

Activated Pancreatic Stellate Cells Enhance the Warburg Effect to Cause the Malignant Development in Chronic Pancreatitis

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Primary research

Keywords: activated pancreatic stellate cells, the Warburg effect, chronic pancreatitis malignancy, pancreatic ductal adenocarcinoma

Posted Date: May 21st, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-524181/v1>

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Activated pancreatic stellate cells enhance the Warburg effect to cause the malignant development in chronic pancreatitis

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Abstract

Background: Chronic pancreatitis (CP) is a precancerous condition associated with pancreatic ductal adenocarcinoma (PDAC), but its evolutionary mechanism is unclear. Pancreatic stellate cells (PSCs) are closely related to the occurrence and development of CP and PDAC. We aimed to find out whether PSCs play a key role in this "inflammation-cancer transition".

Methods: To evaluate the effect of activated pancreatic stellate cells on normal pancreatic duct epithelial cells and pancreatic cancer cells, pancreatic stellate cells isolated from human tissues were co-cultured with these two cells, respectively. Functional assays assessed the proliferation, migration, and invasion of these two cells. RT-qPCR and western blotting were used to detect the mRNA and protein expressions of glycolytic enzymes in these two cells. Lactate production and glucose utilization assays assessed the aerobic glycolysis level of these two cells. Immunohistochemistry was used to detect the expression of glycolytic enzymes and α -SMA, and the correlation between the two was analyzed in human tissues.

Results: Our research found that co-culture with activated PSCs promoted the proliferation, migration and invasion of normal pancreatic duct epithelial cells and pancreatic cancer cells. At the same time, activated PSCs had a significant effect on the expression of the glycolytic enzymes PKM2 and LDHA in normal pancreatic duct epithelial cells and pancreatic cancer cells and increased lactic acid production and glucose consumption in these two cells. In vivo experiments showed that the expression of the glycolytic enzymes PKM2 and LDHA in pancreatic duct epithelial cells and the marker protein (α -SMA) of activated PSCs in the pancreatic duct peripancreatic interstitium were higher in pancreatic cancer tissues and chronic pancreatitis tissues than in normal pancreatic tissues in both animals and humans. In addition, analysis of human tissue specimens showed that there is a correlation between the expression of PKM2/LDHA and α -SMA.

Conclusion: These findings indicate that activated PSCs play an important role in the development and progression of chronic pancreatitis into pancreatic cancer by regulating and promoting aerobic glycolysis. Our research provides a new theoretical basis for further understanding the mechanism of CP malignancy and the selection of targets for reversing CP malignancy.

Keywords activated pancreatic stellate cells, the Warburg effect, chronic pancreatitis malignancy, pancreatic ductal adenocarcinoma

Background

Pancreatic cancer is a highly malignant tumour with a poor prognosis. Its mortality ranked seventh among all malignant tumours in 2020, and its incidence is increasing annually.[1] Early pancreatic cancer lacks specific clinical symptoms. Nearly 85% of patients have lost the chance for radical surgical resection by the time they consult a doctor, and their 5-year survival rate is less than 6%. Even after radical surgery, the 5-year survival rate of patients is less than 25%.[2] Understanding the mechanism of pancreatic cancer will help to guide research on the prevention, early diagnosis and treatment of pancreatic cancer. The literature points out that pancreatic cancer is related to smoking, drinking, obesity, age, chronic pancreatitis, etc.[3] Among these factors, chronic pancreatitis is considered a precancerous condition associated with pancreatic cancer. Chronic pancreatitis is an inflammatory process in which the endocrine and exocrine glands of the pancreas are progressively and irreversibly damaged and the pancreatic parenchyma is replaced by fibrous tissue, which ultimately leads to various morphological and functional changes in the pancreas and triggers a series of clinical signs of inflammation.[4] Clinical studies have shown that patients with chronic pancreatitis have a much higher risk of pancreatic cancer than normal people.[5, 6] Therefore, exploring the mechanism of pancreatic cancer transformation from chronic pancreatitis and reversing the progression of chronic pancreatitis are important issues that urgently need to be solved.

PSCs are pancreatic-specific stromal cells that are considered to be the targets of various cytokines in chronic pancreatitis and the core of the progression of chronic pancreatitis. Various physical and chemical factors stimulate the conversion of quiescent PSCs into a myofibroblast phenotype characterized by the expression of α -smooth muscle actin (α -SMA), by which they become activated PSCs, which synthesize a large amount of extracellular matrix (ECM) and secrete various cytokines to promote irreversible pancreatic fibrosis in chronic pancreatitis.[7, 8] Not only are PSCs the initiating factors of chronic pancreatitis, a large number of reports also indicate that they play an important role in the occurrence, development and metastasis of pancreatic cancer.[9-11] However, whether PSCs play an important role in the malignant transformation of chronic pancreatitis to PDAC has not been reported. To maintain their abnormal proliferation ability and enhanced invasion characteristics, tumour cells reprogram nutrient transporters and glucose metabolism enzymes to maintain a high level of metabolism. This metabolic characteristic of cancer cells is manifested by a greatly increased rate of glucose uptake and very active glycolysis even in the case of sufficient oxygen supply. This phenomenon is called aerobic glycolysis (the Warburg effect).[12] Currently, some studies have confirmed that PSCs promote glycolytic metabolism in pancreatic cancer,[13, 14] but whether PSCs can promote the malignant transformation of CP by affecting the Warburg effect has not

been reported.

In this study, we first isolated and cultured PSCs from human pancreatic cancer and normal pancreatic tissues and then performed phenotypic identification. We successfully obtained activated PSCs (derived from PDAC tissue, CaPSCs) and quiescent PSCs (derived from normal pancreas tissue, NaPSCs). Subsequently, we co-cultured PSCs from different sources with pancreatic cancer cells/normal pancreatic duct epithelial cells and found that co-culture promoted the proliferation, migration and invasion of these two cell types. Finally, we tested the expression of glycoproteins and glucose metabolism levels related to the Warburg effect in pancreatic cancer cells/normal pancreatic duct epithelial cells and further confirmed these experimental results in vivo. Our findings suggest that activated PSCs can enhance the Warburg effect to promote the malignant transformation of CP. The specific mechanism needs further research.

Materials and Methods

Patients and tissue samples

Primary pancreatic ductal adenocarcinoma tissue, chronic pancreatitis tissue and normal pancreatic tissue were obtained from 40 patients with pancreatic head cancer, 19 patients with chronic pancreatitis and 20 patients with duodenal benign tumours between January 2012 and January 2018 at the First Affiliated Hospital of University of Science and Technology of China. All tissue specimens were fixed in formalin for 24 h and then embedded in paraffin. The study was approved by the Ethics Committee of the Anhui Provincial Hospital of the First Affiliated Hospital of the University of Science and Technology of China, and informed consent was obtained from each participant.

Cell lines and cell culture

PANC-1 cells were obtained from Shanghai Cell Bank. hTERT-HPNE cells (a human normal pancreatic ductal epithelial cell line) were obtained from the American Type Culture Collection. PANC-1 cells were cultured in DMEM (HyClone Laboratories Inc, USA) containing 10% foetal bovine serum (Biological Industries, ISR). hTERT-HPNE cell lines were cultured in the recommended complete growth medium, which included 5% foetal bovine serum, 75% DMEM without glucose, 25% Medium M3 Base, 10 ng/mL human recombinant EGF, 5.5 mmol/L D - glucose (1 g/L) and 750 ng/mL puromycin. We collected surgical resection of PDAC tissues from 5 patients with pancreatic head cancer and normal pancreatic tissues from 5 patients with benign space-occupying duodenum between January 2019 and December 2019 in the Department of Biliary and Pancreatic Surgery of the First Affiliated Hospital of University of Science and Technology of China and then used a previous method to isolate and culture PSCs.[15] Then, CaPSCs derived from pancreatic cancer and NaPSCs derived from normal pancreas were obtained. All cell lines were cultured in a 37°C, 5% CO₂ incubator. Transwell co-culture system (6-well plate, 0.4µm, 3412#, Corning, USA) was used. The upper chamber was seeded with 3×10⁵ PANC-1 or hTERT-HPNE, and the lower plate was seeded with 3×10⁵ human pancreatic stellate cells from different sources in a 5% incubator at 37 °C for 24 h.

Immunofluorescence

The specific operation process was the same as before.[15] Isolated PSCs from different sources were inoculated into a six-well culture plate equipped with a cover slip, cultured under standard conditions for 48 hours, and then incubated with primary antibody (rabbit monoclonal anti- α -SMA, 1:100, Abcam, USA). The cells were detected and imaged with a fluorescence microscope, and the purity of PSCs was analysed.

Cell proliferation assay

PANC-1 and HPNE cells co-cultured with PSCs from different sources were routinely digested and seeded in a 96-well plate at 3000 cells per well. The Cell Counting Kit-8 (CCK-8, Beyotime, Shanghai, China) method was used to detect the proliferation of pancreatic cancer cells, and cells that were not co-cultured were used as controls. Briefly, premixed medium (10 μ L of CCK-8 solution, 100 μ L of medium) was added to each well, and after incubation in the dark at 37°C for 3 h, a microplate reader was used to measure the light absorption value at 450 nm to evaluate cell viability.

