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Original Research Article

PLOD1 contributes to proliferation and glycolysis in hepatocellular carcinoma by regulating E2F1

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Abstract

Purpose: *To evaluate the effect of procollagen-lysine 1,2-oxoglutarate 5-dioxygenase 1 (PLOD1) in hepatocellular carcinoma (HCC).*
Methods: *HCC cells were subjected to loss of function assays via transfection with siRNA targeting PLOD1. Colony formation and cell counting kit 8 (CCK8) were used to determine cell proliferation. Cell cycle was evaluated by flow cytometry while extracellular acidification rate (ECAR) levels, glucose consumption, and lactate production were determined to investigate aerobic glycolysis.*
Results: *PLOD1 was significantly up-regulated in HCC tissues and cells compared to normal tissues and cells ($p < 0.001$). Silencing of PLOD1 significantly repressed cell proliferation ($p < 0.001$) and induced cell cycle arrest in HCC at the G1 phase. ECAR levels, glucose consumption, and lactate production in HCC were reduced by knockdown of PLOD1. Loss of PLOD1 down-regulated the expression of E2F1, while over-expression of E2F1 attenuated PLOD1 knockdown-induced decreases in cell viability, glucose consumption, and lactate production in HCC.*
Conclusion: *Knockdown of PLOD1 inhibits cell proliferation and aerobic glycolysis in HCC via down-regulation of E2F1. Thus, PLOD1 may help in developing an effective strategy for the management of liver cancer.*

Keywords: *PLOD1, Hepatocellular carcinoma, Proliferation, Glycolysis, E2F1*

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a common cancer and a leading cause of cancer-related death worldwide [1]. Aflatoxin B1 intake, non-alcoholic fatty liver disease, alcohol consumption, and virus infection are common risk factors for HCC [2]. Therapeutic strategies, such as liver transplantation, immunotherapy, surgical resection, kinase inhibitors, and the anti-hepatitis vaccine, are widely used in the management of HCC [2]. However, the prognosis of HCC patients is still poor due to postoperative recurrence and metastasis [2]. Therefore, there is a need for new approaches to improve the clinic outcomes for patients with HCC. Collagens are components of extracellular matrix, and the interaction between collagens and cells is essential for proliferation, differentiation, and migration in normal tissues [3]. Procollagen-

lysine 1,2-oxoglutarate 5-dioxygenases (PLODs) catalyze hydroxylation of lysine and contribute to the cross-linking and deposition of collagens [3]. Cross-linking and deposition of collagens are important for tumor cell proliferation, adhesion, invasion, and migration, so PLODs are involved in progression and metastasis of cancers [4]. PLOD1 has been shown to promote tumor progression in gastrointestinal carcinoma [5], and knockdown of PLOD1 represses cell growth and metastasis of osteosarcoma [6]. Although PLOD1 has been identified as a neighboring gene relevant to the cell division associated with HCC [7], the mechanism of PLOD1 in HCC is still unclear. E2F transcription factors are widely known as critical regulators in cell differentiation, apoptosis, proliferation, and cycle progression of HCC [8]. One member of the E2F family, E2F1, is recognized as a tumor promoter as it promotes anti-apoptotic activity and mediates cell cycle progression in HCC [9]. PLOD1 has been shown to promote the activation of E2F1 and contribute to lung cancer cell proliferation [10]. It is possible, therefore, that PLOD1 may promote tumorigenesis of HCC via regulation of E2F1. As such, we investigated the effects of PLOD1 on cell proliferation, cell cycle, and aerobic glycolysis in HCC.

EXPERIMENTAL

Cell culture

Liver sinusoidal endothelial cells (LSECs) and HCC cells (Huh7, MHCC97-H, BEL-7405, MHCC97-L) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) (Thermo Fisher, Waltham, MA, USA). Huh7 and MHCC97-H cells (2×10^4/well) were seeded in 96-well plates, and transfected with si-NC (negative control), si-PLOD1-#1, or si-PLOD1-#2 (GenePharma, Suzhou, China) via Lipofectamine 2000 (Thermo Fisher) for 48 h. The cells were also co-transfected with si-PLOD1-#1 and pcDNA-E2F1 (OE-E2F1).

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

RNA was isolated from the cells using TRIzol (Sigma-Aldrich, St. Louis, MO, USA) and then reverse-transcribed into cDNA. PLOD1 and E2F1 mRNA expression was determined using SYBR Green Master Mix (Roche, Mannheim, Germany) and analyzed using the $2^{-\Delta\Delta Cq}$ method. The Primers used are shown in Table 1.

