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RNA interference-mediated silencing of the *polo-like kinase 1* gene enhances chemosensitivity to gemcitabine in pancreatic adenocarcinoma cells

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Abstract

Gemcitabine is the first-line chemotherapeutic agent for advanced adenocarcinoma of the pancreas; however, chemoresistance to gemcitabine remains a major cause of failure for the clinical treatment of this disease. Polo-like kinase 1 (Plk-1) is highly expressed in pancreatic cancer cell lines and pancreatic tumour tissues, and is involved in a wide variety of cell cycle processes. Nevertheless, its biological role and implication for gemcitabine resistance are not clearly defined. In this study, we used RNA-interference (RNAi)-mediated depletion of Plk-1 to determine its potential for sensitizing pancreatic tumour cells to gemcitabine. We showed that the level of Plk-1 protein was correlated significantly with gemcitabine resistance in human pancreatic adenocarcinoma cells and that overexpression of Plk-1 reduced sensitivity to gemcitabine in these cells. In addition, small interfering RNA (siRNA)-mediated knockdown of *Plk-1* caused cell cycle arrest at G2/M and the reduction of cellular proliferation. More importantly, the treatment of pancreatic cancer cells with Plk-1 siRNA followed by exposure to gemcitabine dramatically decreased cell viability and increased cellular apoptosis, as compared with treatment with either agent alone. These observations indicate that down-regulation of *Plk-1* expression by RNAi enhances gemcitabine sensitivity and increases gemcitabine cytotoxicity in pancreatic tumour cells. This is the first demonstration that the combination of *Plk-1* gene therapy and gemcitabine chemotherapy has synergistic anti-tumour activity against pancreatic carcinoma *in vitro*. This combination treatment warrants further investigation as an effective therapeutic regimen for patients with resistant pancreatic cancer and other tumours.

Keywords: polo-like kinase 1 • RNA interference • siRNA • gemcitabine • cell cycle • G2/M arrest • apoptosis • pancreatic cancer

Introduction

Pancreatic adenocarcinoma is now the fourth most common cause of cancer-related death in adults in China and the United States. The high mortality rate seen in patients with pancreatic cancer reflects both the difficulty in early diagnosis and the lack of effective chemotherapy to augment surgery [1, 2]. Patients suffering from pancreatic carcinomas have a life-span of only 4–5 months after diagnosis, and the 5-year survival rate is less than 5% [3]. In the past few years, the use of the deoxycytidine analogue gemcitabine has been shown to result in improved clinical benefit and slightly longer mean survival time. Thus, it is not surprising that gemcitabine has become the first-line chemotherapy for pancreatic adenocarcinomas [4, 5]. However, published results indicate that prolonged exposure to gemcitabine leads to acquired resistance in some pancreatic cancer cells [6]. Chemoresistance to gemcitabine is also a major cause of treatment failure for pancreatic adenocarcinoma. Many chemotherapeutic drugs act primarily by induction of apoptosis. Consequently, the development of cancer cell resistance to

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cytotoxic drugs may be attributable to resistance to apoptosis [7]. Several strategies for overcoming the apoptotic resistance of neoplastic populations have been developed and hold potential therapeutic value, and many new approaches to the treatment of pancreatic adenocarcinoma are currently being investigated [5, 8]. Several approaches focus on combination therapies in which gemicitabine is combined with a second cytotoxic agent (*e.g.* auristatin-PE) [9] or on targeted biological therapy (*e.g.* OSI-774/Tarceva and the anti-Epidermal growth factor receptor (EGFR) antibody C225) [10–12].

Polo-like kinase 1 (Plk-1), a mitotic cyclin-independent serine-threonine kinase, is a member of the family of polo-like kinases involved in a wide variety of cell cycle processes. Polo-like kinase was first identified in *Drosophila melanogaster,* where mutants display abnormal mitotic and meiotic divisions caused by failure to properly organize the mitotic spindle [13]. In mammalian cells, Plk-1 is primarily localized at the centrosome, where it is responsible for centrosome separation and maturation [14]. Plk-1-specific antibodies introduced into HeLa cells by microinjection prevent centrosome separation and reduce γ-tubulin accumulation [14]. In addition to its role in regulating centrosome function, Plk-1 is involved in the timing of mitotic entry and exit [15] and is one of the most important regulators of mitotic progression in mammalian cells [16]. It has been implicated in the targeting of cyclin B1 to the nucleus during prophase [17], the activation of Cdc25C phosphatase [18], and the inactivation of the Cdk1-cyclin B complex required for mitotic exit [15]. Plk-1 is also a target of the G2 DNA damage checkpoint, where it undergoes ubiquitin-dependent proteolysis mediated by the checkpoint protein Chfr; this implicates the loss of Plk-1 function as an important response to DNA damage during the G2 phase of the cell cycle [19].

The elevation of Plk-1 expression occurs in a broad range of human tumours [20–23]. The activity of Plk-1 is elevated in tissues and cells with a high mitotic index, including cancer cells [24, 25], and a close correlation between mammalian Plk-1 expression and carcinogenesis has recently been documented. Overexpression of the *Plk-1* gene has been found in pancreatic cancer cell lines and neoplastic tissues [26]. It has recently shown that its overexpression has been identified as a common and early event in pancreatic cancer progression [27], and the depletion of Plk-1 by small interfering RNA (siRNA) has been shown to dramatically inhibit cell growth and induce apoptosis *in vitro* [28, 29]. Furthermore, constitutive expression of Plk-1 in NIH3T3 cells causes oncogenic focus formation and induces tumour growth in nude mice [30]. Taken together, these observations suggest that Plk-1 is an important player in cell growth and proliferation and may be a potential target for cancer therapy.

RNA interference (RNAi) is emerging as a strategy for highly specific post-transcriptional suppression of gene expression, both *in vitro* and, more recently, *in vivo* [31–35]. It has been shown that siRNAs targeting the M2 subunit of ribonucleotide reductase exhibit therapeutic synergism with gemcitabine, resulting in the retardation of tumour growth, the reduction of tumour mass by 87%, and the absence of hepatic metastasis in a nude mouse xenograft model [36, 37]. The presence of low *Plk-1* expression in normal adult tissues and high Plk-1 levels in tumour tissues suggest that this gene therapy would afford high specificity with low toxicity. In the current study, we hypothesized that directly targeting *Plk-1* with the RNAi technique would enhance the chemosensitivity of pancreatic adenocarcinoma to gemcitabine. To test this hypothesis, we took advantage of recently developed vector-based siRNA technology [38] to specifically deplete Plk-1 in three pancreatic adenocarcinoma cell lines (AsPC-1, PANC1 and BxPC3). Plk-1 protein is normally expressed at high levels in these cells, and therefore they may be good *in vitro* models for studying the effect of Plk-1 on gemcitabine chemotherapy in pancreatic cancers. Here, we show that *Plk-1* gene therapy combined with gemcitabine chemotherapy has a synergistic effect on the induction of apoptosis in these model systems, suggesting that Plk-1 is potentially an important therapeutic target for pancreatic adenocarcinomas.

Materials and methods

Construction of vectors and design of siRNAs

(*i*) The Plk1-PBD^{WT} (aa 326-603) was cloned in-frame by PCR into a pcDNA3.1 vector encoding a amino-terminal 3xmyc-tag (Invitrogen, Carlsbad, CA, USA) using primers 5´-CCG GAA TTC CAG ATC TTC GAT TGC TCC CAG CAG CCT GG-3´ and 5´-CCG CTC GAG TTA GGA GGC CTT GAG ACG GTT GC-3´. Plk-1 cDNA was cloned into the pcDNA3.2-DEST vector (Invitrogen), and the resulting expression plasmid (pcDNA3.2-DEST-Plk1) was verified according to the reference sequence (GenBank accession No. NM_001621). (*ii*) The plasmid pBS/U6-Plk-1 was constructed as described previously (38). The plasmid pBS/U6-Plk1-1st half (sense strand) was used as a scrambled control vector. This control vector produces RNA that cannot form a hairpin structure to generate interfering RNAs. (*iii*) This study also used four *Plk-1* (GenBank accession No. NM_005030) siRNAs, targeting different regions of the Plk-1 transcript. The following siRNA sequences targeting *Plk-1* (National Center for Biotechnology Information accession No. X75932) were synthesized: siRNA1, corresponding to positions 178–200 of the *Plk-1* open reading frame; siRNA2, to positions 362–384; siRNA3, to positions 1416–1438 and siRNA4, to positions 1572–1594. A scrambled version of siRNA3, called siRNA3S, was synthesized and used as a control. All siRNAs were 21 nucleotides long and contained symmetric 3´ overhangs of two deoxythymidines. All sequences were submitted for a BLAST search to ensure that only the *Plk-1* gene was targeted by the Plk-1 siRNAs and that the control sequences were not similar to any known gene sequences.

