**FOLIA HISTOCHEMICA ET CYTOBIOLOGICA**
Vol. 60, No. 1, 2022
pp. 55–65

ORIGINAL PAPER

VIA MEDICA

# 5-fluorouracil suppresses stem cell-like properties by inhibiting p38 in pancreatic cancer cell line PANC-1

**Jin Zhao, Xueying Shi*, Changcheng Dong, Rui Liu, Lifu Su, Cuixia Cao**

Department of General Surgery, Baogang Hospital, Baotou, Inner Mogolia, China

**Abstract**

**Introduction.** Suppressing the phenotype of cancer stem cells (CSCs) is a promising treatment strategy for cancer. P38 mitogen-activated protein kinases (MAPK, p38) play an important role in the occurrence, development, and stemness maintenance of tumors. The aim of the current study was to investigate the effect of p38 on the stemness maintenance of CSCs in pancreatic cancer cell line PANC-1.

**Material and methods.** PANC-1 human pancreatic cancer cells were treated with 5-fluorouracil (5-FU) at 0.5 IC50, IC50, and 2 IC50 for 24 h. PANC-1 cells were treated for 24 h with 5-FU at 0.5IC50, IC50, and 2IC50 with or without VX-702, p38 phosphorylation inhibitor. Cells were resuspended in DMEM supplemented with 20 ng/ml epidermal growth factor, 2% B27, 5 $\mu$g/ml insulin, 20 g/ml basic fibroblast growth factor, and 10 $\mu$g/ml transferrin. Cells were seeded in ultra-low adhesion 6-well dishes to observe tumor spheroidization. The expression of CDK2, cyclin B1, cyclin D1, OCT4, SOX2, Nanog, and p38 was measured by Western blot. The mRNA expression of p38, OCT4, Nanog, and SOX2 was measured by RT-PCR. Flow cytometry was performed to evaluate the cell cycle, apoptosis, and proportion of CD44+CD133+ PANC-1 cells.

**Results.** 5-FU decreased cell viability and increased apoptosis. 5-FU suppressed the stemness maintenance of CSCs in PANC-1 cells, as demonstrated by the inhibition of tumorsphere formation, the decrease in CD44+CD133+ cells' fraction, and downregulation of OCT4, Nanog, and SOX2 expression. In addition, 5-FU inhibited the phosphorylation of p38 in PANC-1 cells. The phosphorylation of p38 was subsequently suppressed by VX-702, p38 mitogen-activated protein kinase inhibitor, which exhibited similar effects as those of 5-FU treatment. The effect of VX-702 on PANC-1 cells was further enhanced by 5-FU treatment. Thus, p38 inhibitor decreased the viability and increased the apoptosis of PANC-1 cells. P38 inhibitor suppressed the stemness maintenance of CSCs in PANC-1 cells, as demonstrated by the inhibition of tumorsphere formation, the decrease in CD44+CD133+ cells, and the downregulation of OCT4, Nanog, and SOX2 expression.

**Conclusions.** These findings indicate that the inhibition of p38 phosphorylation suppresses the stemness maintenance and 5-FU resistance of PANC-1 cells, providing a potential therapeutic target for the prevention and treatment of pancreatic cancer. *(Folia Histochemica et Cytobiologica 2022, Vol. 60, No. 1, 55–65)*

**Key words:** PANC-1 cells; tumorspheres; cancer stem cells; stemness markers; p38; apoptosis

## Introduction

Pancreatic cancer is one of the deadliest cancers worldwide, exhibiting a poor prognosis with a five-year survival rate of only 7% and a median survival time of 6 months [1, 2]. Because of the lack of effective early diagnosis indicators, most patients are already in the middle and advanced stage when they are diagnosed, having missed the best treatment period [3, 4]. Chemotherapy is the major treatment for pancreatic cancer at present. However, its therapeutic effect is severely limited by the drug resistance of cancer cells, which results in tumor metastasis and recurrence, the main cause of greater than 90% of cancer-associated deaths [5, 6]. Therefore, it is necessary to identify

***Correspondence address:** Dr. Xueying Shi,
Department of General Surgery, Baogang Hospital,
20th Shaoxian Street, Kundulun District,
Baotou, Inner Mongolia, China
e-mail: shi13314852553@163.com


Folia Histochem Cytobiol. 2022
10.5603/FHC.a2022.0004
ISSN 0239-8508, e-ISSN 1897-5631

www.journals.viamedica.pl/folia_histochemica_cytobiologica

potential diagnostic markers and therapeutic targets for the prevention and treatment of pancreatic cancer.

Tumor cells with stem cell-like features, known as cancer stem cells (CSCs), often have great multi-directional differentiation potential and self-renewal ability [7]. CSCs not only are a possible cause of tumor occurrence, but also explain tumor heterogeneity, metastasis, and recurrence [8]. In addition, CSCs are identified as vital targets that mediate tumor resistance [6, 9]. Previous studies have demonstrated that CSCs promote the drug resistance and metastasis of pancreatic cancer by regulating the occurrence of epithelial-mesenchymal transition [10]. Thus, suppressing the CSC phenotype is a promising treatment strategy for cancer [11, 12].

Mitogen-activated protein kinases (MAPKs) are widely expressed serine/threonine kinases containing three subtypes that participate in various signal transduction pathways: extracellular signal-regulated kinases, p38 MAPK, and c-Jun NH2-terminal kinases [13, 14]. Evidence has demonstrated that p38 MAPK (p38) plays an important role in tumor occurrence, development, and drug resistance. P38 promotes tumorigenesis by mediating tumor cell invasion and metastasis [15]. In addition, p38 inhibition suppressed the stemness maintenance of CSCs in pancreatic cancer and reduced the number of circulating tumor cells in the body [16]. However, the relationship between p38 and the stemness maintenance of CSCs in pancreatic cancer has been rarely reported.

To address this, PANC-1 human pancreatic cancer cells were treated with 5-fluorouracil (5-FU). The effect of 5-FU on the stemness maintenance of pancreatic CSCs and the expression of p38 were evaluated. Then, the expression of p38 in 5-FU-treated PANC-1 cells was suppressed by VX-702, a specific inhibitor of p38, to investigate the effect of p38 on the stemness maintenance of pancreatic CSCs and the drug resistance of pancreatic cancer cells.

## Materials and methods

**Cell culture and treatment.** The human pancreatic cancer cell line PANC-1 was purchased from the Shanghai Institutes for Biological Sciences, Chinese Academy of Science. The cells were cultured in Dulbecco's modified eagle medium (DMEM, Hyclone, UT, USA) containing 10% fetal bovine serum (FBS, Gibco, Gaithersburg, MD, USA) and maintained at 37°C with 5% $CO_2$. When the confluence reached 80–90%, the cells were treated with 5-FU (SigmaAldrich, St. Louis, MO, USA) at 0, 5, 10, 20, 50, and 200 $\mu$M to obtain the median inhibitory concentration (IC50) using a logit model. The cells were then treated for 24 h with 5-FU at 0.5 × IC50, IC50, and 2 × IC50 to investigate the effect of 5-FU on the biological behavior and stemness maintenance of PANC-1 cells. The cells were subsequently treated with VX-702, p38 mitogen-activated protein kinase inhibitor (p38MAPK) (2 mM) (Selleck, Houston, TX, USA) [17], an inhibitor of p38, combined with 5-FU at the concentration of 0.5 × IC50, IC50, and 2 × IC50 for 24 h to explore the effect of p38 on 5-FU-treated PANC-1 cells.

**Cell Counting Kit-8 (CCK-8) assay.** PANC-1 cells were seeded into 96-well plates at 5 × $10^3$ cells/well and maintained at 37°C with 5% $CO_2$ overnight. The cells were then treated for 24 h with (1) 5-FU at 0, 5, 10, 20, 50, and 200 $\mu$M; (2) 5-FU at 0.5 × IC50, IC50, and 2 × IC50; (3) VX-702 (2 $\mu$M) [17] combined with 5-FU at 0.5 × IC50, IC50, and 2 × IC50. After treatment, 10 $\mu$l of CCK-8 solution (Bioswamp, Wuhan, China) was added to each well and the cells were further incubated at 37°C for 4 h. Finally, the absorbance of each well was detected using a microplate reader (Allsheng, Hangzhou, China) at 450 nm.

