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Original Article

Inhibition of mTORC1 signaling sensitizes hepatocellular carcinoma cells to glycolytic stress

Xin Zhao*, Peng Jiang*, Xiang Deng, Zhonghu Li, Feng Tian, Fei Guo, Xiaowu Li, Shuguang Wang

*Department of Hepatobiliary Surgery Institute, Southwest Hospital, Third Military Medical University, Chongqing 400038, PR China. *Equal contributors.*

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Abstract: Reprogrammed glucose metabolism, especially glycolysis, is profoundly implicated in tumor development or metastasis. As the interconnectedness and flexibility of metabolic signaling, targeting a metabolic signaling molecule may have limited anti-tumor effects. Here, Gene set enrichment analysis (GSEA) was used to explore the accompanied effectors of glycolysis in hepatocellular carcinoma (HCC). Based on the expression of lactate dehydrogenase A (LDHA), a key enzyme in catalyzing pyruvate into lactate, the glycolytic ability of HCC was defined as low group and high group. GSEA of two independent GEO datasets showed that mTORC1 signaling was the most striking metabolic alternations in high group. Pharmacological inhibition of mTORC1 signaling with rapamycin decreased LDHA level and glycolytic capacity of six HCC cell lines. Furthermore, c-Myc was identified as a downstream target of mTORC1 signaling and mediated mTORC1-induced LDHA expression. Importantly, rapamycin sensitized HCC cells to the glycolysis inhibitor 2-deoxyglucose (2-DG) *in vitro* and *in vivo*. Meanwhile, genetic silencing several other downstream targets of mTORC1 signaling (TFEB, SREBP-1 and SKAR) failed to enhance or faintly influenced the cytotoxic effects of 2-DG. These results demonstrate that combining rapamycin with 2-DG holds significant promise as prospective clinical treatment in HCC.

Keywords: mTOR signaling, glycolysis, lactate dehydrogenase a, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is one of the most common human malignancies, especially in developing countries. It is the second leading cause of cancer mortality and the 5th most frequent cancer worldwide [1]. Numerous approaches have been developed for the treatment of HCC, such as surgical resection, chemotherapy, radiotherapy and orthotopic liver transplantation [2]. However, the prognosis of the current therapies is still unsatisfactory. High incidence of intrahepatic and distant metastasis, tumor recurrence, and chemoresistance contribute to the poor outcome of HCC patients [3]. Therefore, it is imperative to explore the oncogenic cellular signalings of which are implicated in the malignant phenotype of HCC [4].

Abnormal glucose metabolism is emerged as a hallmark of cancer cells [5]. Cancer cells utilize glucose through glycolysis instead of more energetically efficient mitochondrial respiration even in the presence sufficient oxygen, which is known as Warburg effect. Despite with lower efficiency in generating ATP, Warburg effect enhances biosynthesis and cell proliferation and, therefore, facilitates the initiation and progression of cancers [6]. Emerging studies have implicated that targeting glycolysis is a promising treatment in cancers [7-9]. 2-deoxy-D-glucose (2-DG), an analog of glucose, is particularly well-suited to exploit because it can simultaneously induce multiple forms of metabolic stress through its action as a glycolytic inhibitor [10, 11]. However, extensive crosstalks between the deregulated metabolic network and oncogenic cellular signaling are being revealed. Therefore, targeting a branch flux of cancer metabolism has limited anti-cancer effects. And developing new combined therapeutic strategies based on cancer metabolism may provide inspired promise.

Lactate dehydrogenase A (LDHA), catalyzes the conversion of L-lactate and NAD to pyruvate

and NADH, is an indicator of glycolysis. In this study, we aimed to uncover the prominent oncogenic signaling involved in the glycolysis of HCC cells. By using gene set enrichment analysis (GSEA) based on the expression profile of LDHA, we revealed that mTORC1 signaling was critically implicated in glycolysis through regulating LDHA in a c-Myc-dependent manner. And excitingly, pharmacological inhibition of mTORC1 signaling increased the cytotoxic effect of 2-DG.

Materials and methods

Cell culture and reagents

All HCC cell lines used in this study were purchased from Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Cells were maintained in DMEM medium with 10% (v/v) fetal bovine serum and 25 U/mL penicillin and 25 μg/mL streptomycin (Invitrogen, NY, USA) in a humidified incubator at 37°C with 5% CO_2 atmosphere. 2-DG was purchased from Sigma (Shanghai, China). Rapamycin was obtained from Cell Signaling Technology (#9904, MA, USA). All reagents were diluted with preferable solution before use.

Gene set enrichment analysis

GSEA was performed using the GSEA program provided by the Broad Institute (http://www.broadinstitute.org/gsea/index.jsp). GSEA was used for comparing the expression profiles in gene sets named h.all.v5.1.symbols.gmt [Hallmarks] from Molecular Signature Database) between LDHA low group and LDHA high group, and assesses the enrichment score.