Cell viability, proliferation, and cell cycle assays

Post transfection, Huh7 and MHCC97-H were seeded in 96-well plates for 24, 48, or 72 h and then incubated using a cell counting kit 8 (CCK8; Beyotime, Beijing, China) for another 2 h. Absorbance at 450 nm was measured using a Microplate Autoreader (Thermo Fisher). To detect cell proliferation, Huh7 and MHCC97-H (1×10^3/well) were seeded in 6-well plates and cultured in DMEM for 10 days. Cells were fixed with 4% paraformaldehyde and then stained with crystal violet. Cells were observed under a microscope (Olympus, Tokyo, Japan), and the number of colonies was calculated using ImageJ software. To evaluate cell cycle, Huh7 and MHCC97-H were harvested and incubated with propidium iodide using a BD Cycletest Plus DNA Reagent Kit (BD Bioscience, San Diego, CA, USA). Cells were analyzed using a FACSCanto II flow cytometer (BD Bioscience) to determine the percentage of cells in G1, S, and G2-M phases.

Determination of extracellular acidification rate (ECAR), glucose consumption, and lactate production

Huh7 and MHCC97-H cells (1×10^6) were seeded into Seahorse plates overnight and then incubated with Seahorse buffer with oligomycin, glucose, and 2-deoxyglucose (2-DG). The level of ECAR was analyzed using XF-96 wave software (Seahorse Bioscience, Billerica, MA, USA) and a Seahorse XFe96 Extracellular Flux Analyzer. To determine glucose consumption, Huh7 and MHCC97-H cells were cultured in glucose-free medium in 96-well plates for 24 h. Cells were then treated with 100 μM 2-NBDG (Sigma-Aldrich), and a microplate reader was used to measure the fluorescent intensity of 2-NBDG. The level of lactate in the supernatants of the cultured medium was measured using a Lactate Assay Kit (Sigma-Aldrich).

Table 1: Primer sequences used for PCR

Gene	Forward	Reverse
PLOD1	5'-GGTCATTCTCTTCGCAGACAG-3'	5'-CCACCGGATACTTGGTCTCCA-3'
E2F1	5'-CATCCCAGGAGGTCACTTCTG-3'	5'-GACAACAGCGGTTCTTGCTC-3'
GAPDH	5'-GCACCGTCAAGGCTGAGAAC-3'	5'-TGGTGAAGACGCCAGTGGA-3'

Western blot

Cells were lysed in RIPA buffer (Beyotime), and protein concentration was determined using a BCA Protein Assay Kit (Beyotime). Protein samples were separated using SDS-PAGE and then transferred onto nitrocellulose membranes. The membranes were blocked in 5% dry milk. Membranes were incubated overnight at 4 °C with primary antibodies, including anti-PLOD1, anti-E2F1, and anti-GAPDH (1:2000), anti-cyclin E and anti-CDK2 (cyclin dependent kinase 2, 1:3000), and anti-GLUT1 (glucose transporter 1) and anti-LDHA (lactate dehydrogenase A, 1:4000). The membranes were then incubated with secondary antibodies (1:4500) at 4 °C for 2 h and then chemiluminescence was measured with a commercially available kit (Beyotime). All the antibodies were purchased from Abcam (Cambridge, MA, USA).

Statistical analysis

All the data are expressed as mean ± standard error of the mean (SEM) and were analyzed using either Student's t test or one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered statistically significant.

RESULTS

Up-regulation of PLOD1 in HCC

Bioinformatic analysis, including TIMER (https://cistrome.shinyapps.io/timer/) (Figure 1A), UALCAN (http://ualcan.path.uab.edu/) (Figure 1 B), and GEPIA (http://gepia.cancer-pku.cn/) (Figure 1 B), indicated that PLOD1 was significantly elevated in HCC tissues compared to normal tissues. Moreover, HCC cells demonstrated higher expression of PLOD1 than normal LSEC (Figures 1 C and D; $p < 0.001$).

PLOD1 promoted HCC proliferation

Huh7 and MHCC97-H cells were subjected to loss-of-function assays through transfection with si-PLOD1 #1 or #2 (Figure 2 A). Transfection with si-PLOD1 #1 or #2 reduced viability of Huh7 and MHCC97-H cells (Figure 2 B). Cell proliferation of both Huh7 and MHCC97-H cells was also suppressed by silencing PLOD1 (Figure 2 C; $p < 0.001$).

