

Knockdown of serine/threonine protein phosphatase 5 enhances gemcitabine sensitivity by promoting apoptosis in pancreatic cancer cells *in vitro*

JINHUI ZHU¹, YUN JI², YUANQUAN YU², YUN JIN², XIAOXIAO ZHANG²,
JIALE ZHOU² and YAN CHEN¹

Departments of ¹General Surgery and Laparoscopic Center, and ²General Surgery, Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, Zhejiang 310009, P.R. China

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Abstract. The targeting protein of serine/threonine protein phosphatase 5 (PPP5C) has been reported to be present in various malignancies. However, its functional role in pancreatic cancer (PC) remains unknown. In the present study, the function of PPP5C in PC cells treated with the first-line drug gemcitabine (GEM) was investigated. Short hairpin (sh)RNA targeting PPP5C was constructed to knockdown PPP5C in PANC-1 cells. Cell cycle and apoptosis analyses were performed in order to investigate the mechanisms underlying the effects induced by PPP5C silencing combined with GEM treatment. Western blot analysis was applied to detect the expression of certain key regulators of cell apoptosis in PANC-1 cells treated with GEM. shRNA against PPP5C effectively suppressed the proliferation of PANC-1 cells treated with GEM. Additionally, cell cycle analysis indicated that PPP5C knockdown resulted in a higher number of PANC-1 cells treated with GEM in G₀/G₁ phase arrest. Knockdown of PPP5C increased the expression of associated apoptotic markers, including cleaved caspase 3, poly (ADP-ribose) polymerase and phosphorylated (p)-p53. In addition, the combination of treatment with GEM and PPP5C silencing significantly increased the apoptosis of PANC-1 cells by affecting the expression levels of p-c-Jun N-terminal kinases and p-p38. The present study suggests that PPP5C may be a potential target for the treatment of PC and that it may enhance the gemcitabine sensitivity of PC cells.

Introduction

Pancreatic cancer (PC) is one of the most lethal malignancies of the human digestive system due to its rapid progression, high recurrence rate and strong chemoresistance (1,2). PC is rarely diagnosed at an early stage due to the fact that patients with localized PC have no recognizable signs or symptoms. Therefore, the majority of PC patients do not receive a definitive diagnosis until late, once the PC cells have metastasized to other organs (3,4). Even in cases where surgical resection is performed, the local recurrence rate of PC is high and the 5-year survival rate remains at only 5% following surgery. Despite recent progress in chemotherapeutics and understanding of the molecular biological mechanisms of PC, limited progress has been made in therapeutic methods for metastatic disease. Due to the fact that the incidence of PC has been markedly increasing over recent years (5), it is necessary to find novel therapies for PC (6).

Gemcitabine (2',2'-difluoro-2'-deoxycytidine, dFdC; GEM), a pyrimidine analog, which is phosphorylated to diphosphate and triphosphate forms to inhibit DNA polymerase and ribonucleotide reductase (7), is now used as the standard palliative treatment for PC (8-11). Originally, GEM was approved as the first-line treatment for PC by the US Food and Drug Administration in 1997 based upon the study by Burris *et al* (9). Almost 2 decades later, GEM was approved for the treatment of advanced PC. GEM has demonstrated marked effects on the survival time of PC patients when used in various forms of therapy, including GEM monotherapy, combination treatment with GEM and in a number of other active cytotoxic agents or regimens (12-15). However, there are a number of factors that have been reported to cause GEM resistance (16).

The reversible phosphorylation of proteins serves a crucial function in regulating numerous biological responses. In mammalian cells, >99% of this phosphorylation occurs on serine or threonine (Ser/Thr) residues. This type of protein phosphatase dephosphorylates a range of proteins involved in a wide range of cellular processes, including apoptosis, cell differentiation, cell survival, the response to DNA damage, and the regulation of ion channels and

Correspondence to: Dr Yan Chen, Department of General Surgery and Laparoscopic Center, Second Affiliated Hospital of Zhejiang University School of Medicine, 88 Jiefang Road, Hangzhou, Zhejiang 310009, P.R. China
E-mail: chenyan1256@126.com

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circadian rhythms (17,18). The reversible phosphorylation of proteins may also impact on several signaling pathways, including those controlled by kinases, such as apoptosis signal-regulating kinase 1/mitogen-activated protein kinase kinase kinase 5 (ASK1/MAP3K5), protein kinase, DNA-activated, catalytic polypeptide and RAF1 (19-21). The targeting protein of Ser/Thr protein phosphatase 5 (PPP5C), a member of the phosphoprotein phosphatase (PPP) family of Ser/Thr phosphatases, is an enzyme encoded by the PPP5C gene (22). PPP5C is broadly expressed and has distinct structural properties compared with other phosphatases in the PPP family (23,24). PPP5C belongs to the protein phosphatase-5 subfamily and contains only 1 single polypeptide chain (25-27). It has been reported that PPP5C functions upstream of p53 and that it phosphorylates p53 to regulate the induction of p21 (WAF1/Cip1), as well as to mediate the growth arrest pathway (25,28). PPP5C has been demonstrated to interact with ASK1 (19), cryptochrome circadian clock 2 (29), guanine nucleotide-binding protein subunit α -12 (30) and Ras-related C3 botulinum toxin substrate 1 (31). ASK1 could activate c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinases (MAPK) in a Raf-independent manner in response to a number of stresses. ASK1 is additionally associated with cancer, diabetes, and cardiovascular and neurodegenerative diseases (32).

