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Research Article

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Targeting *TRMT5* suppresses hepatocellular carcinoma progression via inhibiting the HIF-1α pathways

Qiong ZHAO[1,2], Luwen ZHANG[1,2], Qiufen HE[1,2], Hui CHANG[1,2], Zhiqiang WANG[1,2], Hongcui CAO[3], Ying ZHOU[4], Ruolang PAN[5]✉, Ye CHEN[1,2]✉

[1]*Department of Genetics, and Department of Genetic and Metabolic Disease, The Children's Hospital, Zhejiang University School of Medicine, National Clinical Research Center for Child Health, Hangzhou 310052, China*
[2]*Zhejiang Provincial Key Laboratory of Genetic and Developmental Disorders, Institute of Genetics, Zhejiang University, Hangzhou 310058, China*
[3]*State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, National Clinical Research Center for Infectious Diseases, the First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310003, China*
[4]*Xiangshan Hospital of TCM Medical and Health Group, Ningbo 315700, China*
[5]*Zhejiang Provincial Key Laboratory of Cell-Based Drug and Applied Technology Development, Hangzhou 311121, China*

Abstract: Accumulating evidence has confirmed the links between transfer RNA (tRNA) modifications and tumor progression. The present study is the first to explore the role of tRNA methyltransferase 5 (TRMT5), which catalyzes the m1G37 modification of mitochondrial tRNAs in hepatocellular carcinoma (HCC) progression. Here, based on bioinformatics and clinical analyses, we identified that TRMT5 expression was upregulated in HCC, which correlated with poor prognosis. Silencing *TRMT5* attenuated HCC proliferation and metastasis both in vivo and in vitro, which may be partially explained by declined extracellular acidification rate (ECAR) and oxygen consumption rate (OCR). Mechanistically, we discovered that knockdown of *TRMT5* inactivated the hypoxia-inducible factor-1 (HIF-1) signaling pathway by preventing HIF-1α stability through the enhancement of cellular oxygen content. Moreover, our data indicated that inhibition of TRMT5 sensitized HCC to doxorubicin by adjusting HIF-1α. In conclusion, our study revealed that targeting *TRMT5* could inhibit HCC progression and increase the susceptibility of tumor cells to chemotherapy drugs. Thus, *TRMT5* might be a carcinogenesis candidate gene that could serve as a potential target for HCC therapy.

Key words: Transfer RNA (tRNA); tRNA methyltransferase 5 (TRMT5); Hepatocellular carcinoma (HCC); Hypoxia-inducible factor-1α (HIF-1α)

1 Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver cancer worldwide (Forner et al., 2012) and the third leading cause of cancer-related deaths based on the latest GLOBOCAN data (https://gco.iarc.fr). Despite advances in diagnosis and treatment options for HCC during the past decades, the prognosis of HCC patients is still unfavorable, with a low five-year survival rate (Ji and Wang, 2012; Zuo et al., 2021). Studies have proved that hepatocarcinogenesis is a complex multistep process that involves the deregulation of various signaling pathways (McCubrey et al., 2016; Akula et al., 2019; Dimri and Satyanarayana, 2020), which leads to increased difficulties in targeted therapies. In recent years, emerging immunotherapies have revolutionized the therapeutic landscape for various solid tumors (Yoest, 2017; King et al., 2018; Bergman, 2019). Nevertheless, only a few HCC patients benefit from this therapy (Liu and Qin, 2019; Giraud et al., 2021; Zhong et al., 2021). Therefore, there is an urgent need to explore the underlying mechanisms of HCC progression and develop new therapeutic agents for effective prevention and treatment.

Transfer RNAs (tRNAs), the critical components of translation, are the most abundant molecules in a cell (Kirchner and Ignatova, 2015). Defects in tRNA

✉ Ye CHEN, yechency@zju.edu.cn
Ruolang PAN, panrl@zju.edu.cn
Ye CHEN, https://orcid.org/0000-0003-3671-2504

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expressions and modifications have been linked to various tumorigenesis and progression types. For instance, Yamamoto et al. (2019) reported that the 2-methylthio-N^6-isopentenyl modification of adenosine (ms^2i^6A) in mitochondrial (mt)-tRNAs by Cdk5 regulatory subunit-associated protein 1 (CDK5RAP1) promoted the maintenance of glioma-initiating cells. Rosselló-Tortella et al. (2020) proved that the epigenetic inactivation of tRNA-modifying enzyme TYW2 promoted primary colorectal tumor progression and resulted in poor clinical outcome by inducing ribosome frameshift. He et al. (2020) demonstrated that FtsJ RNA 2'-*O*-methyltransferase 1 (FTSJ1) regulated tRNA 2'-*O*-methyladenosine modification and suppressed the malignancy of non-small cell lung cancer. Thus, tRNA-modifying enzymes represent promising novel targets for drug discovery.

The tRNA methyltransferase 5 (*TRMT5*) is a nuclear gene (MIM*611023) that encodes a mitochondrial protein responsible for the m1G37 modification of various mitochondrial tRNAs to prevent ribosomal frameshift errors and enhance translational efficiency/fidelity (Suzuki et al., 2011). Accumulating studies have identified the abnormal expression of tRNA methyltransferases contributing to various types of tumorigenesis and progression (Endres et al., 2019; Ma et al., 2021). In this study, for the first time, we revealed that TRMT5 was highly expressed in HCC tumors and this correlated with poor prognosis. Furthermore, we proved that targeting *TRMT5* not only suppresses HCC growth but also improves the efficacy of doxorubicin-mediated chemotherapy.

2 Results

2.1 Upregulation of TRMT5 in HCC

In order to evaluate whether TRMT5 is involved in HCC progression, the expression profile of *TRMT5* in HCC was assessed in published databases and clinical tissues. We first analyzed the messenger RNA (mRNA) expression of *TRMT5* in HCC by The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) datasets. The TCGA data showed that the mRNA of *TRMT5* was overexpressed in HCC tumor tissues compared to adjacent non-tumor tissues (Fig. 1a). Consistent results were observed in three GEO datasets (Figs. 1b–1d). Moreover, we found that the mRNA expression level of *TRMT5* in metastatic HCC was higher than that in primary HCC according to the data from GSE40367 (Fig. 1e). The survival data from the Gene Expression Profiling Interactive Analysis (GEPIA) demonstrated that patients with high *TRMT5* levels exhibited worse overall survival (Fig. 1f). Besides, the TRMT5 protein expression in clinical tissues was evaluated by IHC. Consistent with the previous findings for open databases, we showed that TRMT5 expression was upregulated in HCC tumor tissues (Figs. 1g and S1).

2.2 Attenuated HCC cell proliferation by knockdown of *TRMT5*

In order to explore the function of TRMT5 in HCC cells, we knocked down *TRMT5* in the human HCC cell line HepG2 and the mouse HCC cell line Hepa1-6 via lentivirus-mediated short hairpin RNA (shRNA). The knockdown efficiency was demonstrated by western blotting. As shown in Figs. 2a and S2, both shRNA1 and shRNA2 effectively inhibited the expression of TRMT5 in HepG2, and only shRNA1 was effective in Hepa1-6. Then, we compared the cell proliferation between these cell lines using cell counting kit-8 (CCK8) assay and colony formation assay, as well as Ki67 immunofluorescence staining (Figs. 2b–2d). The results suggested that cell proliferation was attenuated in these *TRMT5*-knockdown HCC cells. Furthermore, we detected the cell cycle distribution by flow cytometry. It turned out that *TRMT5* knockdown significantly decreased the proportion of cells in the S phase but increased the proportion of cells in the G1 phase (Fig. 2e), confirming the role of *TRMT5* in promoting cell growth by regulating cell cycle progression in HCC cells.

