Fucoxanthin suppresses pancreatic cancer progression by inducing bioenergetics metabolism crisis and promoting SLC31A1-mediated sensitivity to DDP

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Received September 6, 2024; Accepted January 16, 2025

DOI: 10.3892/ijo.2025.5737

Abstract. Pancreatic cancer (PC) is one of the most malignant tumors, with a 5-year survival rate <10%. Chemosynthetic drugs are widely used in the treatment of PC; however, their toxicity and side effects often reduce the quality of life for patients. MTT and colony formation assay were performed to detect cell growth and viability in PC cells. Levels of ROS in whole cell and mitochondria were analyzed through flow cytometry. ATP production was evaluated using an ATP Assay Kit. Cellular bioenergetics were analyzed with a Seahorse XFe96 Analyzer, and changes in target molecules were monitored by western blotting. The present study reports that fucoxanthin (FX), a carotenoid derived from aquatic brown seaweed, significantly inhibits PC by inhibiting cell proliferation and inducing cell death via the non-classical pathway. FX switches mitochondrial respiration to aerobic glycolysis in PC cells. Furthermore, FX decreases whole-cell ATP levels, which indicates that promotion of glycolysis does not compensate for FX-induced ATP depletion in mitochondria. Moreover, FX decreased the reduced glutathione/oxidized glutathione ratio observed under glucose-limited conditions. These alterations caused by FX may decrease metabolic flexibility, indicating higher sensitivity to glucose-limited (GL) conditions. FX increased the cytotoxicity of cisplatin (DDP) and the expression of solute carrier family 31 member 1 (SLC31A1) in PC cells. Furthermore, the knockdown of SLC31A1 can attenuate cytotoxicity caused by the combination of FX and DDP. It was inferred that FX increased the sensitivity of PC cells to DDP), potentially by upregulating SLC31A1 expression. In conclusion, FX exhibited potent antitumor effects by reprogramming energy metabolism and inducing a distinct form of regulated cell death. Therefore, combining FX with GL treatment or DDP presents a promising therapeutic strategy for PC.

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Key words: pancreatic cancer, fucoxanthin, GSH/GSSG, glucose-limited condition, SLC31A1, energy metabolism

Introduction

Pancreatic cancer (PC) is one of the most common malignant tumors of the digestive tract. The 5-year survival rate for pancreatic cancer is estimated to be 10% after diagnosis, making it one of the malignancies with the poorest prognosis (1). In the clinical setting, the common treatment strategy for PC is local surgical resection and adjuvant chemotherapy. However, toxicity and side effects of chemotherapeutic drugs often decrease quality of life (2). Therefore, the discovery, identification and development of novel antitumor drugs from safe, natural products are urgently needed to improve treatment and prognosis of patients with PC.

Fucoxanthin (FX) is a xanthophyll carotenoid extracted from marine aquatic brown seaweed such as *Hizikia fusifarme* and *Laminaria japonica*. It has antioxidant, anti-inflammatory, anti-obesity and antidiabetic properties (3). Furthermore, FX is incorporated as a functional ingredient in certain food, cosmetic and pharmaceutical products, suggesting it is a safe therapeutic natural extract with no adverse effects on the human body (3). FX has antitumor activity against various types of cancer (4). It has also been found to inhibit

tumor progression by inducing cell cycle arrest and apoptosis *in vitro* and suppress carcinogenesis *in vivo* in various types of cancer, such as colorectal and breast cancer, lymphoma and leukemia (5). Moreover, FX triggers LC3-dependent autophagy, which may be associated with apoptosis in cervical, gastric and nasopharyngeal cancers. However, the precise role of autophagy in different types of cancer remains controversial (6-8). FX has been shown to prevent tumorigenesis of murine PC by suppressing the chemokine (C-C motif) ligand 21/chemokine receptor 7 axis, T-lymphocyte attenuator, tumor microenvironment, epithelial-mesenchymal transition and adhesion (9). The metabolite of FX, fucoxanthinol (FxOH), has been shown to promote human PC PANC-1 cell proliferation while proliferation of MIA PaCa-2 PC cells (10). This suggests that cell heterogeneity may contribute to the differential anti-tumor effects of FXOH, potentially associated with genetic background of the cells. However, the effects of FX on human PC and its mode of action remain unclear. Therefore, the present study aims to investigate the potential antitumor effects of FX on human PC.

One potential mechanism by which FX may promote anticancer effects is altering cancer cell metabolism. Metabolic alterations are a key hallmark of cancer cells, enabling them to meet biosynthetic and energetic requirements (11). Therefore, targeting tumor metabolism may represent a promising strategy for cancer treatment. Tumor cells preferentially rely on anaerobic glucose metabolism (aerobic glycolysis) over mitochondrial oxidative phosphorylation (OXPHOS) to meet energy needs, even in the presence of oxygen (12). Most cancer cells rely on glycolysis for energy and macromolecule synthesis to sustain uncontrolled proliferation, contributing to decreased or dysfunction of mitochondrial OXPHOS (13,14). However, studies show that numerous cancer cell subpopulations rely on OXPHOS for bioenergetic and biosynthetic processes (15,16). For example, certain types of cancers (such as leukemia, lymphoma and PC) have increased mitochondrial function, even during active glycolysis (14-17). Furthermore, cancer cells retaining mitochondrial OXPHOS metabolism use glucose-derived pyruvate and also derive energy from alternative substrates, such as fatty acids and glutamine (Gln), in mitochondria (18). These cancer cells can upregulate the uptake of Gln and its conversion into a-ketoglutarate. Gln metabolism sustains the tricarboxylic acid (TCA) cycle, thereby supporting mitochondrial generation of the nucleotides and amino acids necessary for tumor progression (19-24). Overall, these observations suggest that aerobic glycolysis and mitochondria-associated metabolism (including OXPHOS and Gln metabolism) are key for development of certain types of tumor.

Aerobic glycolysis and mitochondria-associated metabolism are crucial for cancer cell proliferation, with Gln serving as an alternative metabolite during glucose limitation, suggesting that inhibiting either process may be a potential treatment. However, although this strategy may work in *in vitro* cultured cells, its clinical efficiency for *in vivo* cancer therapy is unsatisfactory (25). Therefore, it was hypothesized that the *in vivo* therapeutic effect of PC treatment could be enhanced by a targeted metabolic strategy combining dietary glucose restriction and drug-induced inhibition of mitochondrial metabolism. FX can attenuate the reprogramming of energy metabolism during activation of hepatic stellate cells, suggesting a potential role of FX in metabolism regulation (26). To the best of our knowledge, however, FX regulation of the bioenergetic metabolism of cancer cells, especially PC cells, has not been investigated. The present study aimed to investigate the effects of FX on aerobic glycolysis, mitochondrial OXPHOS and Gln metabolism to develop a safe, natural and efficient therapeutic strategy for PC.

Materials and methods

Cell lines and culture. PATU-8988T (cat. no. CL0254) and PATU-8988S (cat. no. CL0908) cell lines were purchased from Hunan Fenghui Biotechnology, whereas PANC-1 (cat. no. CL-0184) and 293T (cat. no. CL-0005) cell lines were obtained from Procell Life Science & Technology and Cell Bank of the Chinese Academy of Science, respectively. All cell lines were cultured in a humidified incubator with 5% CO_2 at 37˚C in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (BIOVISTECH; cat. no. SE100-B) and penicillin-streptomycin.

The genetic background of PATU-8988T and PATU-8988S is similar in k-ras; for example, the mutation site of K-RAS is the same (k-ras-p.G12V) between the two cell lines. PANC-1 is a k-ras-p.G12D mutated cell line. Therefore, PATU-8988T and PATU-8988S were used for experiments.

Primary antibodies and reagents. The primary antibodies and reagents are all listed in Tables SI and SII, respectively. FX was obtained from Shanghai Macklin Biochemical Co., Ltd. (cat. no. F861277; purity, 98%).