Migration and invasion assays

The effect of activated PSCs on the migration and invasion of pancreatic ductal cells was evaluated using Transwell chambers. CaPSCs and NaPSCs were inoculated in a 24-well plate (24-well plate, 8 μ m, 3422#, Corning, USA) at a density of 5×10^4 cells/well. After 24 h, the medium was replaced with 750 μ L of 10% FBS DMEM. The chambers were placed in a 24-well culture dish, and HPNE (2×10^4) and PANC-1 (2×10^4) cells resuspended in 200 μ L of serum-free medium were then seeded into the upper compartments containing either uncoated or Matrigel-coated membranes. After incubation for 24 h, the compartments were removed for cleaning, and cells on the lower surface were fixed with methanol and stained with crystal violet. The migrated cells on the lower surface were counted to assess migration and invasion.

RNA isolation and quantitative real - time PCR

Cellular RNA was extracted by an RNA extraction kit (Takara, Japan), and cDNA was synthesized by a reverse transcription kit (Takara, Japan). qPCR was performed on a real-time PCR system (Roche, Switzerland) using a SYBR Green PCR kit (Takara, Japan). All primers (Table 1) were synthesized by Sangon Biotech (Shanghai, China). The RNA levels were normalized to human ACTB(β -actin) levels.

Table 1 Primer sequence

Gene name	Primer sequences, 5'-3'
PKM2	F: TGACGAGAACATCCTGTGGC
	R: AGCACAGATGACAGGCTTCC
LDHA	F: GCCTGTATGGAGTGGAATGAA
	R: CCAGGATGTGTAGCCTTTGAG
ACTB	F: CTGGAACGGTGAAGGTGACA
	R: AAGGGACTTCCTGTAACAACGCA

Western blotting

Briefly, RIPA lysis buffer (Beyotime, Shanghai, China) was applied to fully lyse the cells and extract total protein. After the protein concentration was determined, loading buffer (1:4, Beyotime) was added and then denatured in boiling water. SDS-PAGE was used after protein loading. After electrophoresis, proteins on the gel were transferred to PVDF membranes by the wet transfer method. After full sealing with the blocking solution, the membrane was transferred to a primary antibody (PKM2, LDHA rabbit polyclonal antibody, Abcam, USA) diluted 1:1000 and incubated at 4°C overnight. After washing the membrane, it was transferred to the secondary antibody diluted at an appropriate proportion and incubated at room temperature for 2 h. After washing the membrane again, immunoreactions were visualized by chemiluminescence.

Lactate production and glucose utilization assays

Human pancreatic stellate cells (3×10^5 cells/well) from different sources were seeded into 6-well plates for later use. Transwell co-culture system (6-well plate, 0.4 μ m, 3412#, Corning, USA) was used. The upper chamber was seeded with 3×10^5 PANC-1 or HPNE, while the lower chamber was not seeded with cells. After 12h culture with 10%FBS medium, the cells were replaced with serum-free medium for starvation overnight. Subsequently, the upper chambers were moved to six-well plates pre-inoculated with human pancreatic stellate cells from different sources, and co-cultured for 24h. According to the instructions, a glucose detection kit and lactic acid detection kit (Jiancheng Bioengineer Institute, Nanjing, China) were used to detect the glucose and lactic acid contents in the culture medium of HPNE and PANC-1 cells co-cultured from different PSC sources, and normalization treatment was conducted according to the number of cells. Three replicates were established for each group, and the experiment was repeated three times. Glucose intake = (glucose content in the original medium - glucose content in the medium after 24 h of co-culture)/number of cells; lactic acid secretion = (lactic acid content in the medium after 24 h of co-culture - lactic acid content in the original medium)/number of cells.

Animals and animal models

Adult male SD rats weighing 180–200 g were used. They were maintained in accordance with the guidelines of the Committee on Animal Care of the First Affiliated Hospital of University of Science and Technology of China. Twenty rats in the CP model group were injected with Dibutyltin dichloride (DBTC, Sigma, Germany) solution (8 mg/mL/kg) through the tail vein, and six rats in the control group were injected with an equal volume of normal saline. After 6 weeks of modelling, the rats were anaesthetized and killed by cervical dislocation. Twenty rats in the PDAC model group were fasted for 24 h before surgery (water was provided). After intraperitoneal injection of 3% pentobarbital sodium 1.5 ml/kg, the pancreas was exposed through a median incision in the upper abdomen, and then the pancreatic capsule was cut open at the flat and firm part of the pancreas. The model group was implanted with 6 mg 7,12-Dimethylbenz[a]anthracene (DMBA, Sigma, Germany), and six rats in the control group underwent no other operations. Then, the abdomens were closed after the capsules were sutured. After 16 weeks of modelling, the rats were anaesthetized and then killed by cervical

dislocation. All tissue specimens were fixed with formalin for 24 h and then embedded in paraffin. The animal models were confirmed by pathological diagnosis.

Immunohistochemistry

The paraffin-embedded sections were dewaxed with xylene, hydrated with gradient ethanol and boiled in citrate buffer for 20 min. Endogenous peroxidase activity was blocked by 3% H₂O₂. Rabbit polyclonal antibodies (Abcam, USA) against PKM2, LDHA and α -SMA were added at a concentration of 1:100 and incubated overnight at 4 °C. After incubation with the secondary antibody for 1 hour, DAB was used for colour development. Finally, the sections were re-stained with haematoxylin and fixed by dehydration. For the negative control group, the main antibodies were replaced with PBS under the same conditions. Semi-quantitative method was used to score the results: staining area: 0, no staining; 1 point, 10%; 2 points, 10%-30%; 3, 30% staining; Dyeing intensity: 0, none; 1 points, light yellow; 2 points, yellow; 3 points, brown and yellow. The total score was the product of the staining area and staining intensity score. Low expression was defined as a total score ≤ 3 , and high expression was defined as a total score > 3 . All immunohistochemical slides were scored independently by two experienced pathologists, and differences were adjusted for by a joint assessment.

Statistical analysis

Statistical data are presented as the means \pm SDs (n = 3). Student's t test, chi-square test and Spearman correlation analysis were used for statistical analysis. P values of <0.05 were accepted as statistically significant.

Results

Immunofluorescence shows that CaPSCs had the characteristics of activated PSCs

We used previous methods to obtain CaPSCs and NaPSCs from pancreatic cancer and normal pancreatic tissues, respectively.¹⁵ Phenotypic identification of CaPSCs and NaPSCs was performed by immunofluorescence labelling of α -SMA, a marker protein of activated pancreatic stellate cells. Although quiescent PSCs will gradually activate and express α -SMA in vitro, immunofluorescence can distinguish them from activated PSCs. The expression of α -SMA in CaPSCs and their phenotypic characteristics were significantly different from NaPSCs, indicating that CaPSCs had the characteristics of activated PSCs. (Figure 1)

Co-culture with CaPSCs promotes the proliferation, migration and invasion of benign and malignant pancreatic duct epithelial cells

We examined the percentage changes in the proliferation, migration and invasion abilities of PANC-1 and HPNE cells in each co-culture system. The CCK-8 proliferation assay showed that the proliferation ability of HPNE and PANC-1 cells in the CaPSC co-culture group was significantly improved compared with that in the blank groups. (Figure 2a, b). Transwell experiments showed that the migration and invasion abilities of HPNE and PANC-1 cells in the CaPSC co-culture group were significantly improved compared with those in the NaPSC and blank groups. (Figure 2c, d)

Co-culture with CaPSCs promotes the Warburg effect in pancreatic duct epithelial cells (increased expression of PKM2 and LDHA; increased lactic acid production and glucose consumption)

To explore why CaPSCs promote the proliferation, migration and invasion of pancreatic cancer cells and normal pancreatic duct epithelial cells, we examined the effect of co-culture on aerobic glycolysis (Warburg effect) in HPNE and PANC-1 cells. First, we detected the changes in the lactic acid production and glucose consumption of HPNE and PANC-1 cells after co-culture, and the results showed that the lactic acid production and glucose consumption of HPNE and PANC-1 cells in the CaPSC group were significantly higher than those in the NaPSC group and blank group. (Figure 3a-d) Subsequently, we used Western blotting and qPCR to detect the expression of two glycoproteins (PKM2 and LDHA) known to play an important role in the Warburg effect, and the results showed that the protein and mRNA expression levels of PKM2 and LDHA in PANC-1 and HPNE cells in the CaPSC co-culture group were significantly increased compared with those in the NaPSC and control groups. (Figure 3e, f)

Establishment of CP and PDAC rats; immunohistochemical analysis showing the activation of PSCs and the enhancement of glycolysis in diseased tissues.