2.3 Metabolic reprogramming induced by *TRMT5* deficiency

In order to characterize the mechanism of *TRMT5* regulation in HCC cell growth, we further assessed the effect of *TRMT5* on the bioenergetic profiling of HCC by examining both extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) in knockdown cells and control cells. The results demonstrated that the knockdown of *TRMT5* led to reduced ECAR and OCR in both HepG2 and Hepa1-6 cells (Figs. 3a and 3b), which also indicated a reduced metabolic transition from glycolysis to mitochondrial

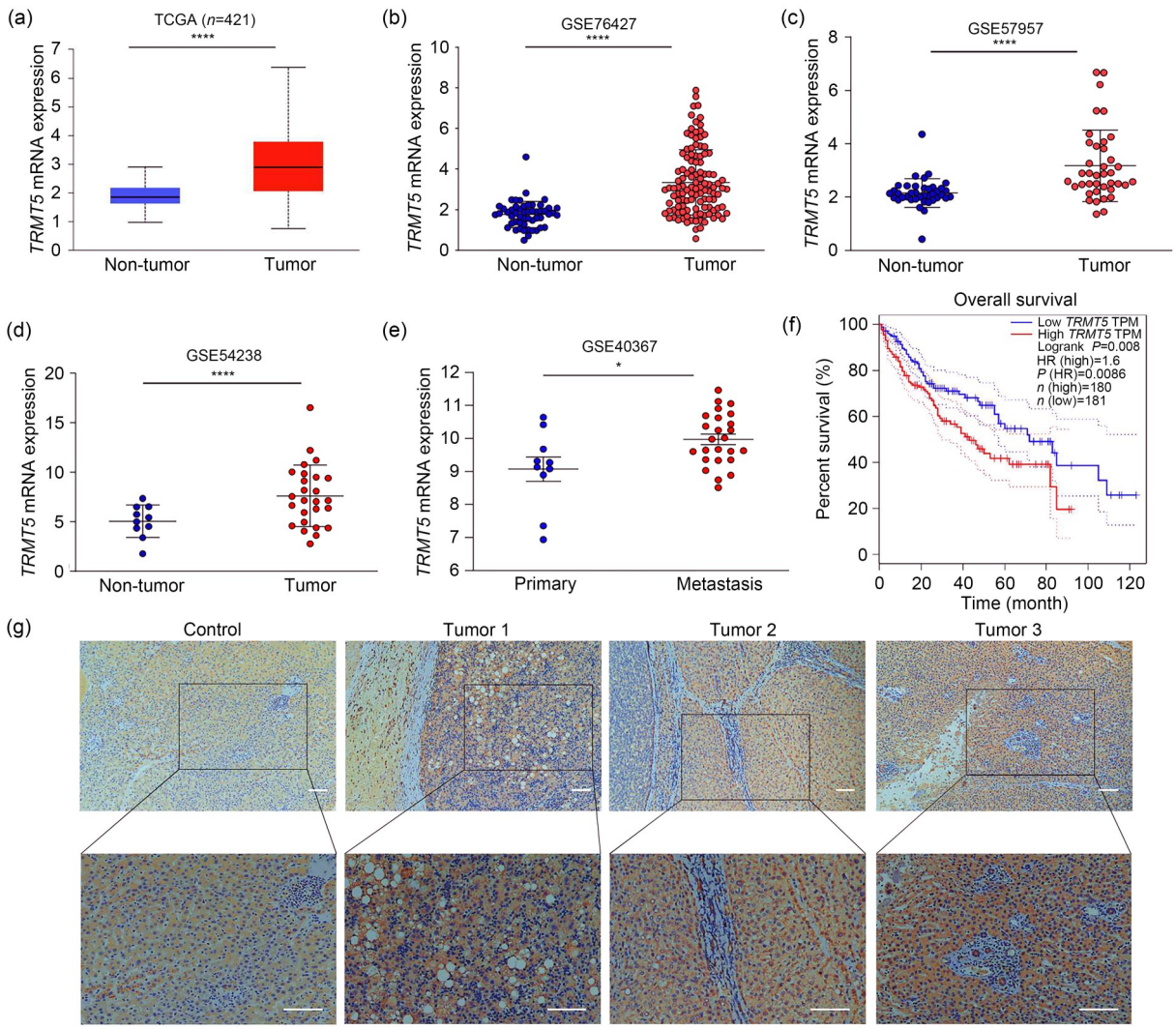

Fig. 1 Upregulation of TRMT5 in HCC, which was correlated with poorer overall survival. (a) *TRMT5* mRNA expression in paired samples of tumor and non-tumor tissues from patients with HCC in TCGA. (b–d) The mRNA expression levels of *TRMT5* in normal and HCC tissues in the dataset from the GSE cohorts. (e) *TRMT5* mRNA expression in primary HCC and metastatic HCC in GSE40367. (f) Survival curves of HCC patients with high or low expression of *TRMT5* based on GEPIA. (g) Immunohistochemical staining for TRMT5 protein was performed in one normal liver tissue and three tumor tissues from HCC patients. *P* indicates the significance: $^{*}P<0.05$, $^{****}P<0.0001$. Scale bar=100 μm. tRNA: transfer RNA; TRMT5: tRNA methyltransferase 5; HCC: hepatocellular carcinoma; TCGA: The Cancer Genome Atlas; mRNA: messenger RNA; GEO: Gene Expression Omnibus; GSE: GEO series; GEPIA: Gene Expression Profiling Interactive Analysis; TPM: transcripts per million; HR: hazard ratio.

oxidative phosphorylation in *TRMT5* knockdown cells. Moreover, lactate production was measured to evaluate the function of *TRMT5* in glycolytic metabolism. As shown in Fig. 3c, lactate production was declined in *TRMT5* knockdown cells compared to controls. We found that adenosine triphosphate (ATP) production was also decreased in knockdown cells (Fig. 3d). These findings confirmed that *TRMT5* deficiency in HCC impaired glycolysis, which is the primary method for cancer cells to produce energy. Thus, we concluded that *TRMT5* knockdown induced metabolic reprogramming in HCC cells and resulted in insufficient energy for HCC progression.

