then, cells were washed three times with PBS and subsequently digested into single cells for the flow cytometry analysis.

2.13. Statistical analysis

Data are expressed as mean ± SEM of at least three independent experiments. Two-tailed Student's t-tests were used to calculate the P values. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Metformin inhibited hepatoma cell proliferation by decreasing the glycolytic flux

To assess the inhibitory effect of metformin on the proliferation of HCC cells *in vitro*, cell proliferation assays were carried out using $HepG_2$ and Huh7 hepatoma cell lines. As shown in Fig. 1a, 24 h after metformin treatment (0, 1, 2, or 5 mM) a dose-dependent inhibition of proliferation in $HepG_2$ and Huh7 cells was evident. Furthermore, to discern the direct effect of metformin on the proliferation of HCC cells, cell proliferation was further monitored for 72 h after the addition of metformin using a real-time cell analysis (RTCA) platform. HCC cells treated with metformin showed markedly lower levels of proliferation than those of the untreated control group (Fig. 1b). Metformin clearly suppressed the proliferation of HCC cells.

The relation between impaired hepatoma cell proliferation on incubation with metformin and the inhibition of glycolytic flux was then investigated. The metabolites produced by glycolysis and the tricarboxylic acid (TCA) cycle were measured using the stable isotope

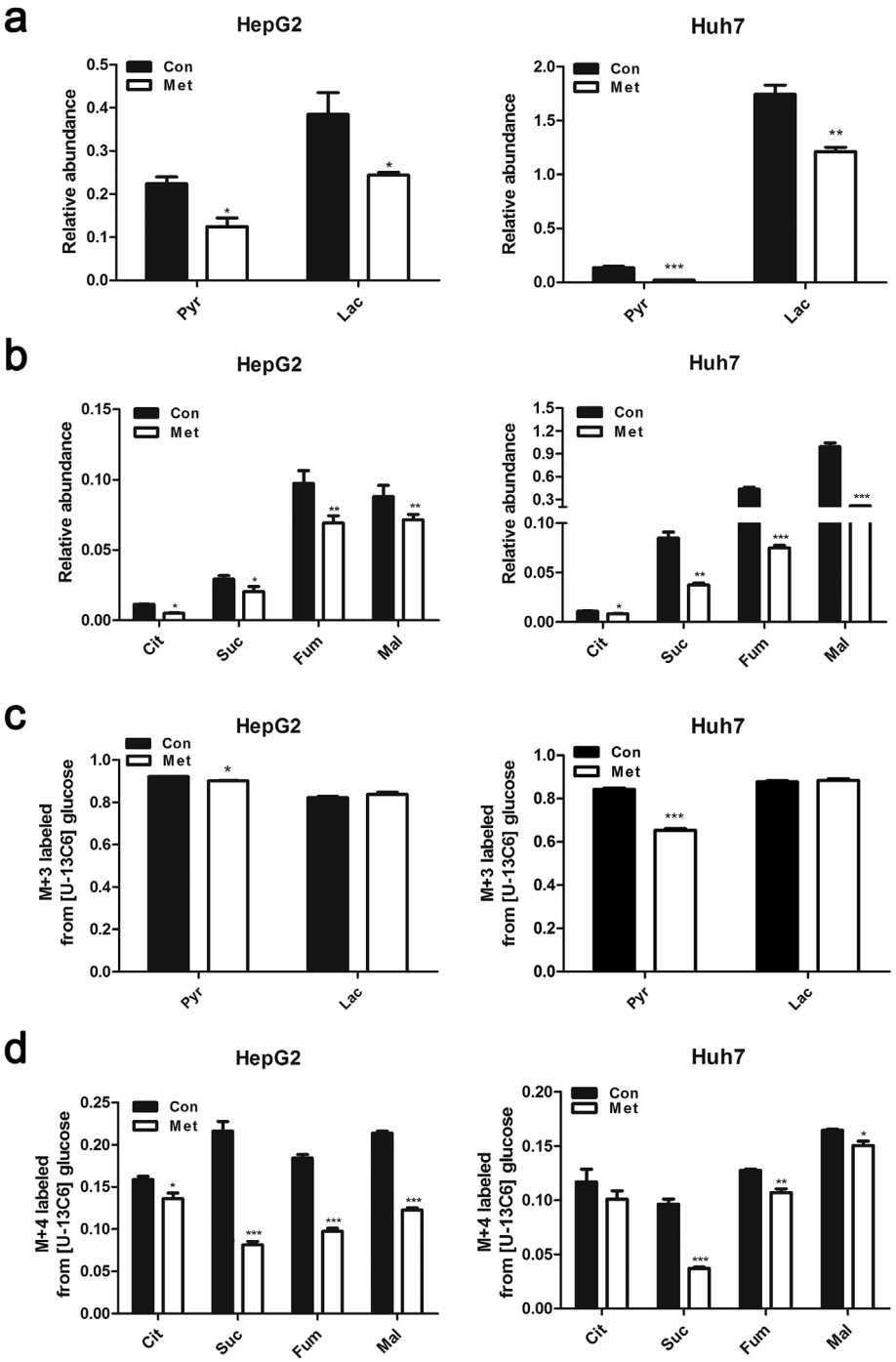