PLOD1 mediated cell cycle progression in HCC

Transfection with si-PLOD1 #1 or #2 induced cell cycle arrest in Huh7 and MHCC97-H cells at G1 phase (Figures 3 A and B). Moreover, protein expression levels of cyclin E and CDK2 were down-regulated in Huh7 and MHCC97-H cells after knockdown of PLOD1 (Figure 3 C).

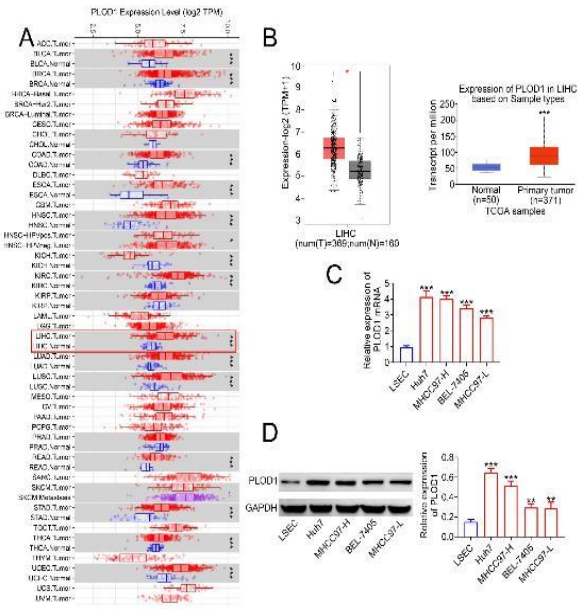

Figure 1: Up-regulation of PLOD1 in HCC. (A) TIMER showed that PLOD1 was significantly up-regulated in CRC tissues compared to normal tissues. (B) UALCAN and GEPIA showed that PLOD1 was significantly up-regulated in HCC tissues compared to normal tissues. (C) The mRNA expression of PLOD1 was up-regulated in HCC cells compared to LSEC. (D) Protein expression of PLOD1 was up-regulated in HCC cells compared to LSEC. *$P < 0.05$, **$p < 0.01$, ***$p < 0.001$

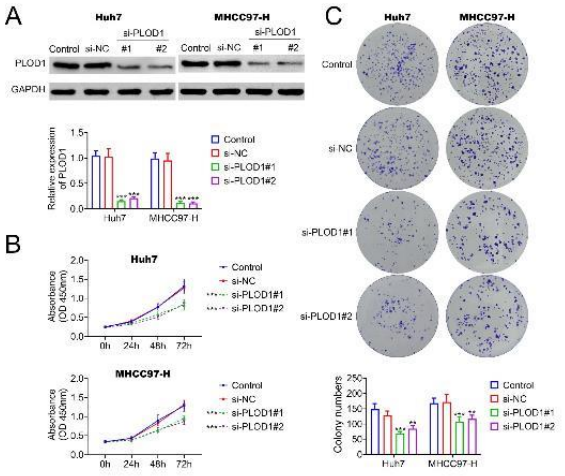

Figure 2. PLOD1 promoted HCC proliferation. (A) Transfection with si-PLOD1 #1 or #2 reduced protein expressions of PLOD1 in Huh7 and MHCC97-H. (B) Transfection with si-PLOD1 #1 or #2 reduced viability of Huh7 and MHCC97-H cells. (C) Transfection with si-PLOD1 #1 or #2 significantly reduced proliferation of Huh7 and MHCC97-H cells vs. si-NC. **$P < 0.01$, ***$p < 0.001$

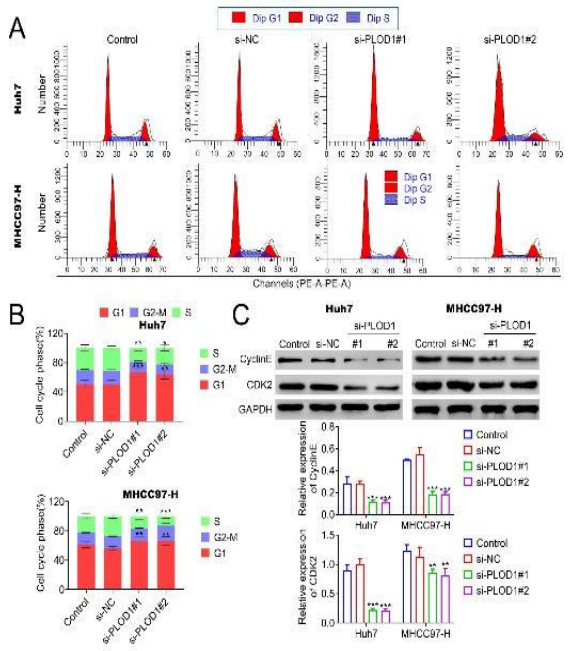

Figure 3. PLOD1 promoted cell cycle progression in HCC. (A) Transfection with si-PLOD1 #1 or #2 induced cell cycle arrest in Huh7 and MHCC97-H cells at G1 phase. (B) Cell cycle phase distribution in Huh7 and MHCC97-H cells after transfection of si-PLOD1 #1 or #2. (C) Transfection with si-PLOD1 #1 or #2 reduced protein expression of cyclin E and CDK2 in Huh7 and MHCC97-H cells. ***P* < 0.01, ****p* < 0.001