Cell culture and plasmid transfection

The human pancreatic adenocarcinoma cell lines AsPC-1, PANC1 and BxPC3, and the normal pancreas cell line HPDE6c7 (an immortalized but not transformed pancreatic epithelial cell line) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and were grown as monolayers in RPMI 1640 medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% foetal bovine serum (FBS) (Hyclone, Logan,

UT, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere supplemented with 5% CO_2. The cells were divided into three groups: untransfected cells were used as a control group, the negative control group used cells transfected with the empty vector pBS/U6 or pcDNA-DEST, or scrambled vector or Plk1-siRNA3S and cells transfected with pBS/U6-Plk-1 or pcDNA-DEST-Plk-1 or Plk1-siRNA3 were the study group. The cells were seeded at a density of about $5 \times 10^4/cm^2$ in 6-cm-diameter culture dishes. The cells were transfected using GenePORTER reagent (GTS, San Diego, CA, USA) as described by the supplier. Briefly, both the GenePORTER reagent and the plasmid DNA or Plk1-siRNA were diluted with serum-free medium by using one-half of the transfection volume. Then, the diluted plasmid DNAs (1 μg) or Plk1-siRNAs (0.1 μm) were added to the diluted GenePORTER reagent, mixed rapidly, and incubated at room temperature for 30 min. The culture medium was aspirated from 80% confluent cells seeded 1 day (24 hrs) before, the plasmid DNA-GenePORTER or Plk1 siRNA- GenePORTER mixture was added carefully to the cells, and the cells were incubated at 37°C. At 4–6 hrs after transfection, the plasmid- or siRNA-containing medium was replaced with normal culture medium containing 20% FBS, and the cells were incubated in a 5% CO_2 incubator at 37°C. At 12, 24 and 48 hrs after transfection, the cells were harvested and stored at −80°C until used for analysis. After checking the efficiency of the transfection, a clone with empty vector alone and a clone with silencing Plk-1 were selected for further experiments. At 2 days (48 hrs) after drug selection, the floating cells were washed away, and the remaining attached cells were used for phenotype analysis following additional incubation in the presence of puromycin.

Treatment with chemotherapeutic agents

The time-points chosen for gemcitabine addition to the transfected cells were based on preliminary experiments testing various times. The preliminary experiments showed that the maximal reduction in the number of viable cells, determined by Trypan blue exclusion assay, occurred when the cytotoxic drugs were added to the cells at 48 hrs after transfection (data not shown). For investigating sensitization to gemcitabine, cells were treated with the IC_{50} dose of gemcitabine (0.1 nM in Dimethyl sufoxide (DMSO)), and with DMSO alone and medium alone as controls, at 48 hrs following transfection. Cell growth and viability analyses were performed at 96 hrs following transfection, using the Trypan blue exclusion assay.

Detection of Plk-1 mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated using Trizol reagent (Gibco, USA), and cDNA was synthesized following the manufacturer's instructions (Promega, Madison, WI, USA). Equal amounts of cDNA were subjected to PCR analysis. The primers used for Plk-1 amplification were 5´-CCCCTCACAGTCCTCAATAA-3´ and 5´-TGTCCGAATAGTCCACCC-3´, which generated a 244-bp amplicon. As an internal control, β-actin was amplified using the primers 5´-GGCTACAGCTTCACCACCAC-3´ and 5´-TACTCCTGCTTGCTGATCCAC-3´, which gave a 498-bp amplicon. The amplification conditions were 30 cycles at 94°C for 30 sec., 52°C for 40 sec., and 72°C for 45 sec. for the *Plk-1* gene; and 20 cycles at 94°C for 30 sec., 55°C for 30 sec., and 72°C for 40 sec. for β-actin. The PCR products were electrophoresed in a 2% agarose gel. Densitometry of the PCR products was analysed with a Kodak digital science 1 D system (Kodak, Toronto, Ontario, Canada).

Western blot analysis

Cells were washed in cold Phosphate-buffered Saline (PBS), and total cellular protein was extracted in 100 μl of RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 1% NP-40, 150 mM NaCl, 1 mg/ml aprotinin, 1 mg/ml leupetin, 1 mM Na_3VO_4 and 1 mM NaF) at 4°C for 30 min. Cell debris was removed by centrifugation at 14,000 × *g* for 20 min. at 4°C, and the supernatants were collected. After the protein concentrations were quantified using a Bio-Rad protein assay, an equal amount of protein (50 μg) from each sample was subjected to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to Polyvinylidene fluoride (PVDF) membranes (Amersham, USA). The membranes were blocked with 5% non-fat milk in Tris-buffered Saline (TBS) buffer at room temperature overnight, followed by incubation for 1 hr with primary antibodies. Anti-Plk-1 and anti-Plk-2 monoclonal antibodies were purchased from Zymed (Zymed, USA) and were used at 1 μg/ml (1:1000 dilution). The membranes were then incubated for 1 hr with horseradishperoxide (HRP)-conjugated goat antimouse or goat anti-rabbit secondary antibody (Jackson, Inc., USA) at 1:3000 dilution. Electrochemiluminescence was performed with a ChemiImager 5500 imaging system (Alpha Innotech Co., San Leandro, CA, USA), according to the manufacturer's instructions. After development, the membrane was stripped and re-probed with antibody against β-actin (1:1000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) to confirm equal sample loading.

Cell survival assay and synergistic analysis

Cell viability was measured using a 3-bis-(2-methoxy-4-nitro-5-sulfenyl)-(2H)-tetrazolium-5-carboxanilide (MTT) assay (Cell Proliferation kit, Roche Molecular Biochemicals, Indianapolis, IN, USA), as described previously [39]. To determine the cytotoxicity of single-drug and combination treatments, cells from each of the three cell lines (AsPC-1, PANC1, and BxPC3) were seeded onto 96-well plates at a density of 1×10^4 cells/well. Twenty-four hours later, the cells were treated with pBS/U6 or pBS/U6-Plk-1 at different concentrations (0.25, 0.5, 1.0 and 2.0 ng/ml). Cells treated with PBS were used as a mock control. Gemcitabine at various concentrations (0.1–10 nM) was also added to each well. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cell growth and viability were analysed by the MTT assay at day 4 (96 hrs) following drug treatment. Cell survival with drug treatment is expressed as a percentage (%) of the survival of untreated control cells. Each experiment was performed in quadruplicate, and each series was repeated at least three times. The data of representative experiments are depicted below in the Results section.

The effects of the combination treatment of pBS/U6-Plk-1 and gemcitabine were analysed with CalcuSyn software (Biosoft, Cambridge, UK) to determine the combination index (CI). Generally, it is considered that a CI < 1.0 indicates synergism, CI = 1.0 indicates an additive effect, and CI > 1.0 indicates antagonism.

Cell cycle and apoptosis analysis by flow cytometry

The effect of Plk-1 siRNA treatment on cell cycle progression and the effect of a combined Plk-1 siRNA and gemcitabine treatment on cellular apoptosis were determined by flow cytometry. In brief, the cells were harvested *via* trypsinization after treatment with Plk-1 siRNA and/or gemcitabine, washed once with ice-cold 0.1 M PBS, fixed with 70% ethanol and stored

at −80°C until the day of assay. Cells were analysed for hypoploidy and cell cycle status by flow cytometry (Epics XL, Coulter, USA) as described earlier [40]. The data were analysed with Win MDI 2.1.3 software. The hypoploid peak ahead of G1 represents the percentage of apoptotic cells relative to the total number of cells distributed in different phases of the cell cycle. The results were compared with control values, modelling 20,000 events per sample.

Apoptosis assay by annexin-V staining

To quantify apoptosis, cells were stained with annexin-V and PI using a Vybrant Apoptosis Assay Kit (Molecular Probes) following the step-by-step protocol provided by the manufacturer. Briefly, cells were harvested by trypsinization after treatment with Plk-1 siRNA and/or gemcitabine, washed once with cold PBS. Cell pellets were re-suspended in 100 μl 1× annexin-binding buffer, 5 μl of Fluorescein isothiocyanate (FITC)-annexin-V (component A) and 1 μl of the 100 μg/ml PI working solution was added to each 100 μl of cell suspension. The cells were incubated on ice for 1 hr and washed with cold PBS once again, re-suspended in 300 μl of 1x annexin-binding buffer, and the stained cells were analysed for apoptosis by flow cytometry as soon as the staining finished.

Statistical analysis of data

Differences between treatments with Plk-1 siRNAs and control siRNAs were analysed using a one-tailed or two-tailed paired t-test for the cell survival assays and cell cycle and apoptosis studies (Statview for PC). The significance level was set at $P < 0.05$.

Results

Plk-1 expression is positively correlated with chemoresistance to gemcitabine in pancreatic adenocarcinoma cells

We first assessed the role of Plk-1 in gemcitabine resistance in our cell models for pancreatic adenocarcinoma. Plk-1 protein expression was analysed in the pancreatic ductal adenocarcinoma cell lines AsPC-1, PANC1 and BxPC3 by Western blotting. Differential expression of Plk-1 protein was observed in the three cell lines, and the level of Plk-1 was correlated with the largest tolerable dose of gemcitabine. As seen in Fig. 1 and Table 1, the highest levels of Plk-1 protein expression occurred in AsPC-1 cells, which also display the greatest gemcitabine tolerance, with an IC_{50} of 50 nM gemcitabine. The lowest expression of Plk-1 protein was found in BxPC3 cells, which were the least tolerance to gemcitabine, with an IC_{50} of 18 nM (Fig. 1; Table 1).

To further assess the correlation between the level of Plk-1 expression and tolerance to gemcitabine, we examined the influence of forced Plk-1 overexpression on gemcitabine sensitivity in each of the three pancreatic tumour cell lines. The AsPC-1, PANC1, and $BxPC_3$ cell lines were transiently transfected with the expression vector pcDNA-DEST Plk-1, respectively. In the transfected cells from each of the three cell lines, the Plk-1 protein level was measured by Western blotting and the 50% inhibitory concentration (IC_{50}) of gemcitabine was determined by MTT assay. Western blot analysis showed that the Plk-1 protein levels were significantly increased in all three cell lines following transfection with the Plk-1 expression construct pcDNA-DEST-Plk-1, compared with the levels in the respective empty vector (pcDNA-DEST) transfected cell lines (Fig. 2). In addition, the MTT assay revealed 3.5- to 4.5-fold increases in the IC_{50} of gemcitabine in Plk-1 transfected cells, compared with that in the empty vector transfected cells, indicating a strong correlation between an elevated Plk-1 protein level and increased chemoresistance (decreased sensitivity) to gemcitabine. Thus, the level of Plk-1 protein is inversely correlated with gemcitabine sensitivity in human pancreatic adenocarcinoma cells.

