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Original Paper

Acetyl-CoA Synthetase 2 Promotes Cell Migration and Invasion of Renal Cell Carcinoma by Upregulating Lysosomal-Associated Membrane Protein 1 Expression

Lv Yao[a,b] Xiaoqiang Guo[a,b,c] Yaoting Gui[a,b]

[a]Department of Urology, Peking University Shenzhen Hospital, Institute of Urology of Shenzhen PKU-HKUST Medical Center, Shenzhen, Guangdong, [b]The Guangdong and Shenzhen Key Laboratory of Male Reproductive Medicine and Genetics, Peking University Shenzhen Hospital, Institute of Urology of Shenzhen PKU-HKUST Medical Center, Shenzhen, Guangdong, [c]State Engineering Laboratory of Medical Key Technologies Application of Synthetic Biology, Key Laboratory of Medical Reprogramming Technology, Shenzhen Second People's Hospital, The First Affiliated Hospital of Shenzhen University, Shenzhen, Guangdong, China

Key Words

ACSS2 • Renal cell carcinoma • Lipid metabolism • Acetate • Cell migration • Invasion

Abstract

Background/Aims: Reprogramming energy metabolism is an emerging hallmark of many cancers, and this alteration is especially evident in renal cell carcinomas (RCCs). However, few studies have been conducted on lipid metabolism. This study investigated the function and mechanism of lipid metabolism-related acetyl-CoA synthetase 2 (ACSS2) in RCC development, cell migration and invasion. ***Methods:*** Quantitative real-time PCR (qRT-PCR) was used to determine the expression of *ACSS2* in cancer tissue and adjacent tissue. The inhibition of *ACSS2* expression was achieved by RNA interference, which was confirmed by qRT-PCR and Western blotting. Cell proliferation and apoptosis were detected by a CCK8 assay and a flow cytometry analysis, respectively. Cell migration and invasion were determined by the scratch and transwell assays. Following the knockdown of ACSS2 expression, the expression of the autophagy-related factor LAMP1 was measured by qRT-PCR and Western blotting. ***Results:*** Compared to adjacent tissues, ACSS2 expression was upregulated in RCC cancer tissues and positively correlated with metastasis. Inhibition of ACSS2 had no effect on RCC cell proliferation or apoptosis. However, decreased ACSS2 expression was found to inhibit RCC cell migration and invasion. ACSS2 was determined to promote the expression of LAMP1, which can also promote cell migration. This pathway may be considered a potential mechanism through which ACSS2 participates in RCC development. ***Conclusion:*** These data suggest that ACSS2 is an important factor for promoting RCC development and is essential for cell migration and invasion, which it promotes by increasing the expression of LAMP1. Taken together, these findings reveal a potential target for the diagnosis and treatment of RCC.

Yaoting Gui
and Xiaoqiang Guo

Department of Urology, Peking University Shenzhen Hospital.
Lianhua Road 1120, Shenzhen, Guangdong (China)
E-Mail guiyaoting2007@aliyun.com, xiaoqiangguo123@163.com

Introduction

Renal cell carcinoma (RCC) is a common urologic tumour that accounts for 2 to 3% of all adult malignancies [1]. Notably, the global incidence of RCC has increased over the past two decades at a rate of 2% per year [2]. Furthermore, RCC is generally resistant to chemotherapy and radiation therapy [3, 4]. Therefore, it is essential to discover new therapeutic strategies for treating RCC.

Abnormal metabolism is one of the typical characteristics of many cancers [5]. The most common metabolic phenotype associated with cancer is the Warburg effect, which consists of increased glycolysis in the presence of oxygen [6]. In the case of RCC, several known oncogenes, including fumarate hydratase (FH), succinate dehydrogenase B (SDHB) and succinate dehydrogenase D (SDHD) are involved in abnormal glucose metabolism [7-9]. However, tumour cells exhibit not only an increased demand for glucose but also an increased dependence on other metabolic substances, such as acetate [10].

Acetate can be converted to acetyl CoA, which is a central metabolic intermediate that is widely used in macromolecule biosynthesis and energy production to support cell growth and proliferation. As a donor of an acetyl group, acetyl CoA is also dynamically associated with the acetylation of protein, which modifies their functions [11]. Therefore, maintenance of the cellular acetyl CoA pool is essential for the regulation of various cellular processes, including cell transformation and development [12]. For rapidly dividing or metastatic cancer cells, acetyl CoA is particularly important [13].