Western blot analysis

Cells were lysed with RIPA buffer containing protease inhibitors cocktail and phosphatase inhibitor cocktail on ice for 30 min. The lysates were clarified by centrifugation at 13,000 g for 15 min at 4°C. Protein samples were separated using SDS/PAGE, transferred on to PVDF membranes, blocked and probed with the primary antibodies against p-S6K (#9204, Cell Signaling Technology), S6K (#9202, Cell Signaling Technology), c-Myc (ab32072, Abcam), LDHA (19987-1-AP, Proteintech), TFEB (ab122910, Abcam), SREBP1 (ab28481, Abcam), SKAR (17-466-1-AP, Proteintech), and a secondary antibody. Equal protein sample loading was monitored using an anti-β-actin antibody (ab6276, Abcam). Blots were visualized using the ECL method.

Transient transfection of HCC cells

Specific small interfering RNA (siRNA) targeting c-Myc, LDHA, TFEB, SREBP1 and SKAR as well as a negative control were chemically synthesized by GenePharma Inc (Shanghai, China). Transient transfection of siRNA was performed with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To inhibit c-Myc and LDHA expression in HCC cells, they were transfected with 50 nmol/L siRNA oligonucleotides or control siRNA oligonucleotides. After transfection for 48 h, the knockdown efficiency was measured by western blotting.

Clinical tissue samples and immunohistochemistry

A total of twenty-four HCC tissues were collected from Department of Hepatobiliary Surgery Institute, Southwest Hospital, Third Military Medical University, China. None of the HCC patients had received radiotherapy, chemotherapy, hormone therapy or other related antitumor therapies before surgery. And all patients recruited were well informed and the process was approved by Ethics Committee of Southwest Hospital, Third Military Medical University, China. Immunohistochemical analysis was performed routinely and the primary antibody for Ki67 (ab16667) was purchased from Abcam.

Quantitative real-time PCR

Total RNA from HCC tissues or cells were extracted by the RNAiso Plus kit (Takara Bio Inc.). Next, 2 μg of total RNA was reverse transcribed primeScript RT Master kit (Takara Bio Inc.) to synthesize cDNA samples. Quantitative real-time RT-PCR was performed with SYBR Green quantitative PCR kit (Takara Bio Inc.) using the ViiA7 System (AB Applied Biosystems). The primers used in this study were as follows: LDHA-F, 5'-TTGACCTACGTGGCTTGGAAG-3', LDHA-R: 5'-GGTAACGGAATCGGGCTGAAT-3'; GAPDH-F, 5'-ACAACTTTGGTATCGTGGAAGG-3', GAPDH-R: 5'-GCCATCACGCCACAGTTTC-3'; TFEB-F,