**Flow cytometry.** Flow cytometry was performed to evaluate the cell cycle, apoptosis, and proportion of CD44+CD133+ PANC-1 cells. To evaluate cell cycle: 1 × $10^7$ cells were centrifuged at 1000 *g* for 5 min and resuspended in 300 $\mu$l of phosphate-buffered saline (PBS) (Bioswamp). After the addition of 700 $\mu$l of absolute ethyl alcohol, the cells were maintained at –20°C for 24 h and centrifuged at 1000 × g for 5 min. The collected cell pellets were then resuspended in 100 $\mu$l of RNase A (1 mg/l). After 30 min of incubation at 37°C, 400 $\mu$l of propidium iodide (PI) (BD, Shanghai, China) was added at 50 $\mu$g/ml and the cells were incubated in the dark for 10 min. Finally, the cells were subjected to flow cytometry (ACEA Biosciences, San Diego, CA, USA). To evaluate apoptosis: 1 × $10^6$ cells were centrifuged at 1000 g at 4°C for 5 min and resuspended in 1 ml of PBS, followed by centrifugation at 1000 *g* at 4°C for 5 min (twice). The cells were resuspended in 200 $\mu$l of binding buffer (Bioswamp), after which 10 $\mu$l of Annexin V-fluorescein isothiocyanate (FITC, BD, Shanghai, China) and 10 $\mu$l of PI were added. The cells were incubated in the dark at 4°C for 30 min and subjected to flow cytometry. To evaluate the proportion of CD44+CD133+ cells: 0.25% Pancreatin was added to the cells and the cells were collected by centrifugation at 1000 × g for 5 min. 1 × $10^6$ cells were resuspended in 100 $\mu$l of PBS. After the addition of 2 $\mu$l of CD133-phycoerythrin and 2 $\mu$l of CD44-allophycocyanin, the cells were cultured in the dark at 4°C for 30 min. The cells were washed twice with 2 ml of PBS, centrifuged at 1000 × g at 4°C for 5 min, resuspended in 400 $\mu$l of PBS, and subjected to flow cytometry.

**Tumorsphere formation.** PANC-1 cells were treated for 24 h with 5-FU at 0.5 × IC50, IC50, and 2 × IC50 with or without VX-702. Then, the cells were resuspended in DMEM supplemented with 20 ng/ml epidermal growth factor (PeproTech, Rocky Hill, NJ, USA), 2% B27 (Gibco), 5 $\mu$g/ml insulin (Bioswamp), 20 $\mu$g/ml basic fibroblast growth


Folia Histochem Cytobiol. 2022
10.5603/FHC.a2022.0004
ISSN 0239-8508, e-ISSN 1897-5631

factor (PeproTech), and 10 $\mu$g/ml transferrin (Bioswamp, Wuhan, China). The cells were seeded at $1 \times 10^5$ cells/ml (2 ml per well) in ultra-low adherent 6-well dishes (Corning, New York, USA) and incubated at 37°C with 5% CO2 for 6 days. Thereafter, tumorspheres were observed under an inverted fluorescence microscope (Leica, Wetzlar, Germany).

**Western blot.** Total proteins were extracted from PANC-1 cells and quantified using a bicinchoninic acid assay kit (Bioswamp, Wuhan, China). 20 $\mu$g of harvested proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). After blocking, the membranes were cultured for 1 h at room temperature (RT) with primary rabbit antibodies against cyclin-dependent kinase 2 (CDK2, 1:1000 dilution), cyclin B1 (1:1000), cyclin D1 (1:1000), octamer-binding transcription factor 4 (OCT4, 1:1000), sex-determining region Y-box 2 (SOX2, 1:1000), Nanog (1:1000), p38 (1:1000), and GAPDH (1:1000). The membranes were then incubated for 1 h at RT with horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibodies (1:20000). All antibodies were supplied by Bioswamp. GAPDH served as an endogenous control.

**Quantitative reverse transcription-polymerase chain reaction (qRT-PCR).** Total RNA was extracted from PANC-1 cells and reversed-transcribed into cDNA. The harvested cDNA was amplified using the SYBR Green PCR kit (KAPA Biosystems, Boston, USA) according to the manufacturer's protocol in a CFX-CONNECT 96 apparatus (Bio-Rad, Hercules, CA, USA). The primer sequences were as follows: p38 forward, 5'-CCCGCTTATCTCATTA-3', reverse, 5'-CAGGCTTTTCCACTCA-3'; OCT4 forward, 5'-CTGGGGGTTCTATTTG-3', reverse, 5'-GGTTCGCTTTCTCTTT-3'; Nanog forward, 5'-GTGTCGCAAAAAAGGA-3', reverse, 5'-CAGGATGTTGGAGAGTTC-3'; SOX2 forward, 5'-GGGTTCGGTGGTCAAGTC-3', reverse, 5'-TGTGAAGTCTGCTGGGGG-3'; GAPDH forward, 5'- CCACTCCTCCACCTTTG-3', reverse, 5'-CACCACCCTGTTGCTGT-3'; GAPDH served as an endogenous control. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [18].

**Statistical analysis.** Data are presented as the mean ± standard deviation (SD). Differences among groups were analyzed using a one-way analysis of variance followed by Tukey test. $P < 0.05$ was considered to be statistically significant.

## Results

### *Effect of 5-FU on the proliferation, apoptosis, and cycle of PANC-1 cells*

PANC-1 cells were treated with various concentrations of 5-FU for 24 h and CCK-8 assay was carried out to assess the cell viability (Fig. 1A). The cell viability decreased with increasing concentrations of 5-FU, and the IC50 of 5-FU on PANC-1 cells was calculated to be 2657 $\mu$M. El-Mahdy *et al.* indicated that after 48 h treatment, the IC50 of 5-FU is 12.66 $\mu$M for PANC1 [19]. However, Cheng *et al.* showed that the cell growth of PANC1 cells showed no change after 500 $\mu$M 5-Fu treatment for 24 h [20], indicating that the IC50 is more than 500 $\mu$M. The high IC50 of 5-FU for PANC1 may be associated with the experimental environment, cellular state, and experimental method. The PANC-1 cells were subsequently treated with 5-FU at concentrations of 0.5 × IC50, IC50, and 2 × IC50. The results showed that 5-FU decreased the viability (Fig. 1B) and increased the apoptosis (Fig. 1C) of PANC-1 cells in a concentration-dependent manner. In addition, 5-FU induced S phase arrest of PANC-1 cells in a concentration-dependent manner, as demonstrated by the decrease of CDK2, cyclin B1, and cyclin D1 proteins' expression (Fig. 1D and 1E).

### *Effect of 5-FU on the stemness maintenance of PANC-1 cells*

Tumorspheres show solid and spherical structures, which are believed to be derivates of CSCs. As shown in Fig. 2A, 5-FU suppressed the tumorsphere formation of PANC-1 cells in a concentration-dependent manner, as demonstrated by the decrease of the mean diameter of 20 tumorspheres. In addition, CD44 and CD133 are specific biomarkers of CSCs [21]. Flow cytometry was performed to evaluate the proportion of CD44+CD133+ cells, in turn determining the proportion of CSCs in PANC-1 cells The results indicated that 5-FU decreased the proportion of CSCs in PANC-1 cells in a concentration-dependent manner (Fig. 2B). Furthermore, the protein and mRNA expression of stemness-related factors (OCT4, Nanog, and SOX2) were measured. Both the protein (Fig. 3A) and mRNA (Fig. 3B) expression of OCT4, Nanog, and SOX2 were reduced after PANC-1 cells were treated with 5-FU. The expression of p38 in PANC-1 cells was also evaluated after 5-FU treatment. Both the phosphorylation (Fig. 4A) and mRNA (Fig. 4B) expression of p38 were decreased by 5-FU in PANC-1 cells, suggesting that the effect of 5-FU on the stemness maintenance of PANC-1 cells might be mediated by the p38 signaling pathway.