Acetyl-CoA Synthetase 2 (ACSS2) is responsible for the production of acetyl CoA from acetate. Recent studies showed that ACSS2 contributes to the development of many cancers, including brain cancer, breast cancer, lung cancer, melanoma and others [14-17]. However, the role of ACSS2 in RCC development has been only sparsely investigated.

In the present study, we discovered that ACSS2 expression was elevated in RCC tissue compared to adjacent tissue, and it was positively correlated with metastasis. Although the inhibition of ACSS2 did not affect cell proliferation or apoptosis, it was found to decrease cell migration and invasion. In particular, the inhibition of ACSS2 was found to reduce the expression of autophagy-related factor LAMP1. LAMP1 knockdown is known to inhibit cell migration. Taken together, these data indicate that ACSS2 is required for the migration and invasion of RCC cells through the promotion of autophagy.

Materials and Methods

Patients and specimens

Cancer tissues and paired normal tissues were obtained from 48 patients with RCC in Peking University Shenzhen Hospital, China from 2008 to 2013. All RCC samples were confirmed by a clinical pathologist. All patients gave signed informed consent, and the research programme was approved by the Ethics Committee Review Broad of Peking University Shenzhen hospital. All methods, including the collection and use of patient samples, were performed in accordance with the relevant guidelines and regulations.

Cell culture

RCC cell lines 786-O and ACHN were purchased from The Cell Resource Centre of Shanghai Institutes for Biological Sciences, Chinese Academy of Science. These cells were cultured in DMEM (GIBCO, Grand Island, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS, HyClone, Logan, USA), 100 U /mL penicillin and 100 mg /mL streptomycin (GIBCO). Cells were maintained at 37 °C in a humidified atmosphere (5% CO_2).

RNA interference (RNAi)

Human ACSS2 siRNA, LAMP1 siRNA and control siRNA were purchased from Genepharma (Shanghai, China), reconstituted in sterile DNase/RNase-free water to a stock concentration of 20 mM, and transfected into 786-O and ACHN cells using Lipo-2000 (Invitrogen, Carlsbad, CA, USA) at a final concentration of 20 nM/

well. The siRNA sequences were as follows: negative control (si-NC): 5'-UUCUCCGAACGUGUCACGU-3'; ACSS2 (si-ACSS2): 5'-CAGGAUUGAUGA CAUGCUCAA-3'; LAMP1 (si-LAMP1): 5'-AUGCGAGCUCCAAAGAAAU-3'. The inhibitory effect of the siRNA transfection was evaluated with qRT-PCR and Western blotting.

Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was extracted using Trizol reagent (Invitrogen) in accordance with the manufacturer's instructions and then reverse transcribed into cDNA with the reverse transcription (RT) reagent Kit (TaKaRa, Dalian, China), according to the manufacturer's protocol. The qRT-PCR reactions were prepared with a PCR kit (TaKaRa) and performed on LightCycler480 System (Roche, Foster City, CA, USA). The primers used were synthesized by Sangon (Shanghai, China), and the sequences were as follows: ACSS2 primers, 5'-AAAGGAGCAACTACCAACATCTG-3' (forward); GCTGAACTGACACACTTGGAC (reverse); LAMP1 primers, 5'-TCTCAGTGAACTACGACACCA-3'(forward); 5'-AGTGTATGTCC TCTTCCAAAAGC-3' (reverse); β-Actin primers, 5'-CCACTGGCATC GTGATGGACTCC-3' (forward); 5'-GCCGTGGTGGTGAAGCTGTAGC-3' (reverse). β-Actin was used as an internal reference gene.

Western blotting

Cells were collected and dissolved in radioimmunoprecipitation assay (RIPA) buffer containing a protease inhibitor cocktail and phenylmethylsulfonyl fluoride (PMSF). Samples containing, on average, 50 μg of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were saturated with 5% skim milk in TBST (50 mM Tris–HCl, 150 mM NaCl, 0.1% Tween-20) and then incubated with primary antibodies at 4 °C overnight. The primary antibodies used in this study included rabbit polyclonal antibodies against ACSS2 (1:1, 000, Sigma-Aldrich, St Louis, USA), LAMP1 (1:1000, Proteintech, Rosemont, USA) and β-Actin (1: 2, 500, Abcam, Shanghai, China). The membranes were incubated with an HRP-conjugated goat anti-rabbit antibody (1:5, 000, Cell Signaling Technology, Danvers, MA, USA) for 2 h at room temperature and then exposed to the enhanced chemiluminescence substrate (Millipore, Rockford, USA). Detection of the bands was performed using a fully automatic chemiluminescence/fluorescence image analysis system (TANON 5220S).