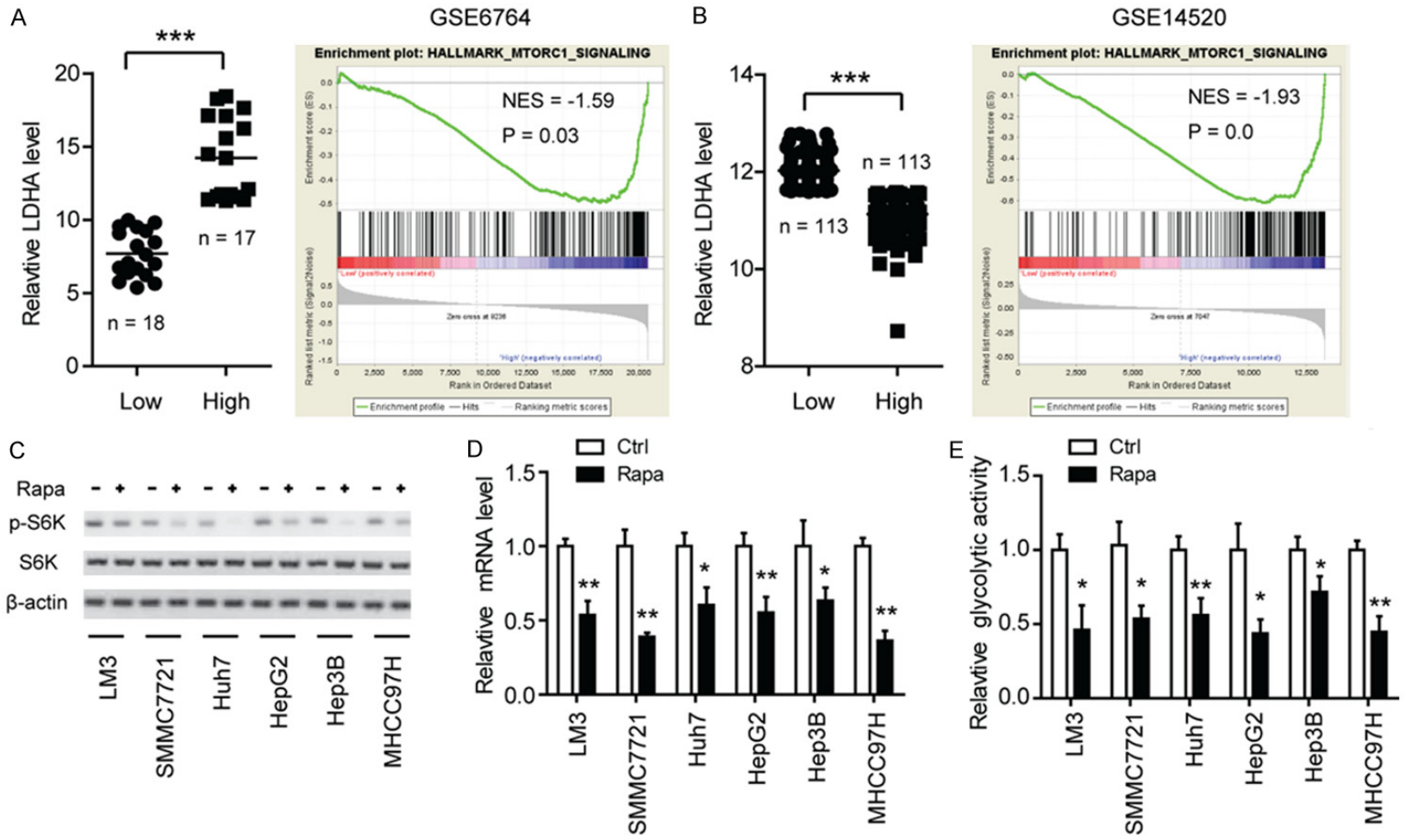

Figure 1. mTORC1 signaling is a key modulator of glycolysis in HCC. GSEA plot of mTORC1 signaling based on the gene expression profiles of low LDHA group versus high LDHA group in GSE6764 (A) and GSE14520 (B). NES, normalized enrichment score. (C) Six HCC cells were treated with 100 nM rapamycin or not for 12 h, then lysed and immunoblotted with the indicated antibodies. (D) The mRNA level of LDHA was detected by real-time qPCR in HCC cell lines upon rapamycin treatment. (E) The glycolytic capacity of HCC cells upon rapamycin treatment was measured by Seahorse XF Analyzers. Data shown represents the mean ± SD. *, $P < 0.05$; **, $P < 0.01$.

5'-ACCTGTCCGAGACCTATGGG-3', TFEB-R: 5'-CGTCCAGACGCATAATGTTGTC-3'; SREBP1-F, 5'-ACAGTGACTTCCCTGGCCTAT-3', SREBP1-R: 5'-GCATGGACGGGTACATCTTCAA-3'; SKAR-F, 5'-GCGAAAGGACGGCTTAATGC-3', SKAR-R: 5'-GCGTGTTGACTGGCTGAGAA-3'.

HCC xenograft model

HepG2 cells (2×10^6 cells) in 100 μL DMEM medium were inoculated subcutaneously into the right flank of 6-week old male nude mice. When bore visible tumors (near 200 mm^3), mice were randomly divided into four groups: control, 2-DG, 2-DG + rapamycin and rapamycin group, n = 5 per group. 2-DG (75 g/L) and rapamycin was treated intraperitoneally at 0.2 ml and 5 mg/kg triweekly. Tumor growth was measured every 5 days and the tumor volume was calculated as follows: tumor volume = (length × $width^2$)/2. Mice were sacrificed at the endpoint and xenografts were harvested. The xenograft experiment was performed according to the Guide for the Care and Use of Laboratory Animals and was approved by Ethics Committee of Southwest Hospital, Third Military Medical University, China.

Measurement of glycolytic capacity

Glycolytic ability was measured using a Seahorse XF96 analyzer (Seahorse Biosciences). Briefly, cells were seeded at 20,000 cells/well in a 94-well cell culture XF microplate (Seahorse Biosciences). After treatment with 100 nM rapamycin for 12 h, HCC cells were washed and incubated with assay medium for 1 h at 37°C in a CO_2-free incubator. Plates were then transferred to the XF24 analyzer. All measurements were recorded at set time intervals and normalized to total protein content. ECAR after oligomycin treatment indicates glycolytic capacity.

Cytotoxicity assay

A total of 3×10^3 indicated HCC cells were seeded onto 96-well plates and cultured for 16-20 h. After drug exposure for 48 h, cell viability and cell apoptosis was performed using Cell Counting Kit-8 (CCK8, Dojindo, Japan) and Caspase-3/7 activity kit (Biovision), respectively. For CCK-8 assay, 10% CCK8 solution was added into the culture medium and incubated for 1 h at 37°C. The absorbance at 490 nm was measured by the multifunctional microplate reader. And Caspase-3/7 activity was measured according to the manufacturer's instructions.

Statistical analysis

Data were shown as the means ± SD. The statistical analysis was performed with GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). The two-sided Student's t test was used to compare data between two groups. $P < 0.05$ was considered statistically significant.