### *Effect of p38 inhibition on the proliferation, apoptosis, and cell cycle of PANC-1 cells*

To investigate the effect of p38 signaling on the behavior of PANC-1 cells, p38 expression was suppressed by its inhibitor, VX-702. As shown in Fig. 5, compared to untreated PANC-1 cell, VX-702 suppressed

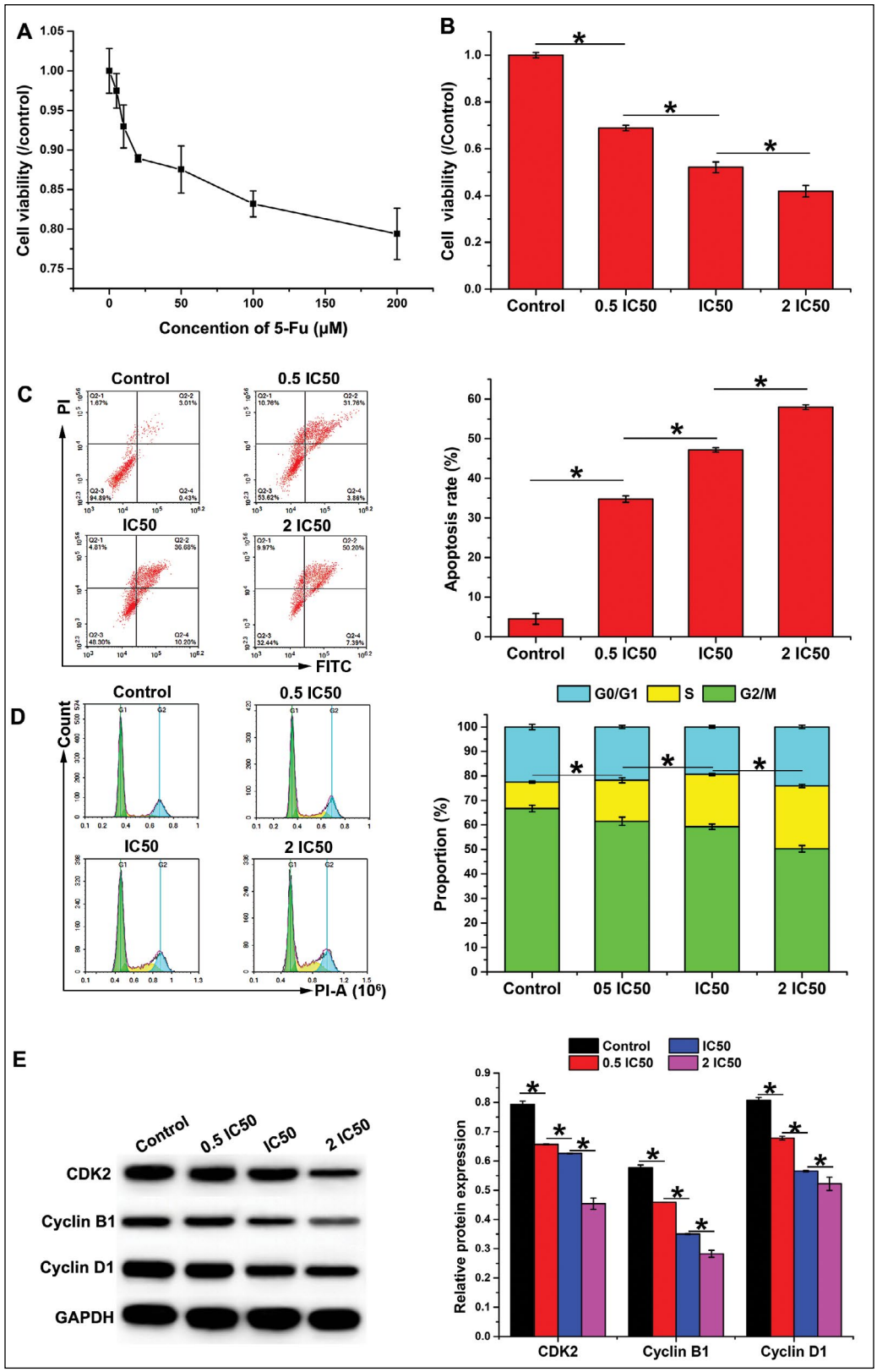


**Figure 1.** Effect of 5-FU on the proliferation, apoptosis, and cell cycle progression in PANC-1 cells. **A.** PANC-1 cells were treated with different concentrations of 5-FU to obtain the IC50 (2657 $\mu$M) using a logit model. Based on the IC50 of 5-FU, PANC-1 cells were treated with 5-FU at 0.5 × IC50, IC50, and 2× IC50 to evaluate the effect of 5-FU on the **(B)** viability, **(C)** apoptosis, **(D)** cell cycle, and **(E)** cell cycle related protein expression. 5-FU decreased cell viability, promoted cell apoptosis, and induced S phase arrest in PANC-1 cells in a concentration-dependent manner. Data are presented as the mean ± SD, n = 3, $^*p < 0.05$.


Folia Histochem Cytobiol. 2022
10.5603/FHC.a2022.0004
ISSN 0239-8508, e-ISSN 1897-5631

