Article

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Hypoxia-Induced Up-Regulation of ACSS2 Drives the PI3K/AKT/mTOR Pathway Through HMGCS1 to Enhance the Proliferation and Stemness of Pancreatic Cancer Cells

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Objective: Pancreatic cancer (PC) is a type of highly malignant tumor associated with poor prognosis, whose progression is driven by hypoxia in the tumor microenvironment. This study aims to explore the effects of hypoxia-induced upregulation of acetyl-CoA synthetase 2 (ACSS2) on the proliferation and stemness of PC cells and its potential molecular mechanism, so as to provide new targets and therapy strategies for the PC.

Materials and Methods: PC cells (PANC-1) were cultured under separate conditions: hypoxic and normoxic. Cell models of ACSS2 overexpression, ACSS2 knockdown and 3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS1) knockdown were constructed using transfection technique. Cell counting kit 8 (CCK-8) and clonal formation assay were used to assess cell viability, and cell stemness was analyzed by means of sphere-formation assay and detection of stem-related markers. A mouse tumor model was established by axilla injection of tumor cells, and tumor growth was evaluated by measuring the volume and weight of the isolated tumors. Relative mRNA and protein levels were analyzed by quantitative real-time polymerase chain reaction, Western blotting, and immunohistochemistry.

Results: Hypoxic condition upregulated the expression of ACSS2 in PC cells. CCK-8 and clonal formation assays showed that upregulation of ACSS2 promoted cell proliferation ($p < 0.001$), while knockdown of ACSS2 inhibited cell proliferation ($p < 0.001$). Sphere formation assay and stemness marker detection showed that ACSS2 upregulation could maintain cell stemness ($p < 0.001$), while knockdown could inhibit it ($p < 0.01$). Through mechanistic studies, we found that ACSS2 activated phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway through HMGCS1. Interference with HMGCS1 inhibited pathway activation caused by ACSS2 upregulation and hindered cell proliferation and stemness. *In vivo* experiments further demonstrated that ACSS2 accelerated PC xenograft tumor growth and promoted tumor stemness.

Conclusion: Hypoxia induces upregulation of ACSS2 and activates PI3K/AKT/mTOR pathway through HMGCS1, thereby enhancing the proliferation and stemness of PC cells. This finding offers a novel perspective for understanding the development mechanism of PC and highlights a potential molecular target for developing targeted therapeutic strategies.

Keywords: pancreatic cancer; ACSS2; HMGCS1; PI3K/AKT/mTOR

Introduction

Pancreatic cancer (PC) is a malignant tumor of the digestive system that have serious implications on human health. Statistical data indicate that it is one of the primary causes of cancer-related deaths globally, with only a 5–10% survival rate [1]. The main reasons for the poor prognosis include the difficulty of early diagnosis, metastasis and spread, the strong resistance to conventional therapies (such as surgery, chemotherapy and radiotherapy), and the immune suppression of the tumor microenvironment [2]. Surgical treatment is the preferred choice for most patients, but the postoperative recurrence rate is very high. Despite their control on tumor progression to a certain extent, chemotherapy and radiotherapy have limited efficacy, and the adverse reactions arising from these treatments are often hardly tolerable by the patients [3].

Tumor microenvironment (TME) plays an important function in the development of PC. Hypoxia in the TME is a hallmark of PC [4]. The formation of such anoxic microenvironment is caused by the rapidly proliferating cancer cells that raise local oxygen demand, and the abnormal tumor angiogenesis that excessively consumes the oxygen supply of other healthy cells [5,6]. Hypoxia can not only induce a series of adaptive changes in PC cells, such as epithelial-mesenchymal transformation and metabolic reprogramming, but also enhance the stemness of cancer cells, making them more capable of self-renewal, differentiation and metastasis, thereby leading to enhanced tumor aggressiveness and treatment resistance [7,8]. Hypoxia can regulate the expression of a sequence of downstream genes by activating hypoxia inducible factors (HIFs), and partake in biological processes of tumor cell proliferation, angiogenesis, metabolism and so on [9,10]. Studies have shown that inhibition of hypoxia-inducing factor exerts an antitumor effect on PC [11,12].

Acetyl-CoA synthetase 2 (ACSS2), which serves a vital function in cell metabolism, has received extensive attention in tumor research in recent years. ACSS2 can catalyze the synthesis of acetyl-CoA, which provides important substrates for tricarboxylic acid cycle, fatty acid and cholesterol synthesis, and protein acetylation modification [13]. In cancer cells, ACSS2 is often upregulated in response to hypoxia to maintain acetyl-CoA levels in biological processes at homeostatic state and promote cell survival and proliferation [14]. Studies have demonstrated that ACSS2 is highly expressed in various of cancers (such as renal cancer [15], colon cancer [16], etc.). In addition, some studies have found that metabolic stress in PC patients may lead to increased expression of ACSS2, which is believed to be involved in poor prognosis of patients [17,18].

The phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway is one of the essential pathways, and abnormal activation of this pathway is a common trait shared by various cancers [19]. In PC, activation of this pathway can promote the growth of tumor cells, resist cell apoptosis, and enhance migration and invasion [20]. It has been reported that hypoxia upregulates ACSS2 to enhance lipid metabolic reprogramming through 3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS1) and promote pancreatic neuroendocrine tumors [21]. However, the mechanism of ACSS2 on PC, especially how it affects the growth of PC cells under hypoxic microenvironment, remains rarely reported. This study aims to investigate whether hypoxia-induced upregulation of ACSS2 drives the PI3K/AKT/mTOR pathway through HMGCS1, thereby enhancing the proliferation and stemness of PC cells. An in-depth study of this molecular mechanism would contribute new insights into the potential therapeutic strategies for PC, which might hold promise for improving the prognosis of patients.

Materials and Methods

Cell Culture

Pancreatic cancer cell lines PANC-1 (SNL-100) were obtained from SUNNCELL (Wuhan, China). The cells were cultured in RPMI 1640 medium (R8758, Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin at 37 °C and 5% CO$_2$. The cells were divided into two groups: hypoxia and normoxia groups. The cells in the hypoxia group were maintained in a tri-gas mixture (8% O$_2$/5% CO$_2$/87% N$_2$, v/v), while the gas composition for the normoxia group—the control group—was 18% O$_2$/5% CO$_2$/77% N$_2$, v/v. The cells were identified by short tandem repeat (STR) prior to the experiment. A negative result in the mycoplasma testing indicates that the cells were not contaminated. PI3K activator (740Y-P, HY-P0175) and PI3K inhibitor (Wortmannin, HY-10197R) were purchased from MedChemExpress (Monmouth Junction, NJ, USA).