The result of PPP5C-knockdown by siRNA or oligonucleotides has revealed that PPP5C is also associated with the stress response and inhibition of the proliferation rate of tumor cells, including ovarian cancer (33), glioma (34) and liver carcinoma (35) cells. Additionally, a previous study demonstrated that an elevated level of PPP5C protein is directly associated with Alzheimer's disease (35,36). However, the function of PPP5C in PC has not been reported prior to the present study. In the present study, shRNA was constructed to silence the expression of PPP5C in the PC PANC-1 cell line and the effects of PPP5C on PANC-1 cells treated with GEM were also investigated.

Materials and methods

Cell lines and cell culture. The human embryonic kidney 293 cell line (HEK 293) and the human PC cell line PANC-1 were used in the present study and were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cell lines were cultured in Dulbecco's modified Eagle's medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA; cat. no. SH30243.01B+), supplemented with 10% fetal bovine serum (Biological Industries, Beit-Haemek, Israel; cat. no. 04-001-1A) and maintained at 37°C in a humidified incubator with 5% CO₂.

Construction of the lentivirus vector for PPP5C short hairpin (sh)RNA and virus packaging. Based upon the sequence of PPP5C (NM_001204284.1), a responsible shRNA sequence (5'-GAGACAGAGAAGATTACAGTACTCGAGTACTGTAATCTTCTCTGTCTCTTTT-3') was generated to target PPP5C and a control shRNA sequence (5'-TTCTCCGAACGTGTCACGTCTCGAGACGTGACACGTTCCGGAGAA-3') was designed and synthesized. The recombinant vectors, PPP5C shRNA (shPPP5C) and control shRNA (shCon), were designated to

carry the corresponding shRNA. T4 DNA ligase (New England BioLabs, Inc., Ipswich, MA, USA) was used to construct shRNA fragments (50 ng), which were cloned into the lentiviral expression vector pGP (Shanghai Hollybio, Shanghai, China) prior to being digested by *EcoRI* and *BamHI* (New England BioLabs, Inc.). Lentivirus was generated by co-transfection of HEK293 cells with recombinant vectors and packaging plasmids (pVSVG-I and pCMV Δ R8.92; Shanghai Hollybio). The supernatants were collected 96 h after transfection to extract the lentivirus that may express PPP5C shRNA or control shRNA. The lentivirus was then purified via ultracentrifugation in a condition of 100,000 x g for 30 min at 4°C. PANC-1 cells were infected with the concentrated virus at a multiplicity of infection of 10 and mock-infected cells were used as negative controls. Since the lentivirus carries a green fluorescence protein (GFP) as a reporter and this GFP is driven by the cytomegalovirus promoter, the titer of lentivirus was determined by counting the number of cells that expressed GFP under fluorescence microscopy under x100 magnification (Olympus Corporation, Tokyo, Japan) following 48 h of infection. The efficiency of PPP5C-knockdown was subsequently measured by reverse transcription quantitative polymerase chain reaction (RT-qPCR) and western blot analysis.

RNA extraction and RT-qPCR. PANC-1 cells were harvested following 72 h of lentivirus infection and all of the RNA from the cultured cells was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA; cat. no. 15596-026) according to the manufacturer's protocols. The purity and integrity of extracted RNA was assessed using spectrophotometry and agarose gel electrophoresis, respectively. The first strand in the complementary DNA of the extracted RNA was synthesized from the aforementioned RNA (2 μ g) using reverse transcription reagents (Promega Corporation, Madison, WI, USA; cat. no. M1705). The primers used in this study were: PPP5C forward, 5'-CCCAACTACTGCGACCAGAT-3' and reverse, 5'-CCCGTCACCTCACATCATTC-3'; and β -actin forward, 5'-GTGGACATCCGCAAAGAC-3' and reverse, 5'-AAAGGGTGTAACGCAACTA-3'. The expression of PPP5C mRNA was evaluated by RT-qPCR using the SYBR Green Core Reagents kit on BioRad CFX96 Touch™ Real-Time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The conditions of the PCR involved incubating all of the samples at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 5 sec, and annealing and extension at 60°C for 20 sec. β -actin was used as the input reference. The relative gene expression levels were quantified using the 2^{- $\Delta\Delta$ C_q} method (37).

Western blot analysis. PANC-1 cells were harvested following 6 days of lentivirus infection. Cells were harvested and washed twice using ice-cold phosphate-buffered saline (PBS) and then lysed in ice-cold 2X SDS Lysis Buffer [100 mM Tris-HCl (pH 6.8), 10 mM EDTA, 4% SDS and 10% glycine]. The protein concentration of cell lysate was determined using the BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.; cat. no. 23235). Extracted proteins (30 μ g) were separated on 10% SDS-PAGE prior to being transferred electrophoretically onto a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Inc.; cat. no. 162-0177).