2.4 HCC cell migration and invasion inhibited by *TRMT5* knockdown

Next, the effects of *TRMT5* on the in vitro migration and invasion of HCC cells were evaluated. The wound healing assay showed that *TRMT5* knockdown significantly reduced the efficiency of wound healing

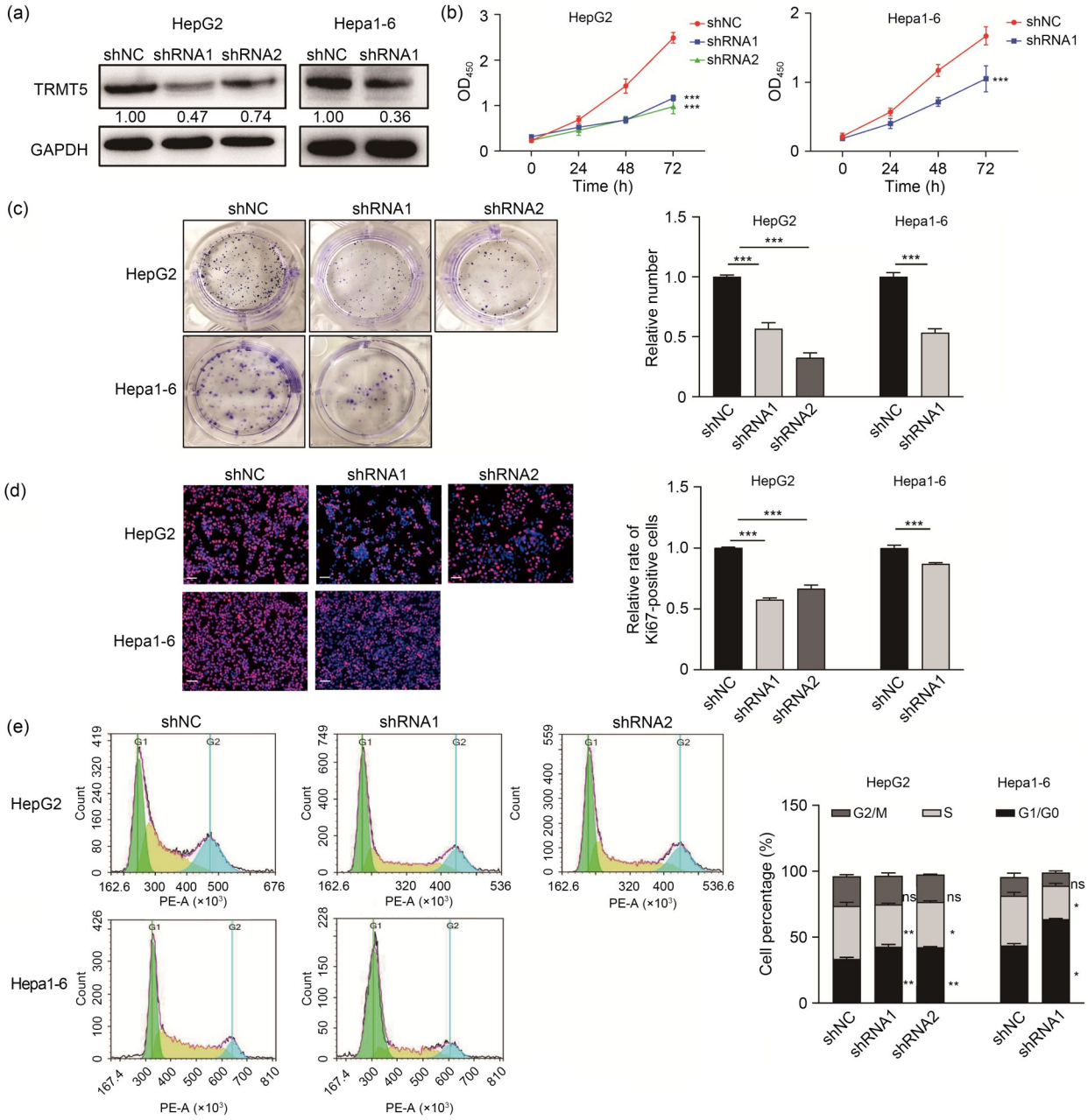

Fig. 2 Inhibition of cell proliferation and cell cycle progression in HCC cells by knockdown of *TRMT5*. (a) Western blot confirmed the successful knockdown of TRMT5 in HepG2 and Hepa1-6 cells. GAPDH was used as a loading control. The expression level of shNC cell was considered as 1, and the values for the silenced cells were expressed as ratio of the control cell value. (b) Cell proliferation ability of HepG2 and Hepa1-6 after *TRMT5* silencing was measured by CCK8 assays at the indicated time points. (c) Analysis of tumorigenicity by colony formation assay in shNC and shRNA HCC cells. (d) Immunofluorescence staining of Ki67 (red) in control and knockdown cells. Nuclei were stained with DAPI (blue). Scale bar=50 μm. Quantification of Ki67-positive rates was determined by ImageJ software. n=10 per group. $^{***}P<0.001$. (e) The cell cycle distribution was detected by flow cytometry. The statistical graphs representing the percentage of cells in G0/G1, S, or G2/M were shown in the right panel. $^{*}P<0.05$, $^{**}P<0.01$, ^{ns} no significant difference, compared to the shNC group according to two-way ANOVA followed by Dunnett's multiple comparison test. Data are expressed as mean±SEM, n=3. tRNA: transfer RNA; TRMT5: tRNA methyltransferase 5; HCC: hepatocellular carcinoma; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; CCK8: cell counting kit-8; shRNA: short hairpin RNA; shNC: shRNA negative control; DAPI: 4',6-diamidino-2-phenylindole; ANOVA: analysis of variance; SEM: standard error of the mean; OD_{450}: optical density at 450 nm; PE-A: phycoerythrin-area (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