Fig. 2. Altered glycolytic flux is involved in metformin-induced inhibition of hepatoma cell proliferation. (a) Relative abundance of metabolites of glycolysis in the control groups and metformin treated groups (normalized by norvaline). Pyr, pyruvate; Lac, lactate; Con, control; Met, metformin. (b) Representative metabolites of the TCA cycle in the control groups and metformin treated groups (normalized by norvaline). Cit, citrate; Suc, succinate; Fum, fumarate; Mal, malate. (c, d) Analyses of metabolite isotope mass abundances M + 3 and M + 4 respectively. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with the control group.

tracing technique with [U–^{13}C] glucose as the tracer. Experiments were carried out for 24 h with HepG$_2$ and Huh7 cells in the presence of 2 mM metformin or without metformin. The levels of several critical glycolytic intermediates, such as pyruvic acid and lactate, were dramatically decreased with the treatment of 2 mM metformin (Fig. 2a). The levels of representative metabolites of the TCA cycle, including citrate, succinate, fumarate, and malate, were also significantly decreased with the administration of metformin (Fig. 2b). Moreover, isotopomer distribution analysis showed that part of the M3 enrichment of glycolysis intermediates (such as pyruvic acid) and M4 enrichment of TCA cycle intermediates (such as succinate, fumarate, and malate) apparently decreased in the presence of metformin (Fig. 2c and d). ^{13}C-labeling experiments revealed a significant decrease of glycolytic flux in HCC cells, suggesting a possible cell-intrinsic metabolic signature of response to metformin-mediated proliferation suppression.

3.2. Metformin suppressed the expression and activity of PFK1 in HCC

To examine the molecular mechanism underlying the inhibition of glycolytic flux in HCC cells, the effects of metformin on the expression