The proteins were blocked in 5% skimmed milk for 1 min at room temperature and probed with specific antibodies at 4°C overnight. The primary antibodies used were rabbit anti-PPP5C (Proteintech Group Inc., Chicago, IL, USA; cat. no. 117515-1-AP; 1:1,000 dilution), and rabbit anti-p-JNK (cat. no. 4668; 1:1,000 dilution), rabbit anti-JNK (cat. no. 9252; 1:1,000 dilution), rabbit anti-p-p38 (cat. no. 9215; 1:500 dilution), rabbit anti-p38 (cat. no. 9212; 1:1,000 dilution), rabbit anti-caspase3 (cat. no. 9661; 1:500 dilution), rabbit anti-PARP (cat. no. 9542; 1:1,000 dilution), rabbit anti-p-p53(Ser315) (cat. no. 2528; 1:500 dilution) and rabbit anti-p53 antibody (Ser15) (cat. no. 11094; 1:500 dilution) (all from Cell Signaling Technology, Inc., Danvers, MA, USA). One rabbit anti-GADPH antibody was used as loading control (cat. no. 11205; 1:1,000 dilution; Proteintech Group Inc.). Subsequently, the membranes were washed 3 times in Tris-buffered saline (TBST) prior to being incubated with goat anti-rabbit Immunoglobulin G horseradish peroxidase-conjugated secondary antibody (cat. no. SC-2054; 1:5,000 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h at room temperature. In addition, the JNK blot was stripped and reprobed with p-JNK, p38 was stripped and reprobed with p-38, and PARP was stripped and reprobed with p-38. Stripping buffers consisted of 0.94 g glycine (cat. no. GB0235; Sangon Biotech Co., Ltd., Shanghai, China) and 5 g sodium dodecyl sulfonate (cat. no. A500228; Sangon Biotech Co., Ltd.) in 500 ml water. The blot was placed into the stripping buffers prior to being agitated in the shaker for 8 min at room temperature. Subsequently, the blot was washed in 1X TBST 3 times, for 5 min each time. The blot was then blocked and reacted with all antibodies indicated as above in the aforementioned manner. The target bands were visualized using an enhanced chemiluminescence kit (GE Healthcare Life Sciences, Uppsala, Sweden) according to the manufacturer's protocols.

MTT assay. Cell proliferation and viability were determined by MTT assay. Cells were plated at a density of 5×10^3 cells/well in 96-well culture plates following 72 h of lentivirus infection and treatment with various concentrations of GEM (1, 5 and 10 μ M). Next, 20 μ l MTT solution (5 mg/ml dissolved in PBS) was added to each well followed by 4 h of incubation at 37°C. Following incubation, 100 μ l stop buffer acidic isopropanol (0.01 M HCl, 10% SDS and 5% isopropanol) was added to each well to stop the reaction. The culture plates with incubated cells were gently agitated for 10 min and analyzed using an Epoch Microplate Spectrophotometer version 2 (BioTek Instruments, Inc., Winooski, VT, USA) at a wavelength of 595 nm.

Cell cycle analysis. Cell cycle analysis was conducted using propidium iodide (PI) according to the manufacturer's protocols. PANC-1 cells were seeded onto 6-cm wide dishes at a density of 5×10^5 cells/well. Following lentivirus infection for 4 days and treatment with 5 μ M GEM, PANC-1 cells were collected, washed, fixed with 75% ethanol at 4°C overnight and then stained with PI. Finally, the distribution of the cell cycle was assessed using a fluorescence-associated cell sorting (FACS) assay with FACSCalibur (Beckman Coulter, Inc., Brea, CA, USA) and the results were analyzed

using ModFit LT 3.1 software (Verity Software House, Inc., Topsham, ME, USA).

Annexin V staining apoptosis analysis. To assess the rate of apoptosis, PANC-1 cells infected with lentivirus and treated with 1 μ M GEM were stained using the Annexin V-APC/7-AAD Apoptosis Detection kit (cat. no. KGA1026; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). The cells were analyzed on a FACSCalibur (Beckman Coulter, Inc.) using the CellQuest Pro software (version 5.1) (BD Biosciences, San Jose, CA, USA) and the percentage of each quadrant was calculated using this software.

Statistical analysis. All results are expressed as the mean \pm standard deviation. Statistical analysis was performed using unpaired Student's t-test or one-way analysis of variance followed by Dunnett's multiple comparisons test. $P < 0.05$ was considered to indicate a statistically significant difference. Statistical analysis was performed using SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA).

Results

Endogenic PPP5C is inhibited by shPPP5C and induced by GEM in the PC PANC-1 cell line. To investigate the role served by PPP5C in PC, the PANC-1 cell line was infected with a lentivirus vector carrying shRNA targeting PPP5C. PPP5C-knockdown efficiency was verified using RT-qPCR and western blot analysis. Compared with that in the negative control cells (shCon), the PPP5C mRNA expression in PPP5C shRNA-infected PANC-1 cells (shPPP5C) was decreased by 94% (Fig. 1A). Likewise, PPP5C protein expression was significantly decreased in the PANC-1 cells infected with shPPP5C (Fig. 1B). In addition, the effect of GEM on PPP5C expression in the PANC-1 cells was detected by qRT-PCR and western blot analysis. The results indicated that GEM could increase intracellular PPP5C mRNA and protein expression at concentrations of 1, 5 and 10 μ M compared with the untreated control (Fig. 1C and D). These results indicate that intracellular PPP5C was successfully silenced and that it could be enhanced by GEM in PANC-1 cells.