Table 1. Primer sequences.

Primer	Sequences (5′–3′)
ACSS2 forward	GGCCCATTCCTTCGGTACAA
ACSS2 reverse	GCTCATTGCCCTCCCAGTAA
HIF-1α forward	AGAGGTTGAGGGACGGAGAT
HIF-1α reverse	GCACCAAGCAGGTCATAGGT
β*-actin* forward	ACACTGTGCCCATCTACG
β*-actin* reverse	TGTCACGCACGATTTCC

ACSS2, acetyl-CoA synthetase 2; *HIF*, hypoxia inducible factor.

Real-Time Quantitative Polymerase Chain Reaction

Total RNA was extracted by the TRIzol solution (DP424) and reverse transcribed to cDNA by FastQuant cDNA First Strand Synthesis Kit (Degenomics) (KR116). Real-time quantitative polymerase chain reaction (qRT-PCR) was operated using SYBR Green Premix (FP205). All operations were according to the manufacturer's manual (TIANGEN, Beijing, China). The primer sequence is shown in Table 1. The statistical method used for quantification was $2^{-\Delta\Delta Ct}$ calculation, with β-actin used as the internal control.

Western Blotting

Western blotting was conducted in accordance with procedures as described previously [22]. The antibodies used in this study included: anti-HIF-1α (ER-1802-41, HUABIO), anti-ACSS2 (16087-1-AP, Proteintech), anti-HMGCS1 (17643-1-AP, Proteintech), anti-Oct4 (EM100306, HUABIO), anti-Sox2 (11064-1-AP, Proteintech), anti-Nanog (14298-1-AP, Proteintech), anti-AKT (10176-2-AP, Proteintech), anti-p-AKT (80455-1-RR, Proteintech), anti-mTOR (81670-1-RR, Proteintech), anti-p-mTOR (28879-1-AP, Proteintech), anti-PI3K (67071-1-lg, Proteintech), anti-p-PI3K (HA721672, HUABIO), anti-β-actin (TA-09, ZSGB-BIO), Goat Anti-Mouse IgG H&L (ZB-2305, ZSGB-BIO), and Goat Anti-Rabbit IgG H&L (ZB-2306, ZSGB-BIO). These antibodies were used at a dilution ratio of 1:1000.

Transfection

ACSS2 overexpression and knockdown plasmid and HMGCS1 knockdown plasmid were acquired from Sangon Biotech Co., Ltd. (Shanghai, China). The overexpression and knockdown sequences are shown in the **Supplemen-**

tary Materials. In this study, Overexpression (OE) was used to indicate overexpression, shRNA knockdown (sh) was used to indicate gene knockdown, and negative control (NC) was used as the empty control. Group names are defined in detail in **Supplementary Table**. Cultured cells that had reached approximately 70% confluence were transfected with plasmid vectors. The transfection protocol was based on the recommendations by the manufacturer (Lipofectamine™ 3000, L3000001, Thermo Fisher, Waltham, MA, USA). The medium containing 5 µg/mL puromycin (A11138, Invitrogen, Carlsbad, CA, USA) was replaced 48 hours later to select for stably transfected cells for subsequent experiments. Effective integration of transfection was detected by means of qRT-PCR and Western blotting.

Immunofluorescence Staining

Cells seeded on the culture slides were fixed using 4% paraformaldehyde for a duration of 20 minutes and subsequently permeabilized with 0.1% Triton X-100 for another 20 minutes. Blocking was carried out with 2% BSA for 60 minutes at 37 °C. Afterward, the samples were incubated with ACSS2 antibodies (ET1702-2, HUABIO) for 2 hours at 37 °C, followed by a 60-minute incubation with secondary antibodies (GB21303, Servicebio) at room temperature. DAPI as a counterstain was used for nuclei staining. Lastly, the samples were analyzed using a fluorescence microscope (CKX53, OLYMPUS).

Cell Counting Kit-8 (CCK-8) Assay

The digested cells were re-suspended in medium and inoculated into 96-well plates with a density of 10,000 cells/well. CCK-8 solution (10 µL) was added for reaction after 24, 48 and 72 hours, respectively. Absorbance value (OD value) was detected at 450 nm, and the cell viability curve was plotted using the OD values recorded.

Colony Formation Assay

After transfection for 48 hours, PANC-1 cell in each group were prepared into 90 cells/mL, and 1 mL from each group was inoculated and cultured in 24-well plates. After 21 days, the medium was discarded, rinsed and fixed, and crystal purple staining was performed. The colony formation of cells was observed under the microscope, and colony number was quantified for each group.

Spheroid Formation Assay

The cells at logarithmic growth stage were collected and resuspended with serum-free suspension medium. The cells were inoculated at the density of 500 cells/well, 1000 cells/well and 2500 cells/well in 24-well plates with low adhesion, and cultured continuously for 7 days to observe the growth of cell clusters. After 7 days, the spheroids collected in a centrifuge tube were subjected to centrifugation (1000 rpm, 5 min). Upon removal of the supernatant, 1 mL pancrease (T1300, Solarbio, Beijing, China) was added for digestion for 5 minutes, and single-cell suspension was prepared by dispersing them, centrifuged to remove the pancrease, washed with 5 mL phosphate-buffered saline (PBS) twice, then resuspended in medium, counted and inoculated into 24-well low-adhesion plates at appropriate density. After 1 week, the cells were observed to form into pellets. After two times of culture in low-viscosity culture dishes, the cells could be cultured into ordinary dishes. A small amount of cells would stick to the wall, but their shape would not change, and the cells could be blown properly. The experiment went through four cycles of pellet formation, digestion, preparation of single cells, and pellet formation; eventually, the cell pellets obtained at the last time were regarded as comprising tumor cells equipped with self-renewal ability.

Tumor Formation Experiment in Nude Mice

Thirty male BALB/c nude mice (18–20 g each) were kept in a temperature range of 22–24 °C, humidity between 40% and 60%, and a 12-hour light/dark cycle. The animals were fed with standard food. All animal experiments were in compliance with institutional ethical guidelines approved by the Experimental Animal Welfare Ethics Committee (Approval No. ZFY20250108). PANC-1 cells with stable transfection of ACSS2 overexpression vectors, ACSS2 overexpression vectors with HMGCS1-targeting knockdown vectors, and control lentiviral vectors were prepared as single-cell suspensions. Following adjustment of cell density to 7×10^6 cells/mL, a 100 µL aliquot of cell suspension was subcutaneously injected into the mice. Tumor dimensions were monitored every 3 days using vernier calipers. Tumor volume was calculated by the formula: Volume = (length × width^2)/2. After 21 days, the mice were euthanized through gradual CO_2 asphyxiation in a sealed chamber (5% CO_2 concentration increment per minute), with death confirmation based on respiratory arrest, cardiac cessation, and ocular pallor. Subsequently, xenograft tumors were excised for histological processing.