Results

mTORC1 signaling is a key modulator of glycolysis in HCC

To demonstrate the oncogenic cellular signaling that implicated in glycolytic phenotype of HCC, Gene set enrichment analysis (GSEA) was performed in two independent HCC GEO datasets (GSE6764 and GSE14520). The glycolytic ability of HCC was defined as low group and high group based on the expression of LDHA. The difference of LDHA mRNA level between low group and high group was significant in both two datasets (**Figure 1A** and **1B**). In GSE6764 dataset (18 low versus 17 high), the most striking accompanied metabolic alternations in high group was mTORC1 signaling (**Figure 1A**). To further confirm this observation, a large scale analysis in GSE14520 (113 low versus 113 high) was done. As expected, mTORC1 signaling was accompanied with high glycolytic capacity in HCC (**Figure 1B**). Next, we tested whether mTORC1 signaling contributes to the glycolytic phenotype of HCC. Treatment HCC cells with 100 nM rapamycin blocked mTORC1 signaling as demonstrated by reduced phosphorylation level of its downstream target, S6K (**Figure 1C**). Surprisingly, rapamycin also decreased the mRNA level of LDHA (**Figure 1D**) and glycolytic capacity (**Figure 1E**) of HCC cells, suggesting that activated mTORC1 signaling is critically implicated with the glycolytic phenotype of HCC.

mTORC1 signaling regulates glycolysis through targeting c-Myc/LDHA axis

c-Myc is a crucial transcription factor involved energy metabolism, especially aerobic glycolysis. And the c-Myc/LDHA axis has implicated in various cancers [12]. To assess the correlation between LDHA expression and MYC target gene

