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Regular paper

Upregulation of SOCS2 causes mitochondrial dysfunction and promotes ferroptosis in pancreatic cancer cells

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SOCS2 exerts oncogenic effects in a variety of tumors, but its role in pancreatic cancer has not been studied. The purpose of this study was to explore the role of SOCS2 in pancreatic cancer. The expression level of SOCS2 and the content of mitochondrial DNA (mtDNA) in the cells were detected by real-time PCR (qRT-PCR), and SOCS2 was overexpressed in PANC-1 and Capan-2 cells by transfection with pcDNA3.2-SOCS2. CCK-8, cell colony formation assay, and flow cytometry were used respectively to detect the cell proliferation rate, cell colony formation ability, and the level of ROS in the cells. The ATP level, glucose consumption level, and Fe^{2+} level in the cells were assessed by biochemical assays. And Western blot determined the protein expression levels of SOCS2 as well as ferroptosis-related proteins, namely, SLC7A11, DMT1, TFRC, and FTH. We found that SOCS2 was significantly down-regulated in pancreatic cancer cells. Overexpression of SOCS2 significantly decreased the viability of PANC-1 and Capan-2 cells, reduced the content of mtDNA and the level of ATP, and caused mitochondrial dysfunction with an accumulation of ROS. Aside from these effects, up-regulation of SOCS2 raised the levels of Fe^{2+}, DMT1 and TFRC, and decreased the level of SLC7A11 and FTH in PANC-1 and Capan-2 cells, thereby inducing the occurrence of ferroptosis. In conclusion, up-regulated SOCS2 may enhance mitochondrial dysfunction and ferroptosis in pancreatic cancer cells, which can be used as a molecular target for the diagnosis and treatment of pancreatic carcinoma.

Keywords: SOCS2, pancreatic carcinoma, mitochondrial dysfunction, ferroptosis

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Abbreviations: DCFH-DA, dichlorofluorescein yellow diacetate; DMT1, Divalent metal transport1; OD, optical density; PVDF, polyvinylidene difluoride; RFS, recurrence-free survival; ROS, reactive oxygen species; DS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SOCS2, cytokine signaling 2; TFRC, Transferrin receptor

INTRODUCTION

Pancreatic carcinoma is a highly aggressive malignancy associated with fatal risk. Compounded by its relative insidious incidence, it accounts for 2% of all new cancers and 5% of cancer-related deaths (Goral, 2015). With the improvement of living standards, the growth of life span, environmental pollution and other factors, recent years have witnessed a growing prevalence of pancreatic carcinoma (Bi *et al*., 2017) with a 5-year survival rate of only 9% (Ferlay *et al*., 2021). At present, the mainstream treatments for the disease are surgery, radiotherapy, chemotherapy and traditional Chinese medicine. Despite great progress in treatments, the overall clinical effect remains unsatisfactory and the overall prognosis is also poor (Su *et al*., 2015; Pereira *et al*., 2020). The development of tumors is affected by a variety of biological factors and environmental factors (Fang *et al*., 2015). Dysregulated gene expression plays a pivotal role in not only the development of tumor growth, spread, and metastasis but also the tumor recurrence and chemo-resistance (Welinsky & Lucas, 2017). Therefore, it is urgent to seek new markers for screening and diagnosis, thus improving the early diagnosis rate of pancreatic carcinoma.

The suppressor of cytokine signaling 2 (SOCS2), a gene located in the 12q21.3-q23 region and whose protein consists of 198 amino acid residues, is a member of the SOCS family (Krebs & Hilton, 2001). SOCS proteins play a role in tumor suppressors by negatively regulating multiple cytokine signaling through the JAK/STAT signaling pathway (Culig, 2013; Elliott *et al*., 2008). Studies have found that SOCS1 and SOCS3 are lowly expressed in tumor tissues where they inhibit the proliferation of tumor cells (Trengove & Ward, 2013; Zhou *et al*., 2007; Niwa *et al*., 2005). Likewise, a number of studies have revealed low expression of SOCS2 in various tumors such as prostate cancer (Qiu *et al*., 2013), hepatocellular carcinoma (Zhu *et al*., 2013) and colorectal cancer (Letellier *et al*., 2014), as SOCS2 inhibits the occurrence and development of tumors by regulating a variety of signaling molecular pathways, including Ras/Erk, PI3K/Akt and FAK. However, the role SOCS2 plays in pancreatic carcinoma is as of now unclear. Therefore, in this study, we explored the role of SOCS2 in the proliferation, colony formation, mitochondrial dysfunction, and ferroptosis of pancreatic cancer cells by conducting in vitro cell experiments, thus providing new markers for the prevention, treatment and early diagnosis of pancreatic carcinoma.