Immunohistochemistry

For immunohistochemistry, after dewaxing and hydration, the sections were subjected to antigen retrieval, incubated with 3% hydrogen peroxide solution for 10 minutes, and blocked with 2% Bovine Serum Albumin (BSA) for 60 minutes. The sections were then incubated with primary antibodies at 4 °C overnight after being washed with Tween-20 phosphate-buffered brine (PBST) for 3 times. Incubation with secondary antibodies was performed for 10 minutes at room temperature. The primary antibodies included anti-Nanog (ET1610-2), anti-OCT4 (ET1612-20) and anti-Sox2 (HA721155), all of which were acquired from HUABIO (Wuhan, China) and were diluted 1:1000. The secondary antibody Horseradish Peroxidase (HRP)-labeled goat anti-rabbit Immunoglobulin G

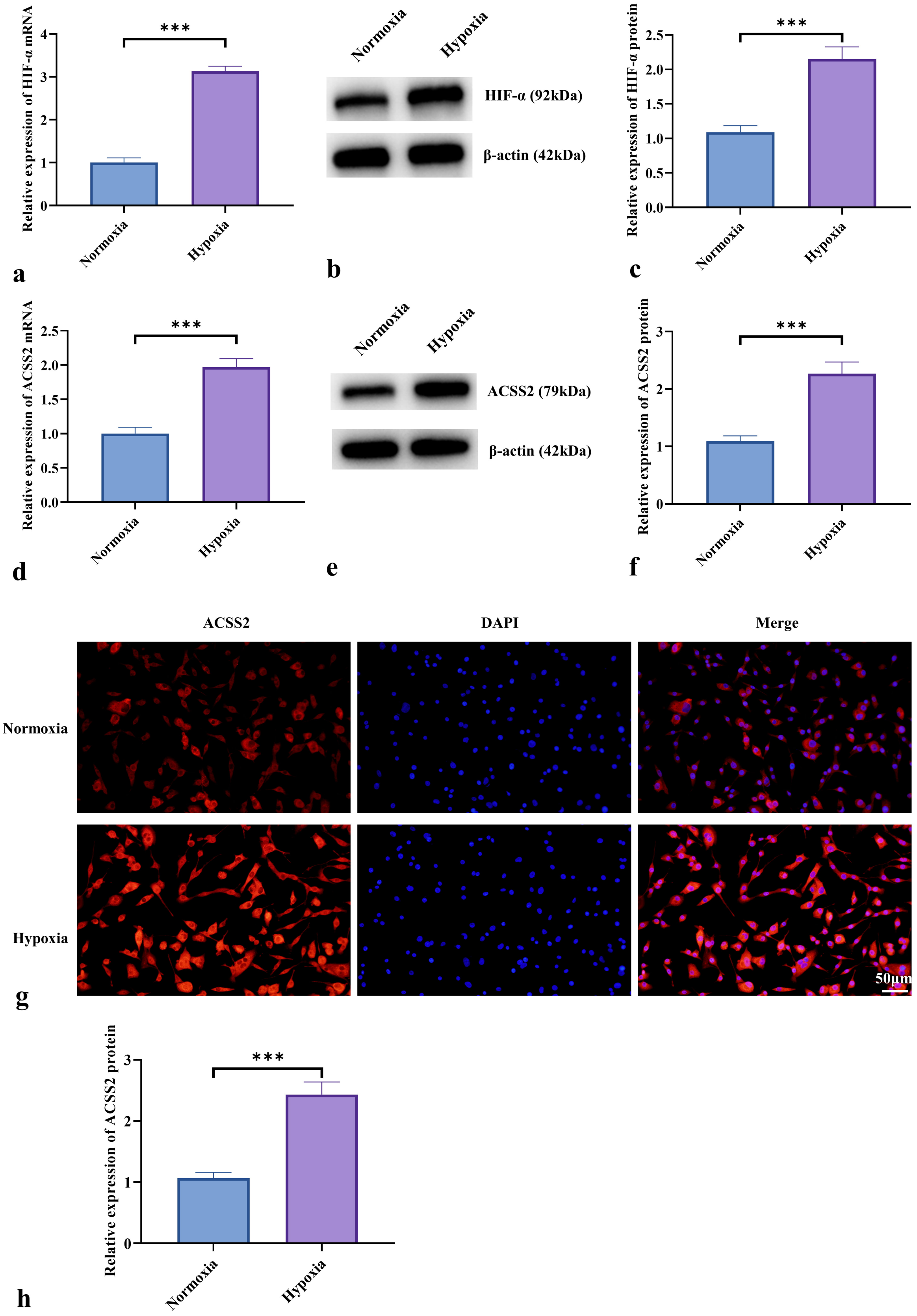

Fig. 1. Hypoxia upregulates the expression of ACSS2 in PC cells. (a) The relative mRNA levels of HIF-1α in PANC-1 cells by qRT-PCR. (b,c) Western blotting analysis of HIF-1α in PANC-1 cells. (d) The relative mRNA levels of ACSS2 in PANC-1 cells by qRT-PCR. (e,f) Western blotting analysis of ACSS2 in PANC-1 cells. (g,h) Immunofluorescence analysis of ACSS2 in PANC-1 cells. $n = 6$; ***$p < 0.001$. ACSS2, acetyl-CoA synthetase 2; PC, pancreatic cancer; HIF, hypoxia inducible factor.