After finding that CaPSCs can promote glycolysis in pancreatic cancer and normal pancreatic duct epithelial cells, we further investigated whether the two are related in animal and human tissues. We successfully obtained animal models of CP and PDAC in rats by means of drug induction. (Figure 4) The statistical results of animal model establishment are briefly listed in Table 2 and Table 3. The CP rat model was established by DBTC caudal intravenous injection, and the incidence of CP in rats was 35% after 6 weeks; the PDAC rat model was established using DMBA surgical implantation into the pancreas, and the incidence of PDAC in rats was 30% after 16 weeks. Subsequently, paraffin sections of CP and PDAC pancreatic tissues were stained with α -SMA and PKM2/LDHA immunohistochemistry, and the staining results were scored. The results showed that PKM2 and LDHA were not expressed or weakly expressed in pancreatic duct epithelial cells and that α -SMA was weakly expressed in peri-pancreatic duct stroma in normal pancreatic tissue of rats. (Figure 5a-d) PKM2/LDHA in pancreatic duct epithelial cells and α -SMA in peripancreatic duct stroma were significantly higher in the CP group than in the normal pancreas group. (Figure 5e-h) This phenomenon has also been observed in rat pancreatic cancer tissues, and protein expression at these sites was higher than that in the CP group. (Figure 5i-j)

Table 2

Statistical results of CP animal model

	DBTC	NS control
CP	7(35.0%)	0(0%)
Pancreatic edema/hemorrhage only	5(25.0%)	0(0%)
Death	2(10.0%)	0(0%)
Normal pancrea	6(30.0%)	6(100%)
Total	20(100%)	6(100%)

Table 3**Statistical results of PDAC animal model**

	DMBA	Surgical control
PDAC	6(30.0%)	0(0%)
PanIN	3(15.0%)	0(0%)
Pancreatic edema/hemorrhage only	5(25.0%)	1(16.7%)
Death	3(15.0%)	1(16.7%)
Normal pancrea	1(5.0%)	4(66.6%)
Total	20(100%)	6(100%)

Immunohistochemical analysis of human CP and PDAC tissues shows that the activation of PSCs is correlated with the high expression of glycolytic proteins.

In immunohistochemical sections of human tissues, we observed findings similar to those in the animal model, namely, activation of PSCs and enhancement of glycolytic metabolism. (Figure 6) Considering the heterogeneity of human pathological sections, we analysed the correlation between α -SMA in the peripancreatic duct interstitium and glycoprotein expression in pancreatic duct epithelial cells in the CP and PDAC groups. We first confirmed that the expression of α -SMA in the CP group and PDAC group was significantly different from that in the normal pancreas group. (Table 4) Correlation analysis (Table 5) showed that there was a correlation between PKM2/LDHA expression in pancreatic duct epithelial cells and α -SMA expression in the peri-pancreatic duct interstitium in both the CP and PDAC groups and that the correlation between α -SMA and PKM2/LDHA expression was higher in the PDAC group than in the CP group.

Table 4**Differential expression of α -SMA in CP/PDAC tissues and normal pancrea tissues**

	α -SMA expression		Total	P-value
	Low	High		
CP	5	14	19	<0.01 [†]
PDAC	5	35	40	<0.001 [†]
Normal pancrea	16	4	20	

[†]: Compared with normal pancrea tissues

Table 5
Correlations of α -SMA expression with PKM2、LDHA in CP /PDAC tissues

Immunoreactivity		α -SMA expression		r-value	P-value
		Low	High		
CP	PKM2 expression				
	Low	4	4	0.459	<0.05
	High	1	10		
PDAC	PKM2 expression				
	Low	4	3	0.520	<0.01
	High	1	32		
CP	LDHA expression				
	Low	3	2	0.457	<0.05
	High	2	12		
PDAC	LDHA expression				
	Low	4	2	0.688	<0.001
	High	1	33		

Discussion

PSCs are pancreatic-specific mesenchymal cells that accumulate around blood vessels and ducts in pancreatic tissue and surround the base of acini. PSCs only account for approximately 4% of pancreatic cells in the normal quiescent state; their cytoplasm contains abundant lipid droplets containing vitamin A, and they are characterized by the expression of GFAP and desmin.[16-18] Quiescent PSCs in normal tissues play an important role in the metabolic homeostasis of pancreatic ECM by regulating ECM-related enzymes. PSCs are activated during pancreatic tissue injury and stress and acquire a myofibroblast phenotype. Lipid droplets rich in vitamin A disappear in their cytoplasm. With the expression of α -smooth muscle actin (α -SMA) as a marker, a large amount of extracellular matrix (ECM) is synthesized, and a variety of cytokines are secreted.[7] Activated PSCs have contractile microfilament-like structures, which can help them migrate to the injured area and secrete ECM to promote pancreatic tissue repair. When the stimulating factor persists, the synthesis of ECM exceeds its degradation, so pancreatic fibrosis occurs.[8] Pancreatic stellate cells are currently considered to be the initiators of chronic pancreatitis and pancreatic cancer. Studies have shown that activated PSCs in the extracellular matrix of pancreatic cancer are cancer-associated fibroblasts (CAFs). CAFs are activated fibroblasts and an important part of tumour stromal cells.[19-21] Through specific communication with cancer cells, CAFs directly promote tumour initiation, progression, and metastasis.[22, 23]

In this study, we successfully obtained activated and quiescent PSCs from pancreatic cancer patients and individuals with a normal pancreas using a method described previously, and phenotypic identification was performed by immunofluorescence. In our previous work, we found that activated PSCs promoted the proliferation, migration and invasion of pancreatic cancer cells.[24] We replicated the results using activated cells isolated in this study. Subsequently, we co-cultured activated PSCs with HPNE cells, a normal pancreatic duct

epithelial cell line that is thought to have the potential to differentiate into cancer cells,[25] and found that activated PSCs also promoted the proliferation, invasion, and migration of HPNE cells. When CP occurs, there are activated PSCs around benign pancreatic duct epithelial cells, and chronic pancreatitis is a precancerous lesion. Does this phenomenon suggest that activated PSCs have a role in promoting the malignant transformation of CP? Studies have shown that chronic pancreatitis is the result of the combined action of cytokines, inflammatory cells and activation of pancreatic stellate cells, among which PSCs are considered to be the target of various cytokines in chronic pancreatitis and the core of the disease progression of CP.[26] In summary, PSCs are closely related to the occurrence and development of chronic pancreatitis and pancreatic cancer, but their role in the carcinogenesis of chronic pancreatitis has not yet been elucidated.

We then began to explore the possible mechanisms by which activated PSCs influence the malignant degeneration of pancreatic duct epithelial cells. As a characteristic of cancer cells, the Warburg effect is a very obvious feature of benign cells after malignant transformation. At present, some studies have confirmed that CAFs are involved in regulating and promoting the glucose metabolism of cancer cells. Shan et al.[13] found that CAFs could promote the glucose metabolism of pancreatic cancer cells and enhance their migration and invasion ability. Zhao et al.[14] found that exosomes secreted by CAFs can regulate glucose metabolic reprogramming of pancreatic cancer cells through the KRAS pathway and enhance their invasion and metastasis abilities. Therefore, will PSCs enhance glycolytic metabolism in CP and eventually lead to malignant transformation of CP? We tentatively detected the changes in glycolytic protein and glucose metabolic capacity of HPNE after co-culture with PSCs. The results showed that co-culture with CaPSCs significantly promoted the expression of the glucose metabolic protein PKM2/LDHA (including mRNA level) in HPNE cells, and concurrently, lactic acid production and glucose consumption were also significantly increased after co-culture. These results suggest that activated PSCs may enhance the Warburg effect in HPNE cells to promote their malignant transformation. Subsequently, we tested this idea in vivo. We established rat models of CP and PDAC using drug induction. To verify the relationship between α -SMA in PSCs and aerobic glycolytic protein in pancreatic duct epithelial cells in the same region, paraffin sections of CP and PDAC tissues were obtained for immunohistochemical identification of α -SMA and glycolytic protein (PKM2 and LDHA), respectively, and compared with those of normal pancreas. The results showed that the expression of α -SMA in both pancreatic duct cells and surrounding stromal cells was low in normal pancreas, but the expression of α -SMA in pancreatic duct epithelial cells and surrounding stromal cells was increased in chronic pancreatitis, and this increase was more obvious in pancreatic cancer. The results showed that the expression of glycolytic proteins in pancreatic duct cells and α -SMA in surrounding stromal cells was low in normal pancreatic tissues, but the expression of these proteins was increased in CP tissues, and the increase was more pronounced in PDAC tissues. Then, we collected pathological sections from clinical patients and explored the correlation between α -SMA and PKM2/LDHA in heterogeneous pathological sections of human CP and PDAC. Compared with that in normal pancreatic tissue, α -SMA expression in the matrix of human CP and PDAC tissue was increased significantly. The correlation between the glycoprotein of pancreatic duct cells and the activation of pancreatic stellate cells in the surrounding matrix was observed in both chronic pancreatitis and pancreatic cancer. This

suggests an association between activated PSCs and the Warburg effect in pancreatic duct epithelial cells.

Conclusion

In summary, we believe that activated pancreatic stellate cells have the ability to enhance the Warburg effect in pancreatic duct epithelial cells and play a supporting and promoting role in the malignant progression of chronic pancreatitis. The idea and process of the whole experiment are shown in Figure 7. The specific influencing mechanism needs to be further studied. Our current study provides a new theoretical basis for further understanding the mechanism of CP malignancy and selecting diagnosis and treatment targets for reversing CP malignancy.

Acknowledgements

None.

Authors' contributions

FM and QH contributed to the conception and design, as well as critical revision of the article for important intellectual content. YT and FS conceived and designed the experiments, performed the experiments and analyzed and interpreted the data, drafted and revised the manuscript. ZL and MC analyzed and interpreted the data.

All authors read and approved the final manuscript.

Funding

This study was supported by funds from Anhui Provincial Natural Science Foundation (2008085QH416 and 2008085QH419) and New Medical Association of University of science and technology of China (WK9110000105).