MATERIALS AND METHODS

Cell culture and transfection

Normal pancreatic duct epithelial cell line HPNE, human pancreatic cancer cell lines PANC-1, SW1990, Capan-2, and MiaPaCa-2 were purchased from the Shanghai Institute of Biochemistry and Cell Biology, China. All cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS, Gibco, USA) and 100 penicillin-streptomycin (Beyotime, China) at 37°C in 5% CO_2, digested with 0.25% trypsin (Beyotime, China), and subcultured.

PANC-1 and Capan-2 cells in the logarithmic growth phase were collected and diluted to 2×10^6 cells/ml, then seeded in 6-well plates. They were transfected when cell culture was performed until the confluence was 80% ~ 90%. In accordance with Lipofectamine 2000 instructions (Thermo, USA), pcDNA3.2 and pcDNA3.2-SOCS2 were transfected into PANC-1 and Capan-2 cells, which respectively fell into the category of vector group and SOCS2 group, and the cells were collected 48 hours after transfection.

qRT-PCR

Total cellular RNA was extracted by Trizol and then reverse transcribed to cDNA according to the instructions of a reverse transcription PCR kit (Takara, Japan). The cDNA was reacted with a real-time PCR kit (Takara, Japan), and the primer sequences used were present in Table 1 with β-globin and GAPDH serving as internal reference genes for mtDNA and SOCS2, respectively. The $2^{-\Delta\Delta Ct}$ method was employed for data analysis (Ma *et al*., 2017).

Table 1. Primer sequences for qRT-PCR

Gene name	Primer sequences 5' to 3"
SOCS2	F TGCAAGGATAAGCGGACAGG
	R CAGAGATGGTGCTGACGTGT
GAPDH	F TGCACCACCAACTGCTTAGC
	R GGCATGGACTGTGGTCATGAG
mtDNA	F CCCCACAAACCCCATTACTAAACCCA
	R TTTCATCATGCGGAGATGTTGGATGG
β-globin	F CGAGTAAGAGACCATTGTGGCAG
	R GCTGTTCTGTCAATAAATTTCCTTC

CCK-8 assay

CCK-8 reagent (Beyotime, China) at 0 h and 24 h of attachment was used for measuring cell proliferation. The cells were seeded in a 96-well plate at 2000 cells/well and 10 μL of CCK-8 solution and 90 μL of fresh complete medium were added to each test well, and the 96-well plate was placed in an incubator for another 1 h. The optical density (OD) at a wavelength of 450 nm was measured by Varioskan™ LUX multimode microplate reader (Thermo Scientific, Waltham, MA, USA) and the cell proliferation rate of each group was calculated.

Cell colony formation assay

The trypsin was used to digest cells at the logarithmic growth phase, which was made into cell suspension. By repeatedly blowing, the cells were fully dispersed and counted. Finally, cell concentration was adjusted. The cells were seeded at 400 cells/well in a 6-well plate, gently shaken to disperse the cells evenly, and cultured in a 37°C, 5% CO_2 incubator for 2–3 weeks with the fresh medium replaced. When macroscopic clones were formed, the culture medium was removed, and 4% paraformaldehyde (Beyotime, China) was added for fixation for 20 min. Then, the sample was stained with crystal violet solution (Beyotime, China) for 15 min and the excess staining was rinsed with PBS. The photos that covered the whole sample were collected using a camera (Nikon, Japan) and the colony formation number was calculated.

Flow cytometry

Cells were collected and washed twice with PBS, and then resuspend with 200 μL of PBS. Subsequently, dichlorofluorescein yellow diacetate (DCFH-DA) at a concentration of 10 μmol/L was added as per the instructions of the ROC reactive oxygen species (ROS) assay kit. The cells were then incubated in a cell culture incubator at 37°C for 20 min, whose intracellular ROS level was determined by flow cytometry.

Biochemical tests

Cells were collected, and the ATP level, glucose consumption level, and Fe^{2+} level in the cells of each group were measured according to the instructions of the corresponding biochemical kit (Nanjing Jiancheng Bioengineering Institute, China).