CCK8 assay

Cell proliferation was examined using the Transdetect Cell Counting Kit (Transgen, Beijing, China) according to the manufacturer's instructions. Briefly, 786-O and ACHN cells were grown in a 96-well plate for 24 h, transfected with ACSS2 siRNA or the negative control and then cultured in normal medium. To measure the cell proliferation at 0, 24, 48, or 72 h after transfection, 10 μl of CCK-8 was added to each well and then the cells were cultured for 1 h. Absorbance was measured at a wavelength of 450 nm. Assays were repeated at least three times.

Flow cytometry analysis

Cell apoptosis was detected using an Alexa Fluor®488 Annexin V/Dead Cell Apoptosis Kit (Invitrogen, Carlsbad, CA, USA) according to the supplier's protocol. Cells were seeded in 6-well plates (5×10^5/well). The cells were harvested 48 h after transfection with RNAi and washed in cold phosphate-buffered saline (PBS). Then, cells were re-suspended in 100 μL of 1 × annexin-binding buffer. Next, 5 μL of Alexa Fluor® 488 annexin V and 1 μL of PI working solution were added to each reaction system and incubated at room temperature for 15 min. The cell apoptosis assay was performed immediately on a flow cytometer. Each experiment was repeated at least three times.

Scratch assay

A scratch assay was performed to determine the migration ability of both 786-O and ACHN cells. Briefly, cells were seeded into 6-well plates (Corning, NY, USA) and treated with RNAi. At 6 h post-transfection, a clean line was created with a sterile 200-μl pipette tip. The cell migration was monitored using a digital camera system and imaged at 0 h and 24 h (for ACHN cells) or 30 h (for 786-O cells). The relative cell migration rate was calculated according to the position of the line.

Transwell assay

A transwell assay was performed to assess the migration and invasion abilities of RCC cells 786-O and ACHN *in vitro*. Transwell chamber inserts (BD Biosciences, FranklinLakes, NJ, USA) with (for cell invasion)

or without Matrigel (for cell migration) were used in the assay according to the manufacturer's protocol. The transfected cells were seeded in the upper chamber of the insert at a density of 2×10^4 cells in 200 μL of serum-free DMEM. The bottom of the inserts contained DMEM with 10% FBS. The 786-O cells were allowed to migrate for 24 h or invade for 36 h. The ACHN cells were allowed to migrate for 36 h or invade for 48 h. The cells that migrated to or invaded the bottom of the inserts were stained with crystal violet and counted using a light microscope.

Statistical analysis

All experimental data were presented as the means ± standard deviation (SD) from three independent experiments. The differences between two groups, including clinical data and cell experiments, were analysed with a paired two-tailed Student's t-test. A *P* value of less than 0.05 was considered to be statistically significant.

Results

ACSS2 is upregulated in RCC tissue

To explore the association between ACSS2 and RCC development, we first measured the expression of *ACSS2* mRNA with qRT-PCR. The results indicated that the mRNA level of *ACSS2* was higher in RCC tissue than in the adjacent normal tissue ($P < 0.05$, Fig. 1A). The mRNA level of *ACSS2* was higher in tumours that had spread to regional lymph nodes (N1) compared with tumours localized only in the kidney (N0) ($P < 0.05$, Fig. 1B) and higher in tumours extending into major veins or perinephric tissues (T3) compared to tumours limited to the kidney (T2) ($P < 0.05$, Fig. 1C). These preliminary data indicate that ACSS2 is a factor that promotes RCC development and may be associated with metastasis.

ACSS2 does not affect cell proliferation or apoptosis

To elucidate the role of ACSS2 in the RCC cell phenotype, we first performed RNAi experiments. The results showed that specific siRNA treatment could significantly decrease the mRNA and protein levels of ACSS2 ($P < 0.01$, Fig. 2A and 2B). Then, CCK-8 assays were performed to investigate cell proliferation. The results showed that suppressing ACSS2 expression (siRNA) did not significantly affect the proliferation of 786-O and ACHN cells compared to the control group (NC) (Fig. 3). We also performed a flow cytometry analysis to determine the effect of ACSS2 on apoptosis. The results showed that there was no difference in the apoptotic cell ratio between the control group (NC) and the ACSS2 RNAi group (siRNA) in 786-O or ACHN cells (Fig. 4A and 4B). This finding suggests that ACSS2 has no significant effect on cell growth.