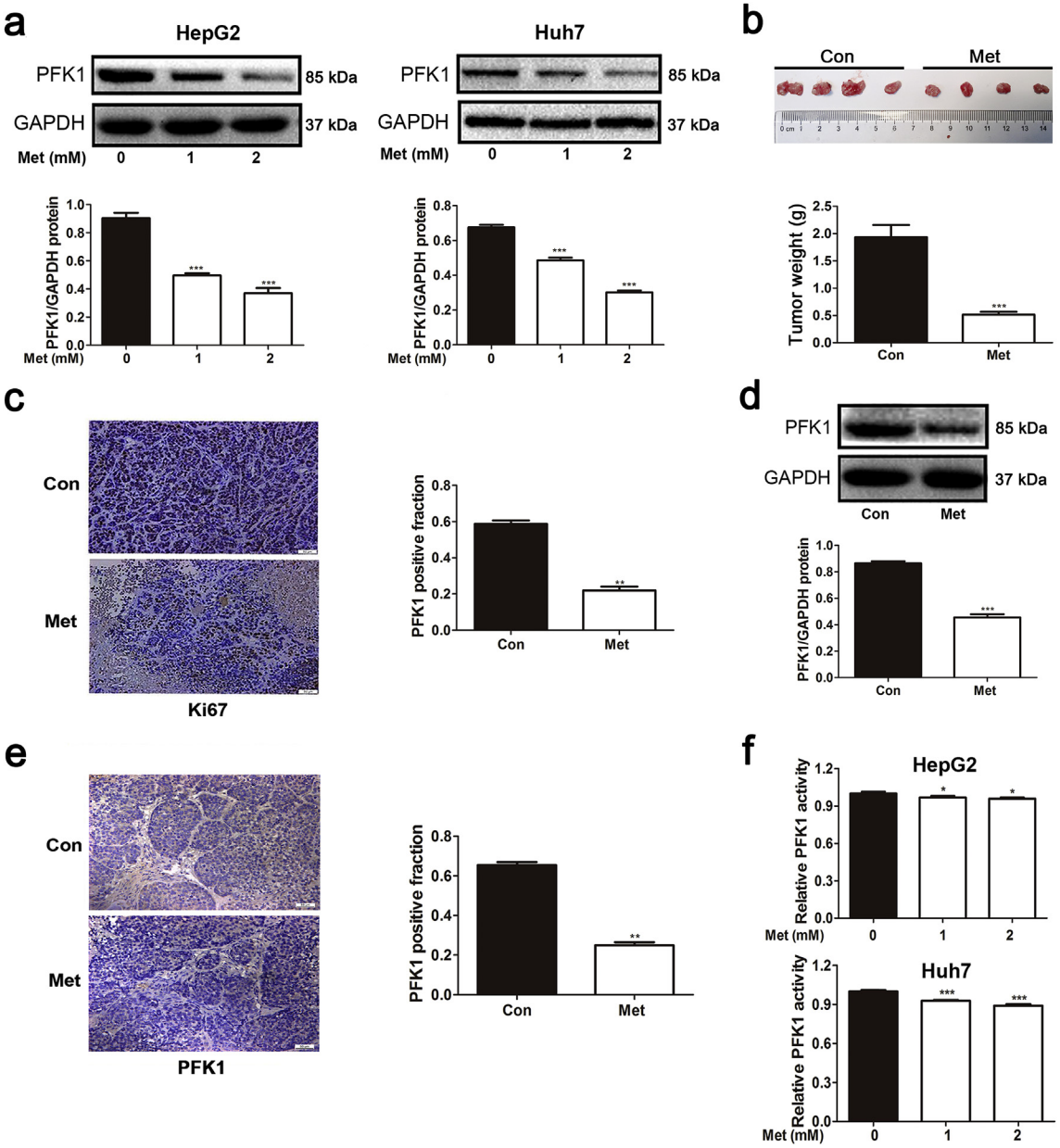

Fig. 3. Effect of metformin on PFK1 expression and activity. (a) Western blot analysis of PFK1 protein in $HepG_2$ and Huh7 cells treated with various concentrations of metformin (0, 1 and 2 mM) for 24 h mM, mmol/L; h, hours; Con, control; Met, metformin. (b) Representative image of tumors and quantification of tumor weight from mice treated with metformin (250 mg/kg/d) vs. the control group. (c) Immunohistochemistry indicating the level of Ki67 protein in tumor sections from mice liver tumor tissues in the control group and metformin-treated group. Scale bars: 50 μm. (d) The expression of PFK1 protein in tumor tissues as assessed by Western blot analysis. (e) Immunohistochemistry of the PFK1 protein expression in tumor sections from mice liver tumor tissues in the control group and metformin treated group. (f) PFK1 activities measured in $HepG_2$ and Huh7 cell lines treated with metformin for 24 h $^*P < 0.05$, $^{***}P < 0.001$ compared with the control group. Error bars obtained from SEM of n = 3 independent measurements.

and activity of PFK1 in HCC cells were assessed. The expression levels of PFK1 protein were significantly decreased in $HepG_2$ and Huh7 cells treated with metformin in a concentration-dependent manner (Fig. 3a). To verify these results in *in vivo* experiments, we generated a mouse xenograft model of HCC by the subcutaneous inoculation of $HepG_2$ cells in nude mice. After tumor formation, mice were treated with a metformin diet (250 mg/kg/day) or a regular chow diet. The *in vivo* tumor growth was significantly impaired in the group of metformin diet compared with the group of regular chow diet (Fig. 3b). Moreover, metformin treatment resulted in inhibition of cell proliferation and reduction of PFK1 protein expression in the mouse liver tumor tissues (Fig. 3c and d). In line with the western blotting results, immunohistochemical assays revealed that the number of PFK1-positive cells was reduced in the tumors of animals that underwent metformin treatment (Fig. 3e).

The activity of PFK is reportedly not always associated with its protein levels [10]. Consequently, we examined the effect of metformin on the activity of PFK1. PFK1 activity was notably inhibited in HCC cells by metformin in a dose-dependent manner (Fig. 3f). Therefore, metformin appears to inhibit glycolytic flux by suppressing the expression and activity of PFK1.