PPP5C silencing enhances the chemosensitivity of PANC-1 cells to GEM. To determine whether or not PANC-1 cells with reduced PPP5C expression were more sensitive to GEM, PANC-1 cells were stably transfected with shPPP5C or shCon using a continuous 72-h MTT assay. As demonstrated in Fig. 2A, shPPP5C markedly inhibited the proliferation of PANC-1 cells compared with shCon. Following addition of GEM at varying concentrations, the cell growth rates were significantly decreased (Fig. 2B-D) compared with shCon cells (Fig. 2A) using a Student's t-test. As demonstrated in Fig. 2E, the inhibition ratio was significantly increased in the shPPP5C group following GEM treatment. In addition, statistical analysis revealed that regardless of whether or not PPP5C was silenced, the inhibitory effect of 5 or 10 μ M GEM exhibited no marked differences at 24, 48 and 72 h, indicating that the effect of GEM had reached saturation (Fig. 2E). Even if the effect of GEM had reached saturation, the shPPP5C

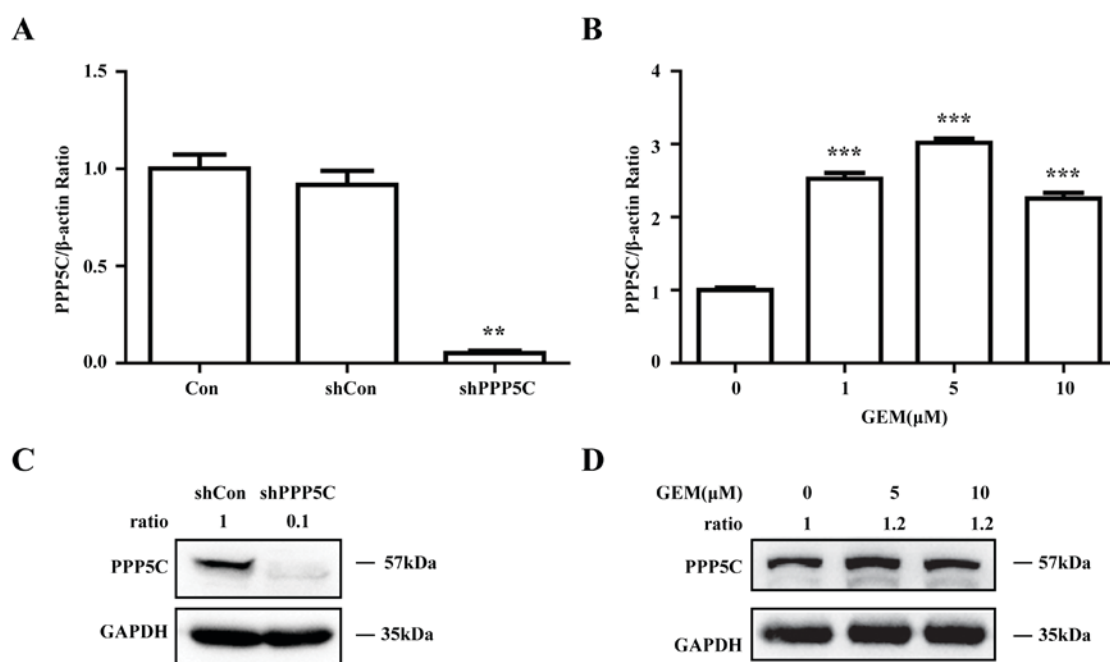


Figure 1. Expression of PPP5C in the PC PANC-1 cell line. (A) Analysis of PPP5C expression in PANC-1 cells transduced with shCon and shPPP5C by RT-qPCR analysis. (B) Analysis of PPP5C expression in PANC-1 cells transduced treated with GEM at different concentrations by RT-qPCR analysis. (C) Analysis of PPP5C expression in PANC-1 cells transduced with shCon and shPPP5C by western blot analysis. (D) Analysis of PPP5C expression in PANC-1 cells transduced treated with GEM at different concentration by western blot analysis. Data are presented as the mean \pm standard deviation of 3 independent experiments. Statistical analysis in A and B were performed using one-way ANOVA followed by Dunnett's multiple comparisons test. ** $P < 0.01$ vs. control, *** $P < 0.001$ vs. 0 μ g GEM. PPP5C, serine/threonine protein phosphatase 5; shCon, short hairpin control; shPPP5C, short hairpin PPP5C; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; GEM, gemcitabine; ANOVA, analysis of variance.

group remained able to further inhibit PANC-1 cell proliferation compared with the shCon group (the inhibition ratio at different GEM concentrations exhibited no dose-response effect; Fig. 2E). These results indicate that PPP5C-knockdown sensitizes PANC-1 cells to GEM treatment.

GEM enhances shPPP5C-induced PANC-1 cell cycle arrest and apoptosis. In order to identify the mechanisms underlying the antiproliferation effect, the distribution of cells in the cell cycle phases was analyzed using flow cytometry (Fig. 3A). The proportion of G₀/G₁ phase cells was markedly increased while the G₂/M phase population was decreased under GEM treatment when compared with PANC-1 cells treated with the single shCon or shPPP5C alone (Fig. 3B and C). These data suggested that the combined treatment could further arrest the cell cycle at the G₀/G₁ phase in PANC-1 cells. In addition, GEM treatment plus PPP5C silencing resulted in a significant increase in the cell population in the sub-G₁ phase (Fig. 3D), suggesting the presence of cell apoptosis.