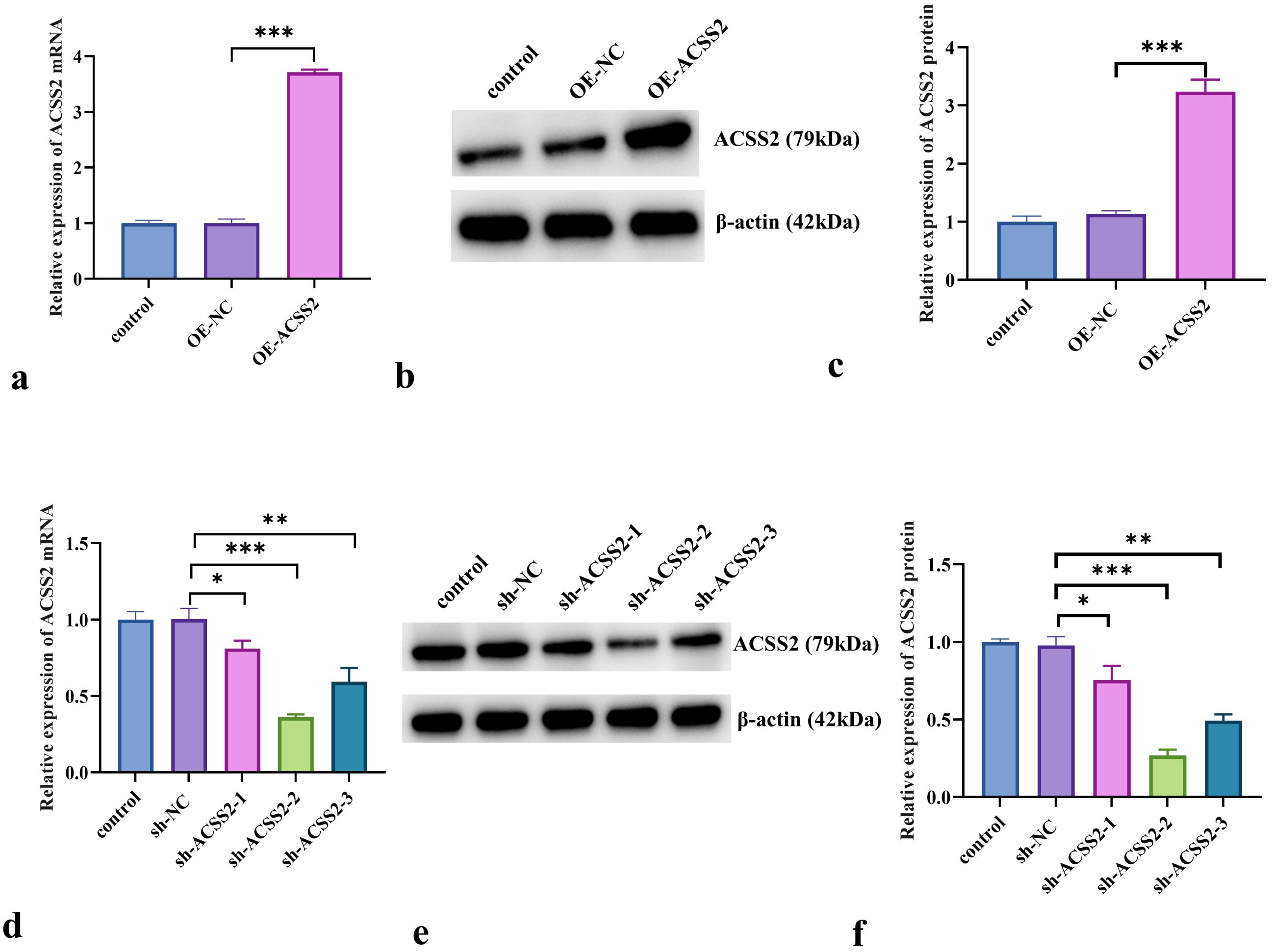

Fig. 2. Transfection efficiency in PC cells. (a–c) The overexpression of ACSS2 was detected by means of qRT-PCR and Western blotting. (d–f) The knockdown of ACSS2 was detected by means of qRT-PCR and Western blotting. $n = 6$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

(IgG) (GB23303) was purchased from Servicebio (Wuhan, China) and were diluted 1:1000. Finally, Diaminobenzidine (DAB) (PA140212, TIANGEN) was used for development, observation and photography under a microscope.

Statistical Analyses

Statistical analysis was carried out with GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). The data are presented as mean ± standard deviation. For comparisons between two groups, a *t*-test was applied, while comparisons across multiple groups were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A difference with $p < 0.05$ was regarded as statistically significant.

Results

Hypoxia Upregulates the Expression of ACSS2 in PC Cells

In order to investigate the role of hypoxia on the expression of ACSS2 in PC cells, PANC-1 cells were cultured under normoxia and hypoxia. Western blotting and qRT-PCR were used to detect HIF-1α and ACSS2 protein and mRNA levels. The results verified that HIF-1α was significantly upregulated after hypoxia treatment, indicating that the hypoxia model of PANC-1 cells was successfully constructed ($p < 0.001$) (Fig. 1a–c). qRT-PCR results showed that *ACSS2* mRNA expression was elevated after hypoxia treatment ($p < 0.001$) (Fig. 1d). Western blotting results showed that ACSS2 protein was increased in the hypoxia group ($p < 0.001$) (Fig. 1e,f). Further immunofluorescence results verified that the expression of ACSS2 under hypoxia condition was higher than that under normoxia condition ($p < 0.001$) (Fig. 1g,h).