Western blot

Cells were lysed in RIPA lysis solution on ice for 20 min, then disrupted by sonication in an ice bath, and centrifuged at 10000 rpm for 20 min at 4°C. The supernatant was collected and the protein concentration was determined using a BCA kit. 20μg of total protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked with 5% skimmed milk for 1 h at room temperature, washed three times with PBST and incubated overnight at 4°C with diluted primary antibodies (anti-SOCS2, anti-SLC7A11, anti-DMT1, anti-TFRC, anti-FTH, anti-GAPDH, Abcam, USA). The diluted secondary antibody (ZSGB-BIO, China) was added for 1-h incubation at room temperature after washing the membranes twice in PBST. After washing the membrane twice with PBST, ECL hypersensitive luminescence solution was added. Then the cells were placed in a chemiluminescence developer for the development and photographic recording. Image pro plus was used to analyze the protein bands with GAPDH used as an internal reference to quantify.

Statistical analysis

The results of experimental data were expressed as mean ± standard deviation (S.D.). SPSS 24. 0 software was used for statistical analysis, with *t*-test for comparison between two groups, and one-way analysis of variance for comparison of multiple groups. $P<0.05$ indicates statistically significant differences.

RESULTS

SOCS2 was down-regulated in pancreatic cancer cells

Using qRT-PCR and western blot, we found that the mRNA and protein expression levels of SOCS2 were significantly decreased in pancreatic cancer cell lines PANC-1, SW1990, Capan-2, and MiaPaCa-2 cells compared with normal pancreatic duct epithelial cells HPNE ($P<0.01$). The SOCS2 expression was low-

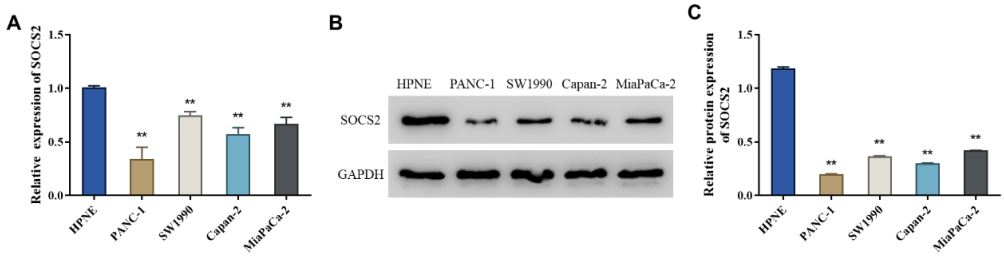

Figure 1. SOCS2 was down-regulated in pancreatic cancer cells.
(**A**) The expression levels of SOCS2 in normal pancreatic duct epithelial cells (HPNE) and pancreatic cancer cells (PANC-1, SW1990, Capan-2, and MiaPaCa-2 cells) were detected by qRT-PCR (n=3). (**B/C**) The protein expression levels of SOCS2 in HPNE, PANC-1, SW1990, Capan-2, and MiaPaCa-2 cells were determined by western blot and the relative expression levels of SOCS2 analyzed with Image pro plus (n=3), ***P*<0.01 *vs*. HPNE.

est in ovarian cancer cell lines PANC-1 and Capan-2 (Fig. 1A–C).

Upregulation of SOCS2 Inhibited the viability of pancreatic cancer cells

To further evaluate the role of SOCS2 in pancreatic carcinoma, we overexpressed PANC-1 and SOCS2 in Capan-2 cells by transfecting SOCS2, and the transfection efficiency was shown in Fig. 2A–C. Compared with the vector group, the SOCS2 group had higher mRNA and protein expression levels of SOCS2 in PANC-1 and Capan-2 cells (*P*<0.01). The proliferation ability and colony formation ability were determined using CCK-8 and colony formation assay. The results demonstrated the decreased proliferation rate and colony formation ability of PANC-1 and Capan-2 cells in the SOCS2 group relative to the vector group (*P*<0.01) (Fig. 2D–F). The above results suggested that overexpression of SOCS2 may significantly inhibit the proliferation and colony formation of pancreatic cancer cells.