Plk-1-specific siRNAs down-regulate Plk-1 mRNA and protein levels in human pancreatic tumour cell lines

Next, we examined the effect of siRNA on Plk-1 expression in our cell models for pancreatic carcinoma. Four different siRNAs, targeting four different Plk-1 sequences (178–200, 362–384, 1416–1438 and 1572–1594; GenBank accession no. NM_005030), were designed to test for the most effective targeting sequence. Of those tested, siRNA3 (siPlk-1; sequence target: 1416–1438), significantly reduced Plk-1 mRNA and protein levels, compared with the other three Plk-1 siRNAs and with the scrambled sequence control for siPlk-1 (siRNA3S). Therefore, siRNA3 (siPlk-1 1416–1438) was selected for subsequent applications.

To determine the effect of Plk1-specific siRNA on mRNA and protein levels of Plk-1 in human pancreatic carcinoma cell lines, we analysed Plk-1 mRNA and protein expression using quantitative RT-PCR and Western blotting, respectively. As shown in Table 2, transfection with 0.1 μm of Plk1-specific siRNA (siRNA3) resulted in maximal decreases (20–43%) of the Plk-1 mRNA level in all three cell lines examined, with no significant toxicity attributable to the transfection reagent. Similar results were obtained for the Plk-1 protein level. Western blot analysis showed that the protein level of Plk-1 was markedly down-regulated by Plk-1 siRNA at 24–48 hrs after administration of the siRNA3, with reductions of 52–81% in the three cell lines (Fig. 3). The target specificity of Plk-1 siRNA was verified by using an anti-Plk-2 antibody, our results clearly revealed that Plk-1 siRNA did not change the level of Plk-2 protein in the three pancreatic tumour cell lines (Fig. 4). Our data for the Plk-1 mRNA and protein levels were then compared with those obtained in HPDE6c7 cells, an immortalized non-transformed pancreatic epithelial cell line [41]. Given that Plk-1 is involved in cell cycle progression and is generally present in proliferating cells, HPDE6c7 cells served as a useful control in our analyses of the Plk-1 expression level in pancreatic cancer cells.

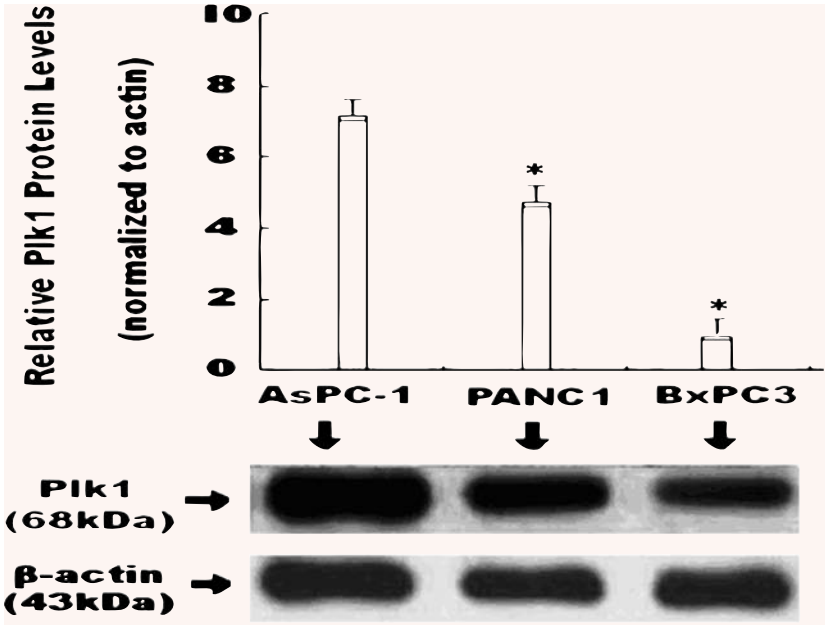


Fig. 1 Western blot analysis of Plk-1 protein levels in human pancreatic ductal adenocarcinoma cells. Total cellular protein was extracted from AsPC-1, PANC1 and BxPC3 cells, and the level of Plk-1 protein was analysed by Western blotting, as described in the Materials and methods section. β-Actin was used as a control to verify equal protein loading and transfer. Shown are Western blot data representative of those obtained from three separate experiments. Densitometry values are means of three independent determinations. * $P < 0.05$ *versus* AsPC-1 group for Plk-1 protein level.

Table 1 Correlation between the Plk-1 protein level and gemcitabine chemoresistance in three pancreatic adenocarcinoma cell lines

Cell line	Gemcitabine IC_{50}	Plk-1 protein level (arbitrary units)
AsPC-1	50 nM	6.8 ± 1.75
PANC1	40 nM	4.3 ± 1.12*
BxPC3	18 nM	1.5 ± 0.62*

* $P < 0.05$ *versus* AsPC-1 group for Plk-1 protein level by Kruskal-Wallis test.
$R^2 = 0.94$ between gemcitabine IC_{50} and Plk-1 protein level by Spearman correlation analysis.

Plk1-siRNA treatment causes cell cycle G2/M arrest and proliferation reduction in pancreatic carcinoma cells

Previous studies have established Plk-1 as an important regulator of cell cycle progression and cell growth; thus, we were interested in the effect of Plk-1 depletion on cell cycle distribution and cell proliferation in Plk1-siRNA vector transfected pancreatic tumour cells (Figs. 5 and 6). In Plk1-siRNA vector transfected AsPC-1 (Fig. 5A), PANC1 (Fig. 5B), and BxPC3 cells (Fig. 5C), a large percentage of cells (57% of AsPC-1, 59% of PANC1, and 60% of BxPC3) moved from G0/G1 to S phase within 24 hrs after transfection with pBS/U6-Plk-1. At 48 hrs after transfection, however, 68% of Plk1-siRNA-transfected AsPC-1 cells, 62% of Plk1-siRNA-transfected PANC1 cells and 45% of Plk1-siRNA-transfected BxPC3 cells were blocked at G2/M, suggesting an inability of these cells to complete mitosis in the absence of sufficient Plk-1 activity. Cells treated with pBS/U6-Plk1-1st-half scrambled control vector showed no apparent changes in their cell cycle profiles throughout the experiment. Consistent with a role for Plk-1 in regulating cell cycle control, the cell proliferation rate was decreased remarkably in AsPC-1 (Fig. 6A), PANC1 (Fig. 6B), and BxPC3 (Fig. 6C) cells transfected with the Plk-1 siRNA expression vector pBS/U6-Plk-1, compared with the rate in cells transfected with the empty vector pBS/U6 control. This reduction in proliferation was associated with a loss of cellular viability. The overall number of attached cells was obviously decreased in the three Plk1-siRNA vector transfected cell lines (data not shown), and again AsPC-1 cells demonstrated a greater response to Plk-1 depletion, with loss of cell viability beginning at 24 h post-transfection (data not shown). Significantly, Plk-1 exhibits differential inhibitory effects on cell cycle progression and cell proliferation between the pancreatic adenocarcinoma cell lines and the pancreatic epithelial cell line. However, there was no distinguishable difference in cell cycle progression or cell proliferation between normal pancreas epithelial cell line HPDE6c7 cells transfected with Plk-1-siRNA vector and cells transfected with the empty control vector (Fig. 5D and Fig. 6D). Although its molecular basis is not yet clear, this phenomenon appears to suggest that pancreatic cancer cells either depend more heavily on Plk-1 function for carrying out mitosis or lack intact checkpoint controls or alternate pathways to overcome the loss of Plk-1.

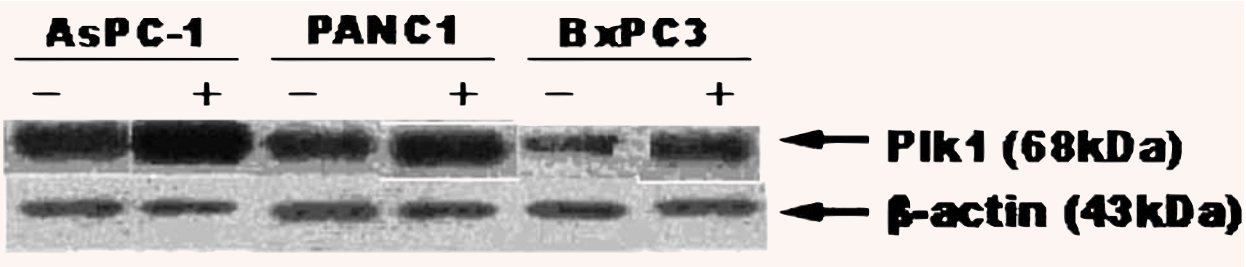


Fig. 2 Effects of Plk-1 overexpression on Plk-1 protein levels in pancreatic cancer cells. AsPC-1, PANC1 and BxPC3 cells were transfected with either the Plk-1 expression construct pcDNA-DEST-Plk-1 (+) or empty pcDNA-DEST vector (–). Total cellular protein was extracted from each of the three cell lines, and the Plk-1 protein level was analysed by Western blotting. β-Actin was used as a control to verify equal protein loading. Shown are Western blot data representative of those obtained from three separate experiments.

Table 2 Suppression of Plk-1 mRNA expression by Plk-1 siRNA in three pancreatic adenocarcinoma cell lines

Cell line	**Plk-1 mRNA level** (arbitrary units)	
	Plk-1 siRNA (–)	Plk-1 siRNA (+)
AsPC-1	2.38 ± 0.19	1.82 ± 0.17*
PANC1	2.14 ± 0.16	1.22 ± 0.12*
BxPC3	1.96 ± 0.14	1.56 ± 0.15**

*$P < 0.01$ and **$P < 0.05$, Plk-1 siRNA (+) group *versus* Plk-1 siRNA (–) group for Plk-1 mRNA levels by Kruskal-Wallis test.

