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ORIGINAL ARTICLE

Inducing apoptosis and enhancing chemosensitivity to Gemcitabine via RNA interference targeting Mcl-1 gene in pancreatic carcinoma cell

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Abstract
Purpose Resistance to chemotherapy is a major cause of treatment failure and poor prognosis in pancreatic carcinoma. Myeloid cell leukemia-1 (Mcl-1) is highly up-regulated in pancreatic carcinoma and is associated with the anti-apoptosis and the resistance to chemotherapy drugs. Suppression of Mcl-1 would be an approach to induce apoptosis and enhance the chemosensitivity.
Methods In this study, three pancreatic cancer cell lines (PANC-1, BxPC-3 and SW1900) stably expressing shRNAs targeting Mcl-1 gene were established and gene expression inhibition was assessed by Real-Time QPCR and Western blotting. The effects of Mcl-1 downregulation mediated by RNAi were explored in vitro and in vivo.
Results We showed that the specific downregulation of Mcl-1 strikingly inhibited cell growth, colony formation, cell cycle arrest and induced apoptosis in pancreatic cancer cells in vitro, and markedly decreased the tumorigenicity in a mouse xenograft model. Moreover, knockdown of Mcl-1 significantly increased the chemosensitivity to Gemcitabine in pancreatic carcinoma cells.
Conclusions Our data suggests that the specific down-regulation of Mcl-1 by RNAi is a promising approach to induce apoptosis and enhance the chemosensitivity for pancreatic carcinoma gene therapy.

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Introduction

Pancreatic cancer is one of the neoplasms with the worst prognosis and higher mortality because of its aggressive invasion, early metastasis, resistance to existing chemotherapeutic agents and radiation therapy [1]. Only 10–14% of patients are eligible for curative surgery to prolong only 1 year's median survival, that still remained palliative [2].

Gemcitabine has become the first-line treatment option for pancreatic cancer because that Gemcitabine can slightly prolong overall survival and ameliorate disease-related symptoms [3]. But the resistance to Gemcitabine is increasing in recent years, and the effectiveness of Gemcitabine is lower than 20% [4]. It is considered that resistance to Gemcitabine treatment is mainly attributed to an altered apoptotic threshold in pancreatic cancer cells due to increased expression of anti-apoptotic members of the bcl-2 family [5].

Increased resistance to apoptosis is a hallmark of many tumor cells. Inhibition of apoptosis is considered as a survival advantage on cells harboring genetic alternations and may promote acquisition of further mutations that induce neoplasm progression and also promote resistance to chemotherapy [6]. Therefore, inhibition of specific anti-apoptotic factors may provide a rational basis for the development of new therapeutic strategies in cancer [7, 8].

Myeloid cell leukemia-1 (Mcl-1) is one novel anti-apoptotic member of the Bcl-2 family, originally identified as an early induction gene during differentiation on myeloid leukemia cells [9]. Mcl-1 plays a crucial role in cell division and malignant tumor development, which is over

expressed in a number of human malignancies, including human pancreatic carcinomas [10–12]. Expression levels of this protein in tumor cells were different depending on the degree of malignancy or the effectiveness of chemotherapy. In the human malignancies, Mcl-1 has been related with the anti-apoptosis and increased resistance to chemotherapy [13–16]. Thus, Mcl-1 is an attractive target for novel gene therapy strategies in different carcinomas [17–19]. Effectively enhancing of chemosensivity by downregulation of Mcl-1 has been confirmed in different tumor cell lines [20–22].

Small interfering RNA (siRNA) is a kind of endogenous gene-silencing mechanism which is currently the most widely employed technique in functional genomic studies and therapeutic gene regulation [23–25]. DNA vector to mediate RNAi by expressing small hairpin RNA (shRNA) has been used to induce stable, long-term, and highly specific gene silencing, which has expanded a broad novel avenue for the analysis of gene function and gene therapy [26, 27]. In this study, we introduced DNA vector pSilencer4.1-CMVneo expressing shRNA targeting to Mcl-1 gene so as to explore the potential efficacy of RNAi on apoptosis induction and enhancing of chemosensitivity in pancreatic carcinoma cells.

Materials and methods

Cell lines and reagents

PANC-1, BxPC-3 and SW1900 human pancreatic carcinoma cell lines were purchased from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum and penicillin (100 Units/ml)/streptomycin (0.1 mg/ml) in 5% CO_2 incubator at 37°C. Gemcitabine was purchased from Eli Lilly Co. (Indianapolis, IN, USA), reconstituted in complete cell culture medium to final working concentrations (0.01–10 μg/ml), and stored in aliquots at −20°C.

RNAi for Mcl-1

The shRNA targeting for Mcl-1 mRNA sequence was chosen using siRNA design software downloaded from internet (http://www.ambion.com). The negative control shRNA (nc) was designed too. BLAST search against EST libraries was performed to confirm that no other human gene was targeted. DNA template encoding siRNA cDNA were designed as follows: 19-nt target sequence as sense strand followed by a 9-nt spacer and complementary antisense strand (Fig. 1a). The shRNAs and polyA sequence were inserted downstream of CMV promoter of pSilencer4.1-CMVneo (Amibion, Austin, TX, USA) between the *Bam*HI and *Hin*dIII enzyme sites, named as pS-sh and pS-nc (Fig. 1b). The pSilencer4.1-CMVneo was used as vector control (named as pS-vc). pS-sh and pS-nc were confirmed by the digestion analysis of restriction endonuclease and all inserted sequences were verified by DNA sequencing.