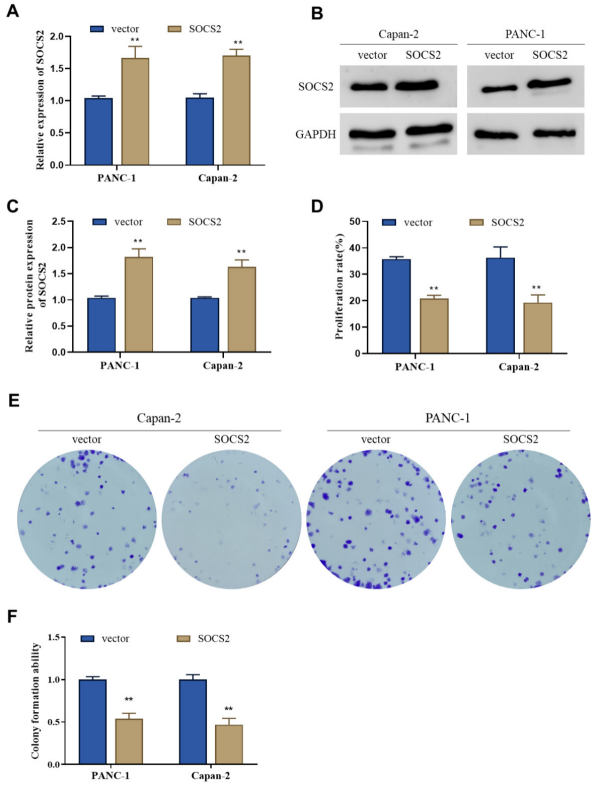

Figure 2. Up-regulation of SOCS2 inhibited the viability of pancreatic cancer cells.
(**A**) The mRNA expression levels of SOCS2 in PANC-1 and Capan-2 cells after transfection with SOCS2 detected by qRT-PCR (n=3). (**B/C**) The protein levels of SOCS2 in PANC-1 and Capan-2 cells after transfection with SOCS2 detected by western blot and the relative protein expression levels of SOCS2 analyzed by image pro plus (n=3). (**D**) The proliferation rate of PANC-1 and Capan-2 cells in each group detected by CCK-8 (n=3). (**E/F**) The colony formation ability of PANC-1 and Capan-2 cells in each group determined by cell colony formation assay (n=3), ***P*<0.01 *vs*. vector group.

Upregulation of SOCS2 caused mitochondrial dysfunction and ROS accumulation in pancreatic cancer cells

Studies have shown the wide appearance of mitochondrial dysfunction triggered by TCA cycle enzyme defects, mitochondrial DNA gene mutations, mitochondrial electron transport chain defects, oxidative stress, and abnormal oncogene and tumor suppressor gene signaling in human cancers, which is closely related to the occurrence and progression of cancer (Luo *et al*., 2020). So

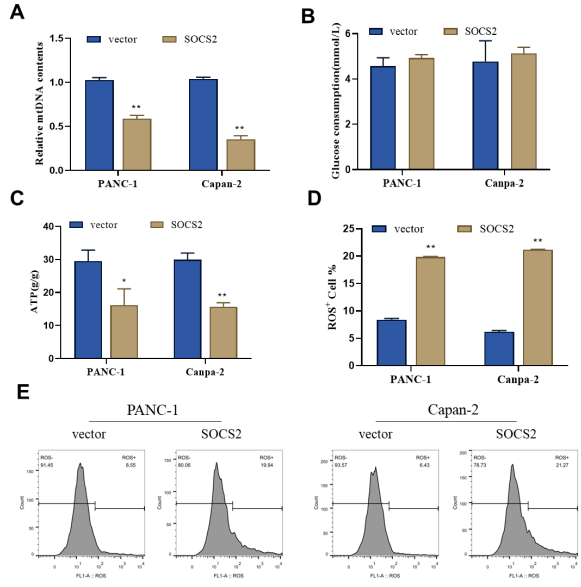

Figure 3. Up-regulation of SOCS2 led to mitochondrial dysfunction accompanied by ROS accumulation.
(**A**) The content of mitochondrial DNA (mtDNA) in PANC-1 and Capan-2 cells detected by qRT-PCR (n=3). (**B**) Biochemical detection of glucose consumption levels in PANC-1 and Capan-2 cells (n=3). (**C**) Biochemical detection of ATP levels in PANC-1 and Capan-2 cells (n=3). (**D/E**) ROS levels in PANC-1 and Capan-2 cells detected by flow cytometry (n=3), *P*<0.05 and ***P*<0.01 *vs*. vector group.