Assessment of cell viability. The half-maximal inhibitory concentration of FX (0, 5, 10, 20, 40, 80, 160 and 320 μM) on PC cells treated for 72 h at 37˚C was determined by MTT Cell Proliferation and Cytotoxicity Assay Kit according to the manufacturer's instructions (Beyotime Biotechnology, C0009S). Furthermore, changes in cells treated for 24 h with FX (25 μM) in the presence or absence of 7.5 mM glutathione (GSH) under glucose-limited (medium containing 1 mM glucose) conditions at 37˚C were also evaluated by MTT assay. In addition, cells treated for 24 h with FX (50 μM) in the presence or absence of 30 μM DDP at 37˚C were also analyzed by MTT assay. Then the purple formazan was dissolved by using Formazan solvent in this Kit and then detected at wavelength 570 nm.

To select concentrations of ZVAD, Z-VAD-FMK (27); Fer-1, Ferrostatin-1 (27); NEC-1, Necrostatin-1 (28); NSA, Necrosulfonamide (28); DFO, Deferoxamine mesylate (29); TTM, Ammonium Tetrathiomolybdate (30,31); 3-MA, 3-Methyladenine (32-34); BafA1, Bafilomycin A1 (35) and GSH, (36), cells were treated with these compounds (ZVAD: 0, 10, 20, 40, and 80 μM; Fer-1: 0, 0.5, 1, 2, and 4 μM; DFO: 0, 25, 50, 100, and 200 μM; NEC-1: 0, 1.25, 2.5, 5, and 10 μM; NSA: 0, 1.25, 2.5, 5, and 10 μM; TTM: 0, 2.5, 5, 10, and 20 μM; 3-MA: 0, 2.5, 5, 10, and 20 mM; BafA1: 0, 5, 10, 20, and 40 nM) in a 12-well plate at 37˚C for 48 h, observed using optical microscope (Nikon Corporation; ECLIPSE TS 100, light) and non-lethal maximum concentrations were selected for subsequent experiments.

Measurement of cell proliferation by real-time cell analysis (RTCA) assay. The suppressive effect of FX on proliferation of PC cell lines was determined using RTCA assay according to the manufacturer's instructions.

Clonogenic cell survival assay. PC cells (1,000 cells/well) were seeded in 6-well plates 1,000 cells/well and treated with or without FX (0, 25, 50, 100 μM) for 2 weeks at 37˚C. The colonies (>50 cells) were washed with PBS, fixed with methanol 4% for 30 min at Room temperature, stained with crystal violet for 30 min at Room temperature, and quantified by Image J (National Institutes of Health (NIH), V1.8.0.112).

FACS (Fluorescence-activated cell Sorting) nalysis of cell death rate. Following 48 h FX (0, 25, 50, 100 μM) monotreatment or 24 h FX (25 μM) treatment in the presence or absence of 7.5 mM GSH under GL conditions at 37˚C, cells were stained with Annexin V-FITC/PI in the dark for 15 min and then analyzed by BD FACSCanto™ Clinical Flow Cytometry System (BD Biosciences, BDCanto II) according to the manufacturer's instruction using Annexin V-FITC/PI apoptosis detection kit (KeyGEN Bio TECH, KGA108). Results were analyzed by Flowjo (BD Biosciences, FlowJo_v10.8.1_CL). Cell death rate was quantified based on the formula as (Q2+Q3+Q4) x100%.

Western blot analysis. PC cells were harvested, lysed with lysis buffer (Beyotime Institute of Biotechnology; cat. no. P0013) for 20 min and centrifuged at 13,400 g for 20 min at 4˚C to collect protein in the supernatant. The proteins were quantified using the Pierce™ BCA Protein Assay kit, according to the manufacturer's instructions, mixed with 5X DualColor Protein Loading Buffer to adjust the protein concentration to 1 μg/μl and heated at 95˚C for 5 min. A total of 20 μg protein/lane were loaded onto 10% SDS-PAGE gel, electrophoresed in running buffer and transferred to a 0.22 μM PVDF membrane in transfer buffer. The membranes were treated with 5% non-fat dry milk to block the non-specific binding at room temperature for 90 min, incubated with primary antibody (GSDME, GSDMD, GSDMC, OPA1, SLC31A1 and GLS; all 1:1,000) overnight at 4˚C and washed three times with 1X TBST at room temperature, followed by incubation with HRP-labeled Goat Anti-Rabbit or Anti-Mouse IgG(H+L) (all 1:1,000) for 90 min at room temperature. After washing the membrane three times with 1X TBST at room temperature, it was visualized using the SuperSignal™ West Pico PLUS kit and UVP ChemStudio PLUS (Jena GmbH, H116457),and quantified using ImageJ software (National Institutes of Health (NIH), V1.8.0.112).

Determination of oxygen consumption rate (OCR). The effect of FX on mitochondrial respiration in PC cells was determined using an XFe96 extracellular flux analyzer according to the manufacturer's instructions. Briefly, cells were seeded into culture plates (20,000 cells/well) and incubated overnight at 37˚C. Adherent cells were treated with FX (0, 25, 50 and 100 μM) for 8 h at 37˚C. The medium was replaced with a Seahorse XF Base Medium (Seahorse Bioscience, 102353-100) containing 25 mM glucose and 2 mM pyruvic acid sodium and cultured for 1-2 h at 37˚C. Working solutions containing oligomycin, FCCP (Carbonyl cyanide 4-(trifluoromethoxy) or rotenone/antimycin A were prepared and added according to the manufacturer's protocol. OCR was detected using the XFe96 extracellular flux analyzer (Seahorse Bioscience, XFe96, S7800B).

Measurement of extracellular acidification rate (ECAR). The effect of FX on aerobic glycolysis in PC cells was measured using the XFe96 extracellular flux analyzer according to the manufacturer's guidelines. Briefly, cells were seeded in a culture plate (20,000 cells/well) overnight and treated with FX (0, 25, 50, 100 μM) for 8 h at 37˚C. The medium was then replaced with Seahorse XF Base Medium containing Gln (2.24 mM), and cells were incubated for 1-2 h at 37˚C. Working solutions containing glucose, oligomycin and 2-DG (2-Deoxy-D-glucose) were added according to the manufacturer's protocol. ECAR was detected using the XFe96 extracellular flux analyzer according to the manufacturer's instructions (Seahorse Bioscience; cat. no. S7800B).

FACS analysis for whole-cell and mitochondrial ROS. Following 48 h FX (0, 25, 50, 100 μM) treatment at 37˚C, cells were stained for 15 min in the dark with DCFH-DA (for whole-cell) or Mitosox (for mitochondrial ROS) at 37˚C. The stained cells were analyzed by BD FACSCanto™ Clinical Flow Cytometry System (BD Biosciences, BDCanto II) according to the manufacturer's instructions using Reactive Oxygen Species Assay kit, Beyotime Biotechnology, S0033; Mitosox Red, Invitrogen M36008). Results were analyzed by Flowjo (BD Biosciences, FlowJo_v10.8.1).

Whole-cell and mitochondrial ROS observed by fluorescence microscopy. Following 48 h FX (0, 25, 50, 100 μM) treatment at 37˚C, cells were stained for 15 min in the dark with DCFH-DA (for whole-cell) or Mitosox (for mitochondrial ROS) at 37˚C and then analyzed by fluorescence microscopy according to the manufacturer's instructions using Reactive Oxygen Species Assay kit (Beyotime Biotechnology, S0033; Mitosox Red, Invitrogen M36008).