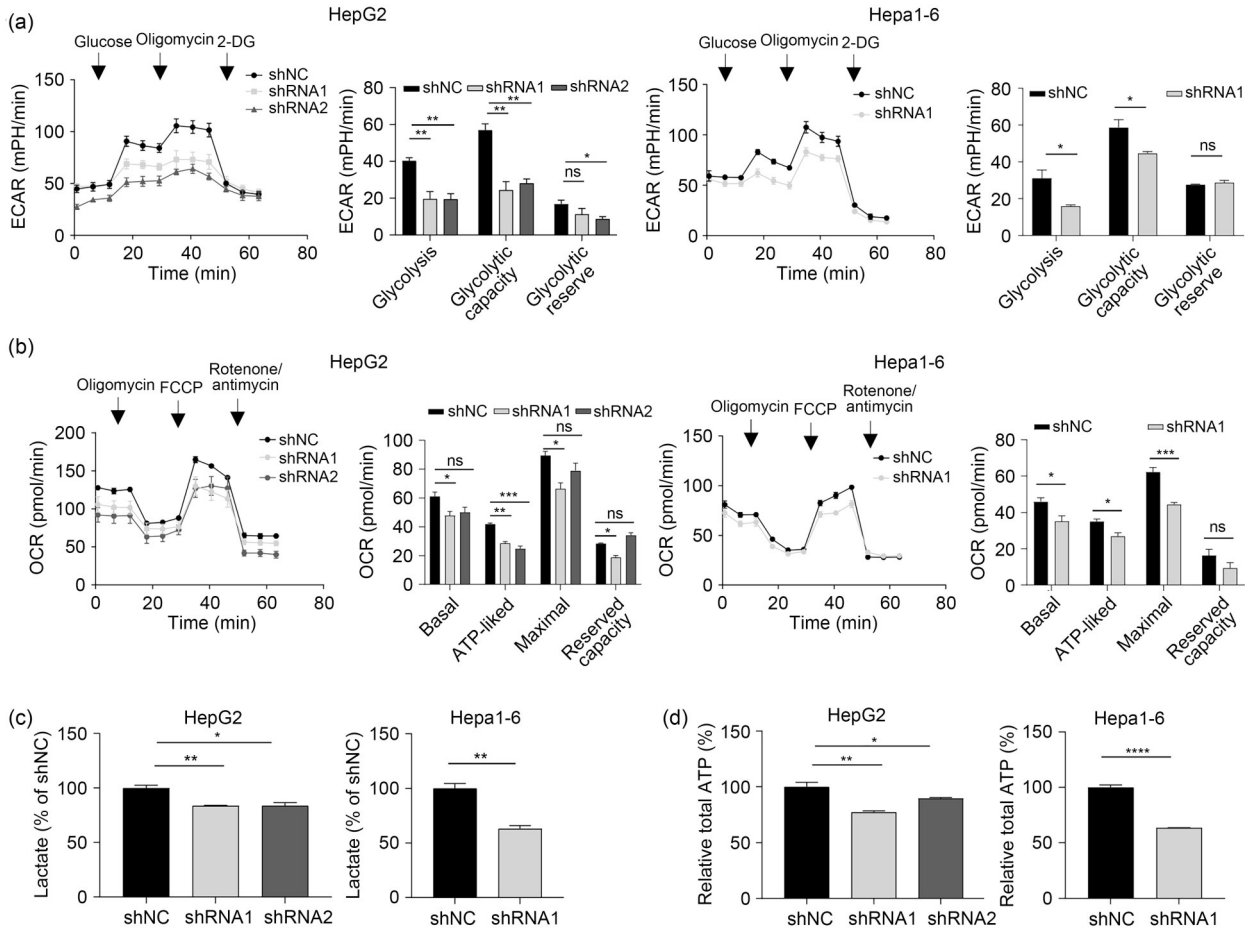

Fig. 3 *TRMT5* deficiency-induced metabolic reprogramming in two kinds of HCC cells (HepG2 and Hepa1-6). (a) The ECAR was assayed by Seahorse analyzer. (b) OCR was analyzed after *TRMT5* knockdown. (c) The relative content of lactate in the medium of knockdown cells and control cells. (d) The relative ATP production. Data are expressed as mean±SEM, n=3. $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001, $^{****}P$<0.0001, ^{ns} no significant difference. tRNA: transfer RNA; TRMT5: tRNA methyltransferase 5; HCC: hepatocellular carcinoma; ECAR: extracellular acid ratio; OCR: oxygen consumption ratio; ATP: adenosine triphosphate; SEM: standard error of the mean; 2-DG: 2-deoxy-D-glucose; FCCP: carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; mPH: milli potential of hydrogen; shRNA: short hairpin RNA; shNC: shRAN negative control.

in both HepG2 and Hepa1-6 cells (Fig. 4a). The cell transwell migration and invasion assays revealed that the migrative and invasive cell numbers were markedly decreased in *TRMT5*-knockdown cells compared to control cells (Figs. 4b and 4c). Additionally, the effect of *TRMT5* on epithelial-mesenchymal transition (EMT) in vitro was detected by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and western blotting (Figs. 4d and 4e). The levels of mesenchymal regulators *N*-cadherin and Vimentin were downregulated, while the epithelial regulator E-cadherin was upregulated in *TRMT5*-knockdown cells relative to control cells. Meanwhile, matrix metalloproteinase 13 (MMP13), which may play a role in cell migration and invasion in tumor cells, was also decreased in *TRMT5*-knockdown cells.

2.5 HCC tumorigenic progression inhibited by *TRMT5* deficiency

In order to further validate the effect of *TRMT5* on HCC tumorigenic progression in vivo, Hepa1-6 cells with stable transfection of shRNA negative control (shNC) and shRNA1 were subcutaneously injected into the left and right flanks of Balb/c nude mice, respectively. Tumor volume was determined for each mouse in Days 3, 5, 8, 10, 12, and 14 after subcutaneous injection. As shown in Fig. 5a, the tumor sizes in the shRNA1 cell group were smaller than those in the