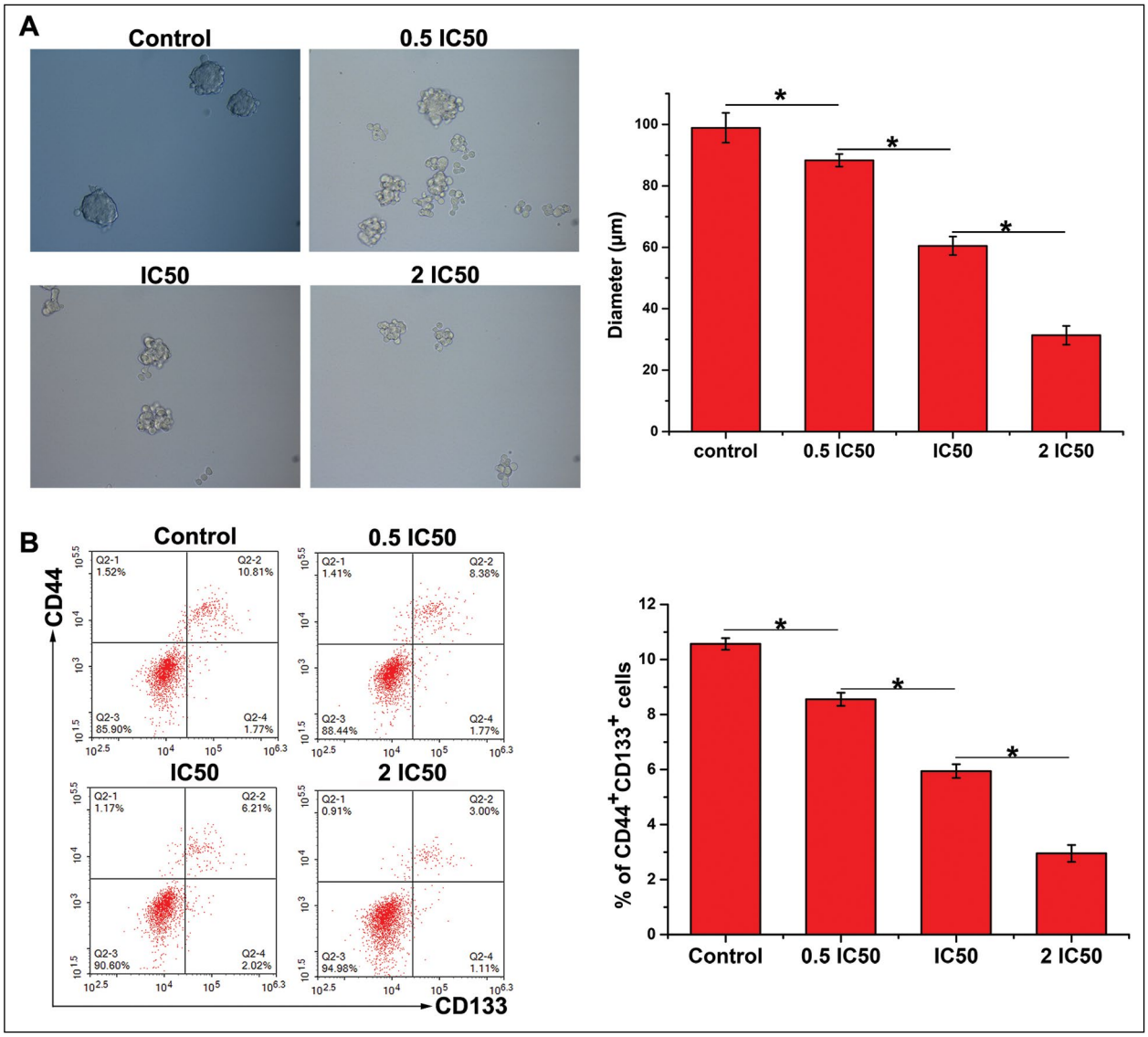


**Figure 2.** Effect of 5-FU on tumorsphere formation and the proportion of CD44+CD133+ cells in PANC-1 cells. PANC-1 cells were treated with 5-FU at 0.5 × IC50, IC50, and 2× IC50 to evaluate the effect of 5-FU on (**A**) tumorsphere formation (magnification ×200) and the mean diameter of 20 tumorspheres in each group. **B.** The proportion of CD44+CD133+ cells. 5-FU suppressed tumorsphere formation and decreased the proportion of CD44+CD133+ cells in PANC-1 cells in a concentration-dependent manner. Data are presented as the mean ± SD, n = 3, $^*p < 0.05$.

cell viability and promoted apoptosis; in addition, VX-702 induced S phase arrest, as demonstrated by decreased expression of CDK2, cyclin B1, and cyclin D1 proteins. These effects were further accentuated with combined 5-FU treatment with VX-702.

### *Effect of p38 inhibition on the stemness maintenance of PANC-1 cells*

VX-702 suppressed the tumorsphere formation (Fig. 6A) and the proportion of CD44+CD133+ cells (Fig. 6B) in PANC-1 cells, which were further suppressed with combined 5-FU treatment. Western blot was performed to evaluate the protein expression of the stemness-related proteins OCT4, Nanog, and SOX2 in PANC-1 cells treated with VX-702, with/without 5-FU (Fig. 7A). p38 inhibitor suppressed the expression of OCT4, Nanog, and SOX2 proteins, and this effect was further downregulated by 5-FU. The effect of p38 inhibition on the mRNA expression of OCT4, Nanog, and SOX2 was similar to that of protein expression (Fig. 7B). Furthermore, the phosphorylation and mRNA expression of p38 were measured using Western blot and qRT-PCR, respectively (Fig. 8). The effect of VX-702 and 5-FU on p38 expression was similar to that on the expression of OCT4, Nanog, and SOX2, suggesting that p38 was involved in regulating the stemness maintenance of PANC-1 cells.

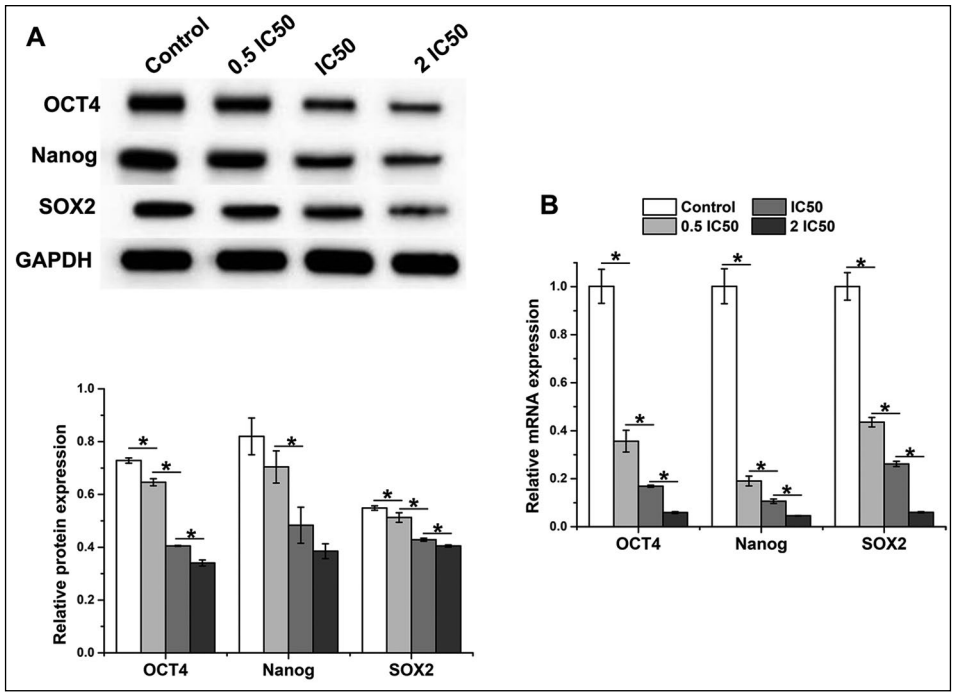


**Figure 3.** Effect of 5-FU on the expression of stemness-related indicators in PANC-1 cells. PANC-1 cells were treated with 5-FU at 0.5 × IC50, IC50, and 2× IC50 to evaluate the effect of 5-FU on the **(A)** protein and **(B)** mRNA expression of OCT4, Nanog, and SOX2. 5-FU downregulated both the protein and mRNA expression of OCT4, Nanog, and SOX2 in PANC-1 cells in a concentration-dependent manner. Data are presented as the mean ± SD, n = 3, $^*p < 0.05$.

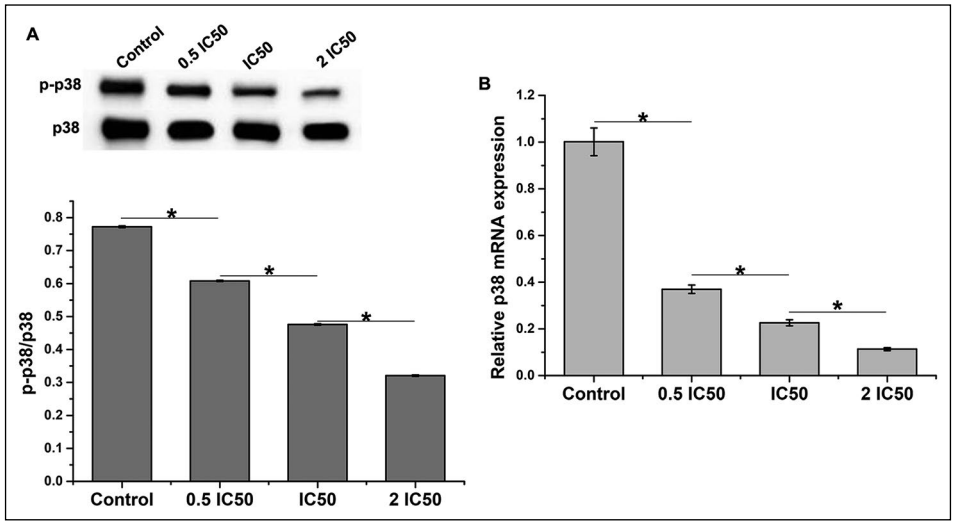


**Figure 4.** Effect of 5-FU on the expression of p38 in PANC-1 cells. PANC-1 cells were treated with 5-FU at 0.5 × IC50, IC50, and 2× IC50 to evaluate the effect of 5-FU on the **(A)** protein and **(B)** mRNA expression of p38. 5-FU downregulated both the protein and mRNA expression of p38 in PANC-1 cells in a concentration-dependent manner. Data are presented as the mean ± SD, n = 3, $^*p < 0.05$.