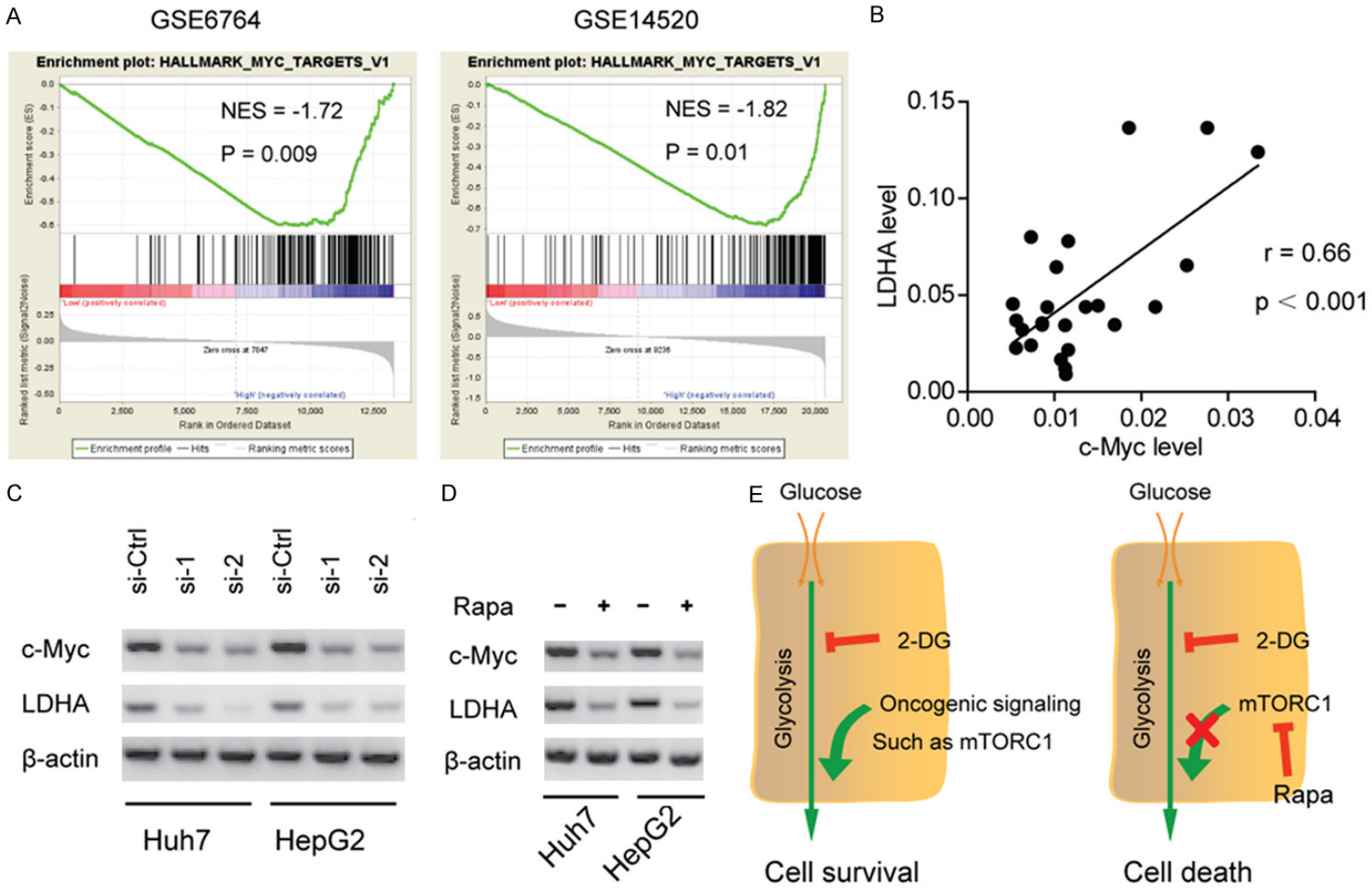

Figure 2. mTORC1 signaling regulates glycolysis through targeting c-Myc/LDHA axis. A. Gene set enrichment analysis of the two HCC dataset based on a signature of MYC target genes and LDHA expression. B. Correlation between c-Myc and LDHA was analyzed in twenty-four HCC tissues. C. Cells were cultured for further 48 h after transfection with LDHA specific siRNAs, then lysed and probed by western blotting with indicated antibodies. D. Cells were cultured for 24 h in the presence of 100 nM rapamycin, then lysed and probed by western blotting with indicated antibodies. E. Hypothesis of dampening glycolysis by combined treatment.

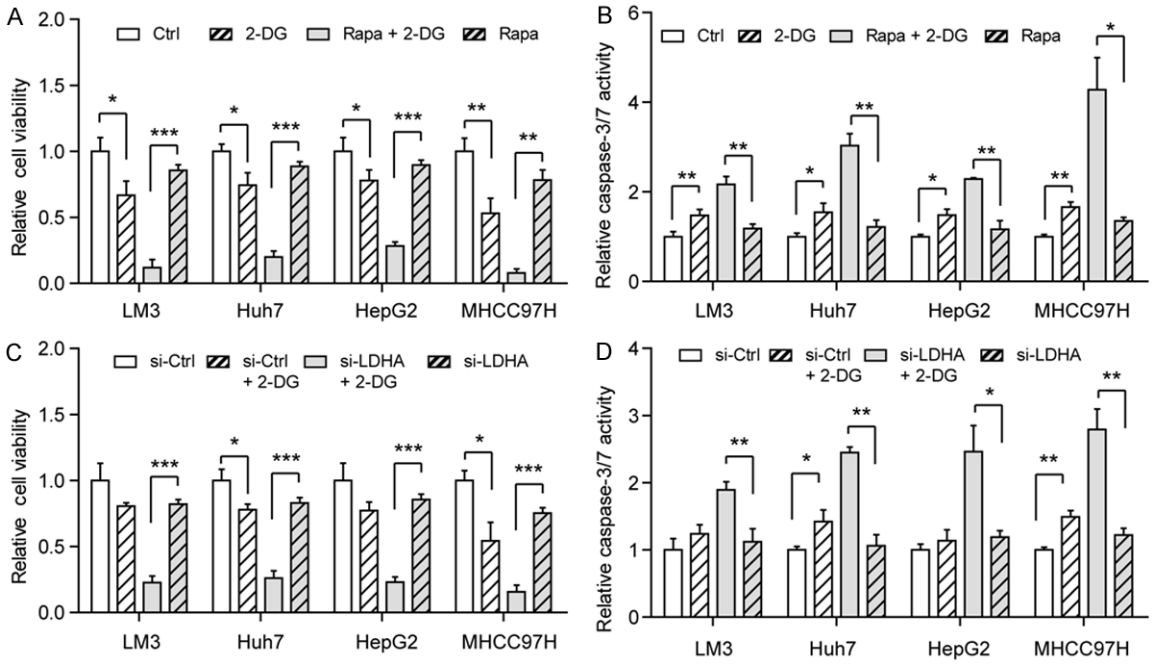

Figure 3. Rapamycin or LDHA knockdown sensitizes HCC cells to 2-deoxyglucose. (A, B) Human HCC cells were treated with 2 mM of 2-DG, 100 nM rapamycin or combined, for 48 h followed by cell viability (A) and caspase-3/7 activity (B) analysis. (C, D) The si-Ctrl or si-LDHA HCC cells were treated with 2 mM of 2-DG for 48 h followed by cell viability (C) and caspase-3/7 activity (D) analysis. Data shown represents the mean ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

signature, GSEA was performed in GSE6764 and GSE14520 datasets. A significant enrichment score was obtained from both two datasets, indicating that a high enrichment in the expression of MYC target gene signature in the tumor subset with high LDHA expression (**Figure 2A**). By real-time PCR analysis the mRNA expression of c-Myc and LDHA in 24 HCC tissues, we noticed a close correlation between c-Myc and LDHA (**Figure 2B**, $r = 0.66$, $P < 0.001$). Consistently, silencing of c-Myc resulted in pronounced decrease in the protein level of LDHA (**Figure 2C**). Because mTORC1 signaling is responsible for c-Myc translation [13], we therefore determined the effect of rapamycin on c-Myc and LDHA expression. Indeed, rapamycin markedly reduced c-Myc and LDHA protein level in both Huh7 and HepG2 cells (**Figure 2D**). Taken together, these data indicate that mTORC1 signaling modulates glycolysis through regulating c-Myc/LDHA axis.