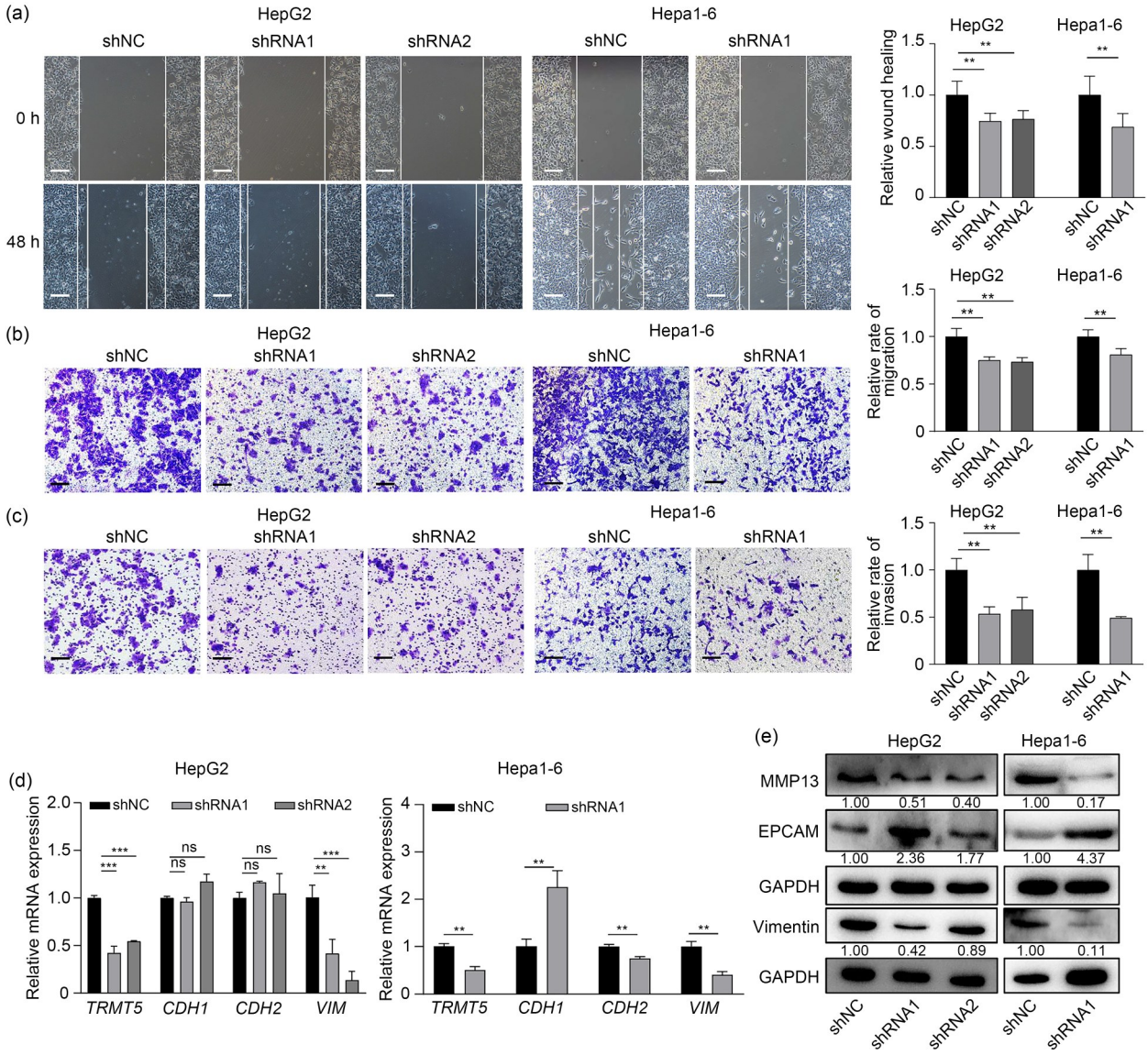

Fig. 4 Inhibition of both the migration and invasion of HCC cells in vitro by knockdown of *TRMT5*. (a) Wound healing assays in *TRMT5*-silenced cells relative to control cells. (b) Cell migration was evaluated by transwell assays. (c) Cell invasion was evaluated by transwell assays. (d) *TRMT5*, *CDH1*, *CDH2*, and *VIM* mRNA expression levels were analyzed by qRT-PCR and normalized to *GAPDH*. (e) The expression of MMP13, EPCAM, and Vimentin proteins was detected by western blot, with GAPDH as a loading control. The expression level of shNC cell was considered as 1, and the values for the silenced cells were expressed as ratio of the control cell values. $^{**}P<0.01$, $^{***}P<0.001$, ^{ns} no significant difference. Each experiment was repeated at least three times, and error bars represent the SEM. Scale bar=400 μm. tRNA: transfer RNA; TRMT5: tRNA methyltransferase 5; HCC: hepatocellular carcinoma; CDH1: epithelial E-cadherin; CDH2: N-cadherin; VIM: Vimentin; mRNA: messenger RNA; qRT-PCR: quantitative reverse transcription-polymerase chain reaction; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; MMP13: matrix metalloproteinase 13; EPCAM: epithelial cell adhesion molecule; SEM: standard error of the mean; shRNA: short hairpin RNA; shNC: shRNA negative control.

shNC group, and this difference increased over time. The mice in each group were then sacrificed, and the tumors were removed and weighed (Figs. 5b and 5c), which further confirmed that the shRNA1 cell group grew slower than the shNC group. Moreover, immunohistochemical analysis revealed that tumors of the shRNA1 group displayed a significantly reduced number of positive Ki67 cells (approximate 34% reduction; Fig. 5d). These data suggested that depressed TRMT5 inhibited HCC tumor growth in vivo. For the HCC metastasis assay, the stably transfected cell lines were injected into the lateral tail veins of nude mice.

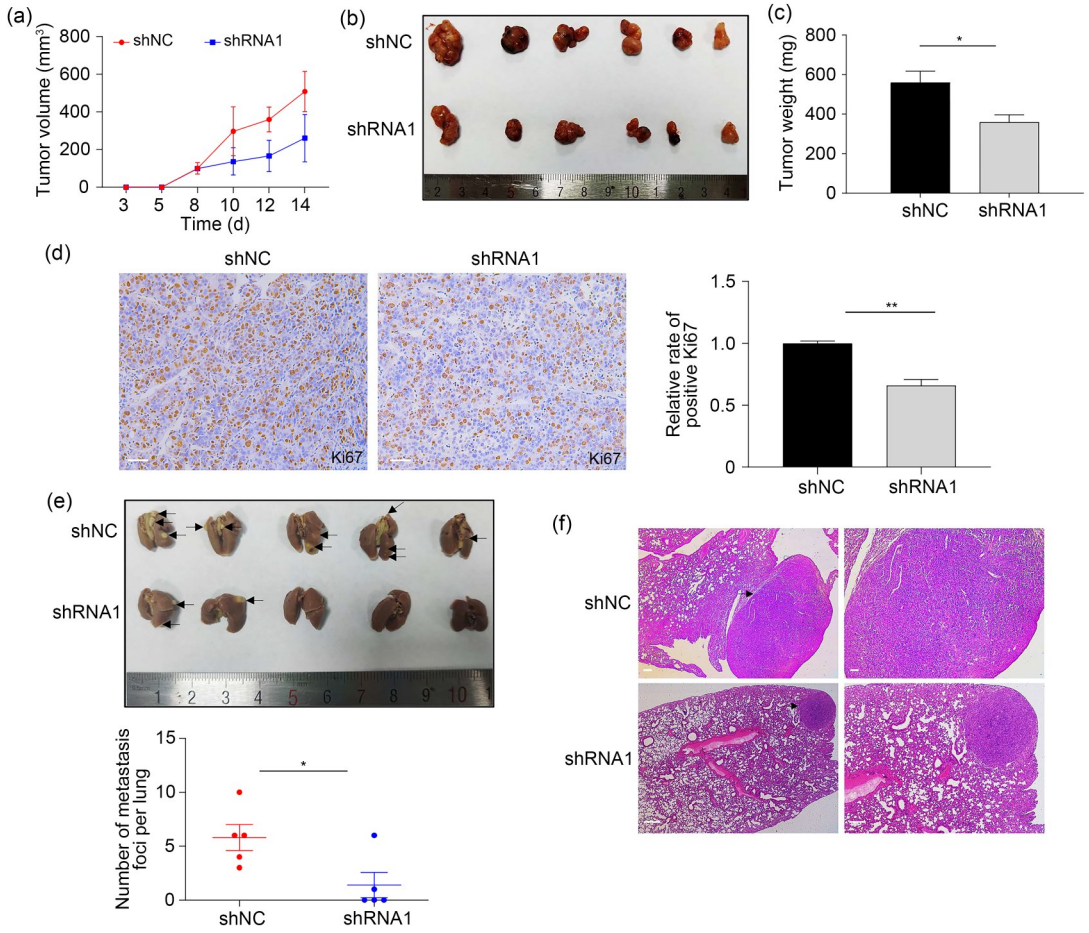

Fig. 5 HCC progression and metastasis suppressed by silencing *TRMT5* in vivo. To establish a murine HCC model, nude mice were subcutaneously implanted with Hepa1-6 cells. (a) The tumor growth was monitored daily. (b, c) Representative mice were sacrificed on Day 14 and the tumor weights were measured (n=6). (d) Cell proliferative activity was evaluated by Ki67 staining. Scale bar=50 μm. (e) Images of nude mice lungs. The black arrows represent metastasis foci. The number of metastatic foci in nude mice lungs was determined in two groups (n=5). (f) Representative HE staining images of metastatic nodules in the lungs of nude mice. The black arrows represent metastasis nodules. Scale bar=200 μm. * P<0.05, ** P<0.01. Data are expressed as mean±SEM. tRNA: transfer RNA; TRMT5: tRNA methyltransferase 5; HCC: hepatocellular carcinoma; HE: hematoxylin-eosin; SEM: standard error of the mean; shRNA: short hairpin RNA; shNC: shRNA negative control.

The mice were sacrificed after eight weeks, and the number of metastatic lung nodules was counted. As shown in Fig. 5e, the metastatic nodules significantly declined in the shRNA1 group compared to the shNC group. Besides, the hematoxylin-eosin (HE) staining results confirmed our findings (Fig. 5f). Taken together, *TRMT5* deficiency was confirmed to inhibit HCC tumorigenic progression in vivo.