Effect of combination treatment with Plk-1 siRNA and gemcitabine on cytotoxicity in pancreatic cancer cells *in vitro*

Given that Plk-1 siRNA has suppressive effects on cell cycle progression and cell proliferation in our cell systems, we next tested the potential application of Plk-1 siRNA for sensitizing pancreatic adenocarcinoma cells to gemcitabine. The effect of combined Plk-1 siRNA and gemcitabine therapy on the experimental pancreatic tumour cells was evaluated *in vitro*. The cells were first transfected with pBS/U6-Plk-1 and then exposed to gemcitabine at various concentrations. Empty pBS/U6 vector was used as a vector control, and cells treated with PBS were used as the mock control. Cell viability was determined for up to 4 days after treatment, using an MTT assay. Even at the lower doses of both agents, the combination of pBS/U6-Plk-1 plus gemcitabine was significantly more effective in killing cells than either pBS/U6-Plk-1 or gemcitabine alone (Fig. 7A). To further evaluate the interaction between *Plk-1* gene therapy and gemcitabine chemotherapy, we determined the CI, which demonstrated a strong synergistic anti-tumour activity at the lower doses of the Plk-1-expressing construct (0.25–0.5 ng/ml) and gemcitabine (0.5–1 nM) in AsPC-1, PANC1, and BxPC3 cells (Fig. 7B–D).

Enhancement of gemcitabine-induced apoptosis by Plk-1 siRNA in pancreatic cancer cells

Finally, we determined the effect of combined Plk-1 siRNA and gemcitabine treatment on the induction of apoptosis in our three cell models. Each pancreatic tumour cell line was treated with pBS/U6-Plk-1 at a dose of 0.5 ng/ml or gemcitabine at a concentration of 0.1 nM, or a combination of the two. Apoptotic cell death was detected by fluorescence-activated cell sorting (FACS) analysis of sub-G1 cells at 48 hrs after the drug treatment. Compared with the cells treated with PBS, pBS/U6, gemcitabine, or pBS/U6-Plk1-siRNA alone, the three pancreatic cancer cell lines treated with the combination of pBS/U6-Plk-1-siRNA and gemcitabine had a dramatically increased population of apoptotic cells (Fig. 8). The combination of Plk-1 siRNA and gemcitabine increased the frequencies of apoptosis in the AsPC-1, PANC1, and BxPC3 pancreatic cancer cell lines from 11, 16 and 15% to 33, 38 and 43% (Fig. 8, panel 5), respectively, compared with the frequencies in the cells treated with gemcitabine as a single agent (Fig. 8, panel 3). Moreover, the control vector pBS/U6 did not increase gemcitabine-induced cell death in our model systems (Fig. 8, panel 4). Not surprisingly, the combination treatment with pBS/U6-Plk-1-siRNA and gemcitabine did not enhance apoptosis induction in the normal pancreas epithelial cell line HPDE6c7 (Fig. 8, bottom row).

To confirm the augmentation of gemcitabine-induced apoptosis by Plk-1 siRNA in pancreatic tumour cells, we performed annexin V staining assays. Phosphatidylserine (PS) translocation occurs early in apoptosis when cell membrane integrity is still intact, and annexin V can bind to PS with high affinity. Flow cytometric analysis using FITC-labelled annexin V is therefore useful as a quantitative measure of early apoptosis. Gemcitabine and/or Plk-1 siRNA

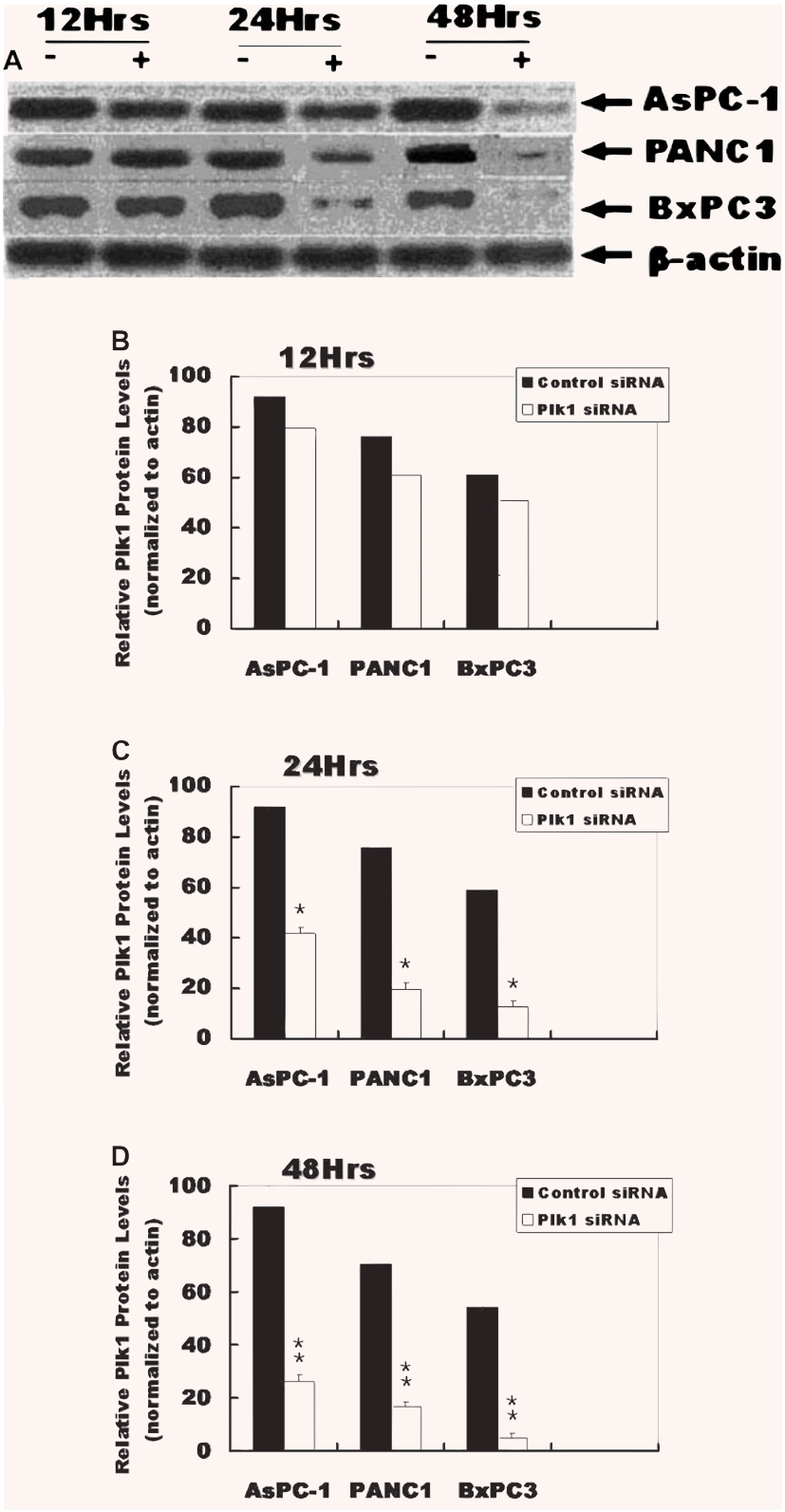


Fig. 3 Plk-1 siRNA down-regulates the Plk-1 protein level in human pancreatic tumour cells. (**A**) AsPC-1, PANC1, and BxPC3 cells were transfected with either 0.1 μm of the siRNA (Plk-1 siRNA3) to the third sequence of the *Plk-1* gene (+) or the inverted (single-base mismatch) sequence scrambled control siRNA3S (–). Total cell lysates were analysed for Plk-1 protein by immunoblotting at 12, 24 or 48 hrs following transfection. β-Actin was used as an internal protein expression and loading control. (**B**, **C** and **D**) The densitometry values shown for Plk-1 protein are means of three independent determinations. $*P < 0.05$ and $**P < 0.01$ *versus* the control siRNA group for the respective cell line.