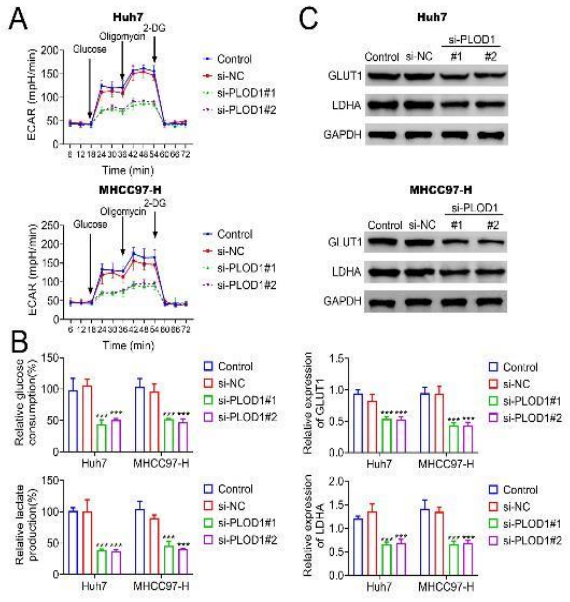

Figure 4: PLOD1 contributed to aerobic glycolysis in HCC. (A) Knockdown of PLOD1 reduced the level of ECAR in Huh7 and MHCC97-H cells. (B) Knockdown of PLOD1 reduced glucose consumption and lactate production in Huh7 and MHCC97-H cells. (C) Knockdown of PLOD1 reduced protein expression of GLUT1 and LDHA in Huh7 and MHCC97-H cells. vs. si-NC, ****p* < 0.001

PLOD1 contributed to aerobic glycolysis of HCC

Knockdown of PLOD1 reduced the level of ECAR in Huh7 and MHCC97-H cells (Figure 4 A). Furthermore, glucose consumption and lactate production were decreased by silencing PLOD1 (Figure 4 B). Loss of PLOD1 also reduced protein expression of GLUT1 and LDHA in Huh7 and MHCC97-H cells (Figure 4 C; $p < 0.001$).

PLOD1 up-regulated E2F1 in HCC

Transfection with si-PLOD1 #1 or #2 decreased E2F1 expression in Huh7 and MHCC97-H cells (Figures 5 A and B), whereas over-expression of E2F1 weakened PLOD1 knockdown-induced decreases in cell viability in Huh7 and MHCC97-H cells (Figure 5 C). PLOD1 knockdown-induced decreases in cyclin E and CDK2 in Huh7 and MHCC97-H cells were also attenuated by E2F1 over-expression (Figure 5 D). The PLOD1 knockdown-induced decrease in cell viability in Huh7 and MHCC97-H attenuated the PLOD1 knockdown-induced decrease in glucose consumption (Figure 5E) and lactate production (Figure 5 F; $p < 0.001$).