ACSS2 promotes cell migration and invasion

We further explored whether ACSS2 contributed to cell migration or invasion. The results of the scratch assay showed that suppressing ACSS2 expression could inhibit the migration capacity of 786-O and ACHN cells (Fig. 5A and 5C). The data analysis showed that there was a

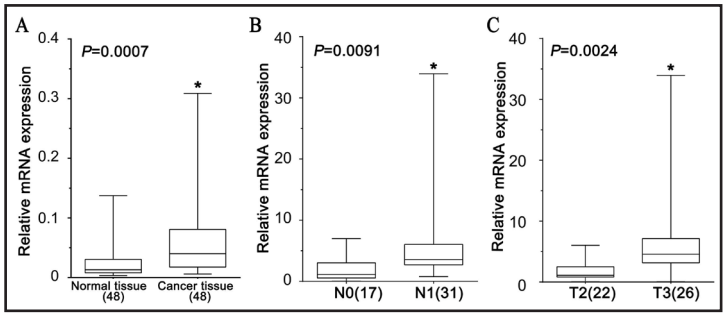

Fig. 1. The expression of ACSS2 in RCC tissue. (A) ACSS2 expression is higher in cancer tissues than normal tissues of RCC patients (n = 48). (B) The relative expression of ACSS2 is higher in RCC patients with tumours already spread to regional lymph nodes (N1, n=31) compared to patients with tumours localized to the kidney (N0, n=17). (C) The relative expression of ACSS2 is also higher in RCC patients with tumours extending beyond the kidney (T3, n=26) compared to patients with tumours detected only in the kidney (T2, n=22). *, $P<0.05$.

significant difference in migration capacity between the ACSS2 RNAi group (siRNA) and the control group (NC) ($P < 0.05$ and $P < 0.01$, Fig. 5B and 5D). A transwell assay was then performed to evaluate the cell migration and invasion abilities. These results also indicated that inhibiting ACSS2 expression could decrease cell migration for 786-O ($P < 0.05$, Fig. 6A and 6B) and ACHN ($P < 0.01$, Fig. 6C and 6D) cells. Furthermore, the transwell assay also demonstrated that reduced ACSS2 expression can lead to a lower invasion ability in 786-O cells ($P < 0.05$, Fig. 7A and 7B) and ACHN cells ($P < 0.01$, Fig. 7C and 7D). These results further confirmed that ACSS2 is a promoting factor of cell migration and invasion in RCC.

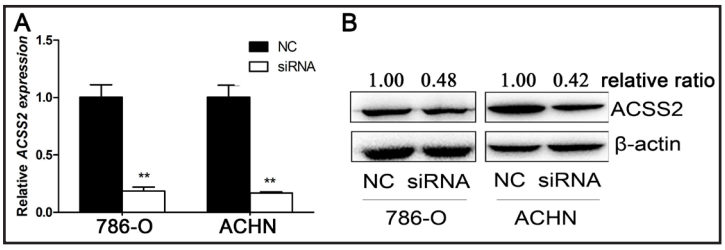

Fig. 2. The effect of inhibiting ACSS2 expression in RCC cells. 786-O and ACHN cells were treated with ACSS2 siRNA (siRNA) or control siRNA(NC), and the inhibitory effect was determined by qRT-PCR and Western blotting. (A) The results of qRT-PCR. (B) The results of Western blotting. **, $P<0.01$.

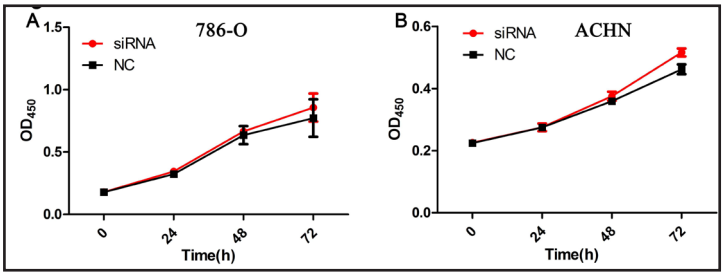

Fig. 3. ACSS2 does not affect cell proliferation. The CCK8 assay was performed to compare cell proliferation between the RNAi group (siRNA) and the control group (NC). (A) The growth curve of 786-O cells. (B) The growth curve of ACHN cells. Values are the mean of triplicate samples from a representative experiment.