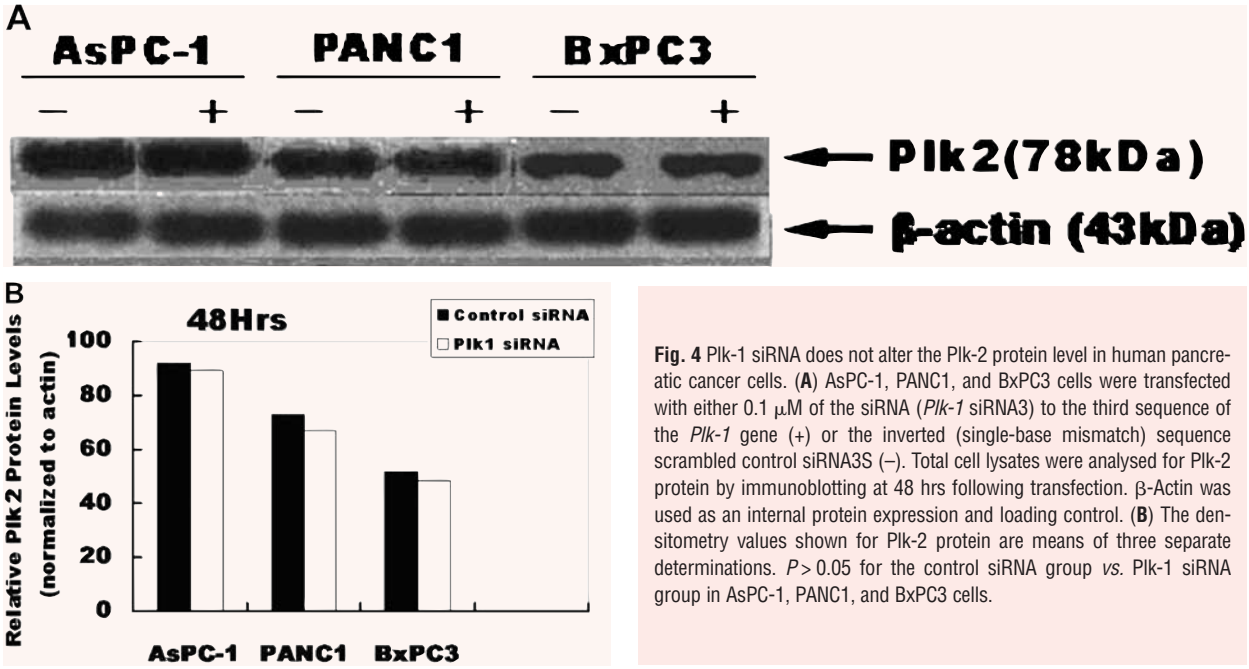


Fig. 4 Plk-1 siRNA does not alter the Plk-2 protein level in human pancreatic cancer cells. (**A**) AsPC-1, PANC1, and BxPC3 cells were transfected with either 0.1 μM of the siRNA (*Plk-1* siRNA3) to the third sequence of the *Plk-1* gene (+) or the inverted (single-base mismatch) sequence scrambled control siRNA3S (–). Total cell lysates were analysed for Plk-2 protein by immunoblotting at 48 hrs following transfection. β-Actin was used as an internal protein expression and loading control. (**B**) The densitometry values shown for Plk-2 protein are means of three separate determinations. $P > 0.05$ for the control siRNA group *vs.* Plk-1 siRNA group in AsPC-1, PANC1, and BxPC3 cells.

induced apoptosis analysis was performed with annexin V/PI staining using the Vybrant Apoptosis Assay Kit, and the results showed that the combination of gemcitabine and Plk-1 siRNA induced significant increases in apoptosis over that of each agent alone in pancreatic cancer cells of the three cell lines (Fig. 9). Taken together with our MTT assay and sub-G1 data, these results suggest that a combination of Plk-1 biotherapy and gemcitabine chemotherapy is potentially useful as a novel therapeutic approach for treating resistant pancreatic adenocarcinomas in the clinic.

Discussion

Pancreatic adenocarcinoma is among the most chemoresistant of malignancies, and there exists an urgent need to establish novel therapeutic approaches. This study was designed to investigate the role of Plk-1 in gemcitabine chemoresistance in human pancreatic adenocarcinomas and its application to the therapy of this disease. We demonstrated that (*i*) the levels of Plk-1 mRNA and protein are correlated positively with gemcitabine resistance in three human pancreatic adenocarcinoma cell lines; (*ii*) forced overexpression of Plk-1 in pancreatic cancer cells significantly increases intracellular levels of Plk-1 protein, which are parallel to enhanced gemcitabine chemoresistance in these cells; (*iii*) RNAi-mediated depletion of Plk-1 mRNA and subsequent reduction in Plk-1 protein levels cause cell cycle arrest at G2/M and growth retardation in pancreatic tumour cells and (*iv*) suppression of Plk-1 expression by siRNA considerably enhances gemcitabine sensitivity and increases gemcitabine cytotoxicity. These results suggest that Plk-1 enhances cell death or reduces cell survival in gemcitabine-treated cells by, at least in part, blocking cell cycle progression, impairing cellular proliferation, and increasing cellular apoptosis. This study strongly supports the hypothesis that Plk-1 is an important determinant for chemoresistance to gemcitabine and documents the first evidence showing that a combination of Plk-1 depletion and gemcitabine therapy has synergistic antitumour activity against pancreatic carcinoma *in vitro*. Further, these findings suggest that Plk-1 depletion may be exploited therapeutically for its potential to sensitize pancreatic tumour cells to gemcitabine in the management of pancreatic cancer patients.

Polo-like kinase-1 (Plk-1) is an important cell cycle regulatory kinase that has been implicated in a multitude of cell cycle events. DNA damage triggers multiple checkpoint pathways to arrest cell cycle progression. Plk-1 controls recovery from a G2 DNA damage-induced arrest in mammalian cells [42]. Human Plk1-deficient cells are unable to separate their centrosomes, fail to form a bipolar spindle, and undergo a Mad2/BubR1-dependent prometaphase arrest [43]. Using RNAi, Liu *et al.* [44] observed the failure of cytokinesis in Plk1-depleted mammalian cells. The protein level, activity and subcellular localization of Plk-1 are tightly regulated during the cell cycle. Plk-1 is undetectable during G1 phase, starts to accumulate during S and G2 phases and reaches a peak during mitosis. It was recently demonstrated that the centrosomal localization of Plk-1 results from the recognition and interaction of the Polo-box domain with phosphorylated peptides [45, 46]. Plk-1 is important in several events during mitosis. Recent reports showed that Plk-1 is involved in both G2 and mitotic DNA-damage checkpoints. There is abundant evidence from *in vivo* and *in vitro* studies showing that Plk-1 plays a crucial role in regulating the proliferation activity of malignant cells. Therefore, Plk-1 has been proposed as a new target for anti-neoplastic treatment strategies.

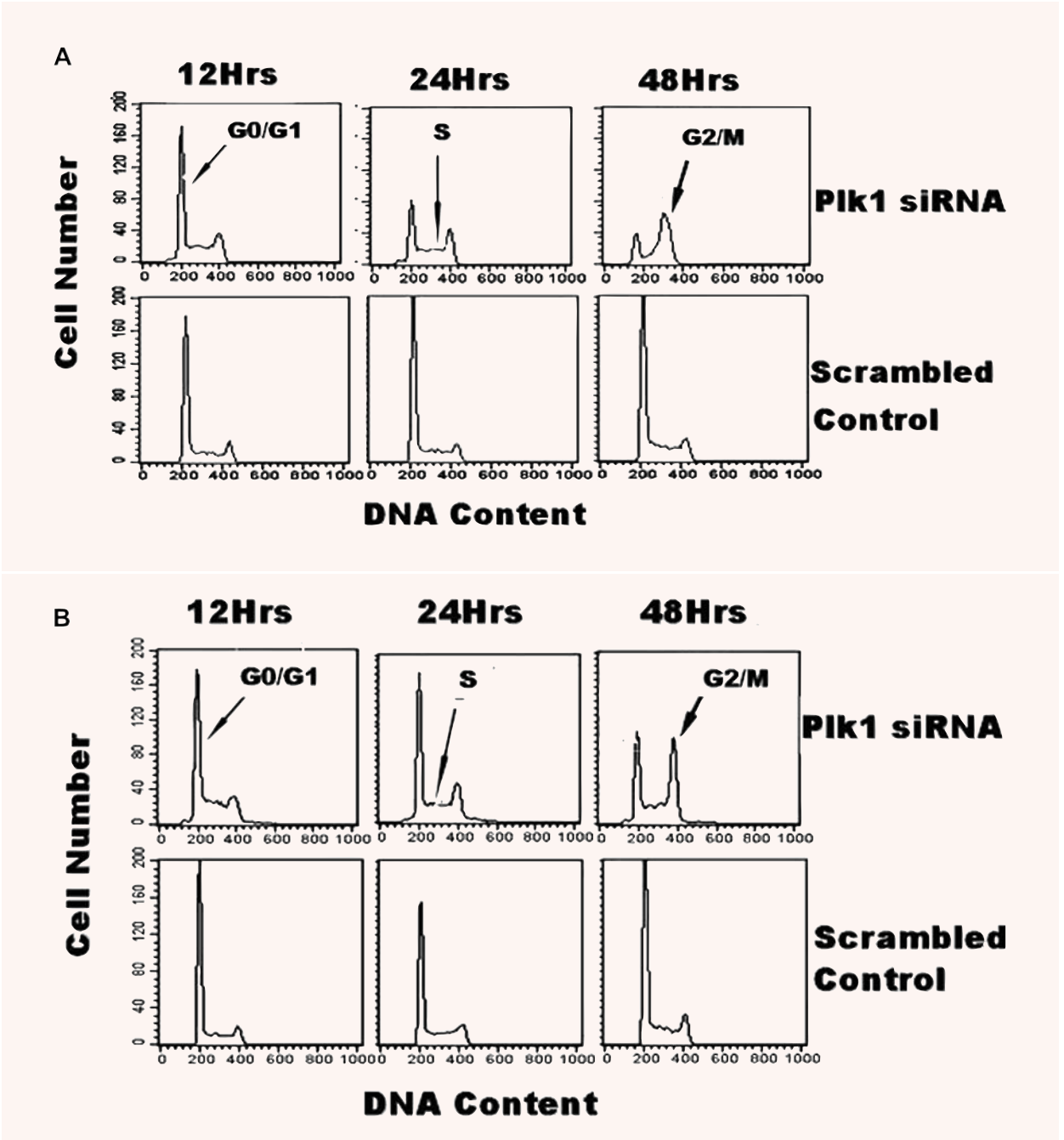


Fig. 5 RNAi-mediated depletion of Plk-1 causes cell cycle G2/M arrest in pancreatic carcinoma cells as analysed by flow cytometry. AsPC-1 (**A**), PANC1 (**B**), BxPC3 (**C**) and HPDE6c7 (**D**) cells were transfected with either the Plk-1 siRNA expression construct pBS/U6-Plk-1 (*Plk-1* siRNA) or the scrambled control vector pBS/U6-Plk1-1st half (*Scrambled Control*). The cells were harvested at 12, 24 and 48 hrs after transfection and stained with propidium iodide. The cell cycle distribution of the propidium iodide-labelled cells was analysed by flow cytometry, as described in the Materials and methods section. The data are representative of three independent experiments. The peaks corresponding to cell cycle phases are indicated by the arrows.