FACS analysis of cell death. Following 48 h FX (0, 25, 50 and 100 μM) monotreatment or 24 h FX (25 μM) treatment in the presence or absence of 7.5 mM GSH under GL conditions at 37˚C, cells were stained with Annexin V-FITC/PI for 20 min in the dark. The stained cells were then analyzed by BD FACSCanto™ Clinical Flow Cytometry System (BD Biosciences, BDCanto II) according to the manufacturer's instructions (Annexin V-FITC/PI apoptosis detection kit, KeyGEN Bio TECH, KGA108). Results were further analyzed by Flowjo (BD Biosciences, FlowJo_v10.8.1).

RNA interference. The lentiviral plasmid of pPLK/GFP + Puro-SLC31A1 short hairpin (sh)RNA was obtained from PPL (Public Protein/Plasmid Library) and 293T cells were used to package lentivirus. The generation system (2nd) was used. A total of 2 target plasmid + 2 μg psPAX2+1 μg pMD2G was transfectd to 293T cells for 6 h at 37˚C to package lentivirus. Collection was performed after 48 h, then centrifuged at 500 g for 5 min at 4˚C, and filtered by using 0.45 μM filter membrane. PATU-8988T cells were infected with packaged lentivirus

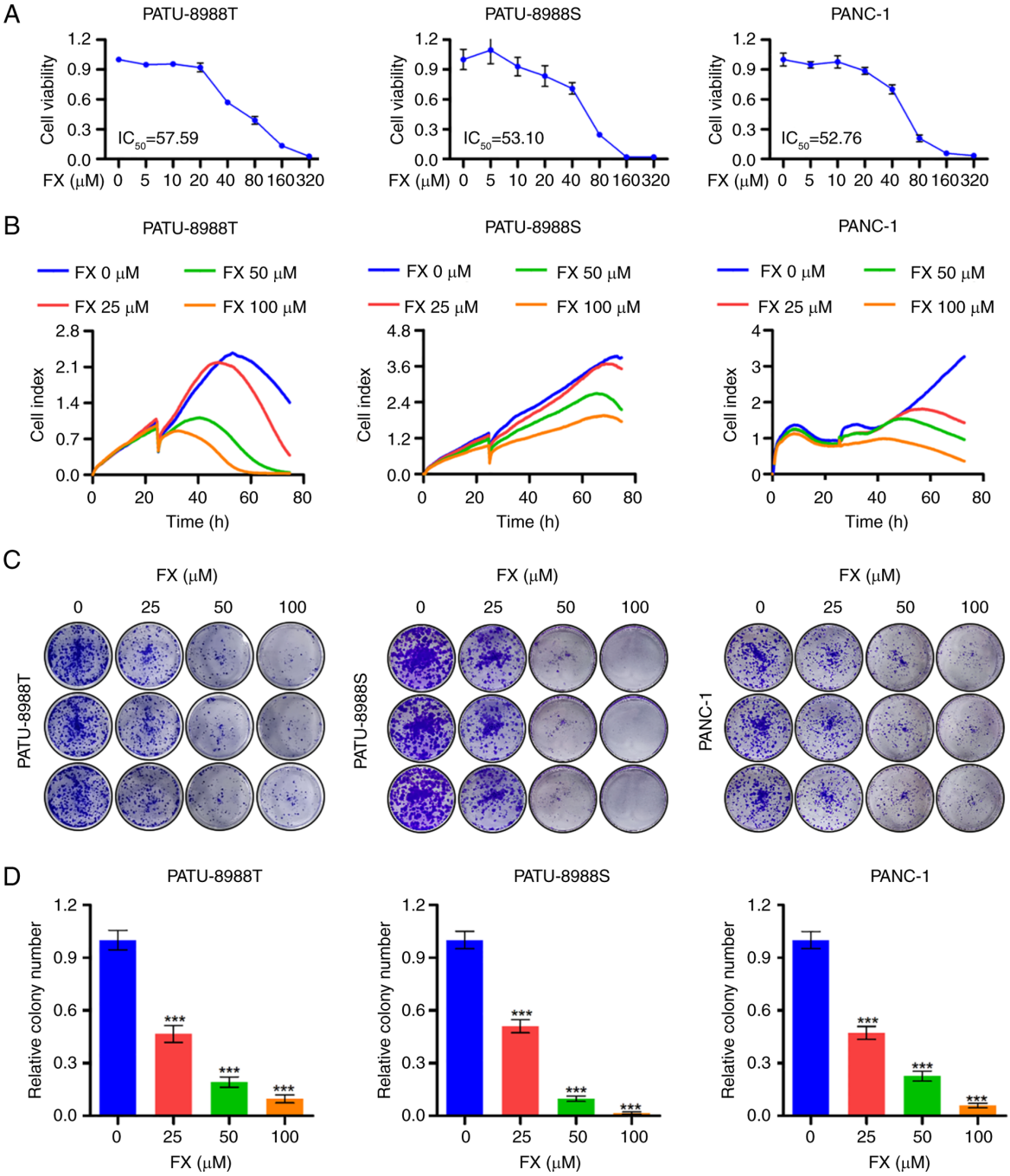

Figure 1. FX inhibits PC cell proliferation. (A) IC_{50} of FX on PC cells was determined using MTT assay. (B) Inhibitory effect of FX on the proliferation of PC cell lines was confirmed by real-time cell analysis. (C) Suppressive effect of FX on the clonogenic activity of PC cells was assessed via (D) colony formation assay. ***P<0.001 vs. 0 μM). FX, fucoxanthin; IC_{50}, half-maximal inhibitory concentration; PC, pancreatic cancer.

(the multiplicity of infection (~3 used in PATU-8988T cells) for 48 h and screened by puromycin (2 μg/ml) for 7 days. The efficiency of transfection was assessed by western blotting. And the 0.5 μg/ml of puromycin was used to maintain the efficiency of transfection. The western blot and MTT assay were performed in transfected PATU-8988T cells to confirm the whether the SLC31A1 knockdown can attenuate cytotoxicity caused by the combination of FX and DDP. Sequences of sh-SLC31A1 clone were as follows: NC, 5'-GTTCTCCGA ACGTGTCACGTT-3'; shSLC31A1#1, 5'-CGGTACAGGATA CTTCCTCTT-3' and shSLC31A1#2, 5'-GATGCCTATGAC CTTCTACTT-3'.

Statistical analysis. All statistical analyses were performed using GraphPad Prism 5 (Dotmatics) and data are expressed as the mean ± SD. One-way ANOVA followed by Tukey post hoc test was performed to analyze differences between >2 groups. Each experiment was repeated ≥3 times. P<0.05 was considered to indicate a statistically significant difference.

Results

FX impedes PC cell proliferation. The structural formula of FX is shown in Fig. S1. The present study demonstrated a dose-dependent reduction in PC cell viability, confirming