To further examine the effect of combined treatment and that of PPP5C silencing alone on cell apoptosis in PANC-1 cells, Annexin V-APC/7-AAD staining was performed. As demonstrated in (Fig. 3E and F), flow cytometry analysis revealed that the percentage of early apoptotic cells (Annexin V⁺/7-AAD⁻) was higher in the shPPP5C group compared with that in the shCon group, while no difference was observed in the percentage of late apoptotic cells (Annexin V⁺/7-AAD⁺) between the shPPP5C and shCon groups. The percentages of early and late apoptotic cells were revealed to be significantly

increased in the shPPP5C group when PPP5C silencing was combined with GEM treatment in comparison with cells treated with shCon, shPPP5C or GEM alone. More specifically, the apoptosis rate (for early and late apoptotic cells) was ~35.08% in the group treated with GEM and shPPP5C, which was significantly higher than that in the shCon (15.57%), shPPP5C (22.46%) or GEM plus shCon (20.64%) groups. These results further corroborated the hypothesis that silencing of PPP5C enhanced the apoptotic effect of GEM *in vitro*.

Mechanism of GEM-enhanced shPPP5C-induced PANC-1 cell apoptosis. To investigate the underlying mechanism of GEM-enhanced shPPP5C-induced PANC-1 cell apoptosis, apoptosis-associated proteins were determined by western blot analysis (Fig. 4). The results demonstrated that the protein expression of cleaved caspase 3 and poly (ADP-ribose) polymerase (PARP) was increased in the shPPP5C group compared with that in the shCon group (Fig. 4A and C). Additionally, the expression of tumor suppressor p53 was also determined and the protein expression ratio of p-p53/p53 was also upregulated (Fig. 4B and C). In addition, the association between MAPK family-associated proteins (JNK, p-JNK, p38 and p-p38) and shPPP5C-mediated apoptosis was investigated. As demonstrated in (Fig. 4A and C), the protein expression ratio of p-JNK/JNK was increased, while almost no change was observed in that of p-p38/p38. These results indicate that the effect of silencing PPP5C on cell apoptosis resulted from the altered expression of the associated anti-apoptotic proteins. Additionally, the combined treatment of GEM and