## Discussion

Chemotherapy is a major treatment strategy for pancreatic cancer, and commonly used chemotherapeutic agents for pancreatic cancer include 5-FU and gemcitabine [22]. Since chemoresistance severely impairs the therapeutic effect, research has been focused on revealing the mechanism and targets that mediate drug resistance and developing better therapeutic strategies for pancreatic cancer [23–25]. However, the underlying mechanism that explains the treatment effect of 5-FU on pancreatic cancer, particularly its



10.5603/FHC.a2022.0004
ISSN 0239-8508, e-ISSN 1897-5631

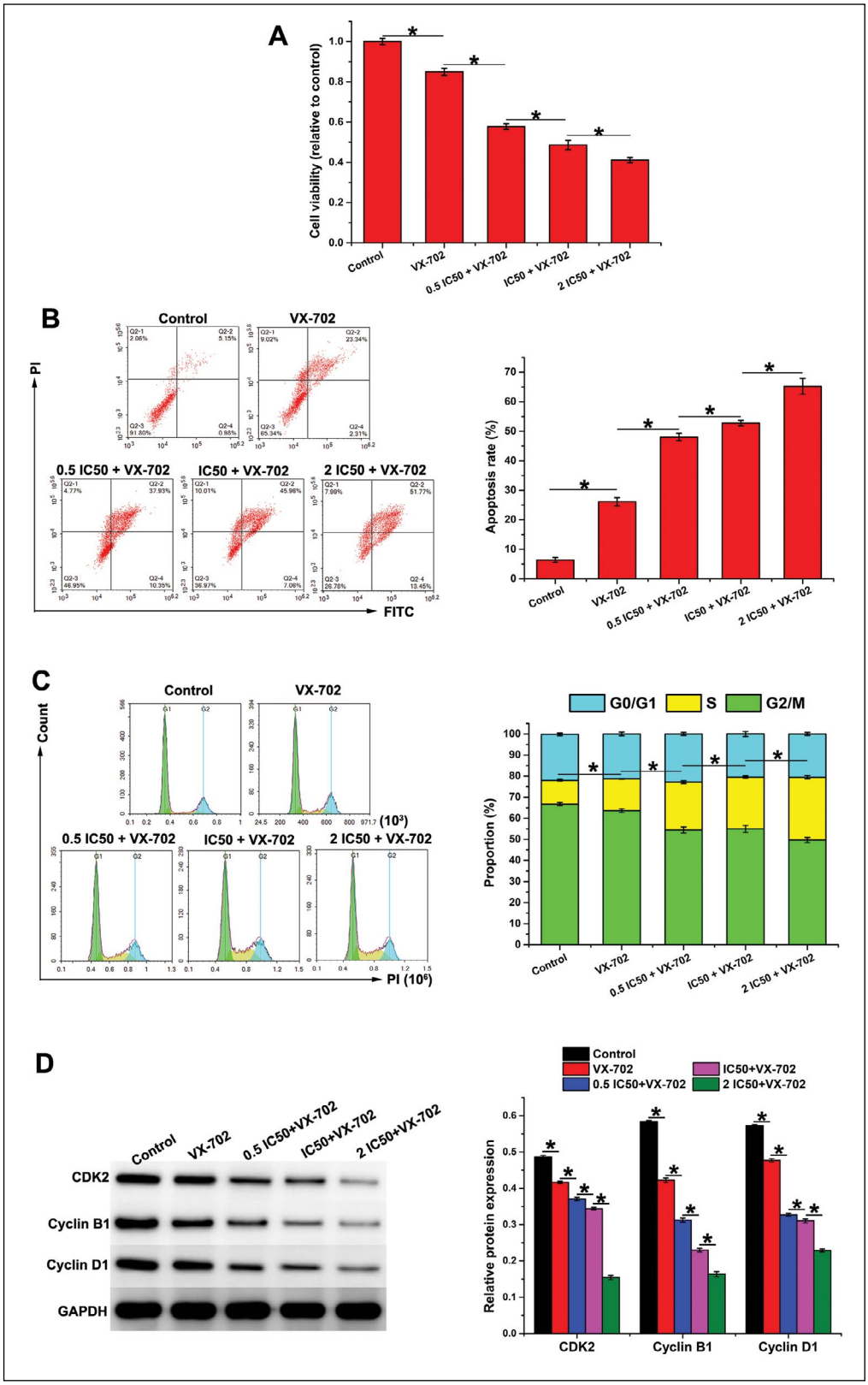


**Figure 5.** Effect of combined p38 inhibition and 5-FU on the proliferation, apoptosis, and cycle cell progression in PANC-1 cells. PANC-1 cells were treated with a p38 inhibitor VX-702 (2 $\mu$M) with 5-FU at 0.5 × IC50, IC50, and 2 × IC50, and cell viability (A), cell apoptosis (B), cell cycle (C) and cell cycle-related protein expression (D) were evaluated. P38 inhibition decreased cell viability, promoted cell apoptosis, and induced S phase arrest in PANC-1 cells. The effect of p38 inhibition was further accentuated by combined 5-FU treatment. Data are presented as the mean ± SD, n = 3, *p < 0.05.


Folia Histochem Cytobiol. 2022
10.5603/FHC.a2022.0004
ISSN 0239-8508, e-ISSN 1897-5631