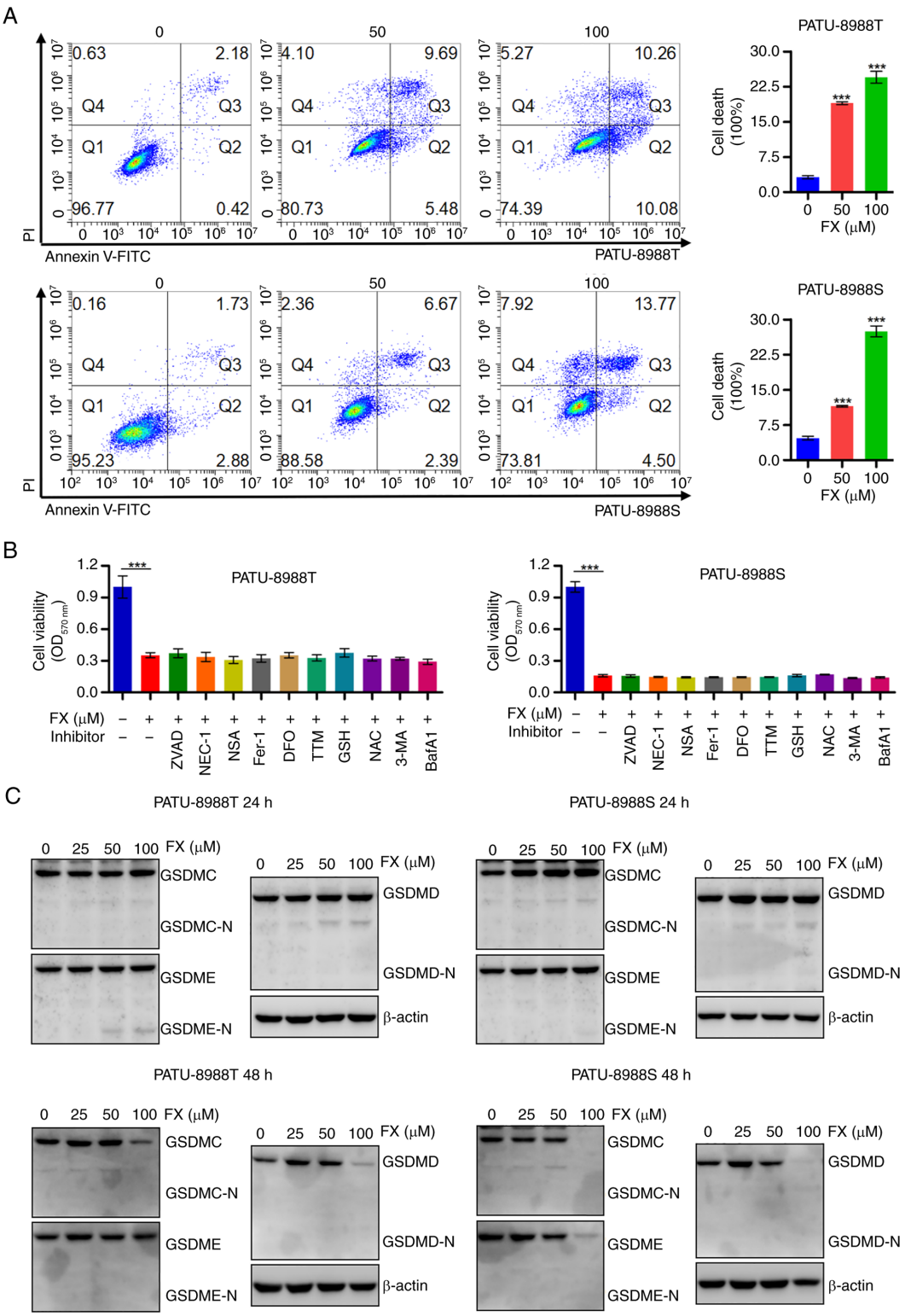

Figure 2. FX induces a distinct form of regulated cell death in PC cells. (A) PC cells were stained with FITC/PI and cell death was analyzed using flow cytometry. (B) Viability was assessed in FX-treated cells in the presence or absence of programmed cell death inhibitors, including inhibitors of apoptosis (ZVAD, 20 μM), ferroptosis (Fer-1, 1 μM; DFO, 50 μM), necroptosis (NEC-1, 5 μM; NSA, 5 μM), cuproptosis (TTM, 5 μM) and autophagic cell death (3-MA, 5 mM; BafA1, 10 nM). (C) Changes in GSDMC, GSDMD and GSDME expression in response to FX treatment were examined using western blot analysis. ***P<0.001 vs. 0 μM). FX, Fucoxanthin; PC, Pancreatic cancer; ZVAD, Z-VAD-FMK; Fer-1, Ferrostatin-1; DFO, Deferoxamine mesylate; NEC-1, Necrostatin-1; NSA, Necrosulfonamide; TTM, Ammonium Tetrathiomolybdate; 3-MA, 3-Methyladenine; BafA1, Bafilomycin A1; GSDMC, Gasdermin C; OD, Optical density; -N, N-terminal.

the tumor-suppressive effects of FX on PC cells (Fig. 1A). RTCA assay further indicated that FX notably inhibited PC cell proliferation (Fig. 1B). Furthermore, FX inhibited the colony-forming ability of PC cells (Fig. 1C and D). Overall, these results suggested that FX significantly inhibited the *in vitro* proliferation of PC cells.