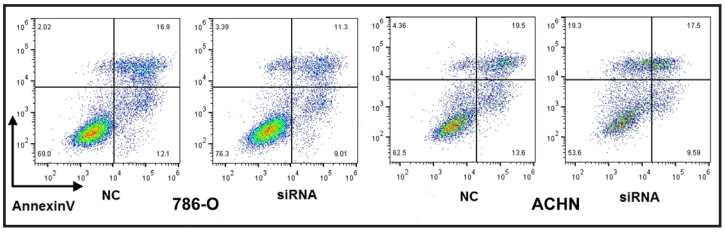

Fig. 4. ACSS2 does not affect cell apoptosis. Flow cytometry was performed to compare the apoptosis ratio between the RNAi group (siRNA) and the control group (NC). (A) 786-O cells. (B) ACHN cells.

ACSS2 increases the expression of autophagy-related factor LAMP1

To investigate the potential mechanism underlying the role of ACSS2 in RCC development, we examined the effect of ACSS2 on LAMP1 expression. The results revealed that the suppression of ACSS2 expression not only reduced the expression of *LAMP1* mRNA (Fig. 8A) but also decreased the protein content of LAMP1 (Fig. 8B). Considering that LAMP1 is an autophagy-related factor, these results suggest that ACSS2 may participate in carcinogenesis by affecting autophagy.

LAMP1 also promotes cell migration

To further investigate the biological effects of LAMP1, we first knocked down the expression of LAMP1 in 786-O and ACHN cells ($P < 0.01$, Fig. 9A and 9B). Then, the scratch assay was performed to assess cell migration. The results showed that the knockdown of LAMP1 inhibited the mobile capacity of 786-O and ACHN cells ($P < 0.01$, Fig. 9C to 9F). Because both ACSS2 and LAMP1 can promote cell migration, these results provided further evidence that LAMP1 may be involved in ACSS2-induced cell migration in RCC.

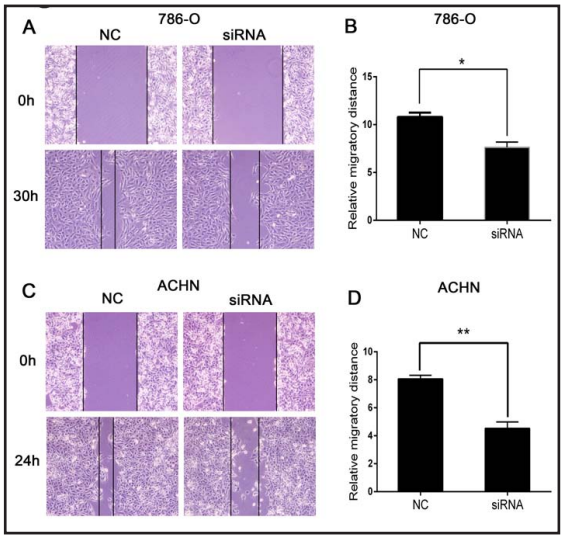

Fig. 5. The inhibition of ACSS2 suppresses cell migration. A scratch assay was performed to investigate the role of ACSS2 in cell migration. (A) The migration of 786-O cells. (B) Quantitative results of 786-O cell migration. (C) The migration of ACHN cells. (B) Quantitative results of ACHN cell migration. Values are the means of triplicate samples from a representative experiment. *, $P<0.05$; **, $P<0.01$.