Availability of data and materials

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

Declarations

Ethics approval and consent to participate

All the clinical and animal experiments were approved by the Ethics Committee of the Anhui Provincial Hospital of the First Affiliated Hospital of the University of Science and Technology of China, and informed consent was obtained from each participant. The experimental animals were maintained in accordance with the guidelines of the Committee on Animal Care of the First Affiliated Hospital of University of Science and Technology of China.

Consent for publication

All the authors agreed to be published

Competing interests

There is no conflict of interest.

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Figure 1:

The expression of α -SMA in NaPSCs and CaPSCs was detected by immunofluorescence, and CaPSCs showed a highly activated state.

a-c: NaPSC immunofluorescence—DAPI, α -SMA, MERGE;

d-f: CaPSC immunofluorescence—DAPI, α -SMA, MERGE.

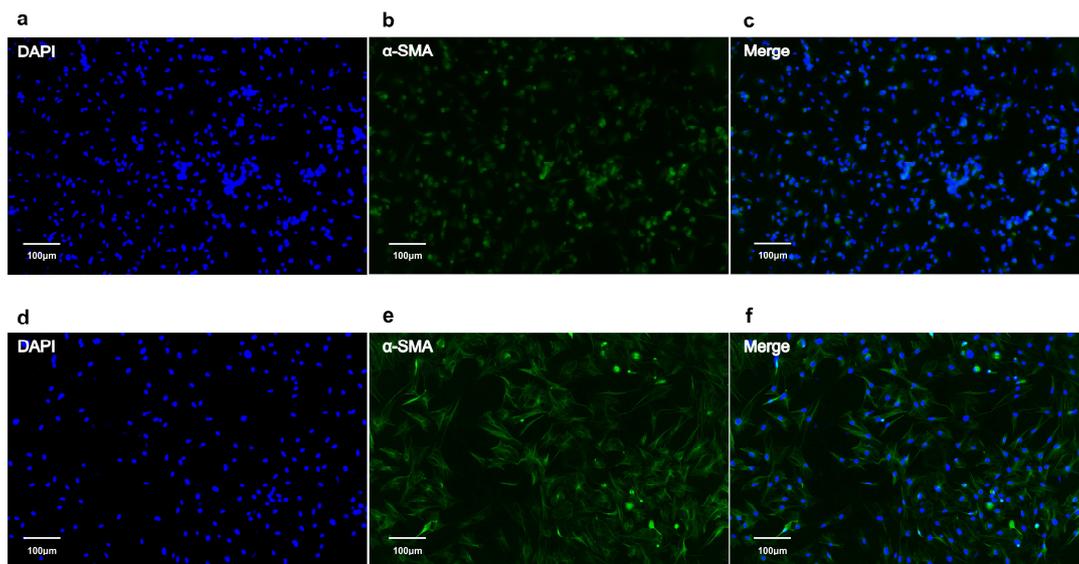


Figure 2:

Co-culture with CaPSCs enhanced the proliferation, invasion and migration of HPNE and PANC-1 cells.

a: CCK-8 proliferation assay after co-culture of HPNE cells with NaPSCs or CaPSCs.

b: CCK-8 proliferation assay after co-culture of PANC-1 cells with NaPSCs or CaPSCs.

c: Transwell migration and invasion assays after co-culture of HPNE cells with NaPSCs or CaPSCs.

d: Transwell migration and invasion assays after co-culture of PANC-1 cells with NaPSCs or

CaPSCs.

*P < 0.05, **P < 0.01, ***P < 0.001

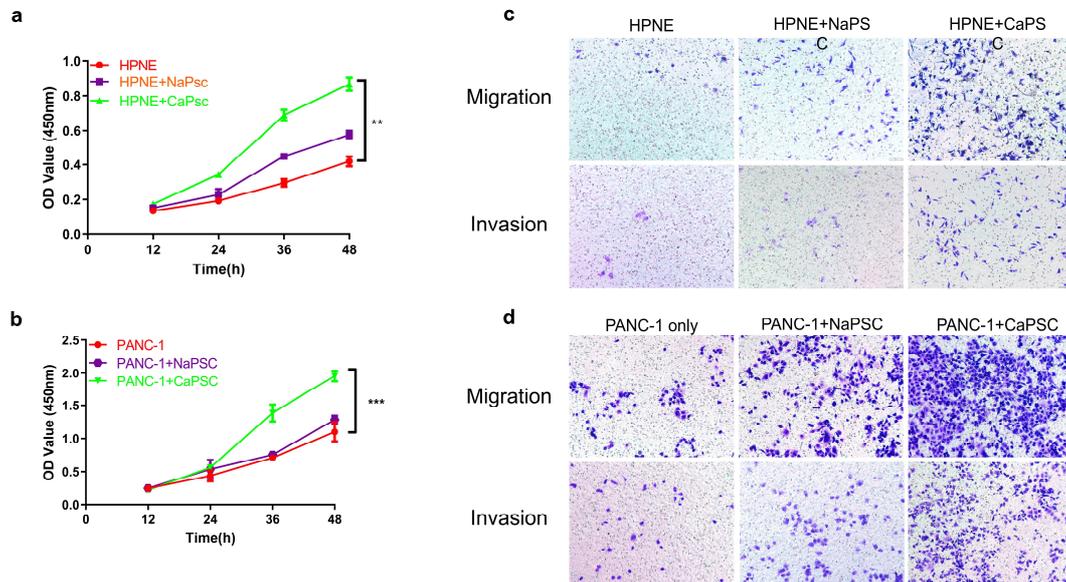


Figure 3:

Co-culture with CaPSCs promoted lactic acid production, glucose consumption and the expression of the glycolytic proteins LDHA/PKM2 in HPNE and PANC-1 cells.

a: Changes in HPNE cell lactic acid production after co-culture with NaPSCs or CaPSCs;

b: Changes in HPNE cell glucose consumption after co-culture with NaPSCs or CaPSCs;

c: Changes in PANC-1 cell lactic acid production after co-culture with NaPSCs or CaPSCs;

d: Changes in PANC-1 cell glucose consumption after co-culture with NaPSCs or CaPSCs;

e: Changes in the protein and mRNA expression of LDHA and PKM2 in HPNE cells after co-culture with NaPSCs or CaPSCs;

f: Changes in the protein and mRNA expression of LDHA and PKM2 in PANC-1 cells after co-culture with NaPSCs or CaPSCs;

*P < 0.05, **P < 0.01, ***P < 0.001

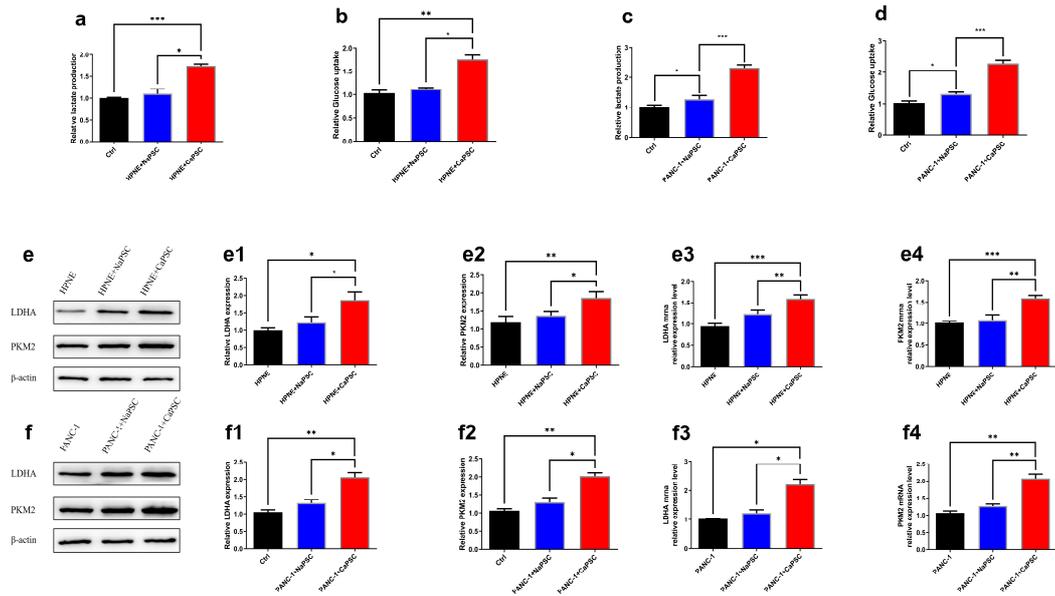


Figure 4:

Establishment and identification of CP and PDAC model rats

a: The rat CP models were established by injecting DBTC solution through the tail vein. B-C: After 6 weeks of modelling, the pancreas of rats in the CP group and the pancreas of rats in the control group (injection of saline) were collected. Severe fibrosis was observed in the pancreas - yellow arrow of the CP group.

b: Rats with orthotopic pancreatic cancer induced by surgical implantation of DMBA;

e-f: Sixteen weeks after modelling, the pancreas of rats in situ PDAC and the pancreas of rats in the sham operation control group. Severe fibrosis and local mass formation were observed at the pancreatic red arrow of the rats in the in situ PDAC group.

g-h: Gross pancreatic specimens and pathological HE staining of rats in the CP group and control group;

i-j: Gross pancreatic specimens and pathological HE staining of rats in the in situ PDAC group and control group.