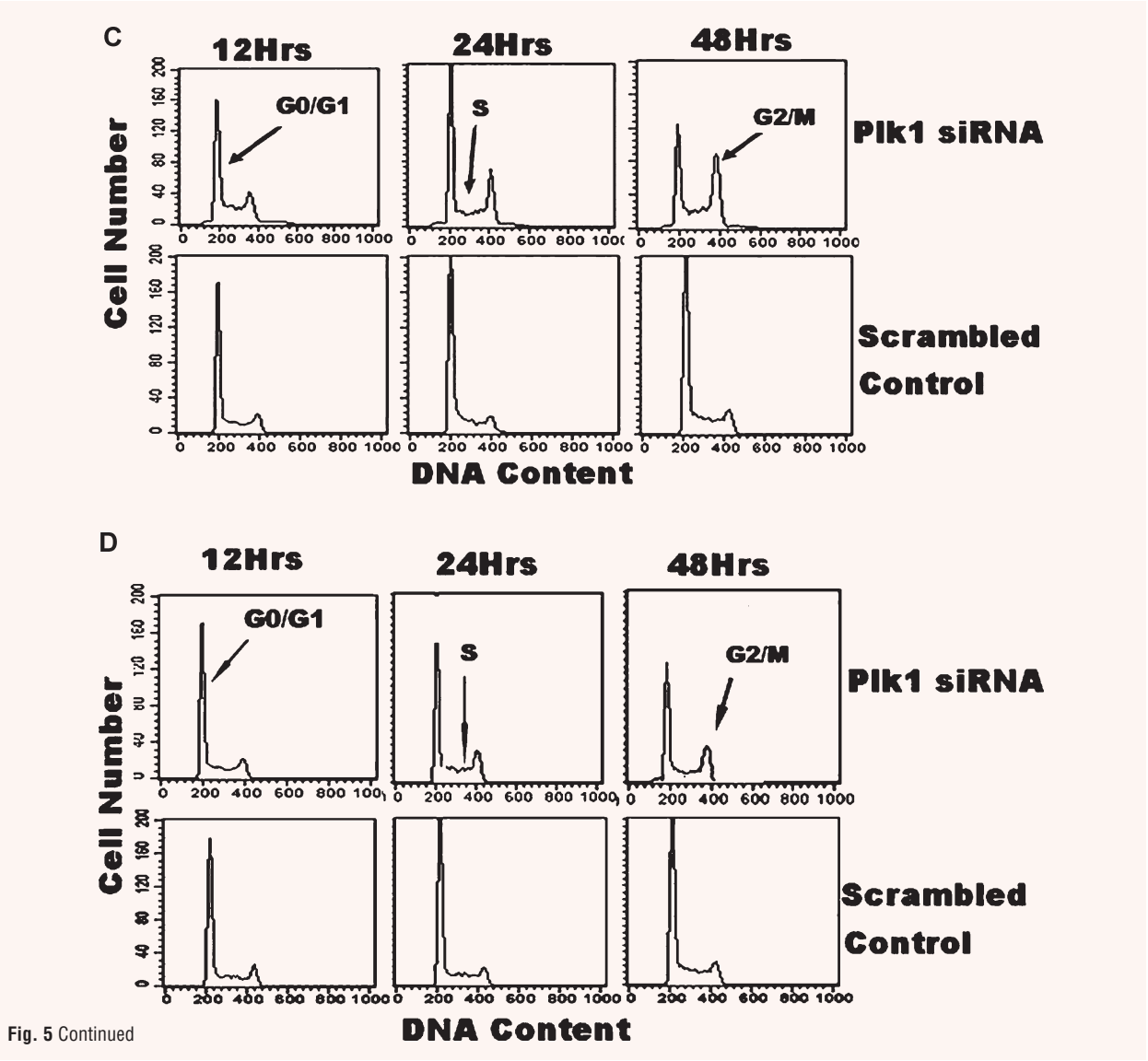


Fig. 5 Continued

Drug resistance to gemcitabine remains a significant problem in the treatment of pancreatic cancer in the clinic. Resistance of tumour cells and the occurrence of dose-limiting toxicities form the most important restrictions to the success of chemotherapy. Therapeutic approaches that will lead to the selective sensitization of tumour cells may overcome these limitations and broaden the therapeutic window. Our increased understanding of the events that drive the onset and progression of pancreatic adenocarcinoma will aid in the development of these therapies. For instance, strong overexpression of the *Plk-1* gene has been reported in pancreatic cancer cell lines and in pancreatic tumour tissues taken directly from patients, which indicates that inhibition of this mitotic key regulator might represent a rewarding approach to the treatment of early and late pancreatic carcinomas. Gray *et al.* [26] reported on an initial validation of Plk-1 as a potential therapeutic target *via* inhibiting Plk-1 expression in pancreatic cancer cells by the use of anti-sense oligonucleotides. Recently, the successful use of siRNAs to down-regulate gene expression in several model systems [29, 31–32, 38, 44, 47] has led to many attempts to explore this methodology in potential therapeutic settings [47].

In the present study, we sought to address the possibility of using Plk-1 as a target for pancreatic cancer treatment and to determine whether RNAi-mediated depletion of Plk-1 might be an effective strategy for enhancing the sensitivity of pancreatic adenocarcinoma to gemcitabine. To this end, we selected three human pancreatic adenocarcinoma cell lines as models for pancreatic cancer. We found that forced Plk-1 overexpression promoted cell proliferation, as well as an increase in G1/S phase cell percentage but

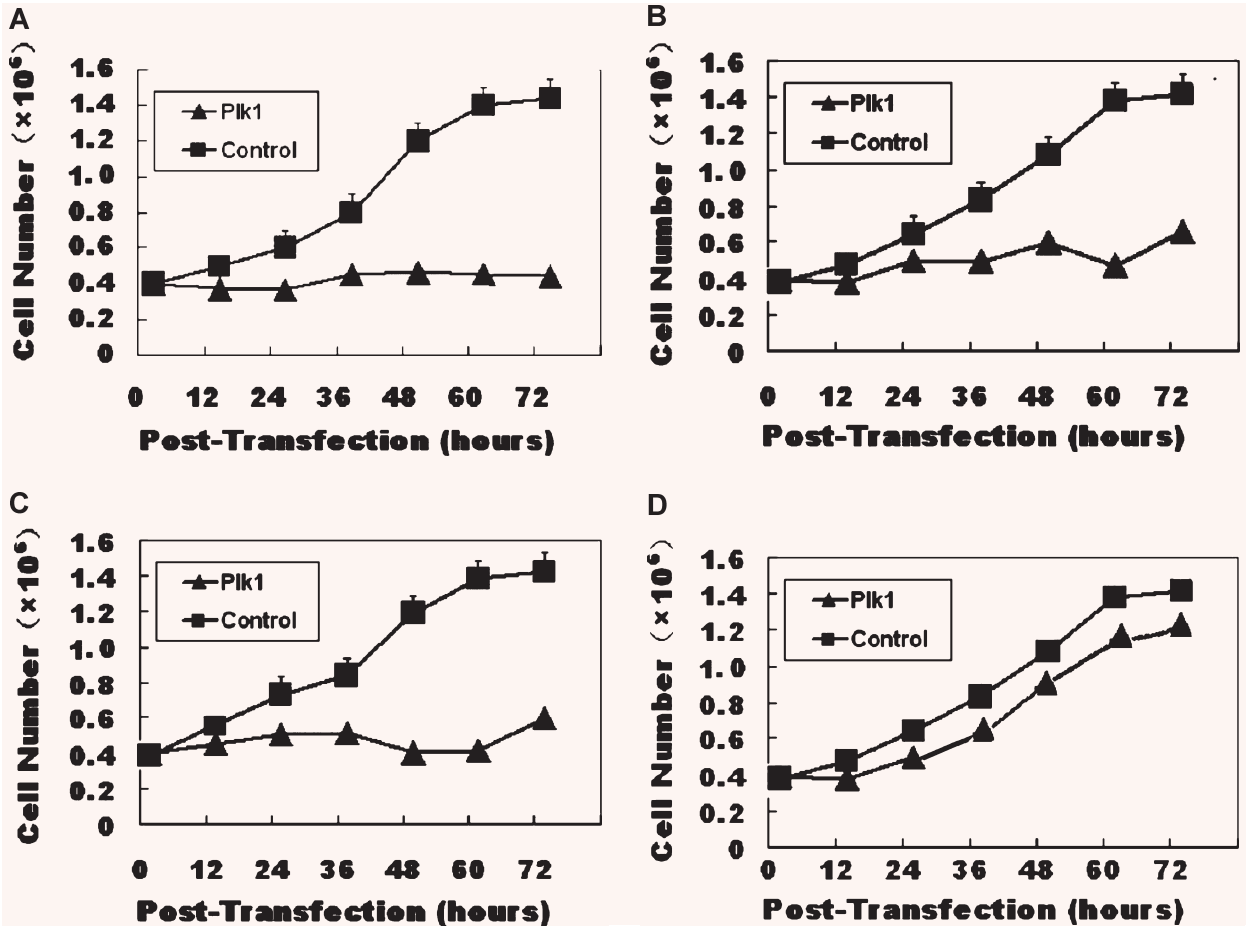


Fig. 6 Inhibitory effect of Plk-1 siRNA on cell proliferation in human pancreatic adenocarcinoma cells as determined by the MTT assay. AsPC-1 (**A**), PANC1 (**B**), BxPC3 (**C**) and HPDE6c7 (**D**) cells were transfected with either the Plk-1 siRNA expression vector pBS/U6-Plk-1 (*Plk-1*) or empty pBS/U6 vector (*Control*). Cell viability and proliferation were assessed by MTT assay at 12, 24, 36, 48, 60 and 72 hrs after transfection, as described in the Materials and methods section. The results represent the means of at least three independent experiments. $P < 0.01$ for the control group *versus* Plk-1 group in AsPC-1, PANC1 and BxPC3 cells. $P > 0.05$ for the control group versus Plk-1 group in HPDE6c7 cells.

a reduction in G2/M phase cell population in these systems (data not shown). Our further studies showed that siRNA-mediated knockdown of Plk-1 expression caused cell cycle G2/M arrest and proliferation inhibition. These observations suggest that the suppression of this mitotic regulator might represent an effective approach to enhancing gemcitabine-induced cytotoxicity. More interestingly, we demonstrated that when pancreatic cancer cells were first treated with Plk-1 siRNA, followed by exposure to gemcitabine, the cell viability and survival were strikingly reduced and apoptotic cell death was considerably increased, compared with cells treated with either agent alone. Thus, the combination of *Plk-1* gene therapy and gemcitabine chemotherapy has a synergistic anti-tumour effect against pancreatic carcinoma.