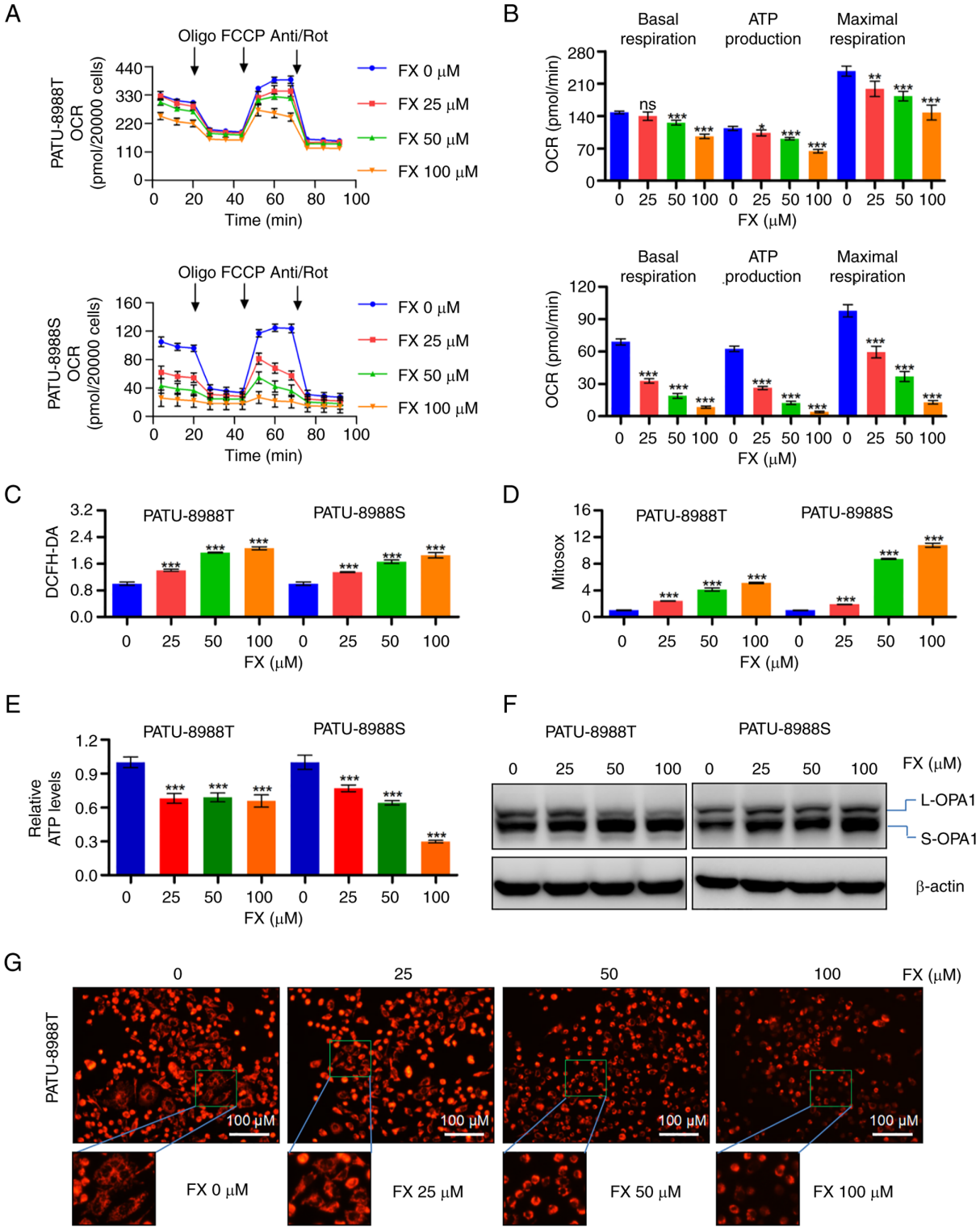

Figure 3. FX impairs mitochondrial homeostasis and causes oxidative stress in PC cells. (A) OCR in FX-treated PC cells was measured using the XFe96 extracellular flux analyzer to evaluate changes in (B) basal respiration, ATP production and maximal respiration in FX-treated PC cells. (C) ROS levels were detected in PC cells stained with DCFH-DA using flow cytometry. (D) Mitochondrial ROS production was measured in PC cells stained with MitoSOX via flow cytometry. (E) Cellular ATP levels. (F) Western blot analysis was performed to evaluate changes in OPA1 expression in response to FX treatment in PC cells. (G) Changes in mitochondrial morphology in PC cells following FX exposure were observed using MitoTracker staining. *P<0.05, **P<0.01, ***P<0.001 vs. 0 μM). FX, Fucoxanthin; PC, Pancreatic cancer; OCR, Determination of oxygen consumption rate; ROS, Reactive oxygen species; OPA1, Optic atrophy 1; ns, no significant; FCCP, Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; Anti/Rot, Antimycin a1/Rotenone; L-, Long-OPA1; S-OPA1, Short-OPA1.

FX induces a distinct form of regulated cell death in PC cells. The present study further investigated the antitumor activity of FX by flow cytometry analysis of PC cells labeled with Annexin V-FITC and PI. There was significant dose-dependent induction of PC cell death by FX (Fig. 2A). However, FX-induced cell death was not prevented by

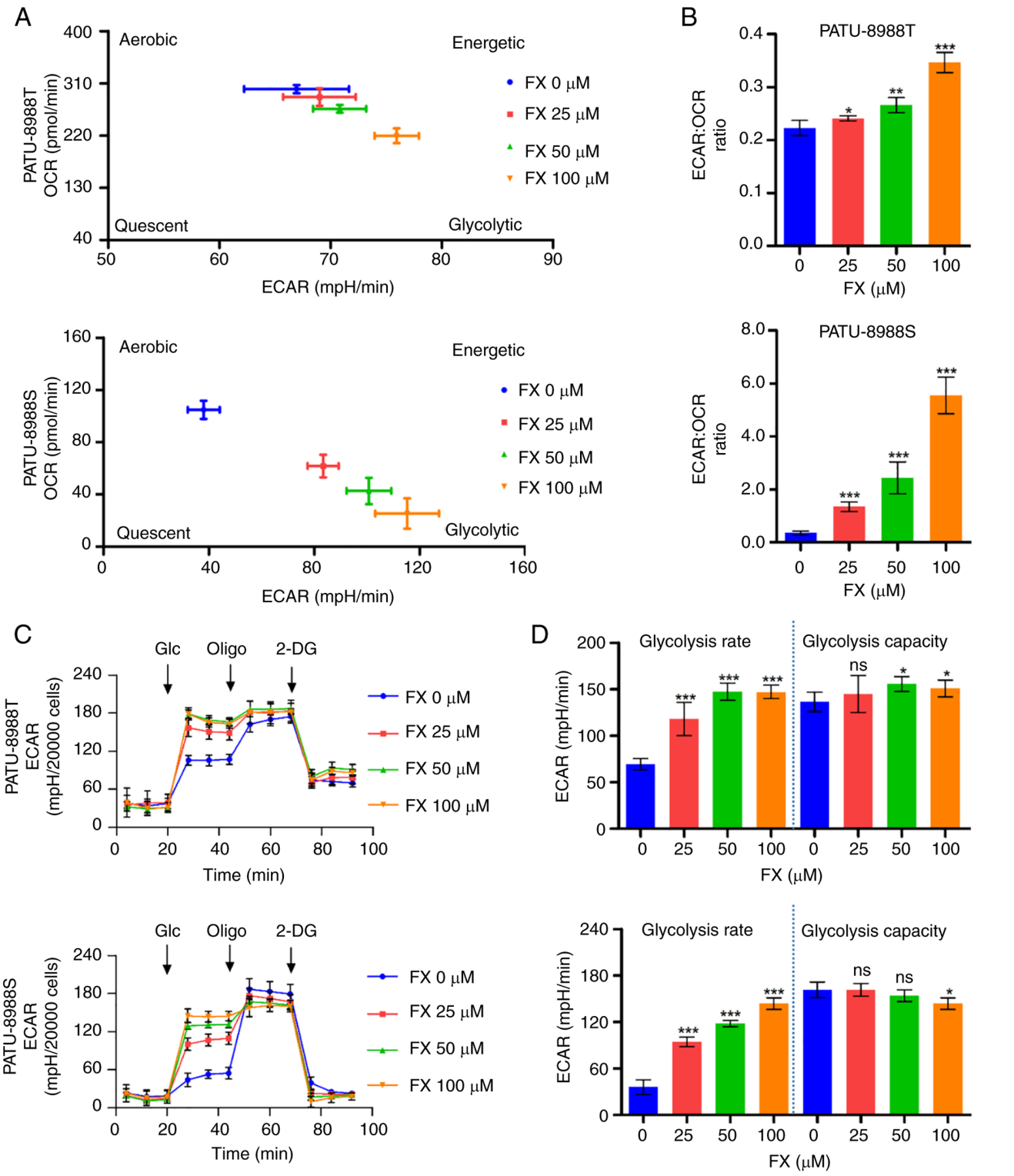

Figure 4. FX initiates reprogramming of energy metabolism to convert mitochondrial respiration to aerobic glycolysis in PC cells. (A) Metabolic phenotype profiles of PC cells representing changes in response to FX treatment. (B) ECAR/OCR ratio was analyzed to confirm changes in metabolic profiles in FX-treated cells. (C) Changes in ECAR in FX-exposed PC cells were measured using XFe96 extracellular flux analyzer. (D) Changes in glycolysis rate and capacity in FX-treated PC cells. *P<0.05, **P<0.01, ***P<0.001 vs. 0 μM. FX, Fucoxanthin; PC, Pancreatic cancer; ECAR, Measurement of extracellular acidification rate; OCR, Determination of oxygen consumption rate; Glc, Glucose; Oligo, Oligomycin; 2-DG, 2-Deoxy-D-glucose; ns, no significant.

inhibitors of cell death mechanisms, including inhibitors of apoptosis (ZVAD), ferroptosis (Fer-1), necroptosis (NEC-1 and NSA), cuproptosis (TTM) and autophagy (3-MA and BafA1; Fig. 2B). Moreover, western blot analysis revealed that FX did not induce the cleavage of GSDME, GSDMD or GSDMC, which are key factors involved in pyroptosis (37). These results suggested that FX stimulated a novel type of cell death in PC cells, distinct from apoptosis, pyroptosis, necroptosis, ferroptosis, cuproptosis or autophagic cell death.