3.3. Metformin suppressed the expression of PFKFB3 independently of AMPK

PFKFB3 encodes the 6-phosphofructo-2-kinase/fructose-2,6-

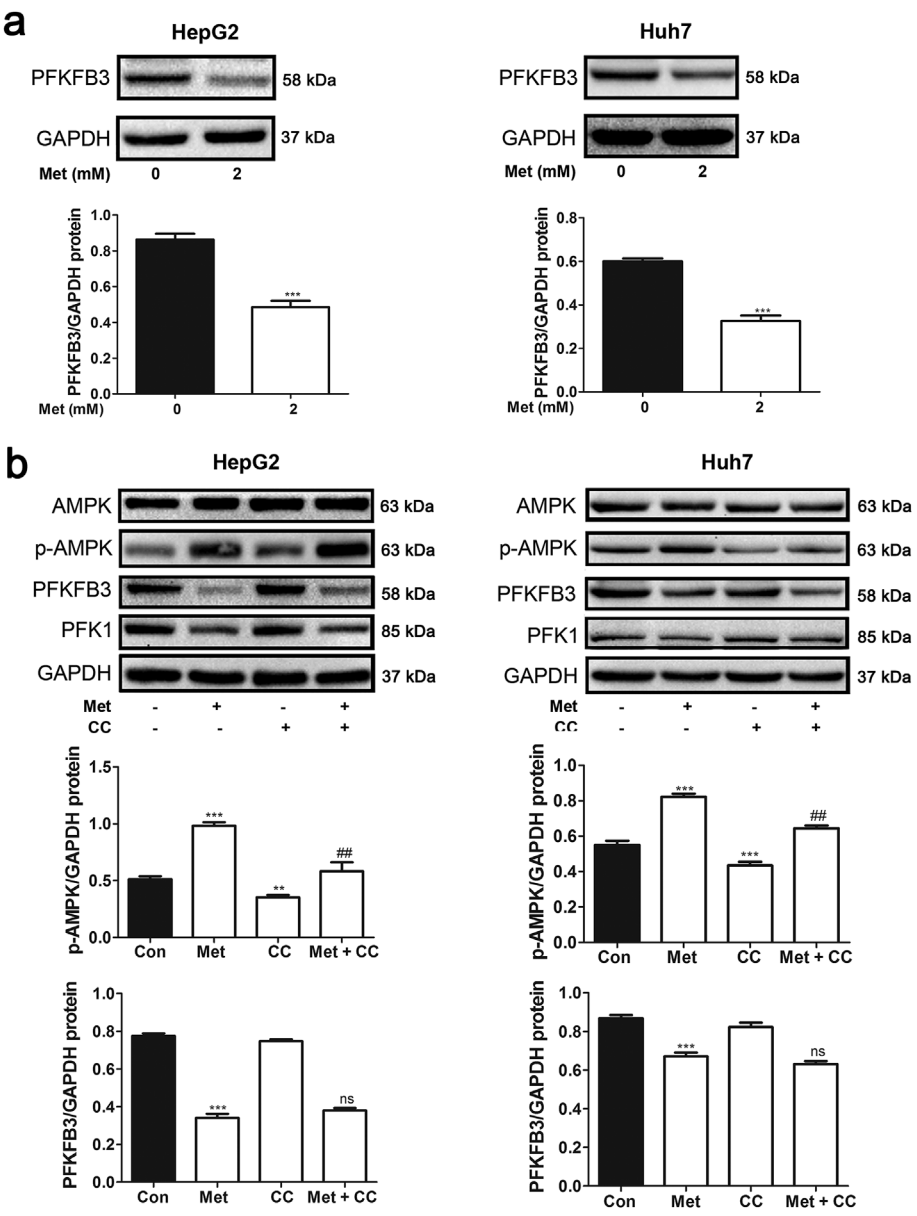

Fig. 4. Metformin suppresses PFKFB3 protein levels independent of AMPK. (a) PFKFB3 protein levels assessed by Western blot in hepatoma cells treated with metformin (2 mM). mM, mmol/L; Con, control; Met, metformin. (b) Western blot analyses of AMPK, phosphorylated AMPK (p-AMPK), PFKFB3 and PFK1. $HepG_2$ and Huh7 cells were treated with AMPK inhibitor Compound C (CC), and then incubated with 2 mM metformin for 24 h under normoxia conditions before immunoblotting with specific antibodies as indicated. h, hours. $^{***}P < 0.001$ compared with the control group. ns, $P > 0.05$ compared with the metformin-treated group. Results from three independent experiments are shown.