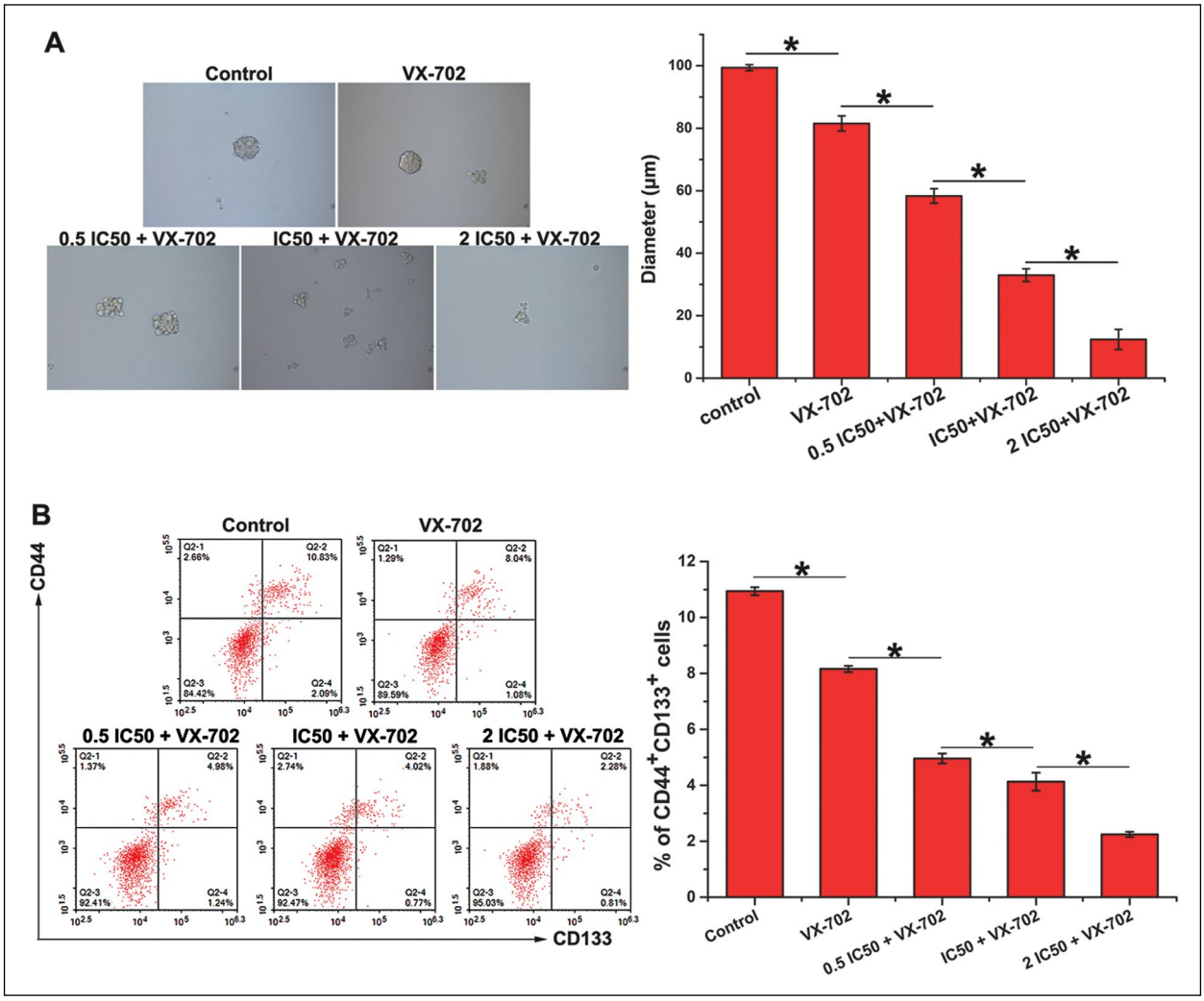


**Figure 6.** Effect of combined p38 inhibition and 5-FU on tumorsphere formation and the proportion of CD44+CD133+ cells in PANC-1 cells. PANC-1 cells were treated with a p38 inhibitor with/without 5-FU at 0.5 × IC50, IC50, and 2 × IC50 and **(A)** tumorsphere formation (magnification ×200) and the mean diameter of 20 tumorspheres in each group. **B.** The proportion of CD44+CD133+ cells. P38 inhibition suppressed tumorsphere formation and reduced the proportion of CD44+CD133+ cells in PANC-1 cells. The effect of p38 inhibition was further accentuated by combined 5-FU treatment. Data are presented as the mean ± SD, n = 3, $^*p < 0.05$.

effect on the stem-like properties, is barely elucidated. CSCs were shown to play an important role in 5-FU resistance [26, 27] but on the contrary, 5-FU exerted anti-tumor effects partially by suppressing the stem-like properties of cancer cells [28]. Cell cycle regulation is one of the key regulatory mechanisms of cancer cell growth, and cell proliferation could be inhibited by inducing cell cycle arrest in the S phase [29, 30]. The current study demonstrated that after 24 h of 5-FU treatment at different concentrations, cell viability was decreased, apoptosis was promoted, and the arrest of the cell cycle at the S phase was induced in PANC-1 cells in a concentration-dependent manner. An obvious fall in the cyclin D1, cyclin B1, and CDK2 protein levels that play an important role in the S phase arrest was shown. In addition, the ability of PANC-1 cells to form tumorspheres, which are initiated from CSCs, was suppressed by 5-FU, and the proportion of PANC-1 cells exhibiting positive expression of CD44 and CD133 (a marker of CSCs (21)) was decreased. Stemness genes NANOG, OCT4, and SOX2 are important transcription factors that regulate stem-cell self-renewal. Down-regulation of stemness genes (OCT4, NANOG, and SOX2) was found to reduce the stemness of mesenchymal stem cells [31]. Furthermore, 5-FU attenuated the expression of the stemness-related indicators OCT4, Nanog, and SOX2 in a concentration-dependent manner, which was consistent with previous studies [31]. Thus, this work suggests that the mechanism *via* which 5-FU

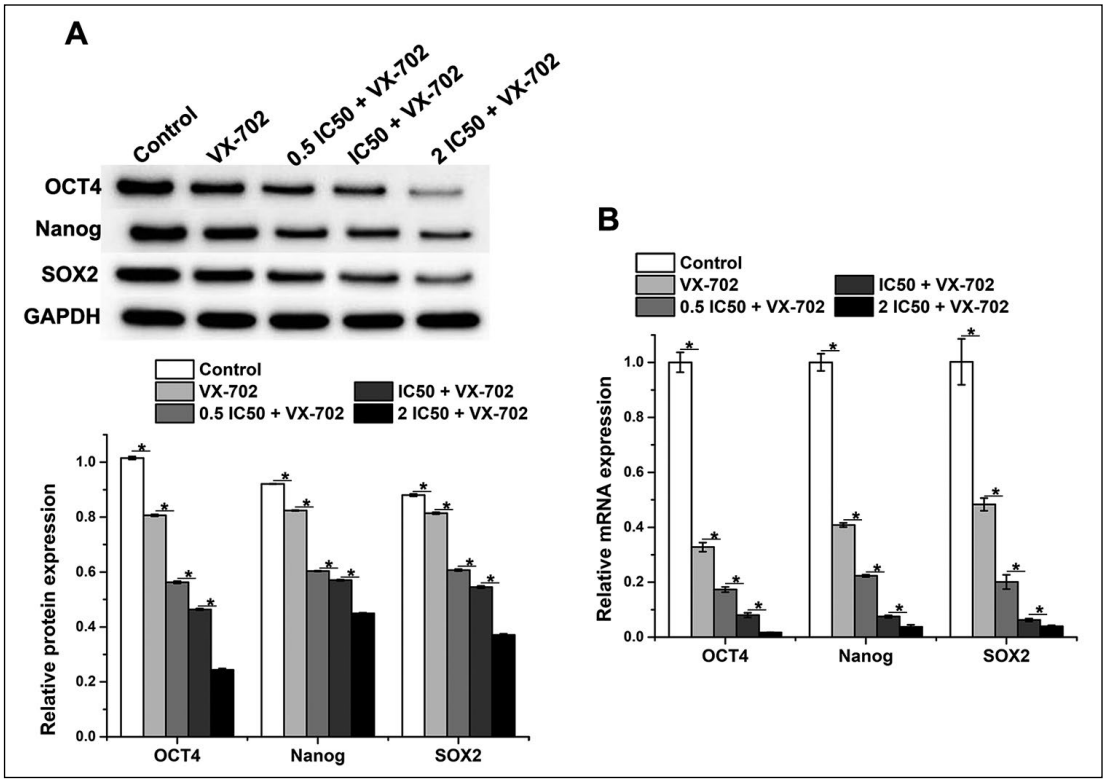


**Figure 7.** Effect of combined p38 inhibition and 5-FU on the expression of stemness-related markers in PANC-1 cells. PANC-1 cells were treated with a p38 inhibitor with/without 5-FU at 0.5 × IC50, IC50, and 2 × IC50 concentrations, and the **(A)** protein and **(B)** mRNA expression of OCT4, Nanog, and SOX2 were evaluated. P38 inhibition downregulated both the protein and mRNA expression of OCT4, Nanog, and SOX2 in PANC-1 cells. The effect of p38 inhibition was further accentuated by combined 5-FU treatment. Data are presented as the mean ± SD, n = 3, $^*p < 0.05$.

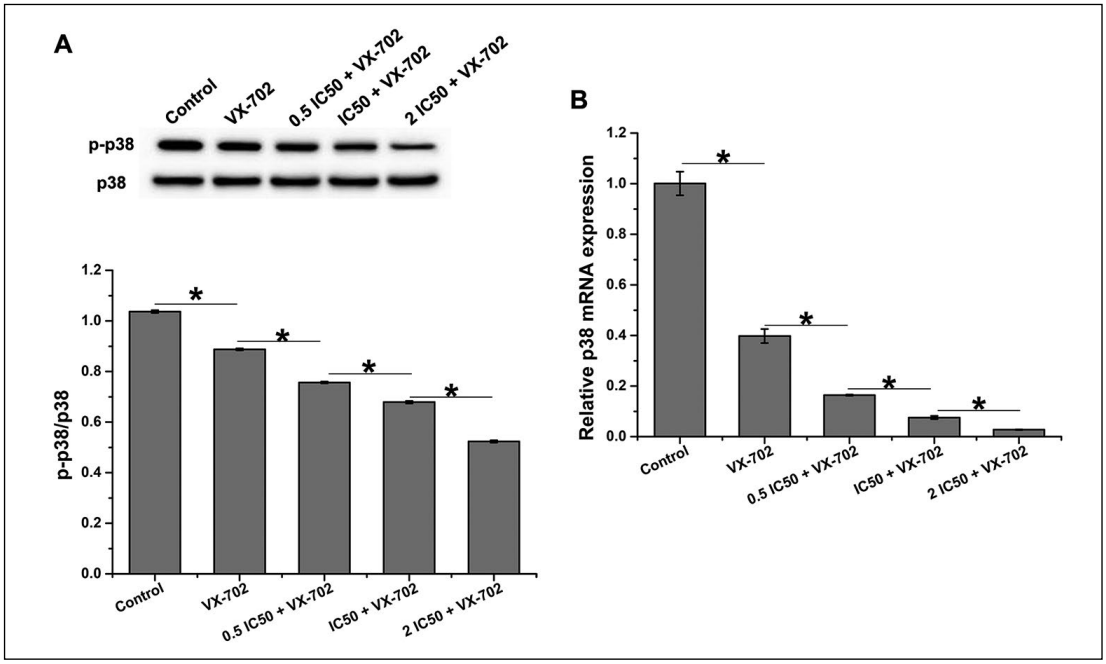


**Figure 8.** Effect of combined p38 inhibition and 5-FU on the expression of p38 in PANC-1 cells. PANC-1 cells were treated with a p38 inhibitor with/without 5-FU at 0.5 × IC50, IC50, and 2 × IC50 concentrations, and the **(A)** protein and **(B)** mRNA expression of p38. P38 inhibition downregulated both the protein and mRNA expression of p38 in PANC-1 cells. The effect of p38 inhibition was further accentuated by combined 5-FU treatment. Data are presented as the mean ± SD, n = 3, $^*p < 0.05$.