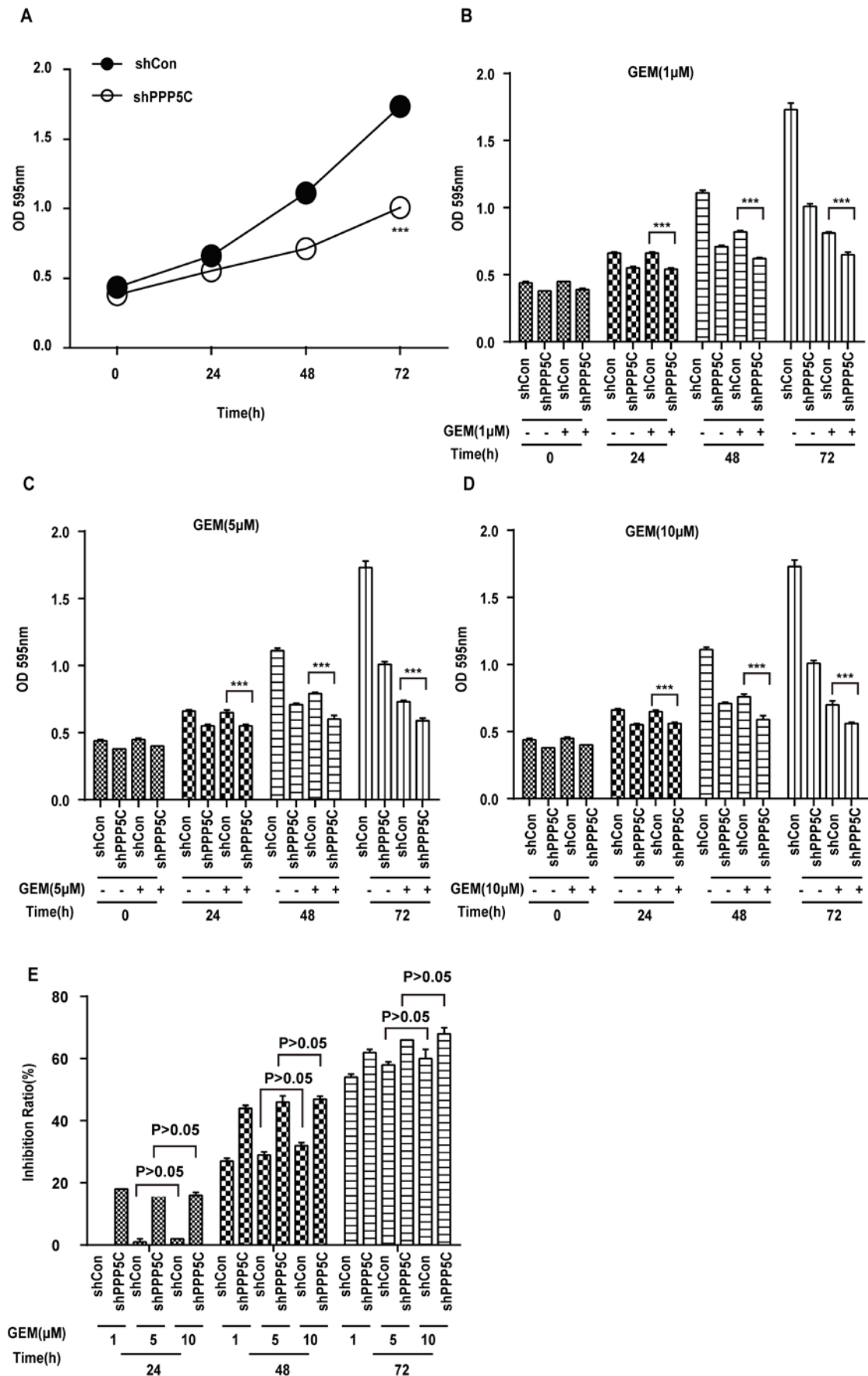


Figure 2. PPP5C silencing increases sensitivity to GEM in PANC-1 cells. (A) The proliferation ability of PANC-1 cells infected with shPPP5C was measured by MTT assay. (B) The cell growth inhibition rate following addition 1 μ M GEM were measured by MTT assay. (C) The cell growth curves following addition 5 μ M GEM were measured by MTT assay. (D) The cell growth curves following addition 10 μ M GEM were measured by MTT assay. (E) Cell growth inhibition rate. Data are presented as the mean \pm standard deviation of 3 independent experiments. *** P <0.001. PPP5C, serine/threonine protein phosphatase 5; GEM, gemcitabine; shPPP5C, short hairpin PPP5C; shCon, short hairpin control; OD, optical density.

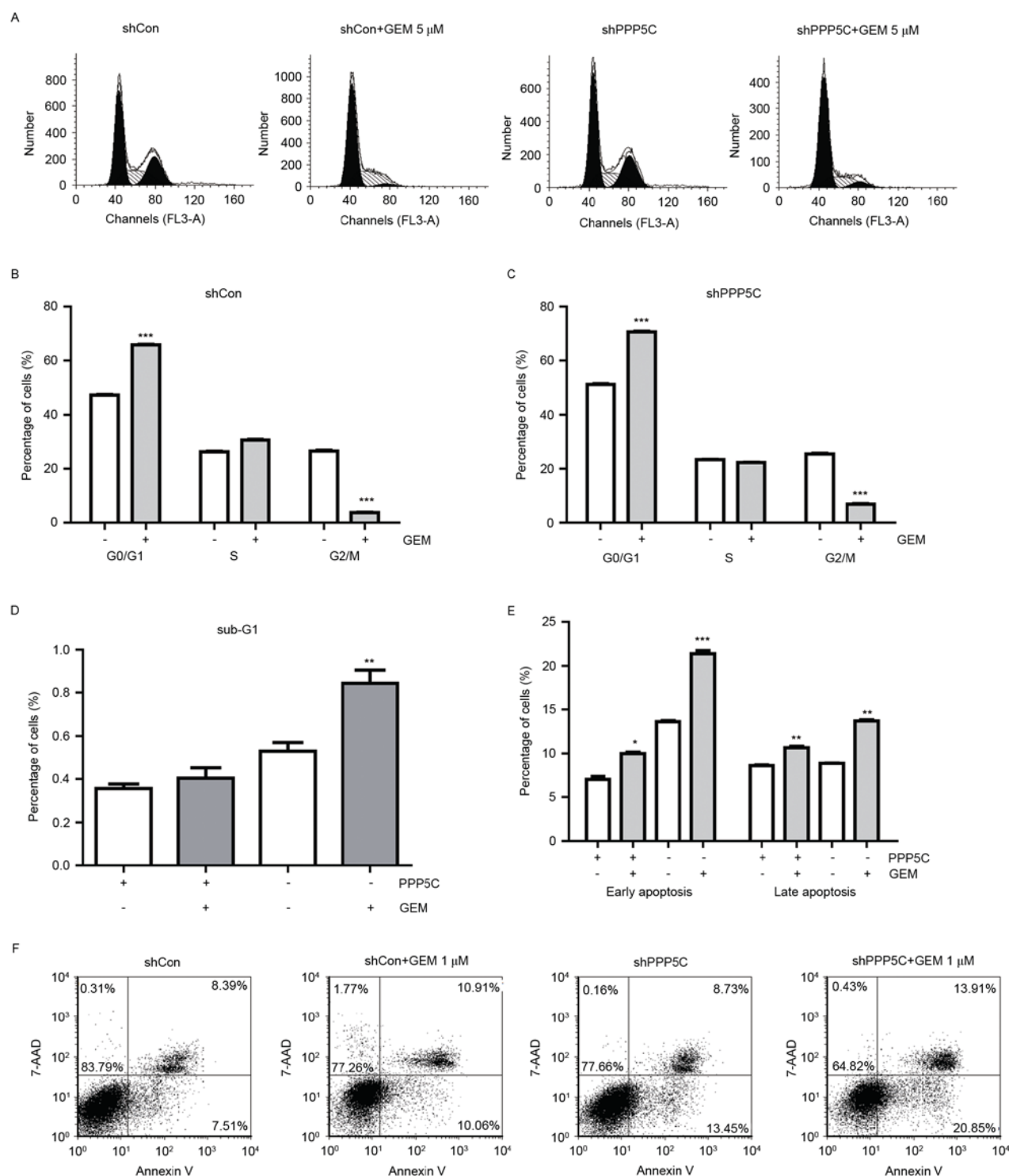


Figure 3. GEM enhances shPPP5C-induced PANC-1 cell cycle arrest and apoptosis. (A) Cell cycle distribution was analyzed by flow cytometry. Representative images of shCon, shPPP5C and the combined treatment of GEM and shCon or shPPP5C measured by fluorescence-activated cells sorting analyses. (B) Quantification and statistical analysis of the cell percentages in the different cell cycle phases in shCon group. (C) Quantification and statistical analysis of the cell percentages in the different cell cycle phases in shPPP5C group. (D) Proportion of cells in sub-G1 phases. (E) Statistical analysis of the proportion of cells in early and late apoptosis. (F) Representative images of the flow cytometry of PANC-1 cells treated with shCon, shPPP5C or combined treatment of GEM and shCon or shPPP5C. Data are presented as the mean \pm standard deviation of 3 independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. GEM, gemcitabine; shPPP5C, short hairpin serine/threonine protein phosphatase 5; shCon, short hairpin control.

shPPP5C resulted in a marked increase in p-JNK/JNK, cleaved caspase 3 and PARP, while the expression ratio of p-p38/p38 and p-p53/p53 exhibited almost no change, suggesting that PPP5C silencing combined with GEM may, to a certain extent, promote further cell apoptosis (Fig. 4C).