biphosphatase 3 enzyme in humans and this enzyme catalyzes the synthesis of F-2,6-BP, which is a potent allosteric activator of PFK1. The expression levels of PFKFB3 protein in metformin-treated HCC cells were then investigated to better understand the underlying molecular mechanism by which metformin inhibits glycolysis. Consistent with the changes in PFK1 expression, Western blot assays demonstrated that PFKFB3 protein levels were significantly reduced by the addition of metformin in $HepG_2$ and Huh7 cells (Fig. 4a). Numerous studies have shown that metformin exerts antitumor effects in an AMPK-dependent manner [9]. To investigate this further, we repeated the experiments with AMPK activity chemically inhibited (Fig. 4b). The inhibition of AMPK with compound C (CC) had no effect on the levels of PFKFB3 protein and failed to modify the inhibitory effects of metformin on PFKFB3 expression in both $HepG_2$ and Huh7 cells (Fig. 4b). Therefore, metformin could suppress the expression of PFKFB3 independently of AMPK.

3.4. Activation of PFK1 by F2,6BP reverses the effect of metformin on hepatoma cell glycolysis

The concentration of F2,6BP in cells is controlled by PFKFB3 which regulates the synthesis and breakdown of F,26BP. F2,6BP strongly activates the glycolysis through allosteric modulation of PFK1. Consequently, we investigated whether activation of PFK1 by F2,6BP would retard metformin-stimulated suppression of cell proliferation. F2,6BP effectively reversed the inhibitory effect of metformin on PFK1 activity (Fig. 5a). Furthermore, cell proliferation (Fig. 5b), lactate production (Fig. 5c), and glucose uptake (Fig. 5d) were significantly enhanced in the presence of F2,6BP in HCC cells treated with metformin. It appears likely that metformin-mediated suppression of PFKFB3 inhibits the glycolytic flux through the inhibition of PFK1.

3.5. Metformin attenuated glycolytic flux through the HIF-1α/PFKFB3/PFK1 pathway

Previous studies have demonstrated that metformin suppressed hypoxia-induced factor 1 (HIF-1α) accumulation and activation independent of AMPK [36]. To further investigate whether metformin inhibited the expressions of PFKFB3 and PFK1 via its effect on HIF-1α, an anaerobic environment was constructed using a hypoxia-mimicking reagent (CoCl2) to explore the effect of metformin on the expression of PFKFB3 and PFK1. Intriguingly, CoCl2 elevated the accumulation of