2.6 Effect of *TRMT5* knockdown on the HIF-1 signaling pathway

In order to further investigate the underlying mechanism of *TRMT5* on HCC progression, we compared the global gene expression profiles between HepG2 cells transfected with either shRNA1 or shNC by RNA sequencing (RNA-seq) analysis. A cluster of genes differentially expressed between shRNA1 and shNC cells was identified based on fold change of >1.5 and adjusted P-value of <0.05 criteria. Among them, 329 genes were downregulated and 204 were upregulated in HepG2-shRNA1 cells (Figs. 6a and S3a). Furthermore, the assessment of biological function by Gene Ontology (GO) analysis indicated that various GO annotations were significantly different between the two groups related to biological processes, which included response to decreased oxygen levels, response to oxygen levels, response to hypoxia, and cellular component and molecular function (Figs. S3b–S3d). The Kyoto

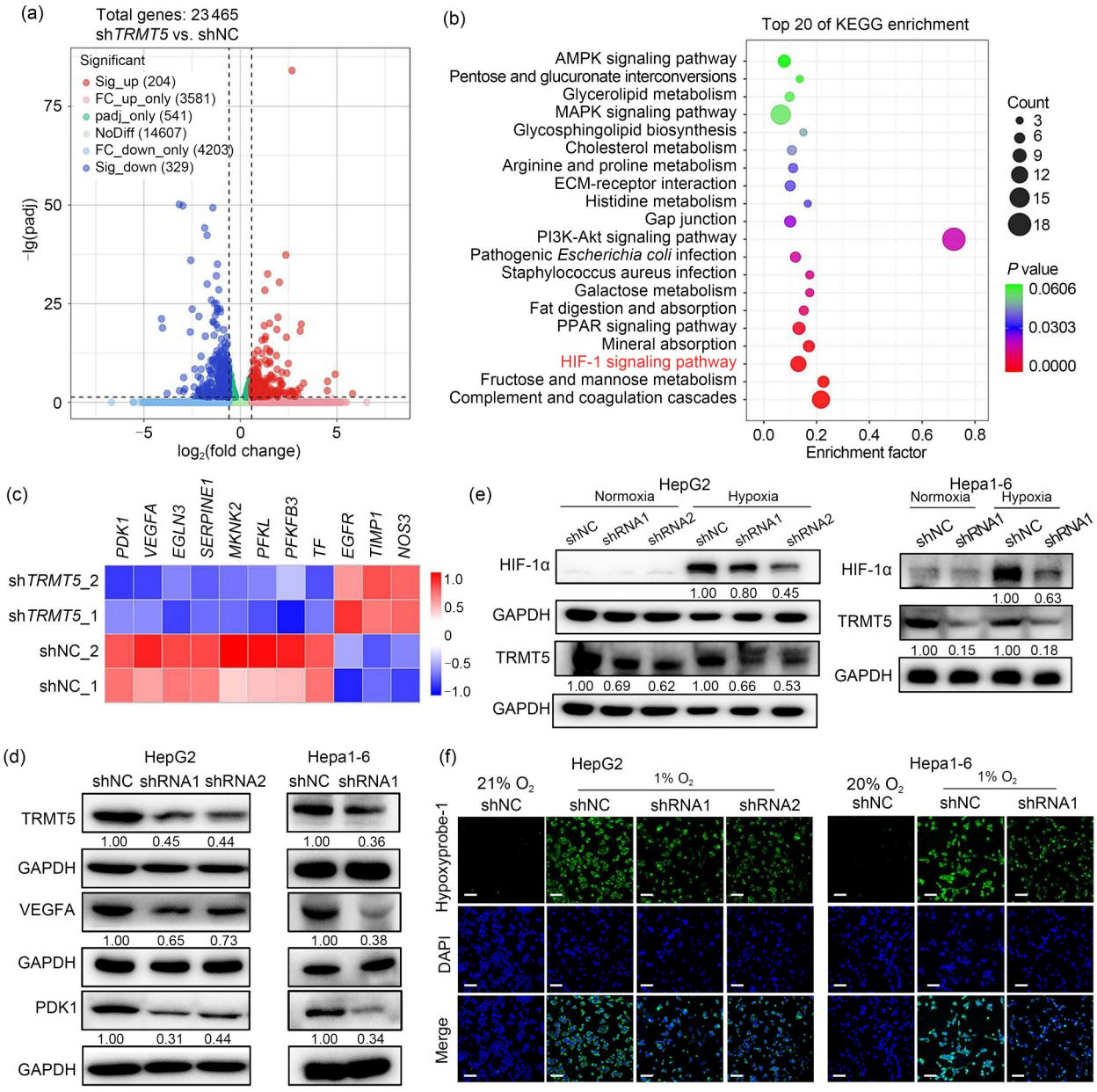

Fig. 6 Effects of knockdown of *TRMT5* on the HIF-1 signaling pathway through increasing the cellular oxygen content. **(a) Volcano plot of differentially expressed genes between shNC and shRNA1 HepG2 cells, where padj represents corrected *P* value. (b) The top 20 significant pathways by KEGG enrichment analysis of RNA-seq data in shRNA1 cells compared to shNC cells. (c) The heat map of dysregulated genes that were enriched in the HIF-1 signaling pathway. (d) Western blot examined the protein expression of PDK1 and VEGFA. (e) The expression levels of HIF-1α in normoxic and hypoxic conditions were detected by western blot, with GAPDH as a loading control. The expression level of shNC cell was considered as 1, and the values for the silenced cells were expressed as ratio of the control cell values. (f) Representative immunofluorescence images for detection of hypoxia using hypoxyprobe-1. Cells were treated with pimonidazole for 3 h under 1% O_2 and 21% O_2. Nuclei were stained with DAPI (blue). Scale bar=200 μm. tRNA: transfer RNA; TRMT5: tRNA methyltransferase 5; HIF-1: hypoxia-inducible factor 1; shRNA: short hairpin RNA; shNC: shRNA negative control; KEGG: Kyoto Encyclopedia of Genes and Genomes; RNA-seq: RNA sequencing; PDK1: pyruvate dehydrogenase kinase 1; VEGFA: vascular endothelial growth factor-A; DAPI: 4',6-diamidino-2-phenylindole; Sig: significance; FC: fold change; NoDiff: no difference; AMPK: adenosine 5'-monophosphate (AMP)-activated protein kinase; MAPK: mitogen-activated protein kinase; ECM: extracellular matrix; PI3K: phosphatidylinositide 3-kinase; Akt: protein kinase B; PPAR: peroxisome proliferators-activated receptor; GAPDH: glyceraldehyde-3-phosphate dehydrogenase (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).**

Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of differentially expressed genes was also performed. The top 20 KEGG pathways were listed in Fig. 6b. Among them, the hypoxia-inducible factor-1 (HIF-1) signaling pathway attracted our attention, which has been proven to regulate various biological processes, including angiogenesis, cellular metabolism, proliferation, and migration (Masoud and Li, 2015; Blatchley et al., 2019). The genes with dysregulated expression enriched in the HIF-1 signaling pathway were shown in Fig. 6c, and included pyruvate dehydrogenase kinase 1 (*PDK1*), vascular endothelial growth factor-A (*VEGFA*), Egl-9 family hypoxia inducible factor 3 (*EGLN3*), serine protease serpin protein E1 (*SERPINE1*), MAP kinase interacting serine/threonine kinase 2 (*MKNK2*), phosphofructokinase (*PFKL*), phosphofructokinase-2/fructose-2,6-bisphosphatase 3 (*PFKFB3*), transferrin (*TF*), epidermal growth factor receptor (*EGFR*), TIMP metallopeptidase inhibitor 1 (*TIMP1*), and nitric oxide synthase 3 (*NOS3*). Then, we confirmed the mRNA expression of some dysregulated genes by qRT-PCR, and the results were consistent with those by RNA sequencing (Fig. S3e). Moreover, we analyzed the correlation between these genes and *TRMT5* according to data from GEPIA. As shown in Fig. S4, *TRMT5* was closely correlated with *PDK1* and *VEGFA* expression (R=0.32, P=3.3×10^{-10} and R=0.33, P=9.4×10^{-11}, respectively; Pearson's correlation test). Therefore, we further examined the protein expression of *PDK1* and *VEGFA*, which were the target genes of HIF-1. Indeed, the protein expression levels of these two genes were downregulated in HepG2 and Hepa1-6 shRNA cells relative to control cells (Fig. 6d). These data suggested that knockdown of *TRMT5* might affect the HIF-1 signaling pathway and then inhibit HCC progression.