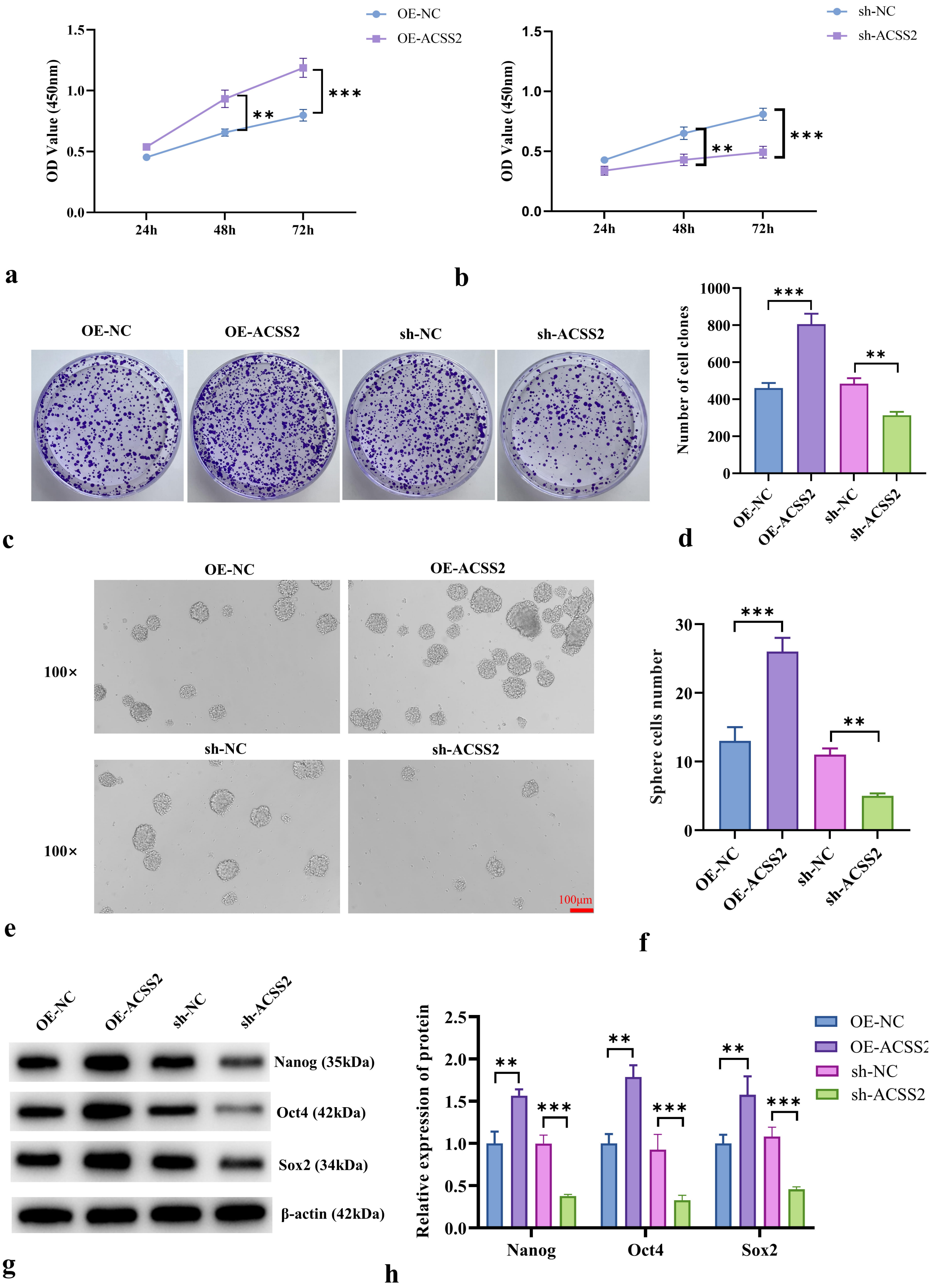

Fig. 3. Upregulation of ACSS2 promotes the proliferation and stemness of PC cells. (a,b) The viability of PC cells was evaluated by CCK-8 assay. (c,d) The proliferative ability of PC cells was tested by colony formation experiment. (e,f) The stemness of PC cells were examined by cell sphere formation experiment. (g,h) The expression of proteins associated with the stemness was detected by means of Western blotting. *n* = 6; $**p < 0.01$, $***p < 0.001$. CCK-8, cell counting kit 8; OE, overexpression; NC, negative control; sh, shRNA knockdown.

Upregulation of ACSS2 Promotes the Proliferation and Stemness of PC Cells

In order to explore the effect of upregulation of ACSS2 on the PC cells, ACSS2 overexpression and knockdown plasmids (OE-ACSS2 and sh-ACSS2) and negative control plasmid (OE-NC and sh-NC) were transfected into PANC-1 cells. Transfection efficiency was detected by means of qRT-PCR and Western blotting. The sh-ACSS2-2 group which had highest overall efficiency was selected for subsequent experiments ($p < 0.001$) (Fig. 2a–f). The cell proliferation ability was detected by CCK-8 assay. The results showed that the absorbance value of the cells in the OE-ACSS2 group was significantly increased after 48-hour ($p < 0.01$) and 72-hour ($p < 0.001$) culture (Fig. 3a). The absorbance of cells in the sh-ACSS2 group was significantly lower than the sh-NC group after 48-hour ($p < 0.01$) and 72-hour ($p < 0.001$) culture (Fig. 3b). Colony formation assay experiment further verifies the ACSS2 effects on cell proliferation. The colony formation ability of the OE-ACSS2 group was stronger than the OE-NC group ($p < 0.001$). The colony formation ability of the sh-ACSS2 group was lower than the sh-NC group ($p < 0.01$) (Fig. 3c,d). In the cell spheroid formation experiment, the number of cell sphere formation was increased in the OE-ACSS2 group ($p < 0.001$), while the number of cell spheroid formation was decreased in the sh-ACSS2 group ($p < 0.01$) (Fig. 3e,f). Further detection of stemness-related protein expression was conducted through Western blotting. In PANC-1 cells, expression levels of Oct4, Sox2 and Nanog ($p < 0.01$) in the OE-ACSS2 group were significantly elevated compared to the OE-NC group, while in the sh-ACSS2 group, the expression levels were significantly decreased ($p < 0.001$) (Fig. 3g,h).

ACSS2 Promotes the Proliferation and Stemness of PC Cells Through HMGCS1

To explore the role of HMGCS1 in the regulation of PC cells by ACSS2, interference with HMGCS1 expression in ACSS2-overexpressing cells was conducted in PANC-1 cells. First, the expression of ACSS2 ($p < 0.001$) and HMGCS1 ($p < 0.001$) was verified through Western blotting analysis (Fig. 4a–c). The cell viability in OE-ACSS2+sh-HMGCS1 was reduced as compared to that in the OE-ACSS2+sh-NC group ($p < 0.001$) (Fig. 4d), aligning with the results obtained in the colony formation assay. As shown in Fig. 4e,f, the proliferative ability in OE-ACSS2+sh-HMGCS1 group was reduced compared to the OE-ACSS2+sh-NC group ($p < 0.001$). According to the cell spheroid formation assay, the spheroids formed in the OE-ACSS2+sh-HMGCS1 group was obviously fewer than those in the OE-ACSS2+sh-NC group ($p < 0.001$) (Fig. 4g,h). In PANC-1 cells, expression levels of stemness-associated proteins Oct4 ($p < 0.01$), Sox2 ($p < 0.05$) and Nanog ($p < 0.01$) were lower in the OE-ACSS2+sh-HMGCS1 group than in the OE-ACSS2+sh-NC group (Fig. 4i,j).

ACSS2 Activates the PI3K/AKT/mTOR Pathway Through HMGCS1

In order to explore whether ACSS2 activates the PI3K/AKT/mTOR pathway through HMGCS1, relevant experiments were conducted in PANC-1 cells. Western blotting analysis suggested that compared to the OE-NC+sh-NC group, the OE-ACSS2+sh-NC group had significantly higher expression of p-PI3K/PI3K, p-AKT/AKT and p-mTOR/mTOR ($p < 0.001$), indicating that overexpression of ACSS2 could trigger the PI3K/AKT/mTOR pathway. In the OE-ACSS2+sh-HMGCS1 group, the expression of the aforementioned pathway protein was significantly decreased ($p < 0.001$), suggesting that interference with HMGCS1 expression can inhibit the activation of this pathway induced by ACSS2 overexpression (Fig. 5a,b). We further treated ACSS2-overexpressing cells with a PI3K inhibitor (Wortmannin, 50 nM, 4 hours) and the results exhibited a significant reduction in phosphorylation of the pathway protein ($p < 0.001$). The cells in the OE-ACSS2+sh-HMGCS1 group treated with PI3K activator (740Y-P, 30 µM, 4 hours) showed significantly elevated levels of phosphorylation of these proteins, indicating that 740Y-P reversed the downregulation of phosphorylation after HMGCS1 knockdown ($p < 0.001$) (Fig. 5c,d).