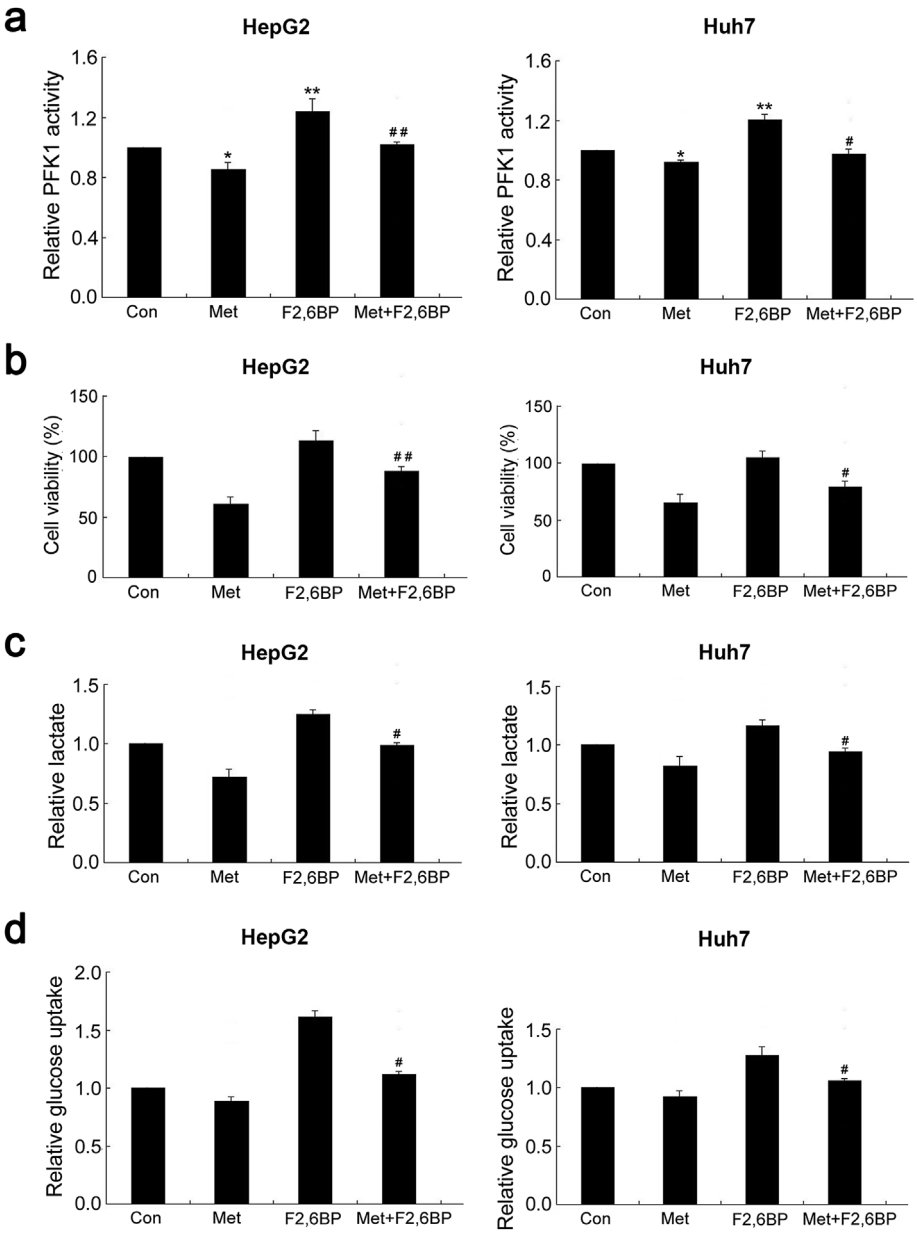

Fig. 5. F2,6BP reverses the effect of metformin on HCC cell proliferation and glycolysis. $HepG_2$ and Huh7 cells treated with metformin with or without F2,6BP (5 μm) for 24 h and followed by assays to measure PFK1 activity (a), cell proliferation (b), lactate production (c), and glucose uptake (d). μm, μmol/L; h, hours. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. untreated control cells; $\#P < 0.05$; $\#\#P < 0.01$ vs. metformin-treated cells. Results from three independent experiments are shown.

HIF-1α and promoted the expression levels of PFKFB3 and PFK1 protein in hepatoma cells HepG2 and Huh7, whereas metformin attenuated the inductive effect of CoCl2 on PFKFB3 and PFK1 (Fig. 6a and b), indicating the inhibitory effect on PFKFB3 and PFK1 may depend on inhibition of HIF-1α signalling. In addition, immunohistochemistry analysis revealed dramtically decreased positive staining of HIF-1α, PFKFB3 and PFK1 in harvested tumors from metformin-treated animals compared with control animals (Fig. 6c). These results demonstrated that metformin could suppress the HIF-1α/PFKFB3/PFK1 pathway in hepatoma cells, and this could be the critical mechanism behind the inhibitory effect of metformin on glycolytic flux in hepatoma cells.

4. Discussion

The uncontrolled proliferation of cells is still the leading cause of cancer and its mortality worldwide. In the context of high proliferation rates, most cancer cells modify cellular glucose metabolism to fulfill their anabolic demands. Metformin, the first-line treatment for type II diabetes, now draws much attention because of its anti-tumor activity. This activity has been confirmed in *in vitro* and *in vivo* studies and in a large number of clinical trials. However, the molecular mechanism of metformin's anti-tumor effect is poorly understood. Metformin seems to retard tumor growth via both AMPK-dependent and AMPK-independent mechanisms [23]. The AMPK-independent mechanism is associated with decreased levels of blood glucose and insulin. Advances in cancer glucose metabolism research have boosted the clinical interest in targeting aberrant metabolic pathways for treating malignant tumors. However, it is still unclear that the extent to which metabolic fluxes based on glucose are used for alternative processes in the presence of metformin. In the present study, we investigated the effect of metformin on glucose metabolites in hepatoma cells cultured *in vitro*. Experiments were done using stable tracing with isotopes (particularly those containing ^{13}C-labling) combined with gas chromatography–mass spectrometry (GC-MS). We found that metformin inhibited the glycolytic