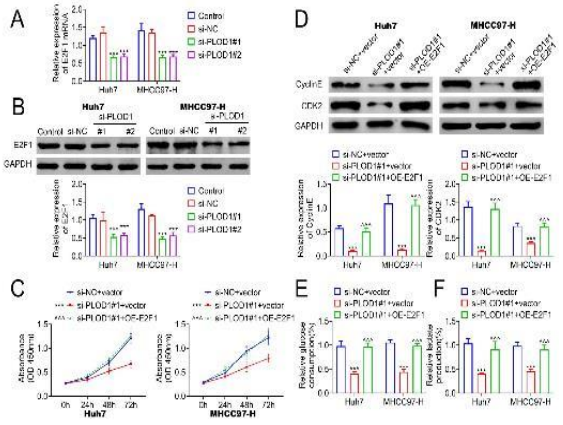

Figure 5: PLOD1 up-regulated E2F1 in HCC. (A) Transfection with si-PLOD1 #1 or #2 decreased mRNA expression of E2F1 in Huh7 and MHCC97-H cells. (B) Transfection with si-PLOD1 #1 or #2 decreased protein expression of E2F1 in Huh7 and MHCC97-H cells. (C) Over-expression of E2F1 weakened the PLOD1 knockdown-induced decrease in cell viability in Huh7 and MHCC97-H cells. (D) Over-expression of E2F1 weakened the PLOD1 knockdown-induced decrease in cyclin E and CDK2 in Huh7 and MHCC97-H cells. (E) Over-expression of E2F1 weakened the PLOD1 knockdown-induced decrease in glucose consumption in Huh7 and MHCC97-H cells. (F) Over-expression of E2F1 weakened the PLOD1 knockdown-induced decrease in lactate production in Huh7 and MHCC97-H cells. ***, ^^^*p* < 0.001

DISCUSSION

PLODs have been reported to be associated with higher tumor grades, poor overall survival, and worse disease-free survival in patients with HCC [11]. PLODs are regarded as potential prognostic biomarkers of HCC [11]. This study found that PLOD1 was elevated in HCC tissues and cells, and knockdown of PLOD1 exerted an anti-proliferative effect, suppressed aerobic glycolysis, and stimulated cell cycle arrest in HCC.

A previous study showed that protein and mRNA expression of PLOD1 were noticeably elevated in HCC tissues [11]. The expression patterns of PLOD1 were also enhanced in HCC cells compared to LSEC. Loss of PLOD1 decreased cell viability and repressed cell proliferation of HCC. However, the effects of PLOD1 on cell invasion, apoptosis, and migration of HCC remain unclear. Specific cyclin/CDK complexes control cell cycle progression, and cyclin E/CDK2 induces entry of S phase through phosphorylation of Rb [12]. Over-expression of cyclin E and CDK2 contribute to HCC tumorigenesis [13]. The results in this study demonstrated that knockdown of PLOD1 reduced protein expressions of cyclin E and CDK2 and stimulated cell cycle arrest at G1 phase. Therefore, PLOD1 functioned as an oncogene in HCC through promotion of cell proliferation and cell cycle progression.

Energy metabolism reprogramming has been reported to be a hallmark of cancer; for example, HCC cells tend to convert glucose into lactic acid through oxidative phosphorylation in the mitochondria, which is known as aerobic glycolysis [14]. Enhancement of aerobic glycolysis contributes to proliferation, growth, immune evasion, metastasis, angiogenesis, and drug resistance in HCC [14]. LDHA converts pyruvate into lactate and is a key enzyme in aerobic glycolysis in HCC [14]. GLUT1 is essential for glucose uptake in HCC, and suppression of LDHA and GLUT1 suppresses aerobic glycolysis in HCC [14]. Over-expression of PLOD1 enhances the ECAR levels, ATP contents, glucose uptake, and lactate production in gastric cancer [15]. The results of this study indicate that knockdown of PLOD1 reduced ECAR levels, glucose uptake, and lactate production; down-regulated protein expression of GLUT1 and LDHA; and suppressed aerobic glycolysis in HCC.

In addition to the anti-apoptotic, proliferative, and pro-metastatic effects of E2F1, E2F1 also promoted the recruitment of Pontin and Reptin to stimulate expression of genes involved in lactate export and glycolysis, thereby contributing to aerobic glycolysis in HCC [16]. Down-regulation of E2F1 reduced cell proliferation and metastasis of HCC [17] and retarded aerobic glycolysis [16]. Research has shown that PLOD1 potentiated the transcriptional activity of E2F1 and strengthened the proliferation of lung cancer cells [10]. This study revealed that knockdown of PLOD1 down-regulated E2F1 expression in HCC, and over-expression of E2F1 attenuated the PLOD1 knockdown-induced decreases of cell viability, cyclin E, CDK2, glucose consumption, and lactate production. Therefore, PLOD1 might contribute to proliferation and aerobic glycolysis in HCC through up-regulation of E2F1. Indeed, E2F1 has been reported to bind to the promoter region and stimulate the expression of PLOD1 [18]. The positive regulation loop between E2F1 and PLOD1 might mediate progression and aerobic glycolysis of HCC.

Taken together, this study indicates an oncogenic role for PLOD1 in HCC and suggests that PLOD1/E2F1 might be a promising target for treatment of HCC. However, the role of PLOD1/E2F1 in *in vivo* tumor growth of HCC should be investigated further.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Jian Chen

and Xing You designed the experiments, and Luke Zhou conducted the experiments. Jie Yang and Hui Xie analyzed and interpreted the data, and Lin Liu and Youwei Li prepared the manuscript with contributions from all co-authors.

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