ACSS2 Promotes Pancreatic Cancer Progression Through HMGCS1 in Vivo

To further validate the findings from the cellular experiments, we constructed a tumor model of PC in nude mice and explored the effects of ACSS2 upregulation on PC cells through HMGCS1 *in vivo*. The results indicated that tumor volume and weight was significantly augmented following ACSS2 overexpression ($p < 0.01$). However, in the OE-ACSS2+sh-HMGCS1 group, tumor growth was significantly inhibited compared to OE-ACSS2+sh-NC group ($p < 0.001$) (Fig. 6a–c). An immunohistochemical analysis of stemness-related gene expression level in tumor tissues revealed that the positive expression of stem-related genes was remarkably increased than the control group after ACSS2 over-expressed ($p < 0.001$). However, the positive expression of these proteins was noticeably decreased in the OE-ACSS2+sh-HMGCS1 group ($p < 0.001$) (Fig. 6d,e), indicating that upregulation of ACSS2 can promote tumor growth, while interference with HMGCS1 expression can inhibit tumor growth.

Discussion

At present, despite the remarkable strides achieved in PC treatment, the incidence still shows no signs of decrease. While PC remains the leading cause of cancer-related death, investigating molecular mechanisms underlying PC pro-

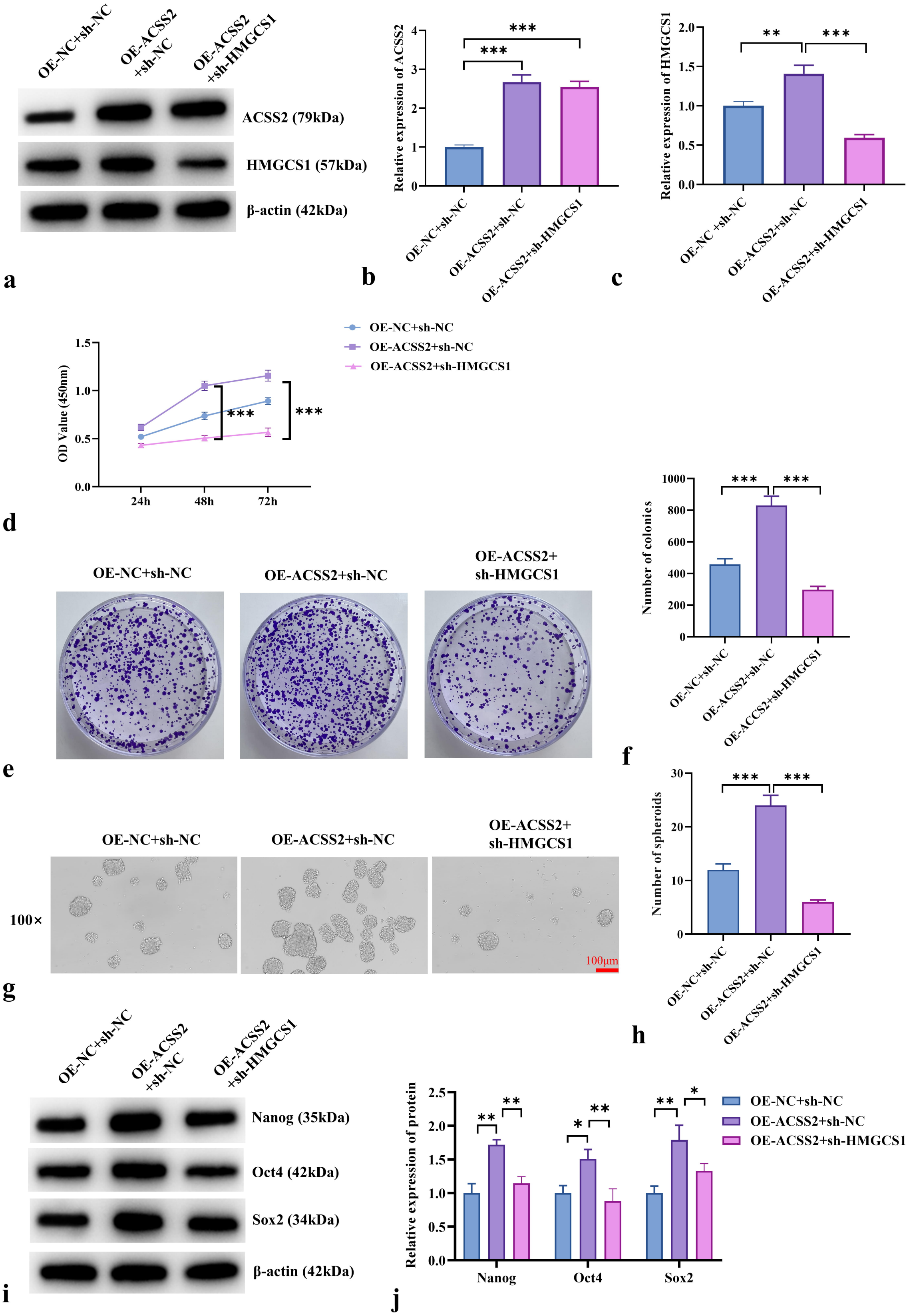

Fig. 4. ACSS2 promotes the proliferation and stemness of PC cells through HMGCS1. (a–c) The expression of ACSS2 and HMGCS1 was analyzed by means of Western blotting. (d) Cell viability was tested using CCK-8 assay. (e,f) The proliferative ability was tested via the colony formation experiment. (g,h) The cell stemness were examined via the sphere formation assay. (i,j) Western blotting was performed for the detection of stemness-related proteins like Oct4, Sox2 and Nanog. $n = 6$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. HMGCS1, 3-hydroxy-3-methylglutaryl-CoA synthase 1; CCK-8, Cell counting kit 8.