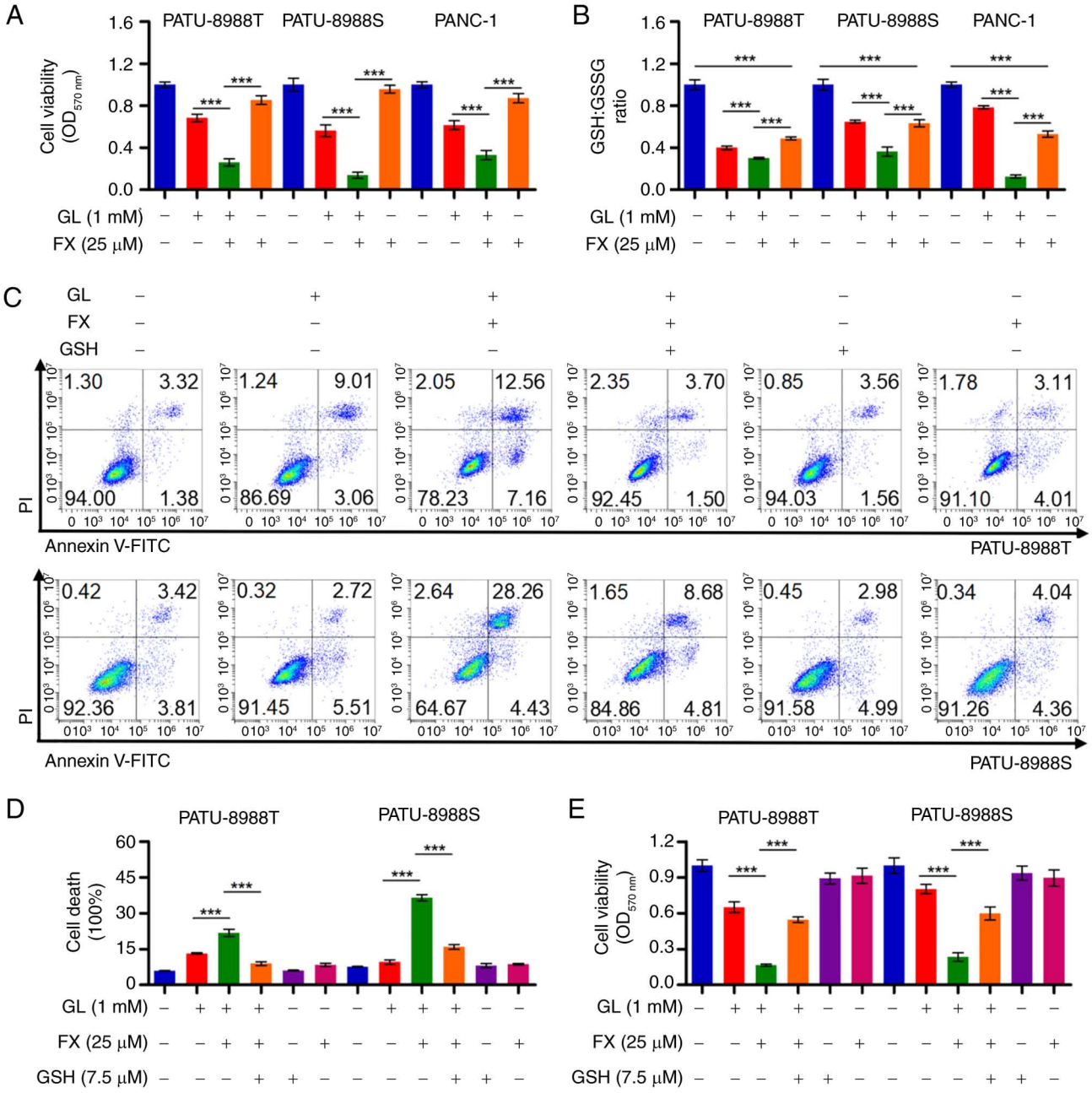

Figure 5. FX sensitizes PC cells to GL conditions by enhancing GSH/GSSG ratio decrease. (A) PC cell viability was measured using MTT assays after 24 h treatment with FX (25 μM) alone or in combination with GL conditions. (B) GSH/GSSG ratios in PC cells. (C) Cell death was assessed by flow cytometry following 24 h treatment with FX alone or combined with GL conditions, with or without 7.5 mM GSH. (D) Cell death were analyzed and quantified. (E) Cell viability was measured using MTT assay. ***P<0.001 vs. 0 μM. FX, Fucoxanthin; PC, Pancreatic cancer; GL, Glucose limited; GSH, glutathione; GSSG, Oxidized glutathione; OD, Optical density.

FX disrupts mitochondrial homeostasis and causes oxidative stress in PC cells. The present study also evaluated mitochondria, responsible for producing cellular ATP and key intermediate metabolites (23), as potential targets of FX-induced proliferation inhibition of PC cells. Seahorse XF Cell Mito Stress test showed that FX treatment significantly inhibited overall mitochondrial OCR (Figs. 3A and S2A) in PC cells and reduced basal respiration, ATP production and maximal respiration (Figs. 3B and S2B). FX triggered accumulation of ROS in whole cells and the mitochondria (Figs. 3C and D and S2C-F). FX significantly decreased the whole-cell ATP production in PC cells (Fig. 3E). An increase in OPA1 cleavage in response to FX supported the observed mitochondrial fragmentation (Figs. 3F and S2G). Mitotracker staining results also confirmed that FX increased morphological fragmentation of PC cells (Fig. 3G). These observations suggested that FX treatment disrupted mitochondrial homeostasis and promoted oxidative stress in PC cells.

FX initiates energy metabolism reprogramming and a switch from mitochondrial respiration to aerobic glycolysis in PC cells. The present study analyzed the overall metabolic profile based on the aforementioned bioenergetics analysis data. FX decreased mitochondrial respiration in PC cells (as determined