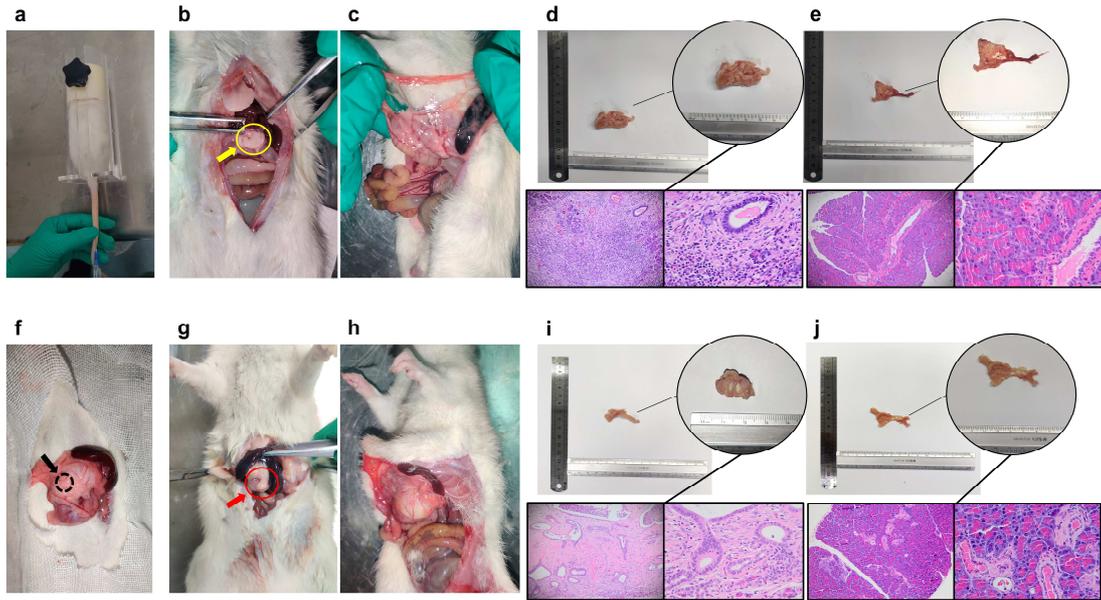


Figure 5:

Immunohistochemistry showed that the α -SMA and glycolytic protein levels in the pancreatic tissues of rats in the normal group, CP group and PDAC group gradually increased

a-d: HE, α -SMA, LDHA, PKM2 in normal rat pancreas

e-h: HE, α -SMA, LDHA, PKM2 in CP rat pancreas

i-l: HE, α -SMA, LDHA, PKM2 in PDAC rat pancreas

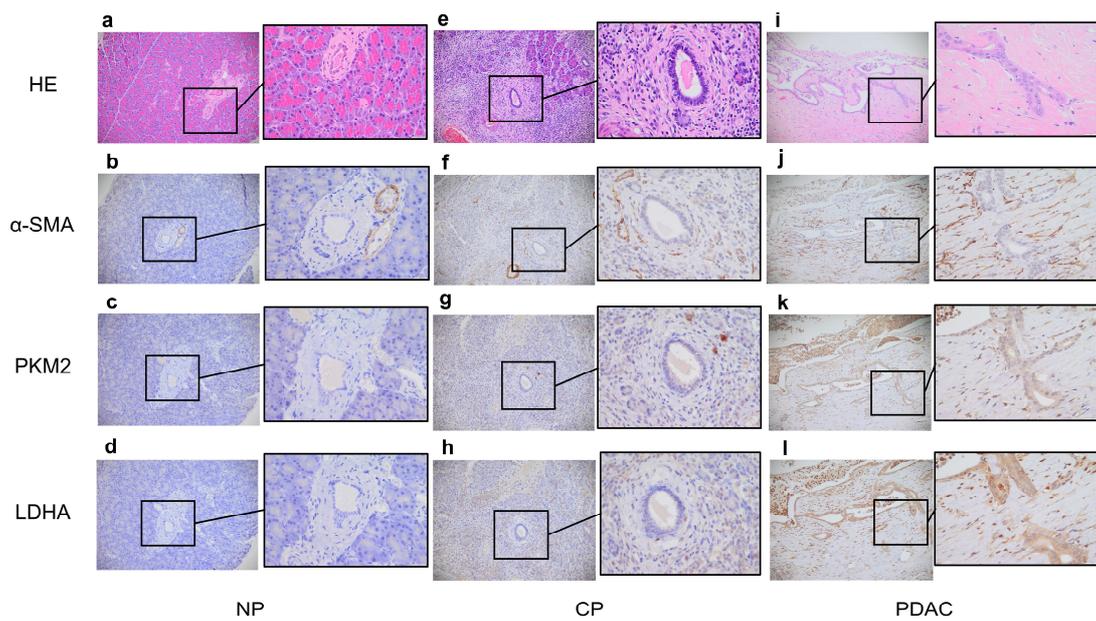


Figure 6:

Immunohistochemistry showed that the α -SMA and glycolytic protein levels in the pancreatic tissues of humans in the normal group, CP group and PDAC group gradually increased

a-d: HE, α -SMA, LDHA, PKM2 in normal human pancreas

e-h: HE, α -SMA, LDHA, PKM2 in CP human pancreas

i-l: HE, α -SMA, LDHA, PKM2 in PDAC human pancreas

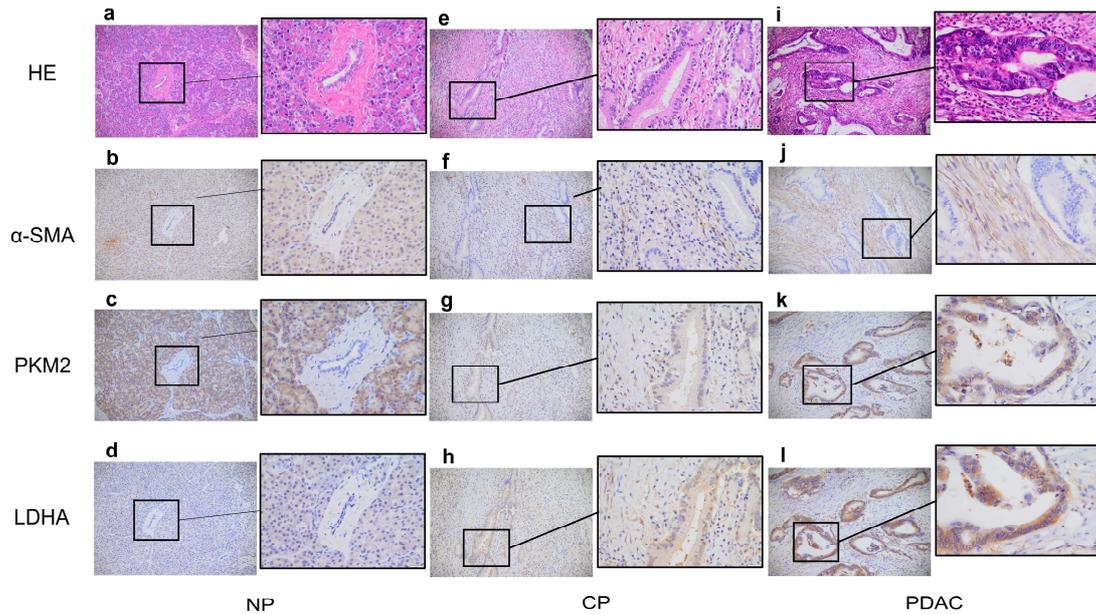
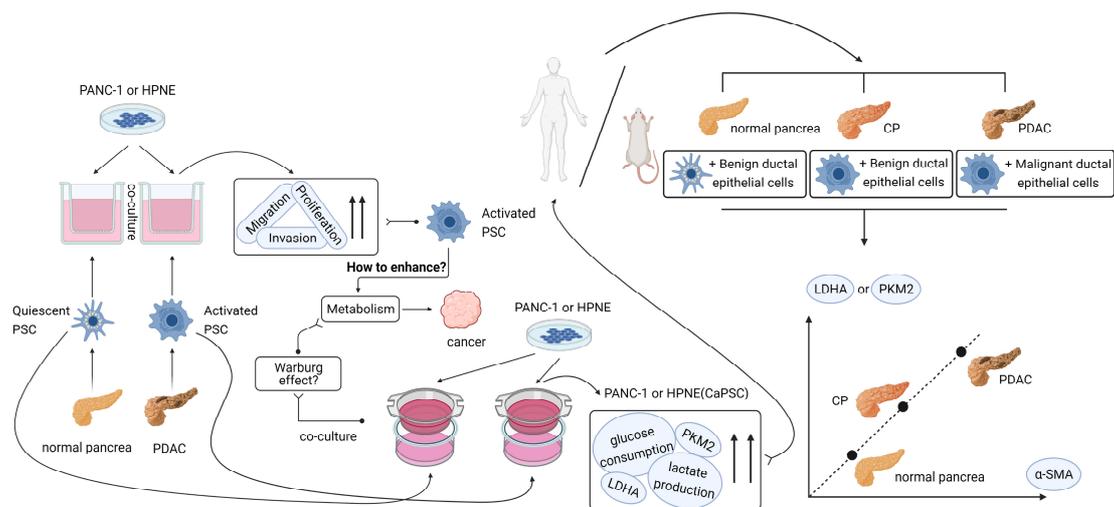


Figure 7:

Design idea and verification flow chart of this study. After finding that activated PSCs of pancreatic cancer origin can promote the proliferation, migration and invasion of benign and malignant pancreatic duct epithelial cells, we hypothesized that activated PSCs may promote the malignant transformation of chronic pancreatitis. In the process of searching for the mechanism, a series of experiments confirmed that the activated PSCs could promote the Warburg effect in benign pancreatic duct epithelial cells in vitro, and the correlation between the glycolytic proteins of pancreatic duct cells and the activated PSCs in the peri-pancreatic duct matrix was confirmed in vivo.