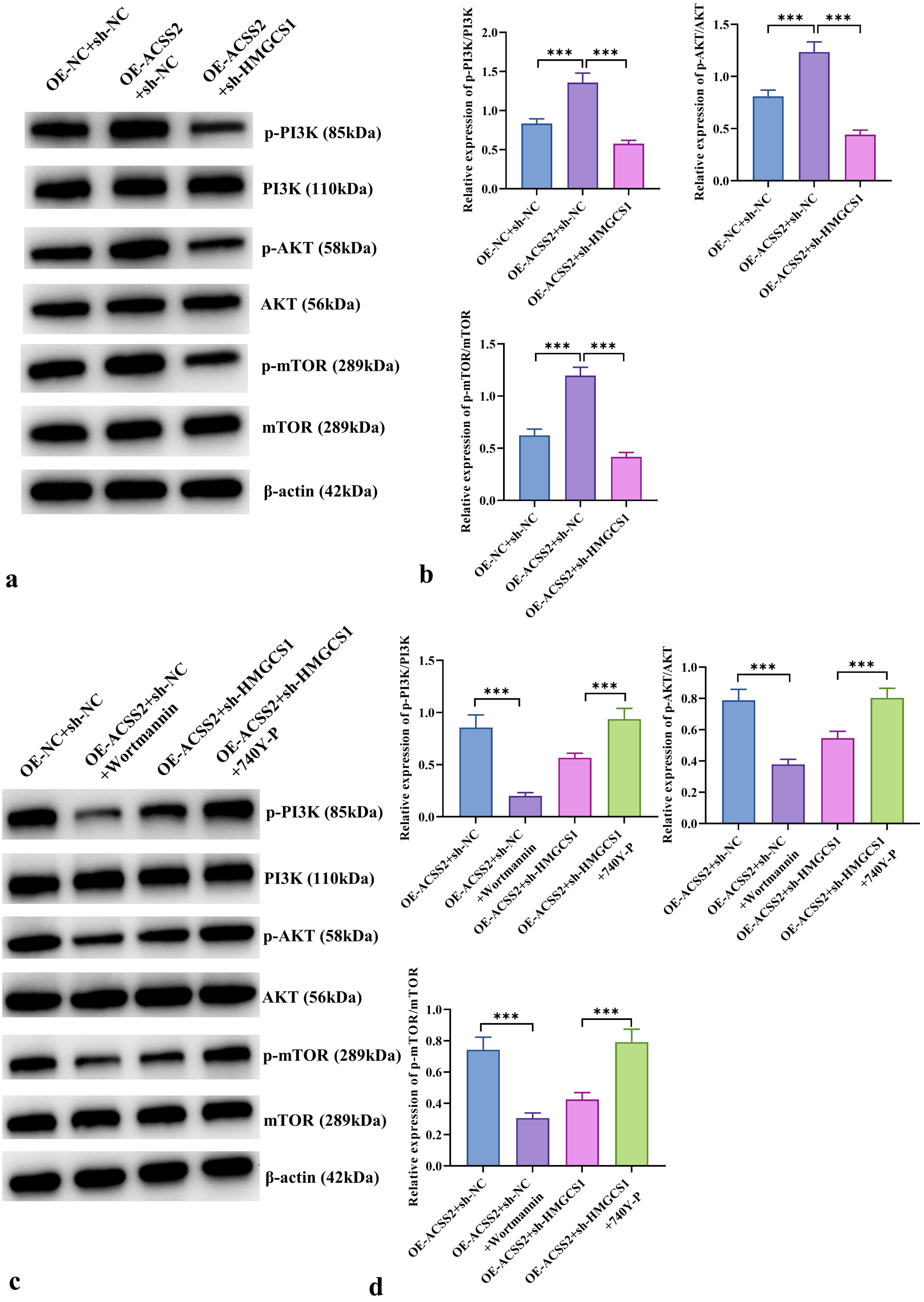

Fig. 5. ACSS2 activates the PI3K/AKT/mTOR pathway through HMGCS1. (a,b) The relative expression levels of p-PI3K/PI3K, p-AKT/AKT and p-mTOR/mTOR were detected by Western blotting after ACSS2 overexpression and HMGCS1 knockdown. (c,d) Western blotting analysis was conducted to quantify the expression levels of p-PI3K/PI3K, p-AKT/AKT and p-mTOR/mTOR after PI3K activator (740Y-P) and PI3K inhibitor (Wortmannin) treatment. *n* = 6; ***$p < 0.001$. PI3K, phosphatidylinositol-3-kinase; AKT, protein kinase B; mTOR, mammalian target of rapamycin.