HIF-1 is a heterodimeric protein that consists of HIF-1α and HIF-1β (Vadlapatla et al., 2013). Since HIF-1α is a master transcription factor regulated in an oxygen-dependent manner, we wondered whether TRMT5 regulates HIF-1α. Therefore, we determined the protein expression of HIF-1α under hypoxia. The results showed that the level of HIF-1α was markedly increased under hypoxic conditions. In contrast, the HIF-1α expression level was significantly lower in knockdown cells compared to shNC cells under hypoxia (Fig. 6e), indicating that TRMT5 plays an essential role in HIF-1α stabilization. HIF-1α is oxygen-sensitive, and oxygen consumption is related to the stabilization of HIF-1α in hypoxia (Ellinghaus et al., 2013; Wheaton et al., 2014). According to our above findings, *TRMT5* deficiency reduced OCR, which could increase cellular oxygen viability. Therefore, we hypothesized that deficient TRMT5 might prevent HIF-1α stabilization by increasing cellular oxygen viability. HCC cells were preincubated with pimonidazole for 3 h, a standard hypoxia marker that forms adducts under hypoxic conditions (Urtasun et al., 1986; Doege et al., 2005). Then, the cells were visualized by immunofluorescence detection of pimonidazole using mouse monoclonal antibody. As anticipated, *TRMT5*-knockdown cells attenuated the accumulation of pimonidazole adducts with a lower fluorescent intensity of hypoxyprobe-1 compared to control cells (Figs. 6f and S5), which indicated that the cellular oxygen tension was increased in these *TRMT5*-knockdown cells. In conclusion, the deficiency of *TRMT5* reduced the OCR and increased the cellular oxygen content, which prevented HIF-1α stabilization.

2.7 HCC sensitivity to doxorubicin increased by inhibition of TRMT5

In order to investigate whether the inhibition of TRMT5 contributed to chemoresistance, we treated HCC cells with doxorubicin, a common antitumor drug and widely used chemotherapeutic agent in HCC treatment. First, we examined the viability of HCC cells treated with doxorubicin for 48 h using CCK8. As shown in Fig. 7a, the viability of *TRMT5* knockdown cells was lower compared to control cells. Besides, the long-term colony formation assays revealed that TRMT5 downregulation increased the sensitivity to doxorubicin in both HepG2 and Hepa1-6 cells (Fig. 7b). Since doxorubicin is an apoptosis inducer, we further detected the apoptosis rate of HCC cells treated with doxorubicin for 48 h by annexin V-fluorescein isothiocyanate (FITC) assay. As shown in Fig. 7c, the knockdown of *TRMT5* in HCC cells increased the sensitivity to doxorubicin (in HepG2 (shNC vs. shRNA1 vs. shRNA2): 1 μmol/L, (10.52±3.31)% vs. (28.14±2.79)% vs. (20.11±2.11)% annexin V$^+$, and 2 μmol/L, (21.44±3.92)% vs. (35.97±5.48)% vs. (29.32±2.38)% annexin V$^+$; in Hepa1-6 (shNC vs. shRNA1): 1 μmol/L, (17.05±0.89)% vs. (26.09±2.76)% annexin V$^+$, and 2 μmol/L, (36.82±2.67)% vs. (65.02±3.66)% annexin V$^+$; P<0.05 for both cells). The western blotting results revealed that doxorubicin reduced