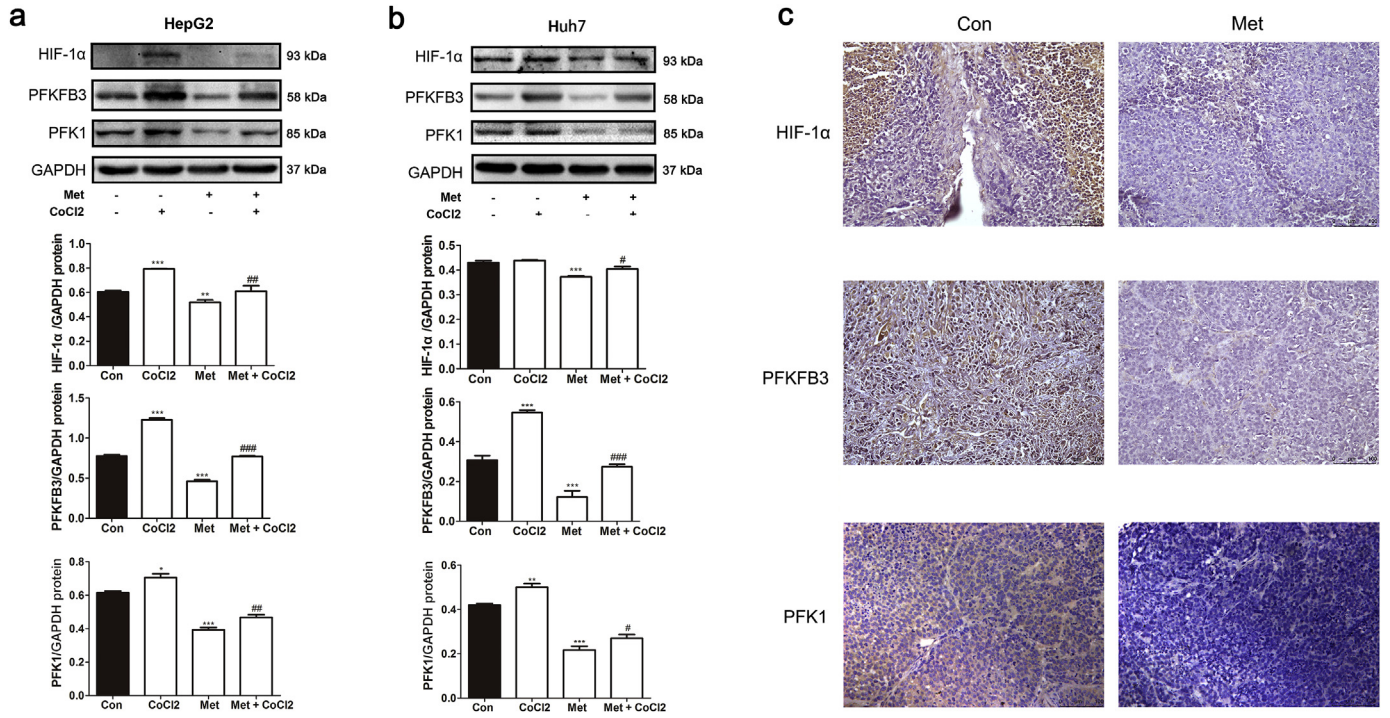

Fig. 6. Metformin inhibited the expression of PFK1 via HIF1-α/PFKFB3 pathway. (a, b) Expression of HIF-1α, PFKFB3 and PFK1 was assayed by western blotting. CoCl2 was used to induce the hypoxia conditions. HepG$_2$ and Huh7 cells were cultured with 100 μmol/L CoCl2 for 3 h before incubation with or without 2 mM metformin for 24 h c Representative immunohistochemistry of HCC xenograft tumor tissues showing the expressions of HIF-1α, PFKFB3 and PFK1. Scale bars: 100 μm mM, mmol/L; h, hours. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ vs. untreated control cells; $\#P < 0.05$; $\#\#P < 0.01$ vs. metformin-treated cells; Results from three independent experiments are shown.