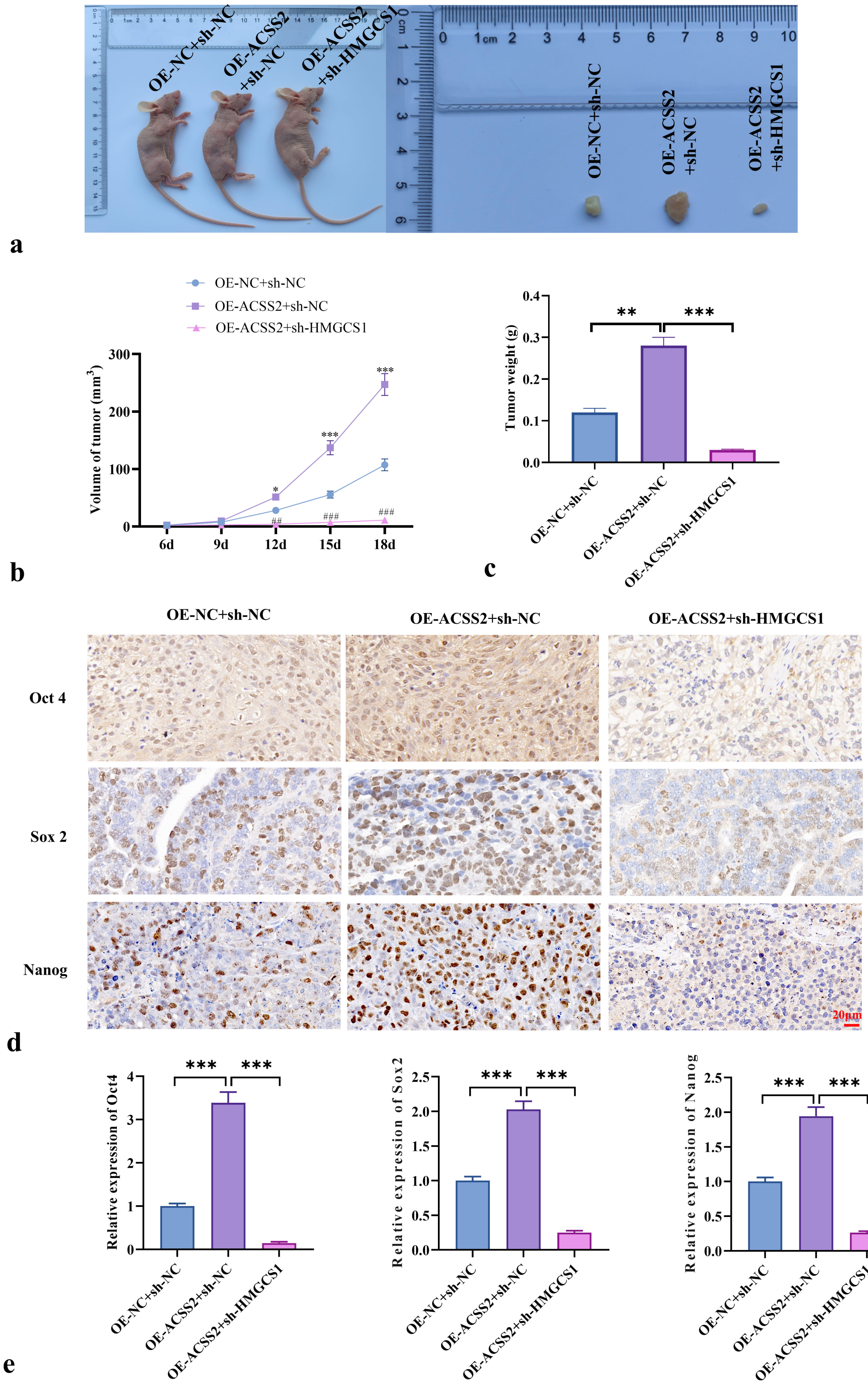

Fig. 6. ACSS2 promotes PC progression through HMGCS1 *in vivo*. (a) Images of isolated PC tumor after ACSS2 overexpression and HMGCS1 knockdown. (b,c) Changes of tumor weight and volume after ACSS2 overexpression and HMGCS1 knockdown. (d,e) Immunohistochemical analysis of expression levels of stemness-related markers in different experimental groups. $n = 5$; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$.

gression, exploring more effective treatment methods, and enhancing therapeutic effects have become the major pillars of approaches that we can undertake to address this prevalent challenge [23].

This study found that hypoxia can upregulate the expression of ACSS2 in PC cells, and hypoxia microenvironment is an important feature of PC development [24]. The upregulation of ACSS2 expression in tumor cells may be an adaptive metabolic regulation mechanism, and the upregulation of ACSS2 may divert the cellular energy utilization pathway to alternative substances such as acetate to maintain the energy metabolism balance of cells [25]. At the same time, acetyl-coA is also an important substrate for substance synthesis and metabolism, which provides the necessary material basis for the proliferation and survival of PC cells. Through the experiments of overexpression and knockdown of ACSS2, we confirmed that the upregulation of ACSS2 can enhance the proliferative ability of PC cells, promote spheroid formation, and increase the expression of stemness-regulating related proteins. Previous studies have found that overexpression of Spindle component 25 (SPC25) promoted tumor cell growth, resistance to chemotherapy drugs *in vitro* and spheroid formation ability. *In vivo* and *in vitro* experiments showed that SPC25 expression was correlated with expression of stemness-related proteins. This further supports the role of SPC25 in promoting tumor growth and regulating stemness [26]. Thus, our experimental results suggest that ACSS2 can promote tumor growth and promote stemness. In addition, study has found that knocking down ACSS2 inhibits the proliferation of PC cells and reduces their ability to survive in acidic environments [18]. This suggests that ACSS2 contributes significantly to the malignant biological behavior of PC.

Through co-immunoprecipitation experiments, it has been found that ACSS2 can interact with HMGCS1, and the interaction between ACSS2 and HMGCS1 enhances lipid metabolism in pancreatic neuroendocrine tumors [21]. HMGCS1 is a key enzyme which catalyzes acetyl-CoA in the cholesterol synthesis pathway. HMGCS1 can promote the growth and xerogenicity of gastric cancer cells [27]. We hypothesize that hypoxic microenvironment leads to upregulation of ACSS2 in PC, and ACSS2 further activates HMGCS1 to promote the growth of PC cells.

The PI3K/AKT/mTOR signaling pathway is among the signaling pathways dysregulated in cancer [28]. In this research, we found that overexpression of ACSS2 can activate the PI3K/AKT/mTOR pathway. Further studies showed that the activation was significantly inhibited after HMGCS1 expression was knocked down, suggesting that HMGCS1 played a key mediating role. According to a previous study, circHMGCS1 was found to regulate the expression of insulin-like growth factor 2 (IGF2) and insulin-like growth factor 1 receptor (IGF1R) by sponging miR-503-5p, affecting the downstream PI3K-AKT pathway, thereby regulating the proliferation and glutamine breakdown of hepatoblastoma cells [29]. This implies that circHMGCS1 was associated with PI3K-AKT axis. Furthermore, PI3K/AKT activation can drive cell cycle progression through a variety of pathways [30], and it has also been found that activation of AKT/mTOR promotes the stemness of cancer stem cells [31,32]. Finally, we confirmed that ACSS2 activates the PI3K/AKT/mTOR signaling pathway through HMGCS1 *in vivo*, and a complex and fine regulatory network is formed, which jointly promotes the growth of PC.

Although this study has made some achievements, there are still some limitations. In terms of cell lines, only PANC-1 cell line was selected. Despite the broad usage in PC research, the PANC-1 cell is not an ideal cell type that can fully represent other subtypes due to its distinctive gene mutation profiles and metabolic phenotypes. In fact, different PC cell lines have significant variations in metabolic pathway activities, signal transduction characteristics, etc. [33]. Future studies can include more PC cell types to more comprehensively explore the mechanism of action of ACSS2, HMGCS1 and PI3K/AKT/mTOR pathways in PC, and enhance generalizability of the study results. Furthermore, integrating multi-omics analyses (such as transcriptomics and metabolomics) helps contribute to a more comprehensive understanding of the mechanisms of related biological processes in the context of PC heterogeneity.

Conclusion

In summary, this study presents a novel mechanism through which ACSS2 augments the proliferation and stemness of PC cells by activating the PI3K/AKT/mTOR pathway, with HMGCS1 playing the mediating role. The revelation of this mechanism provides a considerable theoretical basis for the in-depth understanding of the biological behavior of PC, and lays concrete theoretical groundwork for enabling precise treatment of PC, achievable with identification of potential therapeutic targets and formulation of intervention strategies.

Availability of Data and Materials

The data and materials that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

ZZ: Literature search, experimental studies, statistical analysis, manuscript preparation. GL: Design, data analysis, manuscript preparation. BZ: Data analysis, manuscript editing and review. YZ: Literature search, experimental studies, manuscript editing and review. All authors contributed to important editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All animal experiments were conducted in accordance with the institutional ethical guidelines and were approved by the Animal Experimental Ethical Inspection of the Laboratory Animal Centre, South Zhejiang Institute of Radiation Medicine and Nuclear Technology Applications (Approval No. ZFY20250108).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202537197.93.

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