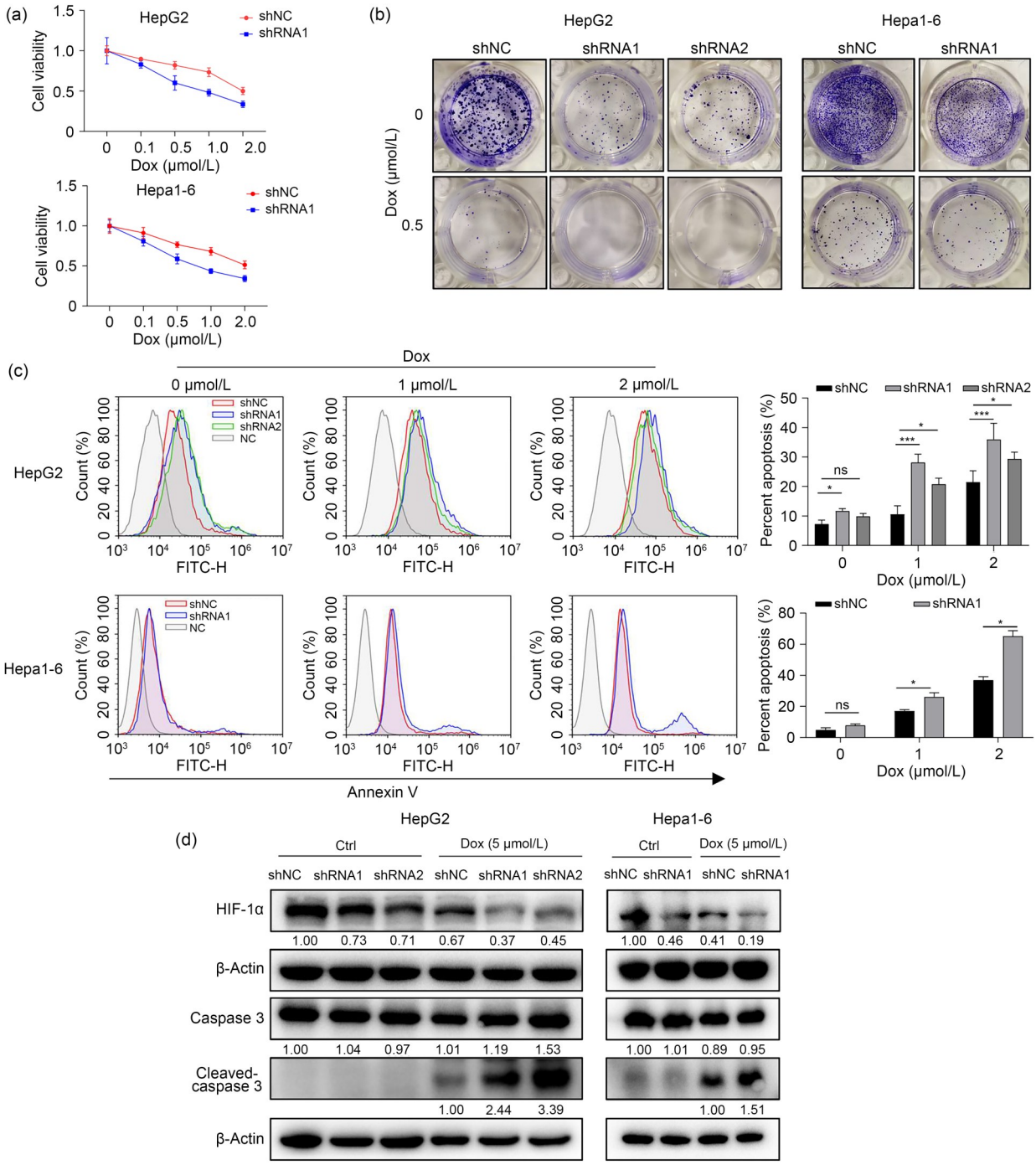

Fig. 7 Inhibition of TRMT5 sensitizes HCC to Dox by adjusting HIF-1α stabilization. (a) CCK8 assay was performed to determine the viability of HepG2 and Hepa1-6 shRNA1 cells and shNC cells treated with different concentrations of Dox. (b) Long-term colony formation assay for the indicated cells treated with Dox at 0.5 μmol/L for 48 h and then grown for 14 d. (c) The levels of cell apoptosis were examined by annexin V-FITC staining after treating cells with 0, 1, and 2 μmol/L Dox. NC represents negative control cells with annexin V-FITC unstained. (d) The HIF-1α stability and apoptosis levels were detected in silenced cells and control cells treated with 5 μmol/L Dox or without Dox (ctrl), with β-actin as a loading control. The expression level of shNC cell was considered as 1, and the values for the silenced cells were expressed as ratio of the control cell values. * P<0.05, *** P<0.001, ^{ns} no significant difference. Data are expressed as mean±SEM, n=3. tRNA: transfer RNA; TRMT5: tRNA methyltransferase 5; HCC: hepatocellular carcinoma; HIF-1α: hypoxia-inducible factor-1α; CCK8: cell counting kit-8; shRNA: short hairpin RNA; shNC: shRNA negative control; SEM: standard error of the mean; Dox: doxorubicin; FITC: fluorescein isothiocyanate; Ctrl: control.

the protein level of HIF-1α in a dose-dependent manner in HCC cells under hypoxia mimicked by $CoCl_2$ (Fig. S6). Furthermore, the dose-dependent upregulation of apoptosis marker cleaved-caspase 3 was observed. These data were consistent with previous research, in which doxorubicin inhibited the accumulation of HIF-1α in HepG2 under hypoxia (Bowyer et al., 2017). Of note, when we treated HCC cells with doxorubicin (5 μmol/L, 24 h), the HIF-1α level was further decreased, and cleaved-caspase 3 was increased in *TRMT5*-deficient cells compared to control cells (Fig. 7d). These data indicated that the inhibition of *TRMT5* sensitizes HCC to doxorubicin by adjusting HIF-1α.

3 Discussion

Recent advances have highlighted the altered epitranscriptomic modifications in tumorigenesis and progression, frequently correlated with poor prognosis (Barbieri and Kouzarides, 2020; Suzuki, 2021). TRMT5 is a highly conserved mitochondrial modified enzyme that catalyzes the m1G37 modification of mitochondrial tRNAs (Christian et al., 2013). Previous research has demonstrated that *TRMT5* mutation causes mitochondrial dysfunction and multiple respiratory chain deficiencies (Powell et al., 2015). In addition, Wing (2020) identified high TRMT5 expression in pancreatic ductal adenocarcinoma cancer (PDAC) tissue but not in surrounding normal fibroblasts. Herein, for the first time, we provided quantitative evidence that TRMT5 was involved with HCC progression. We conducted bioinformatics and clinical analyses to find that *TRMT5* was upregulated in HCC tissues compared to non-tumor tissues, and patients with high levels of *TRMT5* exhibited worse overall survival. Consistently, downregulated TRMT5 attenuated HCC proliferation and inhibited metastasis in vitro and in vivo. These results indicated that *TRMT5* might be a carcinogenesis candidate gene. Moreover, we identified a series of dysregulated pathways in *TRMT5*-deficient HCC cells, which could be the mechanisms linking *TRMT5* deficiency to delayed HCC progression. Among these, the HIF-1 signaling pathway is involved in multiple aspects of tumorigenesis and cancer progression, including proliferation, metabolism, angiogenesis, invasion, metastasis, and therapy resistance (Ke and Costa, 2006; Luo et al., 2014; Li et al., 2022). This information led to our hypothesis that the activation of HIF-1 signaling pathway is responsible for TRMT5-mediated HCC rapid progression.

HIF-1α plays an important role in sensing intratumoral oxygen tension, and its expression is tightly controlled by oxygen tension (Chun et al., 2002). Mitochondrion, the main oxygen sensor, plays a vital role in regulating HIF-1α stability in hypoxia and responses to hypoxic stress by sending retrograde signals to the nucleus that initiate adaptive metabolic responses and maintain the survival of HCC cells (Méndez-Blanco et al., 2019; Yang et al., 2020; Yan et al., 2021). Consistent with our hypothesis, when we suppressed TRMT5 in HCC cell lines, the cells showed mitochondrial defects including declined OCR and ECAR, ATP production, and lactate production. Notably, *TRMT5* deficiency impaired the stability of HIF-1α under hypoxic condition. Thus, we proposed that targeting *TRMT5* inactivates the HIF-1 signaling pathway depending on the attenuation of HIF-1α stabilization. Currently, metastatic HCC is commonly treated by doxorubicin, while drug resistance occurs during therapy, leading to poor prognosis (Wu et al., 2014). Based on the previous investigation, targeting HIF-1α is one of the useful strategies to improve the effectiveness of doxorubicin in a variety of tumors (Ward et al., 2013; Arnason and Harkness, 2015; Prieto-Domínguez et al., 2017). In our study, the knockdown of TRMT5 expression in HCC cells impaired the expression of HIF-1α, and substantially enhanced the sensitivity to doxorubicin. Consequently, *TRMT5* might be a potential target to overcome doxorubicin resistance in HCC.

In conclusion, our study demonstrated for the first time that TRMT5 is overexpressed in HCC tissues, and this phenomenon is correlated with poor prognosis. RNA-seq analysis revealed that TRMT5 promotes HIF-1α stabilization under hypoxia. Furthermore, targeting *TRMT5* in HCC cell lines enhanced their sensitivity to doxorubicin. Thus, our findings provide new insights into the roles of *TRMT5*, as well as clues for developing new therapeutic approaches for HCC.

Materials and methods

The detailed methods are provided in the electronic supplementary materials of this paper.

Availability of data and materials

RNA-seq data have been deposited to Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra), and the data can be accessed by the BioProject ID PRJNA 747936.

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Author contributions

Qiong ZHAO, Ruolang PAN, and Ye CHEN designed the experiments, and monitored the project progression and interpretation. Qiong ZHAO, Luwen ZHANG, Qiufen HE, Hui CHANG, Zhiqiang WANG, Hongcui CAO, Ying ZHOU, and Ruolang PAN participated in the study's data acquisition and analysis. Qiong ZHAO, Qiufen HE, Hui CHANG, Zhiqiang WANG, and Ying ZHOU participated in in vitro experiments. Qiong ZHAO, Luwen ZHANG, and Ruolang PAN completed animal experiments. Qiong ZHAO, Luwen ZHANG, and Hongcui CAO were responsible for histology, image analysis, and quantification. Qiong ZHAO prepared the initial draft of the manuscript. Ye CHEN made the final version of the manuscript. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Qiong ZHAO, Luwen ZHANG, Qiufen HE, Hui CHANG, Zhiqiang WANG, Hongcui CAO, Ying ZHOU, Ruolang PAN, and Ye CHEN declare that they have no conflict of interest.

The study protocol was approved by the Institutional Review Board of Zhejiang University and all animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee at Zhejiang University (application ID: 18021).

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Supplementary information

Figs. S1–S6; Materials and methods