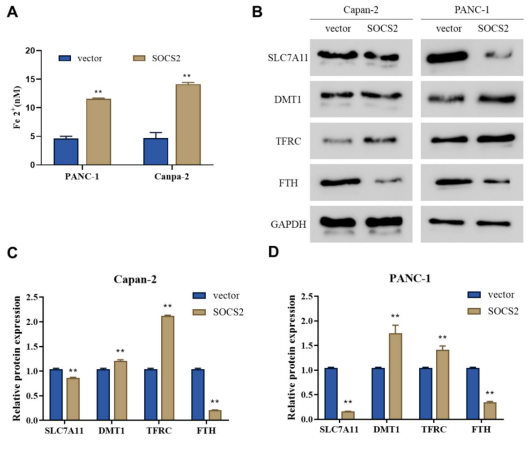

Figure 4. Up-regulation of SOCS2 induced ferroptosis in pancreatic cancer cells.
(**A**) Biochemical detection of Fe2 + levels in PANC-1 and Capan-2 cells (n=3); (**B–D**) western blot detection of protein expression levels of ferroptosis -related proteins SLC7A11, DMT1, TFRC and FTH in PANC-1 and Capan-2 cells and detection of protein levels of SLC7A11, DMT1, TFRC and FTH in pancreatic cancer cells by Image pro plus (n=3), $^{**}P<0.01$ *vs*. vector group.

does SOCS2 affect mitochondrial dysfunction in pancreatic cancer cells? To clarify this, we measured the expression of mitochondrial function-related substances in pancreatic cancer cells overexpressing SOCS2. Of note, the levels of mtDNA and ATP levels were significantly decreased ($P<0.01$) whereas the proportion of ROS-positive cells was significantly increased ($P<0.01$) in PANC-1 and Capan-2 cells in the SOCS2 group. In addition, the level of glucose consumption displayed no significant differences ($P>0.05$) (Fig. 3A–E). These results are indicative of the fact that SOCS2 overexpression resulted in mitochondrial dysfunction and ROS accumulation in pancreatic cancer cells.

Upregulation of SOCS2 induced ferroptosis in pancreatic cancer cells

ROS-induced peroxidation played a key role in ferroptosis (Su *et al*., 2019), which led to the speculation that SOCS2 up-regulation could induce ferroptosis in pancreatic cancer cells. To verify the above speculation, we detected the Fe^{2+} level and the protein expression level of ferroptosis-related markers in each group of pancreatic cancer cells. Elevation of Fe^{2+} level and the protein expression levels of DMT1 and TFRC was found in pancreatic cancer cells upon overexpression of SOCS2 ($P<0.01$), while the protein levels of SLC7A11 and FTH declined ($P<0.01$) (Fig. 4A–D). Taken together, it was shown that SOCS2 overexpression might induce ferroptosis in pancreatic cancer cells.

DISCUSSION

Pancreatic carcinoma constitutes one of the most common causes of cancer-related death and one of the most destructive cancers in China. The incidence and mortality of pancreatic carcinoma in China increased at an annual rate of 1.3% and 1.25% respectively from 2004 to 2015 (Ryan *et al*., 2014; Lin *et al*., 2015). Studies have shown that approximately 10% of pancreatic cancer cases may pass down through families (Xiong *et al*., 2013a). Therefore, early genetic screening and intervention for patients with pancreatic carcinoma may contribute to the early diagnosis and treatment of pancreatic carcinoma. As a member of the SOCS family, SOCS2 is abnormally expressed in a variety of tumors and is found linked with the malignant progression of tumors. For example, a bioinformatics study by Sun and others showed that SOCS2 was significantly under-expressed in breast cancer tissues and its expression was correlated with better recurrence-free survival (RFS) of patients and thus served as an independent prognostic factor for RFS (Sun *et al*., 2021). Hoefer *et al*. found that SOCS2 down-regulation could inhibit the proliferation of prostate cancer cells through the arrest of the cell cycle and the promotion of apoptosis (Hoefer *et al*., 2014). In addition, SOCS2 has also been proven to inhibit the development of gastric cancer by inhibiting H. pylori (Lee *et al*., 2010). The above studies have shown that SOCS2 plays a role as a tumor suppressor in different tumors. This study demonstrated that SOCS2 was significantly underexpressed in pancreatic cancer cells, while overexpression of SOCS2 suppressed the proliferation rate and colony formation ability of pancreatic cancer cells, suggesting that SOCS2 negatively regulates the growth of pancreatic cancer cells.