Rapamycin or LDHA knockdown sensitizes HCC cells to 2-DG

Given the critical function of mTORC1 signaling in regulating glycolysis by modulating c-Myc/LDHA axis, we hypothesized that whether blocking mTORC1 signaling renders tumor cells sensitive to 2-DG treatment (**Figure 2E**). To test this hypothesis, LM3, Huh7, HepG2 and MHCC97H cells were co-treated with 2mM 2-DG and 100 nM rapamycin. As shown in **Figure 3A**, rapamycin itself did not induce significant inhibition of HCC cell viability, but remarkably amplified the toxic effects of 2-DG in all HCC cell lines tested. On the other hand, this cytotoxic effect was also confirmed by caspase-3/7 activity assay (**Figure 3B**). Similarly, silencing of LDHA fully recapitulated the role of rapamycin in improving 2-DG sensitivity (**Figure 3C** and **3D**). Next, we evaluated the in vivo effects of 2-DG and rapamycin on the tumorigenesis of HepG2 cells. Consistent with previous reports [14], treatment 2-DG or rapamycin alone failed to suppress tumor growth (**Figure 4A**) or reduce tumor burden (**Figure 4B**). However, combined treatment (2-DG + Rapamycin) significantly inhibited tumor growth (**Figure 4A** and **4B**). This synergistic effect also accompanied with decreased proliferation index as demonstrated by Ki67 staining (**Figure 4C**).

Because mTORC1 signaling is vital to ribosome synthesis, protein synthesis and lipid synthesis

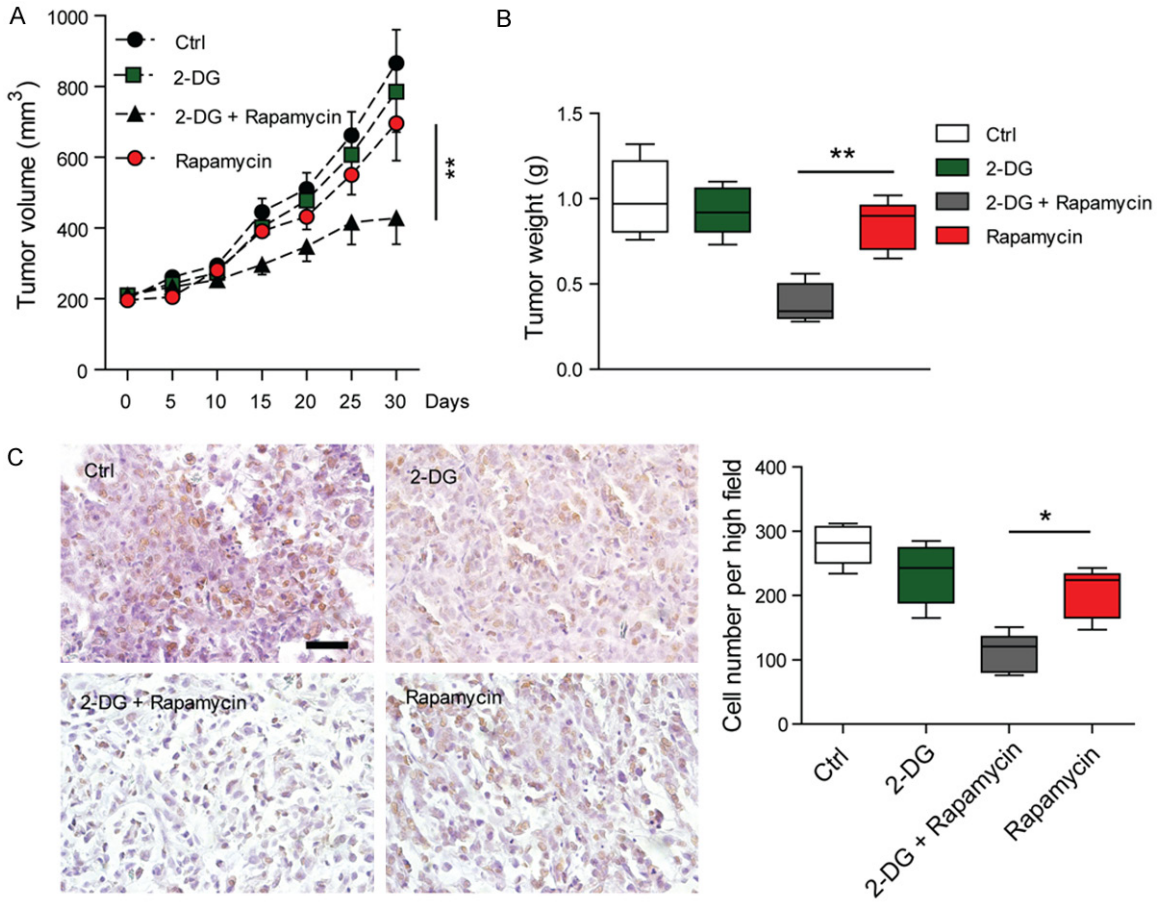

Figure 4. Combined treatment of rapamycin and 2-DG inhibits liver tumorigenesis. A. Tumor growth curve of HepG2 cells upon indicated treatment. B. At the endpoint of animal experiment, mice were sacrificed and tumor weight was calculated. C. Immunohistochemical analysis of Ki67 in indicated treatment group. The mean positive staining cells were calculated with six random fields; Scale bar: 50 μm. *, $P < 0.05$; **, $P < 0.01$.