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Figures

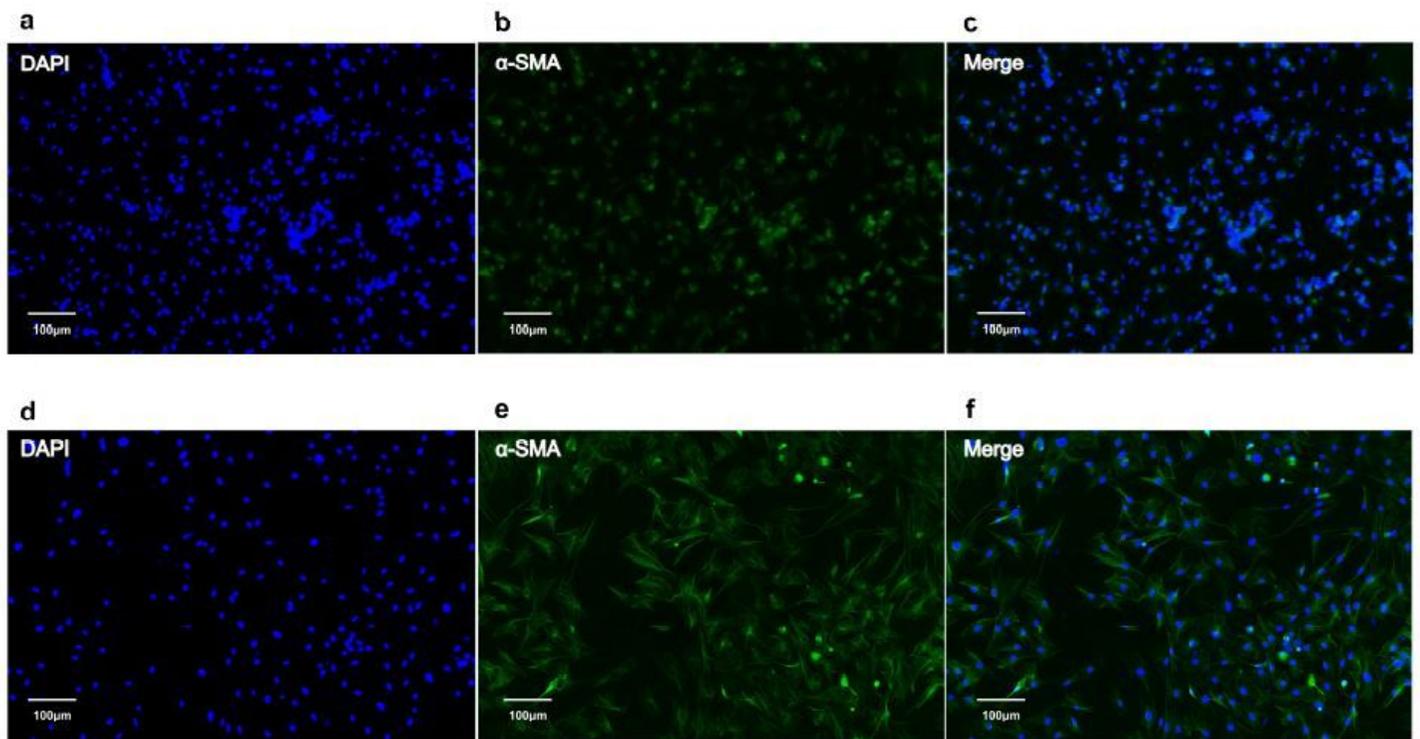


Figure 1

The expression of α -SMA in NaPSCs and CaPSCs was detected by immunofluorescence, and CaPSCs showed a highly activated state. a-c: NaPSC immunofluorescence—DAPI, α -SMA, MERGE; d-f: CaPSC immunofluorescence—DAPI, α -SMA, MERGE.

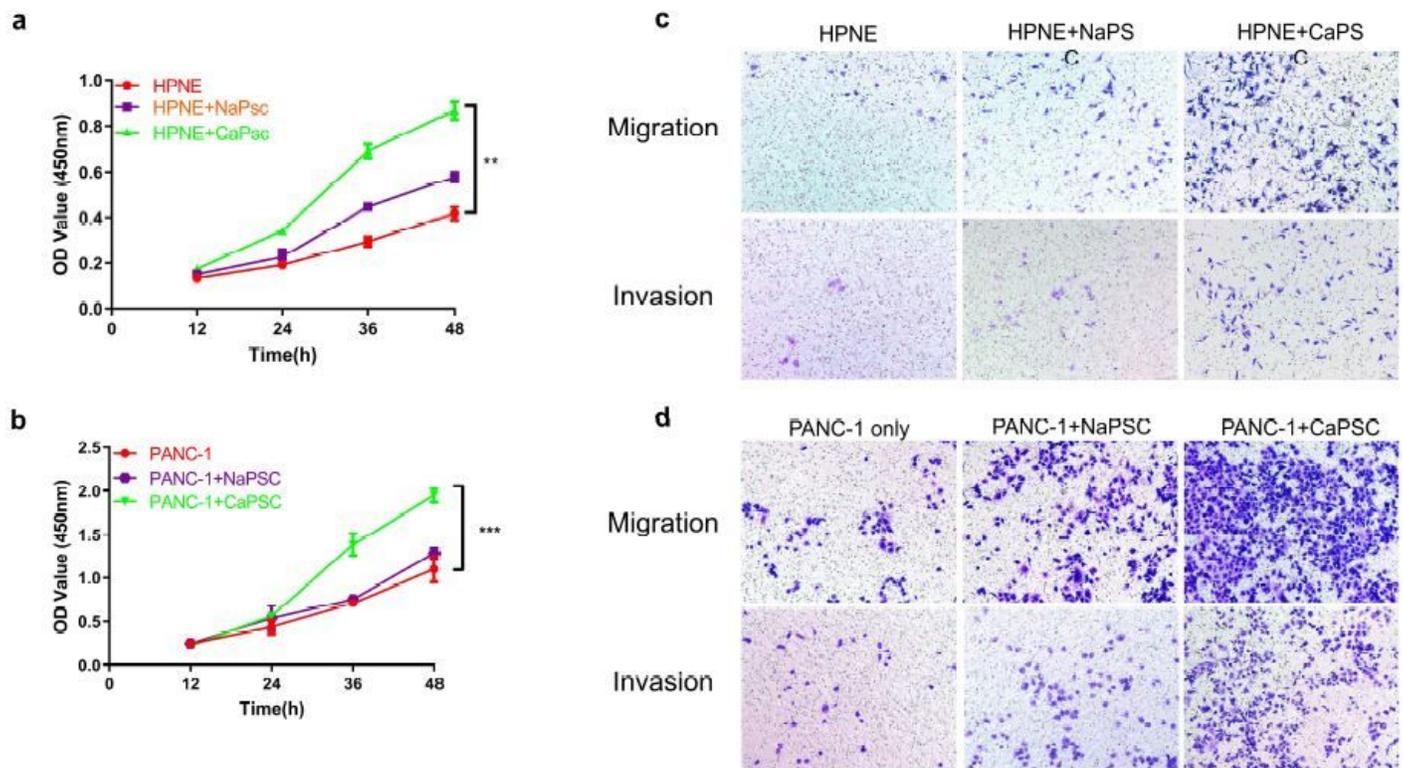


Figure 2

Co-culture with CaPSCs enhanced the proliferation, invasion and migration of HPNE and PANC-1 cells. a: CCK-8 proliferation assay after co-culture of HPNE cells with NaPSCs or CaPSCs. b: CCK-8 proliferation assay after co-culture of PANC-1 cells with NaPSCs or CaPSCs. c: Transwell migration and invasion assays after co-culture of HPNE cells with NaPSCs or CaPSCs. d: Transwell migration and invasion assays after co-culture of PANC-1 cells with NaPSCs or CaPSCs. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