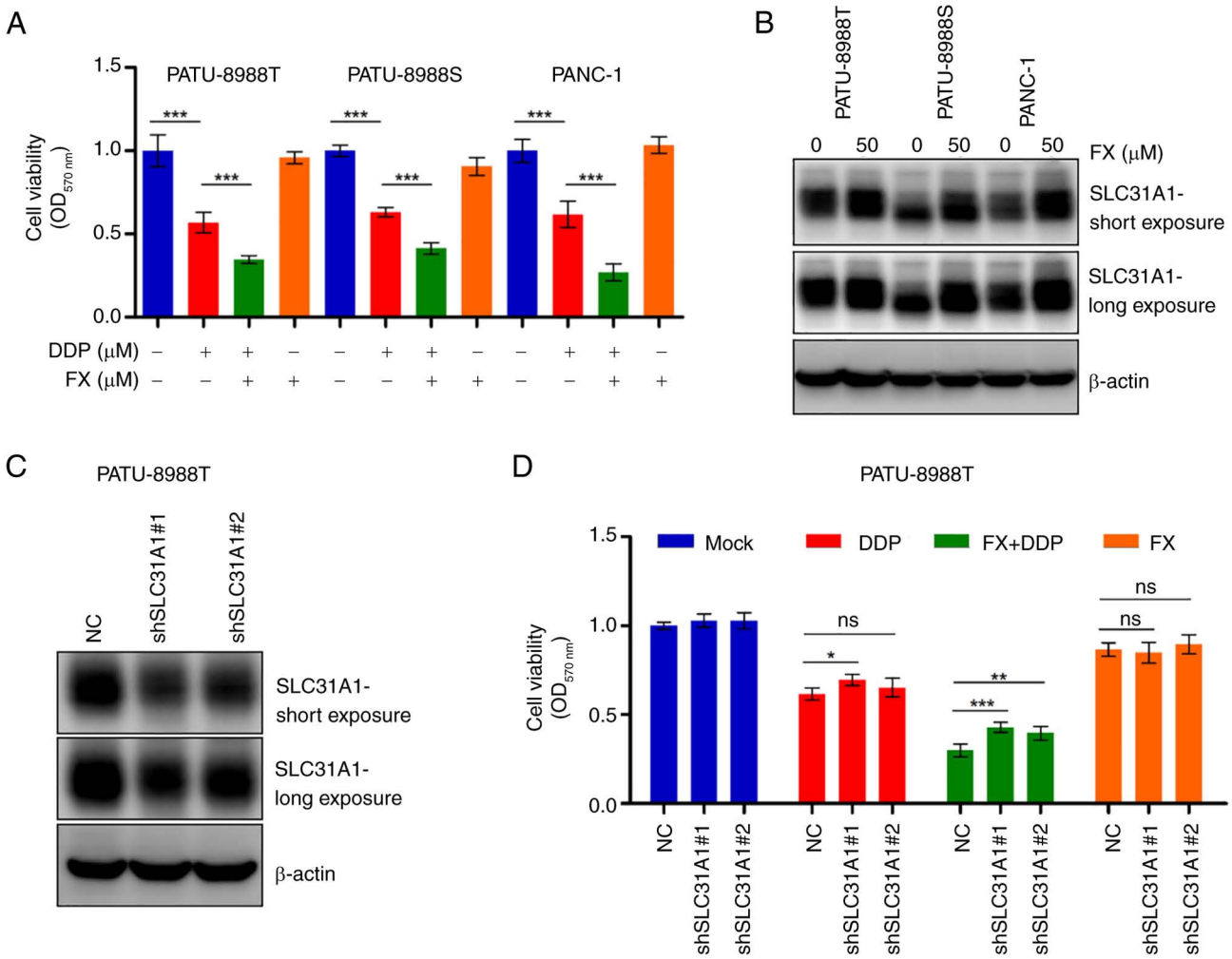

Figure 6. FX enhances cell sensitivity to DDP by upregulating SLC31A1 expression. (A) Cell viability was measured via MTT assay following 24 h treatment with FX (50 μM) and/or DDP (30 μM). (B) Changes in SLC31A1 expression in PC cells after 24 h FX treatment were analyzed by western blotting. (C) PATU-8988T SLC31A1 knockdown cell lines were constructed, and western blot assay was performed to confirm transfection efficiency. (D) Cell viability was assessed using MTT assay following treatment with FX in the presence or absence of DDP for 24 h. *P<0.05, **P<0.01, ***P<0.001 vs. NC. FX, Fucoxanthin; SLC31A1, Solute carrier family 31 member 1; PC, pancreatic cancer; OD, Optical density; sh, short hairpin; NC, Negative control; ns, no significant; DDP, Cisplatin.

by OCR), accompanied by an increase in capability for aerobic glycolysis (as determined by ECAR; Fig. 4A). The overall increase in ECAR/OCR ratio favored glycolysis in PC cells (Fig. 4B). Seahorse XF Glycolysis Stress Test revealed that FX altered aerobic glycolysis and elevated overall ECAR levels in PC cells (Figs. 4C and S3A), which were accompanied by an increase in rate of glycolysis (Figs. 4D and S3B). The aforementioned decrease in whole-cell ATP levels indicated that this glycolysis promotion could not compensate for FX-induced suppression of mitochondrial ATP production. Overall, these results suggested that FX reprograms PC cell metabolism toward glycolysis for ATP production; however, increased glycolysis does not compensate for the ATP reduction caused by FX-mediated inhibition of mitochondrial respiration.

FX sensitizes PC cells to GL condition by enhancing the decrease in GSH/GSSG ratio. FX-induced increased reliance on aerobic glycolysis in PC cells suggests that this shift may decrease metabolic flexibility in response to environmental stress, particularly during glucose deficiency. Under GL conditions, FX significantly decreased cell viability compared with PC cells treated with FX or GL alone (Fig. 5A). There was a significant decrease in GSH/GSSG ratio after the co-treatment (Fig. 5B). GSH prevented the decrease in cell viability and cell death in PC cells co-treated with FX and GL (Figs. 5C-E and S4A). These findings indicated that FX treatment may suppress conversion of GSSG to GSH, thereby increasing oxidative stress under GL conditions and promoting PC cell death.

FX enhances PC cell sensitivity to DDP by promoting expression of SLC31A1. FX has been shown to enhance DDP-induced cytotoxicity in several cancer types (38-40), though the specific mechanism remains unclear. Cell viability assay showed that FX increased the cytotoxicity of DDP in PC cells (Fig. 6A). FX significantly promoted the expression of SLC31A1, a key protein responsible for DDP uptake, in PC cells (Figs. 6B and S5A). Western blotting confirmed SLC31A1 knockdown (Figs. 6C and S5B), which attenuated cytotoxicity caused by the combination of FX and DDP (Fig. 6D). These findings suggested

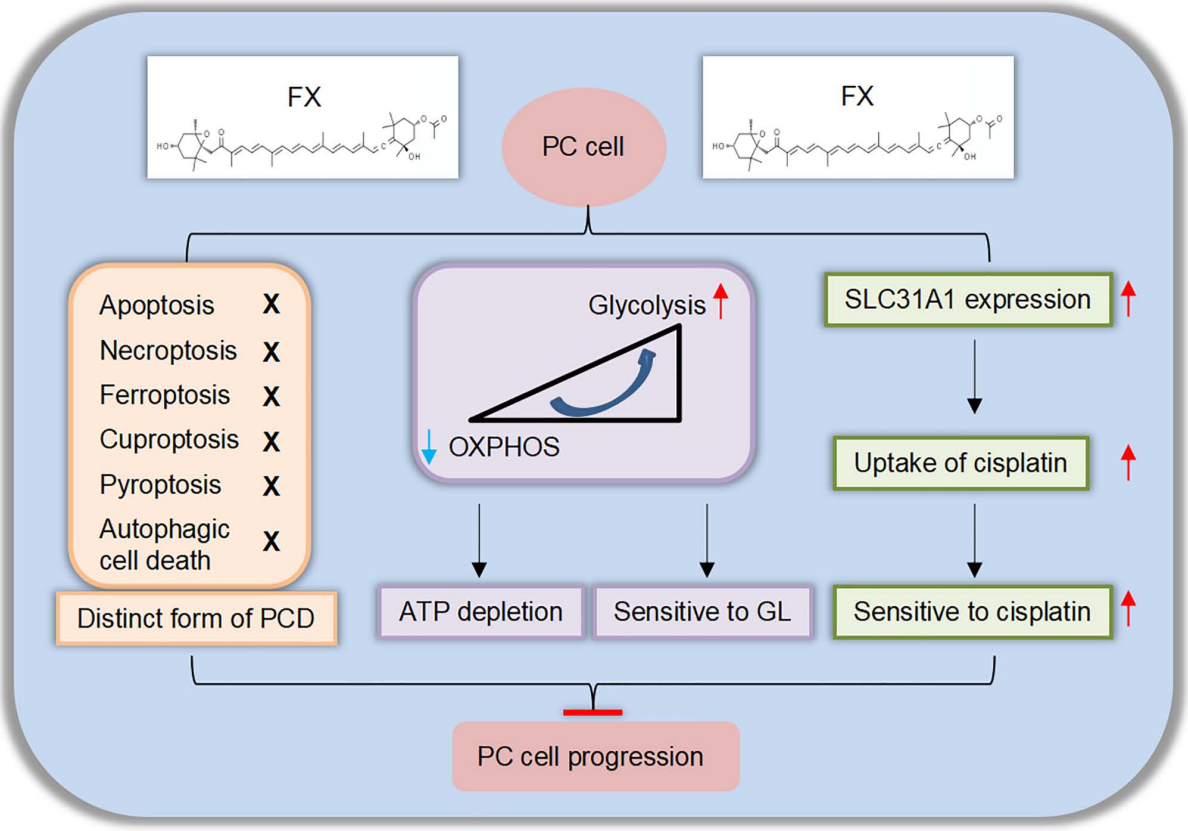

Figure 7. Proposed mechanism underlying antineoplastic properties of FX in PC cells. FX inhibits PC by impeding cell proliferation and inducing cell death via a non-classical pathway. FX switches mitochondria respiration to glycolysis and inhibits GSH metabolism, decreasing metabolic flexibility under GL conditions. FX enhances sensitivity to DDP by upregulating SLC31A1, a key transporter responsible for DDP uptake. FX, fucoxanthin; PC, Pancreatic cancer; SLC31A1, Solute carrier family 31 member 1; PCD, Programed cell death; GL, Glucose limited; OXPHOS, Oxidative phosphorylation..

that FX enhanced the sensitivity of PC cells to DDP by increasing SLC31A1-mediated cellular uptake of DDP.