Discussion

The majority of PC patients exhibit a strong resistance to chemotherapy. The exact mechanisms underlying this remain unknown. One underlying mechanism of chemoresistance

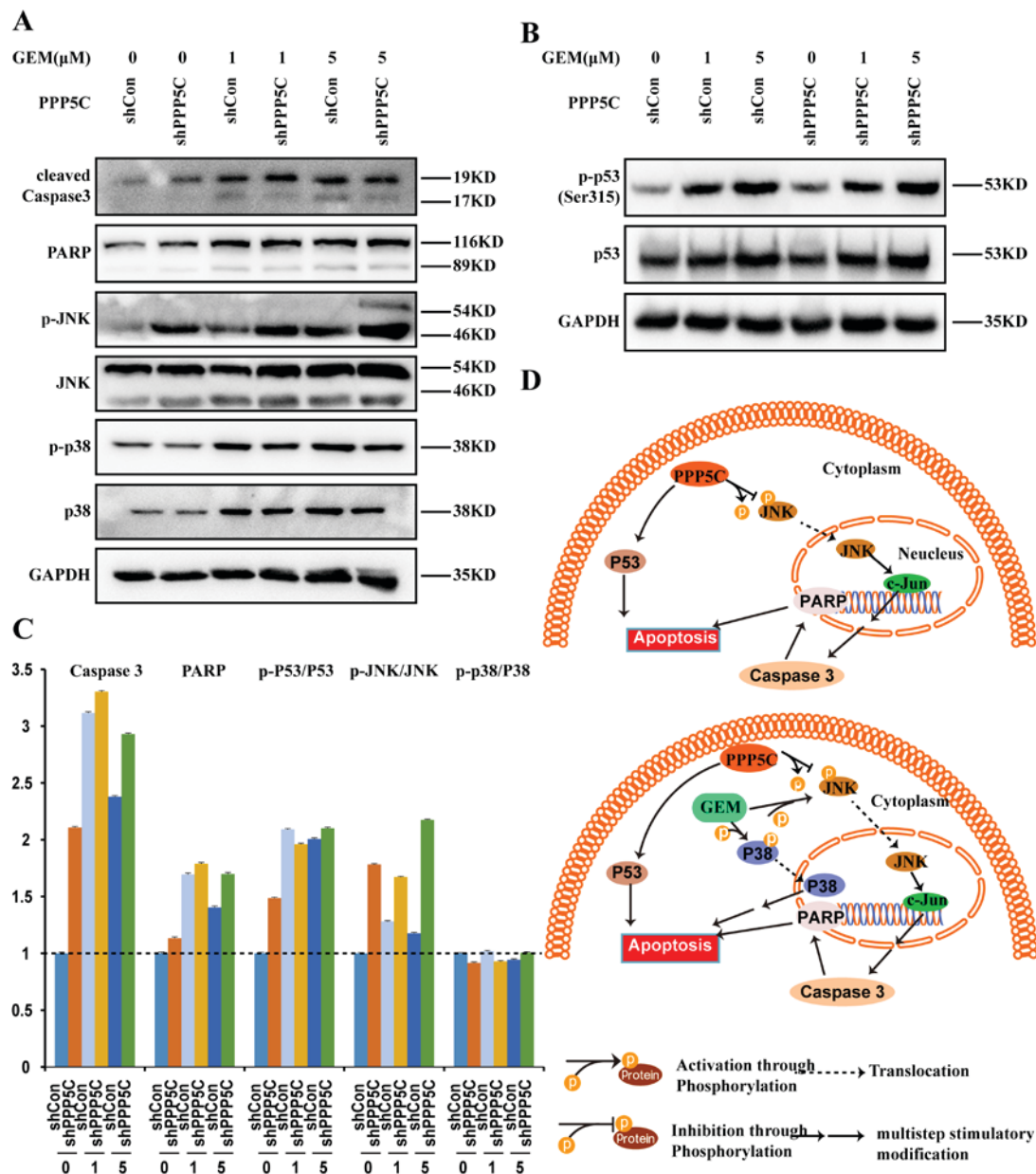


Figure 4. Effects of PPP5C-knockdown on apoptosis pathway-associated molecular expression. (A) The levels of caspase-3, PARP, JNK, p-JNK, p38 and p-p38 protein in PANC-1 cells were detected following PPP5C silencing by western blot analysis. (B) The levels of p-p53 (ser315) and p53 protein in PANC-1 cells were analyzed following PPP5C silencing by western blot analysis. (C) The expression of JNK, p-JNK, p38 and p-p38 proteins in PANC-1 cells transfected with shCon, shPPP5C or the combined treatment of GEM and shPPP5C or shCon was analyzed by western blot analysis. (D) Proposed model of the role served by PPP5C silencing in cell apoptosis. GAPDH was used as an internal loading control. PPP5C, serine/threonine protein phosphatase 5; JNK, c-Jun N-terminal kinases; shCon, short hairpin control; shPPP5C, short hairpin PPP5C; GEM, gemcitabine; p-, phosphorylated; t-, total.