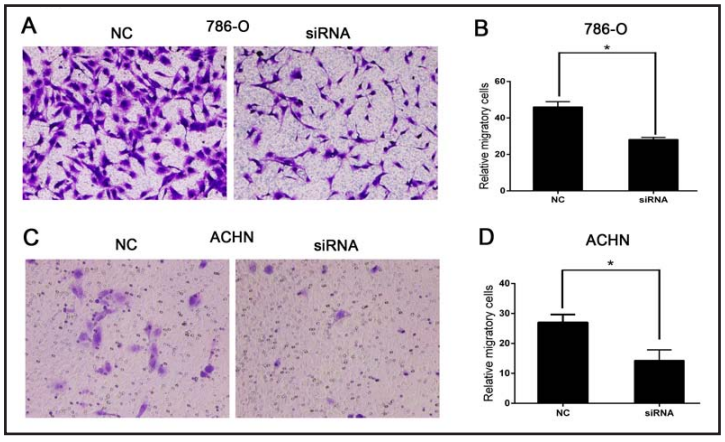

Fig. 6. Low expression of ACSS2 suppresses cell migration. A transwell assay was performed to investigate the role of ACSS2 in cell migration. (A) The migration of 786-O cells. (B) Quantitative results of 786-O cell migration. (C) The migration of ACHN cells. (D) Quantitative results of ACHN cell migration. Values are the means of triplicate samples from a representative experiment. *, $P<0.05$.

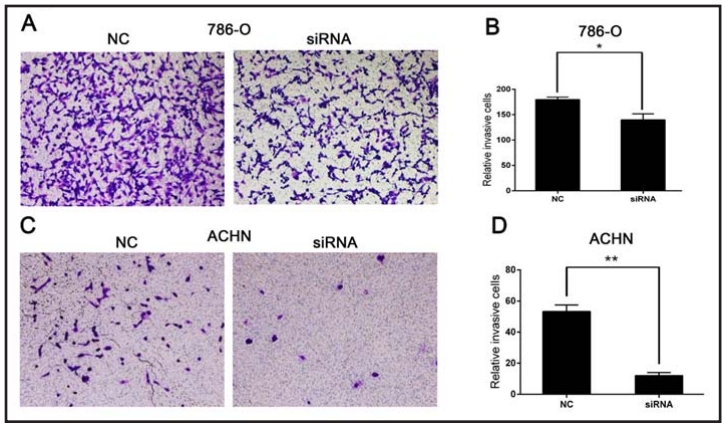

Fig. 7. The inhibition of ACSS2 suppresses cell invasion. A transwell assay was performed to investigate the role of ACSS2 in cell invasion. (A) The invasion of 786-O cells. (B) Quantitative results of 786-O cell invasion. (C) The invasion of ACHN cells. (D) Quantitative results of ACHN cell invasion. Values are the means of triplicate samples from a representative experiment. *, $P<0.05$; **, $P<0.01$.

Discussion

Reprogramming energy metabolism is an emerging hallmark of many cancers because adjusting energy metabolism is essential to fuel cell growth and division [18]. Glucose is one of the principal fuels for developing tumours [19], but alternative fuel sources, such as acetate and amino acids, also play an important role in cancer development [20-22]. Acetate can be

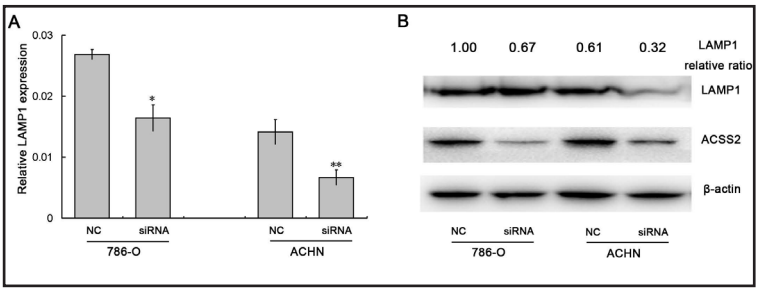

Fig. 8. ACSS2 inhibition suppresses LAMP1 expression. (A) LAMP1 mRNA expression is down-regulated as a result of the knockdown of ACSS . (B) LAMP1 protein levels are reduced in the siRNA group compared to the control group (NC). *, $P<0.05$; **, $P < 0.01$