Folia Histochem Cytobiol. 2022
10.5603/FHC.a2022.0004
ISSN 0239-8508, e-ISSN 1897-5631

suppressed the development of pancreatic cancer may be associated with its inhibitory effect on the stem-like properties of pancreatic cancer cells.

p38 is a major target for chemotherapy in cancer treatment [32]. Additionally, p38 has been found to mediate the properties of CSCs [33] and is activated by 5-FU in colorectal cancer cells, thereby regulating the balance between autophagy and apoptosis [34]. Thus, we proposed that the effect of 5-FU on the stem-like properties of pancreatic cancer cells may be mediated by p38. We revealed that 5-FU inhibited p38 expression in PANC-1 cells in a concentration-dependent manner, which was different from previous results showing that 5-FU upregulated p38 in colorectal cancer cells [34]. This difference could be due to cell-type specificity. The expression of p38 was then suppressed by its inhibitor to investigate the effect of p38 on the stem-like properties of pancreatic cancer cells. Mammalian p38 is inhibited in various conditions, ranging from physiological processes like cell proliferation to pathological states including cancer. Inhibition of p38 often showed different effects depending on the cell type, organ, and pathophysiologic condition [35, 36]. Recently, Xu *et al.* found that p38 knockdown impaired the self-renewal of CSCs in breast cancer cells [33], consistent with the current study showing that p38 inhibition decreased the ratio of pancreatic CSCs.

Previous studies have demonstrated that OCT4, Nanog, and SOX2 are sufficient to reprogram human somatic cells into pluripotent stem cells that show substantial characteristics of embryonic stem cells [37]. Thus, OCT4, Nanog, and SOX2 have been identified as biomarkers of stem cells [38]. OCT4 is an important regulator of CSC pluripotency and self-renewal [28], and its overexpression in breast cancer cells increased tumorsphere formation compared to that in cells with low OCT4 levels and increased the expression of CSC markers including CD34 and CD133 [39]. Co-expression of OCT4 and Nanog, another stem cell marker [40], enhanced tumor metastasis, migration, and invasion through epithelial-mesenchymal transition and promoted the stem cell phenotype in hepatocellular carcinoma [41]. The current study demonstrated that p38 inhibition reduced the proportion of CD44+CD133+ cells and the expression of the stem cell markers OCT4, Nanog, and SOX2.

In conclusion, the current study provides evidence that 5-FU suppresses stem-like properties of pancreatic cancer cells. The underlying mechanism may be associated with the downregulation of p38 signaling. The deeper mechanisms of 5-FU and VX-702 to maintain the stemness of PANC-1 cells need to be further investigated. Thus, targeting p38 may prove to be instrumental in pancreatic cancer therapy.

## Acknowledgments

Not applicable.

## Funding

Not applicable.

## Ethical approval and consent to participate

The experimental protocol does not involve animal or human testing.

## Consent for publication

Not applicble.

## Availability of supporting data

All data generated or analyzed during this study are included in this published article.

## Authors' contributions

JZ and XS were responsible for project design. JZ. CD, RL, LS, and CC performed the experiment and data analysis. JZ drafted the manuscript. XS revised the manuscript.

## Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

## References

1. Siegel RL, Fedewa SA, Miller KD, et al. Cancer statistics, 2015. CA Cancer J Clin. 2015; 65(1): 5–29, doi: 10.3322/caac.21254, indexed in Pubmed: 25559415.
2. Muniraj T, Jamidar PA, Aslanian HR. Pancreatic cancer: a comprehensive review and update. Dis Mon. 2013; 59(11): 368–402, doi: 10.1016/j.disamonth.2013.08.001, indexed in Pubmed: 24183261.
3. Costello E, Greenhalf W, Neoptolemos JP. New biomarkers and targets in pancreatic cancer and their application to treatment. Nat Rev Gastroenterol Hepatol. 2012; 9(8): 435–444, doi: 10.1038/nrgastro.2012.119, indexed in Pubmed: 22733351.
4. Stathis A, Moore MJ. Advanced pancreatic carcinoma: current treatment and future challenges. Nat Rev Clin Oncol. 2010; 7(3): 163–172, doi: 10.1038/nrclinonc.2009.236, indexed in Pubmed: 20101258.
5. Lawson DA, Bhakta NR, Kessenbrock K, et al. Single-cell analysis reveals a stem-cell program in human metastatic breast cancer cells. Nature. 2015; 526(7571): 131–135, doi: 10.1038/nature15260, indexed in Pubmed: 26416748.
6. Prieto-Vila M, Takahashi RU, Usuba W, et al. Drug resistance driven by cancer stem cells and their niche. Int J Mol Sci. 2017; 18(12), doi: 10.3390/ijms18122574, indexed in Pubmed: 29194401.
7. Reya T, Morrison SJ, Clarke MF, et al. Stem cells, cancer, and cancer stem cells. Nature. 2001; 414(6859): 105–111, doi: 10.1038/35102167, indexed in Pubmed: 11689955.