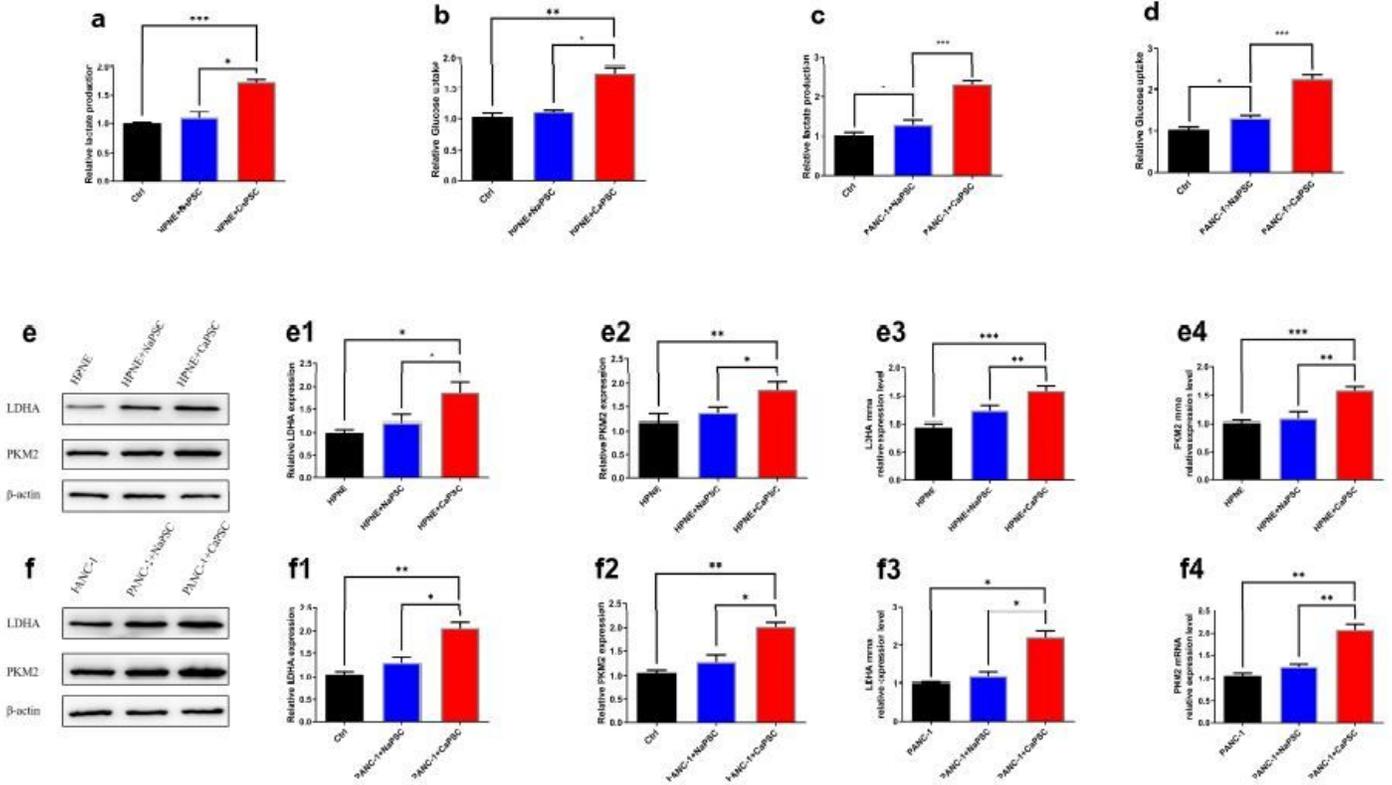


Figure 3

Co-culture with CaPSCs promoted lactic acid production, glucose consumption and the expression of the glycolytic proteins LDHA/PKM2 in HPNE and PANC-1 cells. a: Changes in HPNE cell lactic acid production after co-culture with NaPSCs or CaPSCs; b: Changes in HPNE cell glucose consumption after co-culture with NaPSCs or CaPSCs; c: Changes in PANC-1 cell lactic acid production after co-culture with NaPSCs or CaPSCs; d: Changes in PANC-1 cell glucose consumption after co-culture with NaPSCs or CaPSCs; e: Changes in the protein and mRNA expression of LDHA and PKM2 in HPNE cells after coculture with NaPSCs or CaPSCs; f: Changes in the protein and mRNA expression of LDHA and PKM2 in PANC-1 cells after coculture with NaPSCs or CaPSCs; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

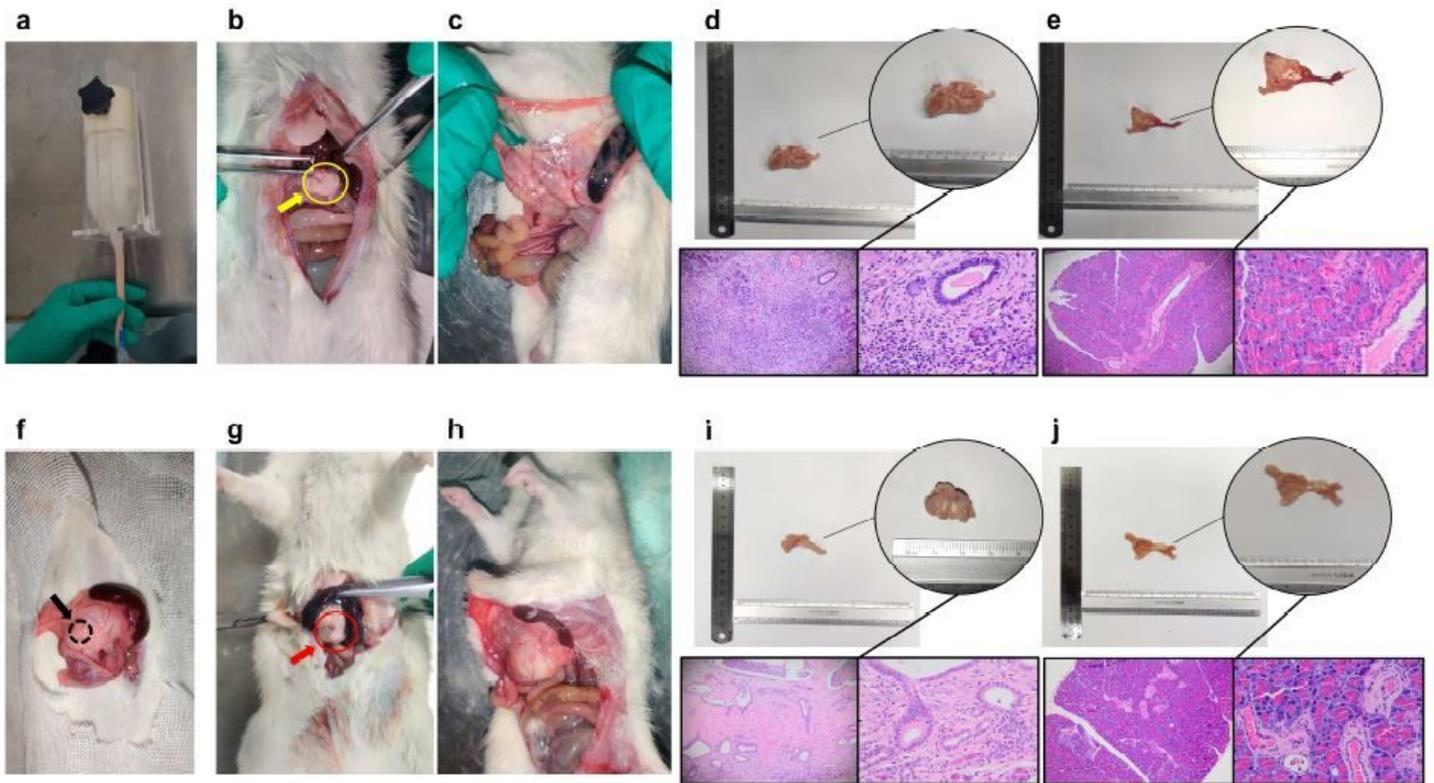


Figure 4

Establishment and identification of CP and PDAC model rats a: The rat CP models were established by injecting DBTC solution through the tail vein. B-C: After 6 weeks of modelling, the pancreas of rats in the CP group and the pancreas of rats in the control group (injection of saline) were collected. Severe fibrosis was observed in the pancreas - yellow arrow of the CP group. b: Rats with orthotopic pancreatic cancer induced by surgical implantation of DMBA; e-f: Sixteen weeks after modelling, the pancreas of rats in situ PDAC and the pancreas of rats in the sham operation control group. Severe fibrosis and local mass formation were observed at the pancreatic red arrow of the rats in the in situ PDAC group. g-h: Gross pancreatic specimens and pathological HE staining of rats in the CP group and control group; i-j: Gross pancreatic specimens and pathological HE staining of rats in the in situ PDAC group and control group.