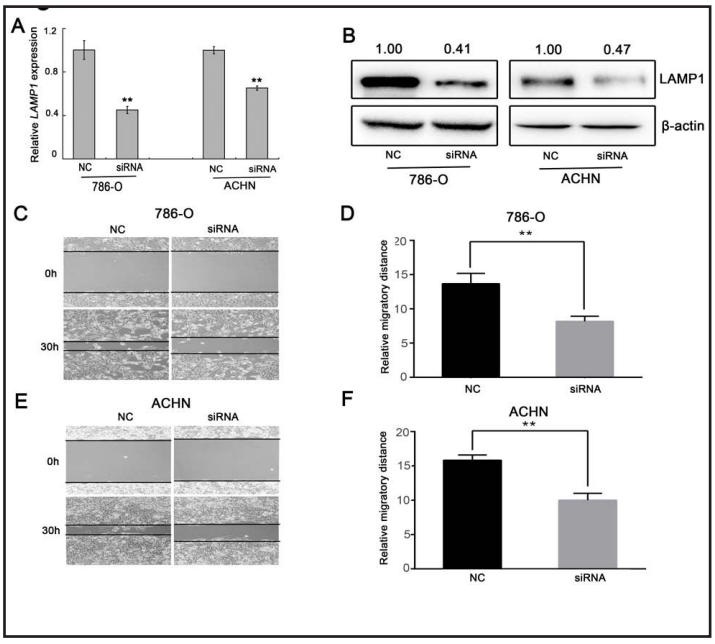

Fig. 9. LAMP1 knockdown inhibits cell migration. 786-O and ACHN cells were transfected with LAMP1 siRNA (siRNA) or control siRNA (NC). The inhibited expression of LAMP1 was confirmed with qRT-PCR and Western blotting, and the biological effect on cell migration was determined with the scratch assay. (A) The knockdown of LAMP1 mRNA. (B) The knockdown of LAMP1 protein. (C) The migration of 786-O cells. (D) Quantitative results of 786-O cell migration. (E) The migration of ACHN cells. (F) Quantitative results of ACHN cell migration. Values are the means of triplicate samples from a representative experiment. **, $P<0.01$.

converted by ACSS2 to acetyl CoA, which is among the most important cellular components facilitating macromolecular biosynthesis and histone modification [23]. The expression of ACSS2 is emerging as one of the key factors that enable cells to maximally utilize acetate as a nutritional source [24].

Many previous experiments have shown that ACSS2 is overexpressed in a large proportion of human cancers, including hepatocellular carcinoma, glioblastoma, breast cancer and prostate cancer [24]. Our results provide further evidence that ACSS2 expression is also abnormally upregulated in RCC cells. This finding indicates that ACSS2 may also play an important role in RCC development.

Previous studies have shown that ACSS2 promotes cancer cell growth [25, 26], which is not consistent with the findings of the present study. One possible reason for the disparity is that ACSS2 is critical for cancer cell survival under metabolic stress, such as hypoxic or low-serum conditions [16, 27]. Therefore, whether ACSS2 is related to cell growth in RCC warrants further study. However, our data also showed that ACSS2 can increase RCC cell migration and invasion *in vitro*. This finding is consistent with preview reports showing that ACSS2 is associated with cancer metastasis [15].

Some studies have also revealed the potential mechanism underlying the role of ACSS2 in cancer. ACSS2 is essential for protein acetylation by producing acetyl CoA, which has an important role in many biological processes. ACSS2 can increase histone H3 acetylation in specific regions, which is vital to autophagy, cell survival and tumourigenesis [28, 29]. In particular, previous studies have shown that ACSS2 can promote brain carcinogenesis by increasing the expression of autophagy-related factors, such as LAMP1. Our results confirmed this finding in RCC. It has been shown that autophagy plays a key role in carcinogenesis

[30-32], especially in cancer metastasis [33]. Therefore, our results are important for the understanding of autophagy abnormalities and RCC development and metastasis.

With a better understanding of cancer-specific metabolic pathways, many important enzymes or factors may be revealed as novel targets for cancer diagnosis and therapy [34]. Therefore, it is possible that the inhibition of ACSS2 may be a potential strategy in the treatment or prevention of cancer [35]. The ACSS2 RNAi data presented here confirmed that possibility, but the feasibility of such a strategy needs to be fully explored.

Conclusion

Our preliminary results indicate that ACSS2 is overexpressed in RCC tissue and may be associated with metastasis. Our data also suggest that the inhibition of ACSS2 expression can suppress cell migration and invasion. Moreover, ACSS2-mediated LAMP1 upregulation may play an essential role in RCC development and metastasis. The detailed mechanism of ACSS2 in the pathogenesis of RCC warrants further research; increasing our understanding of the molecular mechanisms involved in RCC may provide more targets for diagnosis and treatment.

Acknowledgements

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Disclosure Statement

The authors declare no Disclosure Statement.

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