8. Pattabiraman DR, Weinberg RA. Tackling the cancer stem cells—what challenges do they pose? Nat Rev Drug Discov. 2014; 13(7): 497–512, doi: 10.1038/nrd4253, indexed in Pubmed: 24981363.
9. Sun X, Lv X, Yan Y, et al. Hypoxia-mediated cancer stem cell resistance and targeted therapy. Biomed Pharmacother. 2020; 130: 110623, doi: 10.1016/j.biopha.2020.110623, indexed in Pubmed: 32791395.
10. Zhou P, Li Bo, Liu F, et al. The epithelial to mesenchymal transition (EMT) and cancer stem cells: implication for treatment resistance in pancreatic cancer. Mol Cancer. 2017; 16(1): 52, doi: 10.1186/s12943-017-0624-9, indexed in Pubmed: 28245823.
11. Najafi M, Farhood B, Mortezaee K. Cancer stem cells (CSCs) in cancer progression and therapy. J Cell Physiol. 2019; 234(6): 8381–8395, doi: 10.1002/jcp.27740, indexed in Pubmed: 30417375.
12. Phi LT, Sari IN, Yang YG, et al. Cancer stem cells (CSCs) in drug resistance and their therapeutic implications in cancer treatment. Stem Cells Int. 2018; 2018: 5416923, doi: 10.1155/2018/5416923, indexed in Pubmed: 29681949.
13. Pearson G, Robinson F, Beers Gibson T, et al. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev. 2001; 22(2): 153–183, doi: 10.1210/edrv.22.2.0428, indexed in Pubmed: 11294822.
14. Morrison DK. MAP kinase pathways. Cold spring harbor perspectives in biology. 2012; 4(11): a011254–a011254, doi: 10.1101/cshperspect.a011254, indexed in Pubmed: 23125017.
15. del Barco Barrantes I, Nebreda AR. Roles of p38 MAPKs in invasion and metastasis. Biochem Soc Trans. 2012; 40(1): 79––84, doi: 10.1042/BST20110676, indexed in Pubmed: 22260669.
16. Paranjape AN, Soundararajan R, Werden SJ, et al. Inhibition of FOXC2 restores epithelial phenotype and drug sensitivity in prostate cancer cells with stem-cell properties. Oncogene. 2016; 35(46): 5963–5976, doi: 10.1038/onc.2015.498, indexed in Pubmed: 26804168.
17. Kaushik S, Cuervo AM. AMPK-dependent phosphorylation of lipid droplet protein PLIN2 triggers its degradation by CMA. Autophagy. 2016; 12(2): 432–438, doi: 10.1080/15548627.2015.1124226, indexed in Pubmed: 26902588.
18. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25(4): 402–408, doi: 10.1006/meth.2001.1262, indexed in Pubmed: 11846609.
19. El-Mahdy HA, El-Husseiny AA, Kandil YI, et al. Diltiazem potentiates the cytotoxicity of gemcitabine and 5-fluorouracil in PANC-1 human pancreatic cancer cells through inhibition of P-glycoprotein. Life Sci. 2020; 262: 118518, doi: 10.1016/j.lfs.2020.118518, indexed in Pubmed: 33011221.
20. Cheng Y, Diao D, Zhang H, et al. High glucose-induced resistance to 5-fluorouracil in pancreatic cancer cells alleviated by 2-deoxy-D-glucose. Biomed Rep. 2014; 2(2): 188–192, doi: 10.3892/br.2013.211, indexed in Pubmed: 24649094.
21. Yue F, Hirashima K, Tomotsune D, et al. Reprogramming of retinoblastoma cancer cells into cancer stem cells. Biochem Biophys Res Commun. 2017; 482(4): 549–555, doi: 10.1016/j.bbrc.2016.11.072, indexed in Pubmed: 27856246.
22. Cao J, Ma J, Sun L, et al. Targeting glypican-4 overcomes 5-FU resistance and attenuates stem cell-like properties via suppression of Wnt/βcatenin pathway in pancreatic cancer cells. J Cell Biochem. 2018; 119(11): 9498–9512, doi: 10.1002/jcb.27266, indexed in Pubmed: 30010221.
23. Ghadban T, Dibbern JL, Reeh M, et al. HSP90 is a promising target in gemcitabine and 5-fluorouracil resistant pancreatic cancer. Apoptosis. 2017; 22(3): 369–380, doi: 10.1007/s10495-016-1332-4, indexed in Pubmed: 27878398.
24. Wei X, Wang W, Wang L, et al. MicroRNA-21 induces 5-fluorouracil resistance in human pancreatic cancer cells by regulating PTEN and PDCD4. Cancer Med. 2016; 5(4): 693–702, doi: 10.1002/cam4.626, indexed in Pubmed: 26864640.
25. Wang WB, Yang Yu, Zhao YP, et al. Recent studies of 5-fluorouracil resistance in pancreatic cancer. World J Gastroenterol. 2014; 20(42): 15682–15690, doi: 10.3748/wjg.v20.i42.15682, indexed in Pubmed: 25400452.
26. Miyoshi S, Tsugawa H, Matsuzaki J, et al. Inhibiting xCT improves 5-fluorouracil resistance of gastric cancer induced by CD44 variant 9 expression. Anticancer Res. 2018; 38(11): 6163–6170, doi: 10.21873/anticanres.12969, indexed in Pubmed: 30396933.
27. Hu YB, Yan C, Mu L, et al. Exosomal Wnt-induced dedifferentiation of colorectal cancer cells contributes to chemotherapy resistance. Oncogene. 2019; 38(11): 1951–1965, doi: 10.1038/s41388-018-0557-9, indexed in Pubmed: 30390075.
28. Lee JH, Yun CW, Han YS, et al. Melatonin and 5-fluorouracil co-suppress colon cancer stem cells by regulating cellular prion protein-Oct4 axis. J Pineal Res. 2018; 65(4): e12519, doi: 10.1111/jpi.12519, indexed in Pubmed: 30091203.
29. Park S, Kwon W, Park JK, et al. Suppression of cathepsin a inhibits growth, migration, and invasion by inhibiting the p38 MAPK signaling pathway in prostate cancer. Arch Biochem Biophys. 2020; 688: 108407, doi: 10.1016/j.abb.2020.108407, indexed in Pubmed: 32407712.
30. Zhong S, Ji DF, Li YG, et al. Activation of P27kip1-cyclin D1/E-CDK2 pathway by polysaccharide from Phellinus linteus leads to S-phase arrest in HT-29 cells. Chem Biol Interact. 2013; 206(2): 222–229, doi: 10.1016/j.cbi.2013.09.008, indexed in Pubmed: 24060681.
31. Hwang YS, Park SH, Kang M, et al. Stemness and differentiation potential-recovery effects of sinapic acid against ultraviolet-A-induced damage through the regulation of p38 MAPK and NF-κB. Sci Rep. 2017; 7(1): 909, doi: 10.1038/s41598-017-01089-5, indexed in Pubmed: 28424532.
32. García-Cano J, Roche O, Cimas FJ, et al. p38MAPK and chemotherapy: we always need to hear both sides of the story. Front Cell Dev Biol. 2016; 4: 69, doi: 10.3389/fcell.2016.00069, indexed in Pubmed: 27446920.
33. Xu M, Wang S, Wang Y, et al. Role of p38γ MAPK in regulation of EMT and cancer stem cells. Biochim Biophys Acta Mol Basis Dis. 2018; 1864(11): 3605–3617, doi: 10.1016/j.bbadis.2018.08.024, indexed in Pubmed: 30251680.
34. de la Cruz-Morcillo MA, Valero MLL, Callejas-Valera JL, et al. P38MAPK is a major determinant of the balance between apoptosis and autophagy triggered by 5-fluorouracil: implication in resistance. Oncogene. 2012; 31(9): 1073–1085, doi: 10.1038/onc.2011.321, indexed in Pubmed: 21841826.
35. Wada T, Penninger JM. Mitogen-activated protein kinases in apoptosis regulation. Oncogene. 2004; 23(16): 2838–2849, doi: 10.1038/sj.onc.1207556, indexed in Pubmed: 15077147.
36. Wagner EF, Nebreda AR. Signal integration by JNK and p38 MAPK pathways in cancer development. Nat Rev Cancer. 2009; 9(8): 537–549, doi: 10.1038/nrc2694, indexed in Pubmed: 19629069.
37. Yu J, Vodyanik MA, Smuga-Otto K, et al. Induced pluripotent stem cell lines derived from human somatic cells. Science. 2007; 318(5858): 1917–1920, doi: 10.1126/science.1151526, indexed in Pubmed: 18029452.
38. Lee S, Wottrich S, Bonavida B. Crosstalks between Raf-kinase inhibitor protein and cancer stem cell transcription factors (Oct4, KLF4, Sox2, Nanog). Tumour Biol. 2017; 39(4): 1010428317692253, doi: 10.1177/1010428317692253, indexed in Pubmed: 28378634.
39. Kim RJ, Nam JS. OCT4 expression enhances features of cancer stem cells in a mouse model of breast cancer. Lab Anim Res. 2011; 27(2): 147–152, doi: 10.5625/lar.2011.27.2.147, indexed in Pubmed: 21826175.
40. van Schaijik B, Davis PF, Wickremesekera AC, et al. Subcellular localisation of the stem cell markers OCT4, SOX2, NANOG, KLF4 and c-MYC in cancer: a review. J Clin Pathol. 2018; 71(1): 88–91, doi: 10.1136/jclinpath-2017-204815, indexed in Pubmed: 29180509.
41. Yin X, Zhang BH, Zheng SS, et al. Coexpression of gene Oct4 and Nanog initiates stem cell characteristics in hepatocellular carcinoma and promotes epithelial-mesenchymal transition through activation of Stat3/Snail signaling. J Hematol Oncol. 2015; 8: 23, doi: 10.1186/s13045-015-0119-3, indexed in Pubmed: 25879771.


*Submitted: 7 December, 2021*
*Accepted after reviews: 19 January, 2022*
*Available as AoP: 1 February, 2022*