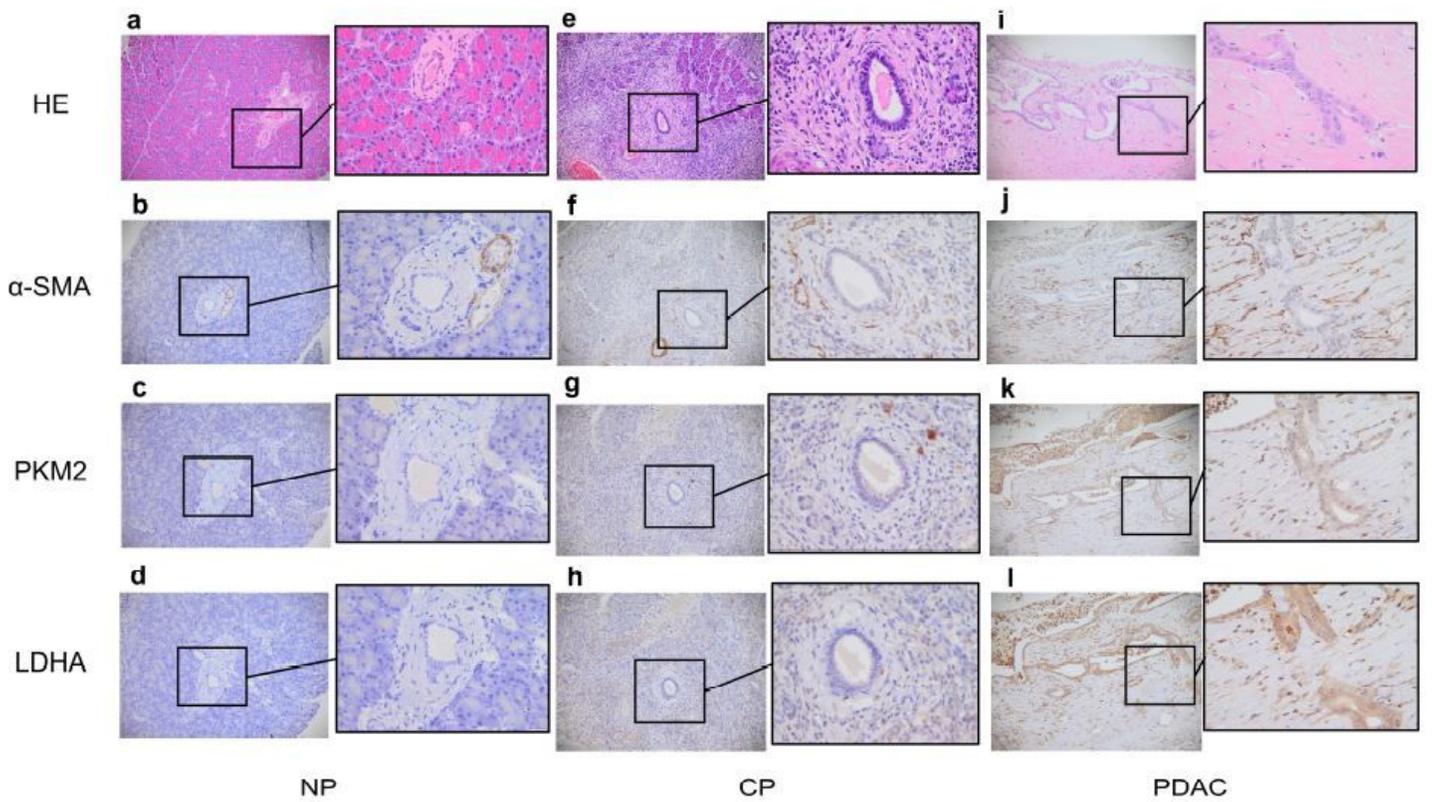


Figure 5

Immunohistochemistry showed that the α -SMA and glycolytic protein levels in the pancreatic tissues of rats in the normal group, CP group and PDAC group gradually increased a-d: HE, α -SMA, LDHA, PKM2 in normal rat pancreas e-h: HE, α -SMA, LDHA, PKM2 in CP rat pancreas i-l: HE, α -SMA, LDHA, PKM2 in PDAC rat pancreas

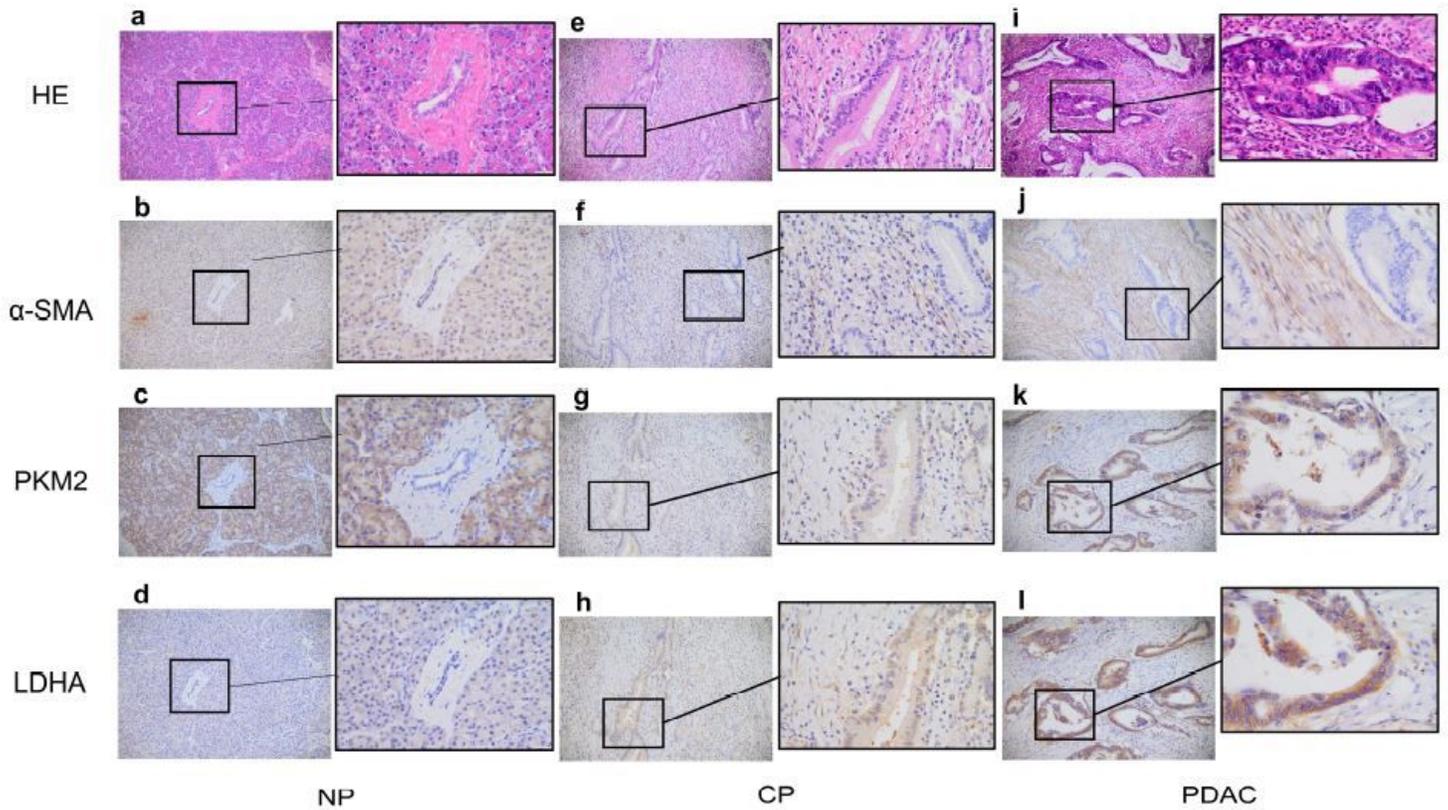


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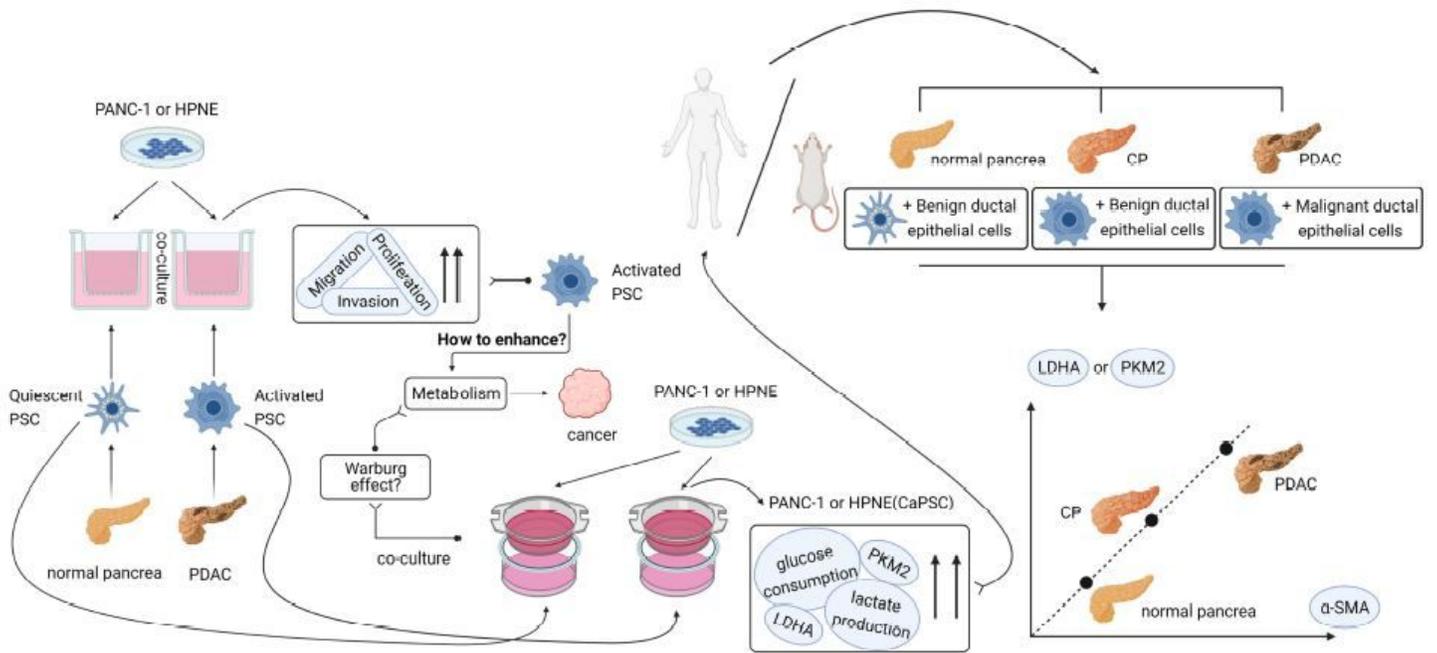


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