and to further certify the function mTORC1/c-Myc/LDHA axis in 2-DG sensitivity, we performed loss-of-function study in several key downstream targets of mTORC1 signaling, including TFEB (autophagy), SREBP1 (lipogenesis) and SKAR (mRNA splicing), in the presence of 2-DG treatment. As expected, rapamycin significantly reduced the mRNA expression of TFEB, SREBP1 and SKAR in three HCC cell lines (**Figure 5A**). And siRNA-mediated knockdown resulted in marked interfere efficiency of all three proteins as demonstrated by western blotting (**Figure 5B**). Silencing of SREBP1 faintly reduced the cell viability upon 2-DG treatment, which is less remarkable than that observed in LDHA knockdown (**Figure 5C**). And no obvious effect was noticed in TFEB and SKAR knockdown (**Figure 5C**). Collectively, those data above suggest that mTORC1/c-Myc/LDHA axis is a mainstream in activating glycolysis.

Discussion

Reprogrammed energy metabolism is critical for the aggressive phenotype of tumor cells and a greater capacity to compete for nutrients. Tumor cells must acquire sufficient nutrients to engage energy metabolism that supports their oncogenic functions. The well-known metabolic phenotype observed in cancers is the Warburg effect, which results in the predominant use of glycolysis to produce ATP instead of oxidative phosphorylation even in the presence of oxygen. Targeting glycolysis by inhibition of HK2, PKM2 and LDHA remarkably suppresses tumor progression in HCC, suggesting the crucial role of the Warburg effect in tumor biology [15-17].

In this study, we firstly showed that mTORC1 signaling is widely implicated the glycolytic phe-