is the insensitivity to drug-induced apoptosis (38). GEM is widely used for several cancer types, not only for PC, but also for lung and bladder cancer types. GEM monotherapy has been the standard treatment for metastatic PC for decades and combination therapy of GEM with a number of other agents has been demonstrated to increase the median survival time to a certain extent (9). However, although GEM is still used as a first-line treatment option, the modest survival benefit of GEM treatment has been revealed to be unsatisfactory (39). GEM is a unique antimetabolite that may inhibit the activity of ribonucleotide reductase and may terminate DNA elongation processes (40). However, the occurrence of drug resistance is common in cancer treatment with various agents due to

multiple factors, including the attenuation of nucleoside transporters, the augmentation of efflux transporters, the acquisition of apoptotic resistance due to overexpression of anti-apoptotic proteins (e.g., heat shock proteins, cyclooxygenase-2, nuclear factor erythroid 2-related factor 2 and B-cell lymphoma 2 family members), and the constitutive activation of survival signaling pathways (16,41). Therefore, GEM resistance is a significant obstacle to effective chemotherapy.

It has been reported that the overexpression of PPP5C has been observed in several solid cancer types (42-44). In addition, overexpression of PPP5C has been demonstrated to promote cell proliferation and tumor progression (35). PPP5C expression is responsive to hypoxia inducible factor-1 and estrogen,

which plays important role in cancer progression. It has been reported that PPP5C serves an important role in glioma metastasis, as downregulation of PPP5C mitigated cell migration in U251 and U373 cell lines (34). In human breast cancer, a strong association was observed between high levels of PPP5C expression and the occurrence of invasive ductal carcinoma in patients with metastatic disease at the time of diagnosis (45). In rats, PPP5C mRNA levels markedly increased in malignant ascites hepatomas (46). Furthermore, a protein microarray analysis of mantle-cell lymphoma also revealed elevated PPP5C expression (47). With regards to PC, increased PPP5C expression was observed in PANC-1 cells treated with GEM in the present study. The association between PPP5C levels and tumor progression requires elucidation in order to find an effective strategy to overcome GEM resistance.

Based upon previous reports of PPP5C in other cancer types and the observation of PPP5C overexpression in PANC-1 cells by GEM treatment, we hypothesized that the suppression of PPP5C expression may serve an important role in PC growth and may promote the effect of GEM treatment on PC cells. In the present study, the effect of PPP5C-knockdown or the combination of this with GEM treatment on the cellular functions of PANC-1 cell was investigated. The results of the MTT assay demonstrated that the proliferation of PANC-1 cells was significantly impaired in the combination treatment group, which indicated that PPP5C silencing enhanced the chemosensitivity of PANC-1 cells to GEM. Additionally, the effects of PPP5C-knockdown alone or the combination treatment of GEM and PPP5C silencing on cell cycle and apoptosis were also studied. The results demonstrated that suppressed PPP5C expression combined with GEM treatment in PANC-1 cells led to cell arrest in the G₀/G₁ phase and increased cell apoptosis. Therefore, these results suggest that PPP5C may serve a central role in the tumor progression process of PC and in GEM resistance.

In particular, PPP5C has been revealed to act as a suppressor of ASK (48,49), p53 and DNA-dependent protein kinase, catalytic subunit (45). PPP5C appears to interact with ASK1, which is associated with the c-JNK and p38 signaling pathways. The present study observed that, in the shPPP5C silencing group, the protein expression ratio of p-JNK/JNK was increased, while almost no change was observed in that of p-P38/P38. Meanwhile, combined treatment with GEM and shPPP5C also resulted in a marked increase in p-JNK/JNK and no change in p-p38/p38. These results indicate that the combined treatment could further induce cell apoptosis via the c-JNK pathway. Furthermore, western blot analysis demonstrated that PPP5C silencing significantly increased the protein levels of cleaved caspase-3, p-p53 and cleaved-PARP, which participate in the cell apoptotic pathway. As the prime activator of PARP is DNA damage and its overexpression has been proven to be associated with the pathogenesis of numerous tumors (50), there may be several links between p53 and PARP. These findings support the hypothesis that the involvement of MAPK signaling pathways affects the results of anticancer therapy on PC cells (51). Combined with the previous findings that the activation of the p38 kinase promotes the activation of p53 and further triggers the activation of caspase-9 and -3 in PC (52), the results of the present study revealed that the downstream target of PPP5C is p53 (Fig. 4D). It is widely acknowledged that MAPKs are responsive to various stress stimuli. Upon

activation, JNK phosphorylates and regulates various cell cycle and apoptotic mediators. p-p53 may initiate the p53 response and may lead to cell arrest, as well as apoptosis within cell cycles (53). However, the detailed mechanisms underlying this remain to be elucidated.

In summary, the present study demonstrated that PPP5C silencing has a potential novel therapeutic function in PC. Further investigation is required to elucidate the precise mechanisms of PPP5C for PC treatment with GEM, such that a novel gene-targeted therapy for PC may be established.

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Competing interests

The authors declare that they have no competing interests.

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