Mitochondria are both the place where aerobic respiration of cells mainly occurs, but also one of the greatest origins of ROS. ROS was capable of interfering with DNA and RNA replication, oxidizing mitochondrial proteins as well as inducing mPTP release, the latter of which was the main culprit of mitochondrial dysfunction and apoptosis (Wang & Liu, 2018). Xiong and coworkers found that increased ROS production could cause oxidative stress damage to mitochondrial structure and thus constitute the main cause of mitochondrial dysfunction (Xiong *et al*., 2013b). In addition, ROS-induced oxidative stress could give rise to mitochondrial DNA mutations, resulting in an impaired coding region of the oxidative phosphorylase gene in the genome. At the same time, the expression and activity of oxidative phosphorylated proteins would be inhibited, ultimately leading to mitochondrial dysfunction (Shokolenko *et al*., 2009). Feng Hao and coworkers figured out that mitochondrial oxidative stress-induced mtDNA damage leads to mitochondrial dysfunction by affecting mitochondrial ATP synthesis, which affects cellular energy metabolism, oxygen free radical generation and apoptosis, finally inhibiting pancreatic carcinogenesis (Feng *et al*., 2014). In the present study, SOCS2 up-regulation was also found to induce mitochondrial DNA depletion, mitochondrial dysfunction and accumulation of ROS, suggesting that SOCS2 acts to promote mitochondrial dysfunction, enhance oxidative stress, and inhibit the progression of pancreatic carcinoma in pancreatic cancer cells. Notably, we observed that SOCS2 up-regulation increased ROS levels of PANC-1 and capan-2 cells in the present study. It is known that ROS is associated with the progression of pancreatic carcinoma (Chang & Pauklin, 2021). ROS level in cells can be regulated by the activity of antioxidant enzymes, including SOS, CAT and GSH-Px (Zhuang *et al*., 2019). In our study, the high level of ROS may be related to the decreased activity of antioxidant enzymes in cells.

Ferroptosis is a newly defined form of programmed cell death induced by iron-dependent peroxidation (Dixon *et al*., 2012). Four pathways have been found to trigger ferroptosis (Feng & Stockwell, 2018): the first by depleting glutathione, the second by directly targeting and inactivating GPX4, the third by depleting GPX4 and CoQ10 through the SQS-mevalonate pathway, and the fourth by increasing unstable iron pools or iron oxide. Transferrin receptor (TFRC) is a key factor in iron me-

tabolism (Basuli *et al*., 2017). When Fe^{3+} is reduced to Fe^{2+} in the nucleosome, it is transported into the cytosol by DMT1. DMT1 acts as the main iron transporter, acting primarily to inhibit tumor growth (Wolff *et al*., 2018). As one of the regulatory ferroptosis pathway marker proteins, up-regulation of FTH1 expression inhibits Fe^{2+}-induced cellular ferroptosis (Wang *et al*., 2019). In this study, SOCS2 overexpression was found to significantly reduce the protein levels of SLC7A11 and FTH, boost protein levels of DMT1 and TFRC in the cells, alluding that overexpression of SOCS2 can induce ferroptosis in pancreatic cancer cells.

In this study, we demonstrated that SOCS2 acts as a tumor suppressor in pancreatic carcinoma by *in vitro* cell experiments, but this conclusion was not validated by in vivo experiments in addition to the failed endeavor to elaborate the mechanism of its role. At present, extant studies have shown that SOCS2 mostly exerts effects in tumors as a downstream gene of non-coding RNAs (ncRNAs), such as lncRNAs SNHG1 (Wang *et al*., 2021), miR-196b (Xu *et al*., 2021), etc. However, little is known about the detailed molecular regulatory mechanism of SOCS2 in malignant tumors. Therefore, all the above need to be further explored by means of cell biology, molecular biology and animal experiments.

CONCLUSION

Collectively, this study first demonstrated that SOCS2 expression is down-regulated in pancreatic cancer cells, and overexpression of SOCS2 inhibits the viability of pancreatic cancer cells, reduces mtDNA content, and promotes mitochondrial dysfunction and ROS accumulation. In addition, it is able to induce ferroptosis in pancreatic carcinoma cells. Therefore, we hypothesize that SOCS2 plays a role as a tumor suppressor gene in pancreatic carcinoma and thus can be used as a molecular target for the diagnosis and treatment of pancreatic carcinoma.

Declarations

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Data Availability. All relevant data can be provided upon request.

Disclosure statement. The authors declare that they have no competing interests.

Authors' contributions. Mingjie He and Huamei Lu conceived and designed the study; Yi Cai and Zhiping Yuan analyzed and interpreted the results of the experiments; Mingjie He and Lixia Zhang wrote the manuscript. All authors have approved the submitted manuscript.

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