Discussion

Although previous studies have shown that FX has anti-tumor activity in various other cancer types (4-10), its effects on human PC cells have not been investigated. The present study showed that FX exerted anti-PC activity by suppressing cell proliferation and inducing non-classical programmed cell death (PCD). FX treatment of PC cells triggered metabolic reprogramming switch from mitochondrial oxidative phosphorylation to aerobic glycolysis, decreasing cellular ATP levels. This FX-mediated metabolic alteration also decreased metabolic flexibility in PC cells, increasing sensitivity to GL conditions. Further investigation revealed that FX treatment decreased GSH/GSSG ratio, resulting in cytotoxicity under glucose-depleted conditions; however, these effects were alleviated by GSH. FX treatment also significantly induced expression of SLC31A1, a crucial execution factor required for DDP uptake (41), indicating FX may improve cell sensitivity to DDP. Taken together, these findings indicated FX exerted its antitumor effects by initiating cellular energy reprogramming and inducing a distinct form of regulated cell death. Therefore, combining FX and either GL or DDP treatment may serve as a potential therapeutic strategy for PC treatment (Fig. 7).

Previous studies have indicated that FX, similar to other antineoplastic drugs, eliminates cancer cells by inducing apoptosis (5,7,42,43). To the best of our knowledge, however, the association between FX and other types of PCD, such as necroptosis (44), ferroptosis (45), pyroptosis (46,47), cuproptosis (48) or autophagic cell death (49), has not been evaluated. Here, FX significantly induced death in PC cells. Furthermore, in PC cells co-cultured with PCD inhibitors, FX induced cell death. Subsequent cell viability assay showed that inhibitors of apoptosis (ZVAD), ferroptosis (Fer-1 and DFO), necroptosis (NEC-1 and NSA), cuproptosis (TTM) or autophagic cell death (3-MA and BafA1) did not attenuate FX-induced cell death. Due to the unavailability of a specific pyroptosis inhibitor, western blotting was used to detect activation of GSDME, GSDMD and GSDMC, the key indicators of pyroptosis (37), in response to FX. FX did not induce cleavage of GSDME, GSDMD or GSDMC in PC cells. These co-culture experiments indicated that FX induced a novel type of cell death that differs from known PCD mechanisms. Mitochondrial dysfunction, oxidative stress and ATP depletion caused by FX occur in parthanatos (50-52), suggesting that cell death caused by FX may be associated with parthanatos. Future studies should assess DNA damage, PARP1 overactivation, oxidative NAD (NAD^+) depletion and apoptosis inducing factor transfer from mitochondria to the nucleus, which are indicative of parthanatos (50-52). Knockdown of PARP1 or related inhibitors is also needed to perform rescue assays

to confirm whether PARP1 mediated parthanatos induced by FX. Overall, further studies are warranted to explore the mechanism of FX-induced cell death.

Mitochondria serve roles in ATP production and macromolecule biosynthesis (28). The role of mitochondria in bioenergetics, dynamics and signal transduction in tumor development has been studied (17). Therefore, it was hypothesized that mitochondria may be a potential FX target in PC cells. FX treatment of PC cells caused mitochondrial-associated changes, including a decrease in mitochondrial respiration, dysfunction of OPA1-mediated mitochondrial dynamics and accumulation of mitochondrial ROS. Moreover, PATU-8988S was more sensitive to inhibition of mitochondrial function by FX compared with PATU-8988T and PANC-1. Daemen *et al* (24) showed that lipogenic subtype PC cells (PATU-8988S) have, on average, higher O_2 consumption and mitochondrial content than glycolytic subtype cells (PATU-8988T and PANC-1) (24). These results indicated that FX may be more effective for PC cells, which highly rely on mitochondria function. Therefore, identifying the key regulatory factors (histopathological markers) that lead to metabolic difference is key for selecting specific treatment for PC, which is consistent with the conclusion of Fraile-Martine *et al* (53) and Ortega *et al* (53,54). FX treatment also stimulated aerobic glycolysis, a key pathway for the generation of energy and intermediate metabolites needed for PC progression (55-57). These alterations in metabolic signatures revealed that FX induced metabolic reprogramming, which switched mitochondrial metabolism to aerobic glycolysis. However, the increased glycolysis was insufficient to compensate for the ATP loss caused by mitochondrial dysfunction. Thus, it was hypothesized that promotion of glycolysis might allow glucose to serve as an alternative anaplerotic metabolite when mitochondria are damaged and increased aerobic glycolysis levels counteract the cytotoxicity of FX (58). The present study confirmed this by culturing PC cells treated with low FX doses in GL conditions. The lower cell viability and a higher cell death rate revealed that GL conditions significantly sensitized PC cells to FX. FX promoted the reduction of GSH, ultimately decreasing the GSH/GSSG ratio in GL conditions. Similarly, the addition of GSH substantially alleviated the cytotoxicity caused by FX, suggesting that FX may sensitize PC cells to GL conditions by promoting consumption of reduced GSH under GL conditions.

The association between FX cytotoxicity and GL conditions smay involve k-ras. K-ras is an oncogene frequently mutated in PC that promotes aerobic glycolysis and Gln metabolism (59,60). Most cancer cells convert Gln into α-ketoglutarate to fuel the TCA cycle, thereby maintaining mitochondria respiration. However, k-ras-mutated PC cells convert Gln-derived aspartate to oxaloacetate and convert malate and pyruvate to fuel the TCA cycle and upregulate the generation of NADPH (12,61). Therefore, whether FX treatment of PC cells causes mitochondrial respiratory depression and oxidative stress by regulating Gln metabolism was evaluated. Western blot analysis of key factors involved in Gln metabolism and Gln synthase (GLS) activity assay revealed significant inhibition of GLS expression in response to FX treatment, indicating decreased Gln metabolism. Based on these results and FX-induced mitochondrial inhibition and oxidative stress, it was hypothesized that FX-induced mitochondrial damage is partly caused by GLS inhibition. However, the specific mechanism requires further exploration.

Drug resistance is an inevitable problem of PC (62). FX promotes cytotoxicity of gemcitabine on PC cells at a safe concentration (63). FX has also been found to increase sensitivity of liver and lung cancer to DDP; however, the underlying mechanism remains undetermined (38-40) and has not been studied in PC. PC cells have also shown a consistent increase in sensitivity to DDP when co-cultured with FX (38-40). FX significantly induces SLC31A1, an important transporter responsible for DDP uptake (41,64), in PC cells. Here, depletion of SLC31A1 can mitigate the inhibition of cell viability caused by combined treatment with FX and DDP . These data indicated that FX promoted chemosensitivity of PC cells to DDP by inducing SLC31A1 expression.

The present study proposed a mechanistic model in which FX significantly inhibited PC by impeding cell proliferation and inducing cell death through a non-classical pathway. FX was also found to switch mitochondrial respiration to glycolysis and inhibit GSH metabolism, decreasing metabolic flexibility under GL conditions. Therefore, FX may be more effective for PC cells that rely on mitochondria, providing novel theoretical support for FX clinical targeted therapy. Moreover, FX treatment induced SLC31A1 expression, promoting DDP uptake and enhancing PC cell chemosensitivity to DDP. These results suggested that combining FX with glucose restriction or DDP may be a potential therapeutic strategy for PC.

Acknowledgements

Not applicable.

Funding

The present study was supported by Project of Zhejiang Provincial Naturel Science Foundation of China (grant nos. LQ23H310006 and LQ20H260003) and Science and Technology Bureau of Wenzhou (grant no. Y20220171).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

FS and HY conceived the study and wrote the manuscript. NM and YC performed experiments and analyzed data. YZ and TM performed experiments. JA and JL analyzed data and edited the manuscript. All authors have read and approved the final manuscript. FS and HY confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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