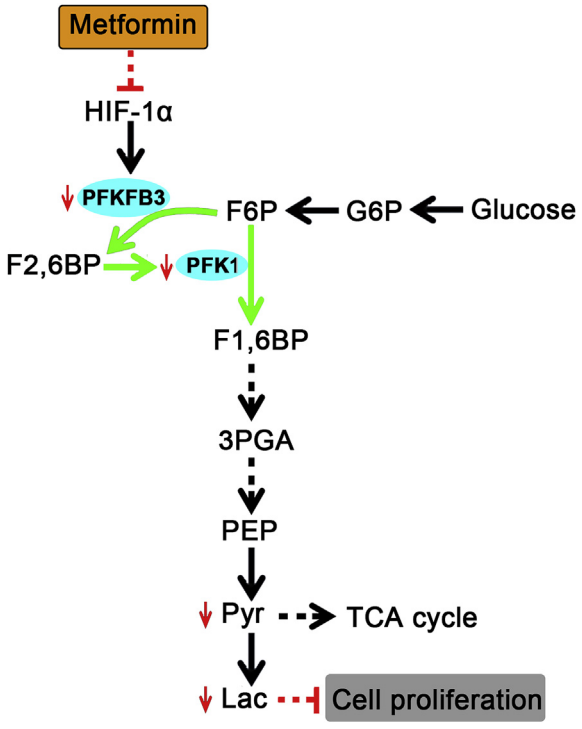

Fig. 7. Proposed model for the role of the HIF-1α/PFKFB3/PFK1 signaling pathway in regulating hepatoma cell glucose metabolism in the presence of metformin. Inactivation of HIF-1α mediated by metformin results in decreased expression of PFKFB3, which further suppress PFK1 activity. Down-regulation of PFK1 facilitates the inhibition of glycolytic flux and cell proliferation in HCC cells. G6P, glucse-6-phosphate; F6P, fructose-6-phosphate; F2,6BP, frucose-2,6-diphosphate; F1,6BP, frucose-1,6-diphosphate; 3PGA, 3-phosphoglyceric acid; PEP, phosphoenolpyruvic acid.

flux, as shown in Fig. 2. Importantly, the levels of intermediates in TCA cycle were significantly decreased, indicating that metformin treatment affects mitochondrial metabolism, which is consistent with the findings of Liu et al. [27]. However, we did not find a significant change in the flux of glucose to lactate (M + 3); indeed, the relative levels of lactate decreased dramatically (Fig. 2a). Notably, previous studies have shown that isotopic steady state can be reached within several hours after the addition of the isotopic tracer [37]. Consequently, the levels of the glycolytic metabolites may have reached isotopic steady state sooner than 24 h [38,39]. This fact may explain why there were no differences in M3 enrichment of lactate between the metformin-treated group and the control group.

Strikingly, our results demonstrated that metformin decreased the relative levels of citrate, succinate, fumarate and malate. Besides, metformin also significantly reduced the abundance of glucose to TCA cycle intermediates such as citrate (M + 4), succinate (M + 4), fumarate (M + 4), and malate (M + 4), which originated from the U–13C labeled glucose.

It was recently observed that hypoxia promoted HCC cell glycolysis [40,41], and that complexes of YAP/HIF-1α could accelerate glycolysis in hypoxia through binding to the PKM2 gene promoter and directly activating its transcription [40]. However, previous study indicated that metformin could improve tumor hypoxia conditions by suppressing the stability of HIF-1α [36] and reducing the oxygen consumption rate [42]. Based on the present findings, we speculated that metformin reduces the expression of PFKFB3 through the HIF-1α/PFKFB3 axis. During normal glucose metabolism, PFKFB3 phosphorylates F6P to F2,6BP. Therefore, the decreased expression of PFKFB3 protein would lead to the suppression of the conversion of F6P to F2,6BP. Because F2,6BP is a strong allosteric activator of PFK1, low concentrations of F2,6BP would result in the inhibition of PFK1 activity. PFK1, the gatekeeper of glycolysis, catalyzes the transformation of F6P to F1,6BP. Consequently, the suppression of PFK1 activity may lead to inhibition of this transformation.

In addition to the above effects, the levels of several intermediates, including sedoheptulose 7-phosphate in the pentose phosphate pathway (PPP), declined with metformin treatment (data not shown). PPP, which is a vital branch of the glycolysis pathway, may also be involved in the mechanism by which metformin affects HCC. However, further research on this point is still needed.

5. Conclusion

The current study provided new evidence for system-wide changes in hepatoma cell glucose metabolism in response to metformin (Fig. 7). Notably, targeting the HIF-1α/PFKFB3/PFK1 pathway, which is pivotal to glycolytic flux control, might provide effective therapeutic targets for suppressing HCC.

Declaration of competing interest

The authors declare that no competing interests exist.

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