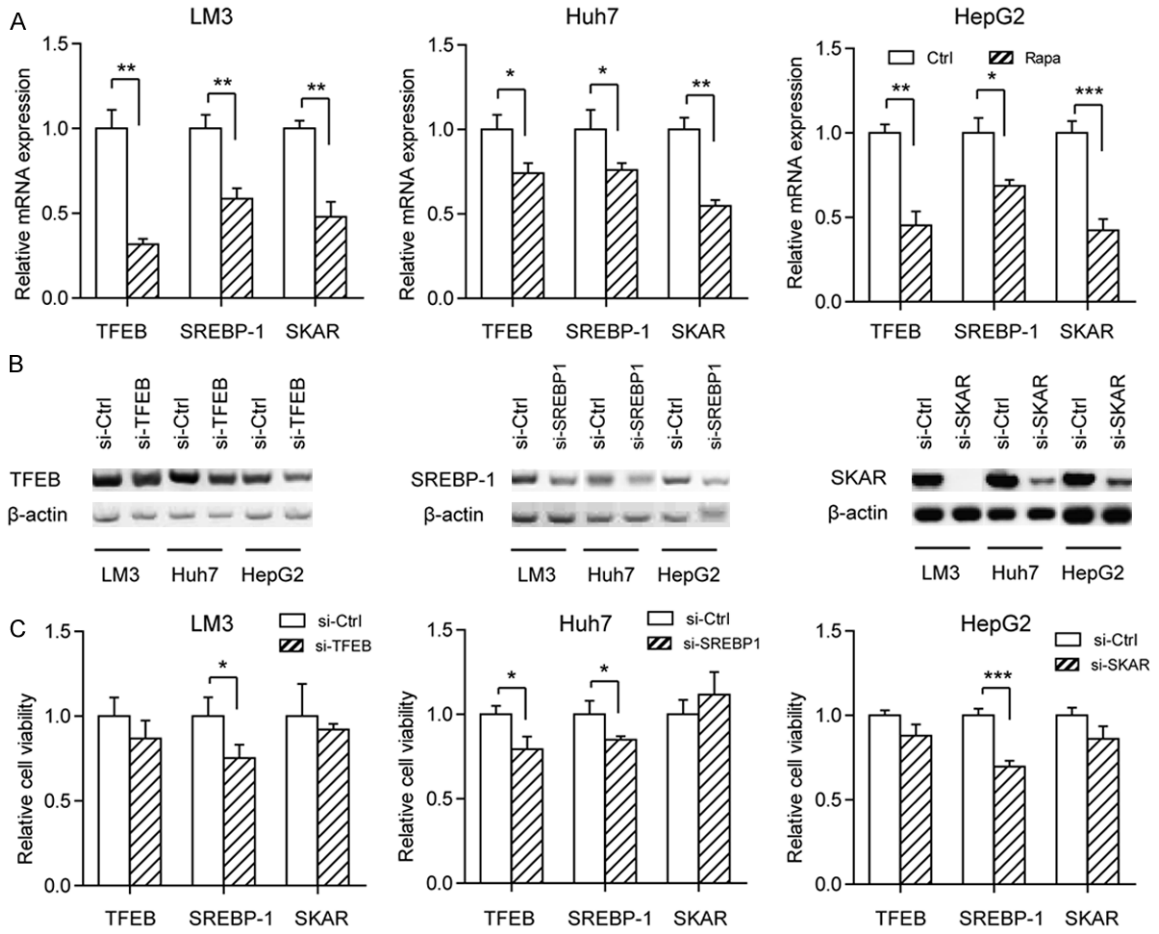

Figure 5. Silencing of TFEB, SREBP1 and SKAR fails to render HCC cells sensitive to 2-deoxyglucose. A. Effects of rapamycin on TFEB, SREBP1 and SKAR mRNA expression in LM3, Huh7 and HepG2 cells. B. Cells were cultured for further 48 h after transfection with indicated specific siRNAs, then lysed and probed by western blotting with indicated antibodies. C. The indicated HCC cells were treated with 2 mM of 2-DG for 48 h followed by cell viability analysis. Data shown represents the mean ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

notype of HCC cells. mTOR is a serine/threonine protein kinase belonging to the PI3K-related kinase family that plays critical roles in the regulation of multiple cellular processes [18]. mTOR acts through two separate complexes, mTORC1 and mTORC2. mTORC1 is a central regulator of cellular growth in response to the availability of nutrients. Aberrant activation of mTORC1 signaling occurs frequently in cancers. Here, by GSEA, we found that mTORC1 signaling is positively associated with high glycolytic capacity of HCC. It has been reported that hyperactivation of mTORC1 signaling enhances glycolytic activity by elevating glycolysis-related genes [19, 20]. Consistently, suppression of mTORC1 signaling with rapamycin decreased LDHA level and glycolytic activity of HCC cells. Next, the specific connection between mTORC1 signaling and LDHA was determined. Activated mTORC1 signaling increases mRNA translation through phosphorylation of the downstream targets p70S6K (S6K) and 4E binding protein 1 (4E-BP1). S6K phosphorylates the S6 component of the 40S ribosomal subunit and further increases translation of mRNA. Phosphorylation of 4E-BP1 regulates translation by preventing association of 4E-BP-1 with the eukaryotic initiation factor 4F (eIF4F) complex [21, 22]. However, no direct evidence demonstrates the regulatory role of mTORC1 signaling in LDHA expression. We therefore focused on c-Myc, a transcription factor involved in the regulation of glycolysis via regulating expression of glycolytic enzymes including LDHA [23]. Indeed, LDHA expression was closely correlated the expression profile of c-Myc targets and silencing c-Myc

reduced LDHA protein level. Notably, c-Myc is regulated by mTORC1-4E-BP1 axis in multiple cancers [24, 25]. A recent report showed that active mTORC1 signaling directs increased glucose flux via the pentose phosphate pathway back into glycolysis [26]. Here we certified this notion that mTORC1 signaling regulates glycolysis through targeting c-Myc/LDHA axis as demonstrated by reduced protein level of c-Myc and LDHA upon rapamycin treatment. However, further experiments are warranted to define the precise regulatory functions of mTORC1 activity in c-Myc/LDHA axis and glycolysis.

Lastly, we revealed that combined inhibition of glycolysis with 2-DG and rapamycin exerts enhanced tumor-suppressive effects *in vitro* and *in vivo*. This effect may mainly dependent on glycolysis itself as LDHA knockdown phenocopied the effect of rapamycin. Inhibition of mTORC1 suppresses ribosome synthesis, protein synthesis and lipid synthesis, however, rapamycin itself does not induce pronounced suppressive role on HCC cells, suggesting that a more complicated network of mTORC1 may be implicated in the glycolytic phenotype of HCC. And we have confirmed that this suppressive role is not largely dependent on autophagy, lipogenesis and mRNA splicing process. Although we cannot fully rule out the possible roles of other mTORC1 downstream targets, our study, at least to some extent, demonstrated that dampening mTORC1 signaling sensitizes HCC cells to 2-DG through targeting LDHA. Decreased LDHA induced by rapamycin may reprogram the acidified tumor microenvironment of HCC, and further influence the glucose utilization and ultimately sensitizes HCC cells to 2-DG treatment. From a therapeutic perspective, targeting mTORC1 activity is of particular promise since its inhibitors have been widely used in clinical treatment. Therefore, our findings, as a proof of principle, provide the rationalities to adopt the combined therapy (2-DG plus mTORC1 inhibitor) in HCC treatment.

Disclosure of conflict of interest

None.

Address correspondence to: Drs. Shuguang Wang and Xiaowu Li, Department of Hepatobiliary Surgery Institute, Southwest Hospital, Third Military Medical University, No. 30 Gaotanyan Street, Shapingba District, Chongqing 400038, PR China. E-mail: prowang0312@163.com (SGW); xiaowuhos@163.com (XWL)

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