However, the molecular mechanism accounting for the anticancer action of this combination therapy is not clear at this point. Growing evidence suggests that Plk-1 depletion can synergize with gemcitabine in the treatment of pancreatic cancer through several mechanisms. First, gemcitabine is well known for its action in blocking cell cycle progression through the G1/S phase boundary, and we showed that treatment with Plk-1 siRNA causes cell cycle arrest at G2/M in pancreatic cancer cells. The G2/M transition is known to be controlled by the cyclin B-Cdk-1 (Cdc2) complex, and the balance between the inhibiting Myt1/Wee1 kinases and the activating Cdc25 phosphatases is a critical regulator of cyclin B-Cdc2 activity and thereby controls mitotic entry [48]. Several lines of evidence indicate that Plk-1 can phosphorylate/activate Cdc25C [18, 48] and mediate nuclear translocation of Cdc25C [49], which leads to the activation of Cdc2 (Cdk-1) protein kinase activity. Plk-1 can also activate Cdc2 through the stabilization of cyclin B [50] or the degradation of Wee1 [42] and by participating in a positive

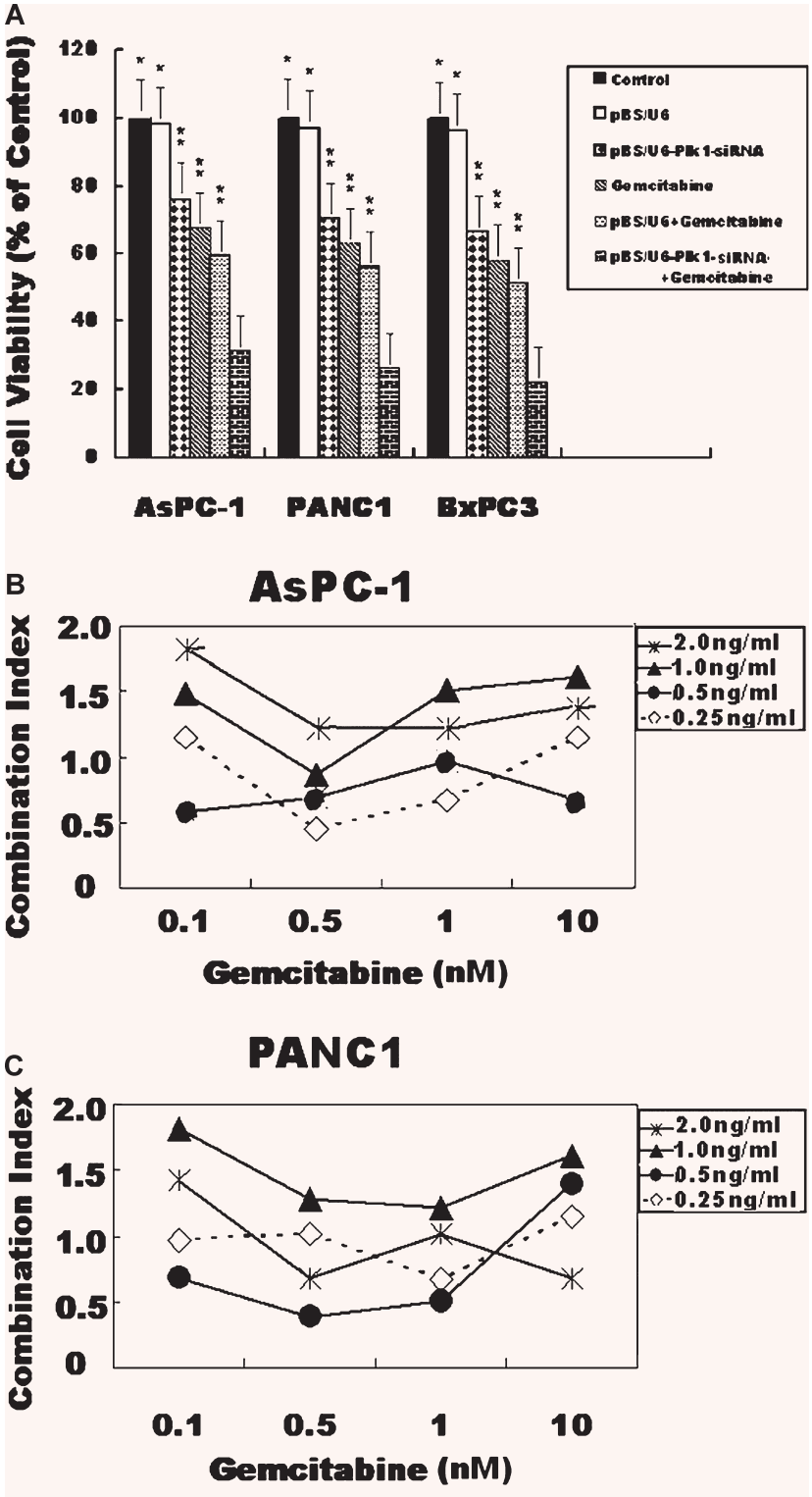
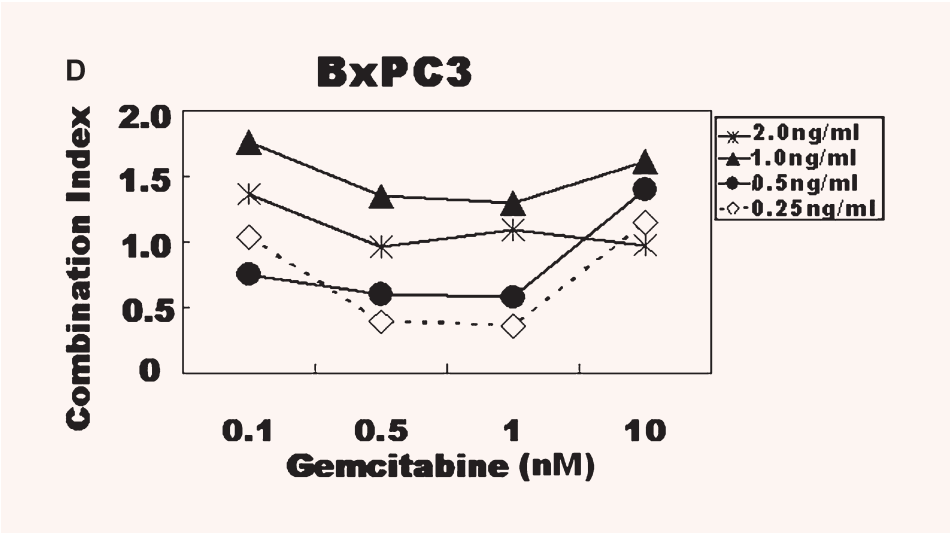


Fig. 7 Plk-1 siRNA enhances gemcitabine sensitivity and increases gemcitabine cytotoxicity in pancreatic tumour cells. (**A**) AsPC-1, PANC1 and BxPC3 pancreatic cancer cells were transfected with only pBS/U6 vector or pBS/U6-Plk-1-siRNA vector at a concentration of 0.5 ng/ml, or were treated with only 0.1 nM gemcitabine or their combination as indicated, for 4 consecutive days (96 hrs). Cell viability and survival were determined by MTT assay. Cells treated with PBS were used as a control, and their viability was set at 100%. The values presented are the means of three quadruplicate assays. $*P < 0.01$ *versus* the pBS/U6-Plk-1-siRNA + gemcitabine group. $**P < 0.05$ *versus* the pBS/U6-Plk-1-siRNA + gemcitabine group. (**B**, **C** and **D**) AsPC-1, PANC1 and BxPC3 cells were transfected with pBS/U6-Plk-1 at different doses (0.25–2.0 ng/ml), followed by treating with different concentrations (0.1–10 nM) of gemcitabine for 96 hrs. Cell viability was assessed by MTT assay, and the combination index (**CI**) for the effect of pBS/U6-Plk-1 and gemcitabine in AsPC-1 (**B**), PANC1 (**C**) and BxPC3 (**D**) cells was determined and analysed using CalcuSyn software. A CI indicating synergism (CI < 1.0) was observed in all three cell lines for the combination of pBS/U6-Plk-1 at concentrations of 0.25–0.5 ng/ml and gemcitabine at 0.5–1.0 nM.



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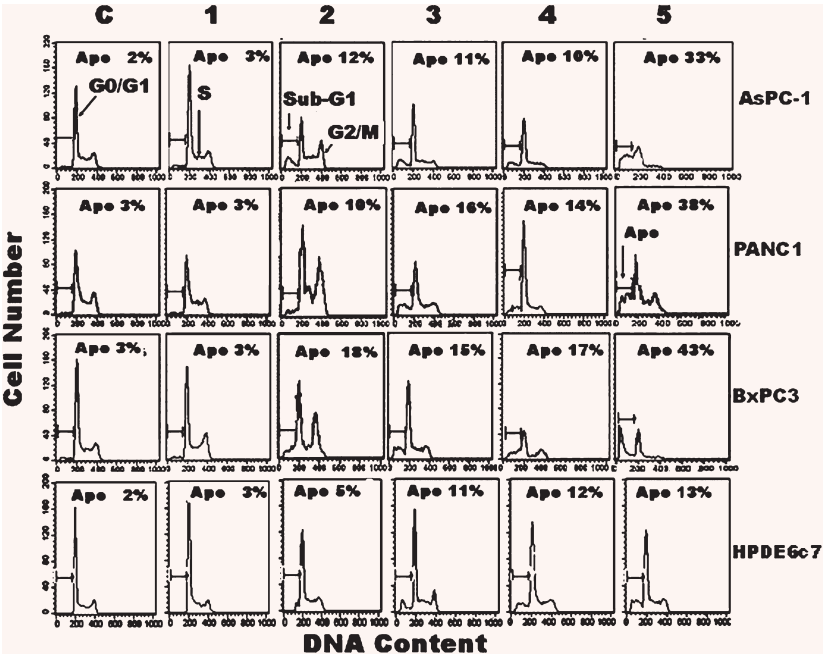

Fig. 8 Enhancement of gemcitabine-induced apoptosis by Plk-1 siRNA in pancreatic cancer cells as assessed by fluorescence-activated cell sorting (FACS) analysis. AsPC-1, PANC1, BxPC3 and HPDE6c7 cells were treated or transfected with PBS (mock control), gemcitabine (0.1 nM), pBS/U6 (vector control), or pBS/U6-Plk-1-siRNA (0.5 ng/ml) alone, or the cells were transfected with pBS/U6 or pBS/U6-Plk-1-siRNA, followed by exposure to gemcitabine, as indicated. Apoptosis induction in the four cell lines was determined at 48 hrs after treatment. The percentage of apoptotic (sub-G1) cells is given in each panel. The data shown are representative of three separate experiments. C, mock controls; 1, pBS/U6; 2, pBS/U6-Plk-1-siRNA; 3, gemcitabine; 4, pBS/U6 + gemcitabine; 5, pBS/U6-Plk-1-siRNA + gemcitabine.

feedback loop [50] that ensures full and irreversible activation of the cyclin B-Cdc2 complex at the G2/M transition. Conversely, knockdown of Plk-1 expression diminishes cyclin B-Cdc2 activity and leads to G2/M arrest by increasing the activity of the Wee1 and Myt1 kinases and inhibiting the activity of the Cdc25 phosphatases. By targeting different phases of the cell cycle, gemcitabine and Plk-1 siRNA may act collaboratively to stop cell cycle progression and inhibit the malignant growth of pancreatic tumour cells.

In addition to its actions in cell proliferation reduction and cell cycle blockade, we have also shown that the suppression of Plk-1 expression by siRNA increases gemcitabine-induced cytotoxicity and apoptosis in pancreatic adenocarcinoma cells. As an antimetabolite, gemcitabine, acts to inhibit DNA synthesis and the depletion of Plk-1 has been shown to induce DNA damage [50] and apoptosis, as evidenced by the appearance of subgenomic DNA in fluorescence-activated cell sorter (FACS) profiles, the activation of caspase-3, and the formation of fragmented nuclei [29]. Moreover,

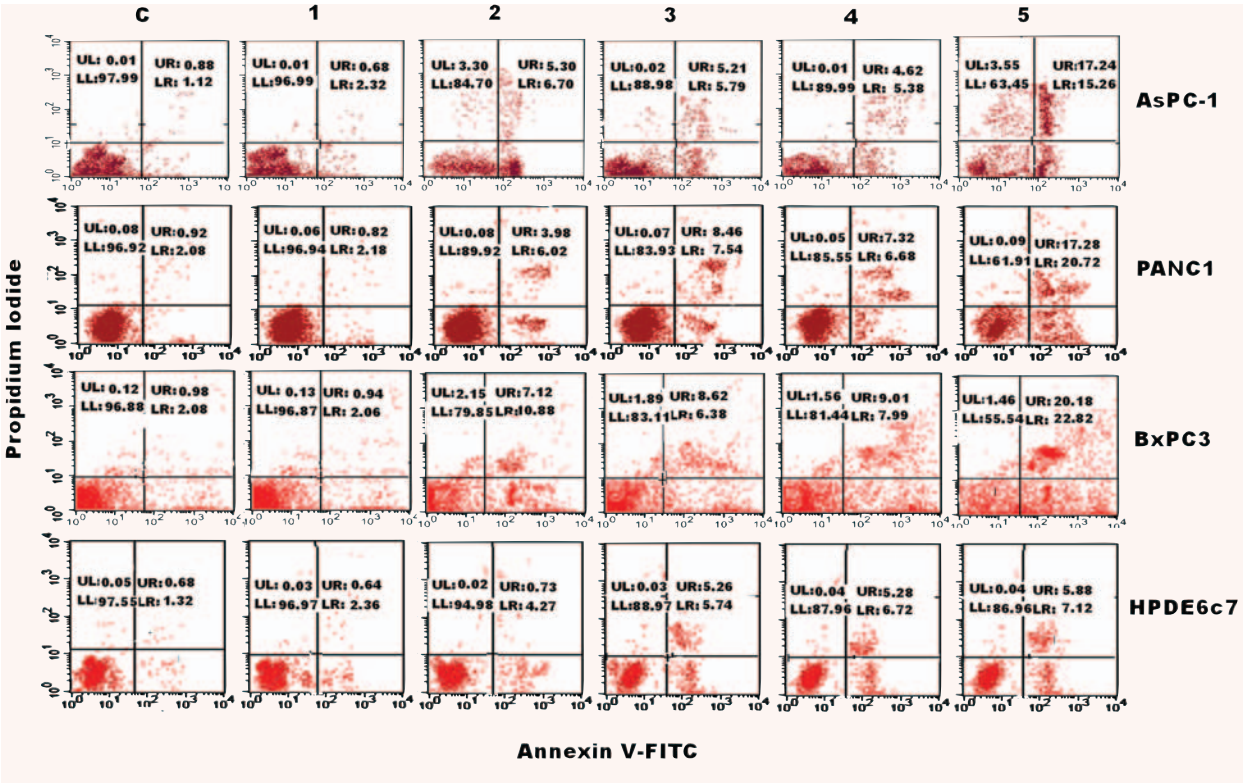

Fig. 9 Augmentation of gemcitabine-induced apoptosis by Plk-1 siRNA in pancreatic cancer cells as assessed by annexin V staining. AsPC-1, PANC1, BxPC3 and HPDE6c7 cells were treated or transfected with PBS (mock control), gemcitabine (0.1 nM), pBS/U6 (vector control), or pBS/U6-Plk-1-siRNA (0.5 ng/ml) alone, or the cells were transfected with pBS/U6 or pBS/U6-Plk-1-siRNA, followed by exposure to gemcitabine. Apoptosis induction in the four cell lines was determined at 48 hrs after the treatments. The cells were then harvested and stained using a Vybrant Apoptosis Assay Kit. Apoptosis was determined by flow cytometry for annexin-V-FITC and propidium iodide (PI) dual labeling, as described in the Materials and methods section. Cytograms of annexin-V-FITC binding (abscissa) *versus* PI uptake (ordinate) show three distinct populations: (*i*) viable cells (low FITC and low PI signal) in gate LL; (*ii*) early apoptotic cells (high FITC and low PI signal) in gate LR and (*iii*) cells that have lost membrane integrity as a result of very late apoptosis (high FITC and high PI signal) in gate UR. Percentages of apoptotic cells (gate LR and gate UR) are indicated on each cytogram. A representative of three separate experiments is shown. C, mock controls; 1, pBS/U6; 2, pBS/U6-Plk-1-siRNA; 3, gemcitabine; 4, pBS/U6 + gemcitabine; 5, pBS/U6-Plk-1-siRNA + gemcitabine.

gemcitabine and Plk-1 depletion block the cell cycle at G1/S and G2/M, respectively, and prolonged and sustained cell cycle arrest eventually activates apoptotic pathways leading to cell death. Therefore, Plk-1 siRNA exhibits therapeutic synergism with gemcitabine by acting on different targets and through different mechanisms, to result in stronger suppression of tumour progression and induction of apoptosis in pancreatic cancer. Further investigations are warranted to evaluate the effect and mechanism by which Plk-1 depletion and gemcitabine therapy co-ordinately control cell growth and cell death to inhibit pancreatic carcinoma progression in *in vivo* models, particularly to elucidate the molecular basis of the synergistic and collaborative anti-tumour activity of these two agents against pancreatic adenocarcinomas through inhibiting cell proliferation, decreasing cell viability, blocking cell cycle progression and inducing cellular DNA damage or cellular apoptosis.

In summary, our findings in this study confirm that Plk-1 is a molecular determinant for chemoresistance to gemcitabine and thus a potential therapeutic target for pancreatic adenocarcinoma. We have shown that overexpression of Plk-1 can protect pancreatic adenocarcinoma cells from gemcitabine-induced cytotoxicity, whereas knockdown of Plk-1 expression has a chemosensitizing effect. Furthermore, we demonstrated that *Plk-1* gene silencing by siRNA is an effective therapeutic adjunct to gemcitabine treatment. This is the first demonstration that the combination of *Plk-1* gene therapy and gemcitabine chemotherapy has synergistic anti-tumour activity against pancreatic carcinoma *in vitro*. This

combination treatment may be a novel therapeutic approach for this highly treatment-resistant disease. Further studies on animal models will pave the way for future combination therapy clinical trials in the development of RNAi-mediated *Plk-1* gene silencing as a target-directed therapeutic strategy to enhance the efficacy of gemcitabine in human pancreatic adenocarcinoma and other tumours.

Acknowledgements

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