Cell culture and transfection

Human pancreatic carcinoma cells (PANC-1, BxPC-3 and SW1900) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum and penicillin (100 Units/ml)/streptomycin (0.1 mg/ml) in 5% CO2 incubator at 37°C. Cells was seeded into six-well plates with antibiotics-free growth medium at a density of 2.0×10^5 cells/well respectively and cultured overnight to 80% confluence prior to transfection. Cells were transfected with 2 μg/well of pS-sh, pS-nc and pS-vc, respectively using the LipofectamineTM2000 reagent (Invitrogen, USA) according to the manufacturer's protocols. The ratio of the plasmids and the transfection reagent was 1 μg:2 μl. Stably transfected cell clones were selected with 600 μg/ml G418 (Sigma, USA) 48 h after transfection, about 2 weeks late individual clones stably expressing shRNA were isolated and maintained with 600 μg/ml G418.

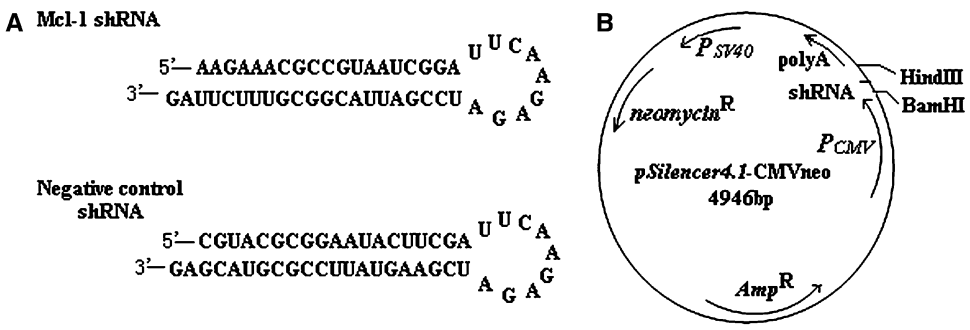

Fig. 1 Design of the shRNA and recombinant eukaryotic vector. **a** Predicted structure of small hairpin RNAs and **b** schematic diagram of the pSilencer4.1-CMVneo vector. shRNA encoding template was inserted between the *Hin*d III and *Bam*H I restriction sites downstream of CMV promoter

Real-time quantitative polymerase chain reaction (RT-QPCR)

Total RNA from stably transfected pancreatic cancer cells was extracted using TRIzol reagent (Invitrogen). One micro gram of total RNA was reverse transcribed using an oligo-dT primer and afterwards analyzed by RT-QPCR (MJ Research Opticon2, USA) using the QuantiTect SYBR Green PCR Kit (Qiagen) and primers as follows: Mcl-1 forward: 5′-*TGGCGGAAGCGCCGGCGC*-3′, and Mcl-1 reverse: 5′-*TTCCGAAGCATGCCTTGG*-3′, β-actin forward: 5′-*CTACAATGAGCTGCGTG*-3′, β-actin reverse: 5′-*GGTCTCAAACATGATC*-3′. The relative increase in reporter fluorescent dye emission was monitored. The level of Mcl-1 mRNA, relative to β-actin, was calculated using the following formula: relative Mcl-1 mRNA expression = 2^[*c*t (Mcl-1 treated) − *c*t (Mcl-1 control) + *c*t (Actin treated) − *c*t (Actin control)], where *c*t is defined as the number of the cycle in which emission exceeds an arbitrarily defined threshold.

Western blotting analysis

Three stably transfected pancreatic cancer cells were lysed in 50 μl lysis buffer (50 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0, 5 mM DTT, 2% SDS) on ice for 30 min, and the resulting lysates were cleared by centrifugation. Proteins were subjected to 10% SDS-PAGE and electroblotted onto nitrocellulose membrane, blocked with Tris buffered saline plus 0.1% Tween-20 (TTBS) containing 5% non-fat milk for 1 h, and probed with anti-Mcl-1 (Merck, GER), anti-poly (ADP ribose) polymerase (PARP) (Beverly, MA, USA) and anti-β-actin (Sigma, USA) antibody, then incubation with horseradish peroxidase–conjugated goat anti-mouse secondary immunoglobulin antibodies (Sigma, USA). Finally, the bands were visualized by chemiluminescence using a chemiluminescence kit (Invitrogen) and the specific bands were recorded on X-ray film. The inhibitory rate of Mcl-1 protein expression was calculated as follows: inhibitory rate = [1 − (shRNA Mcl-1 density/shRNA β-actin density)/(untransfected Mcl-1 density/untransfected β-actin density)] × 100%.

3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay

The MTT assay was performed to assess the effect of Mcl-1 on cell proliferation and chemosensitivity to Gemcitabine. Three stably transfected pancreatic cancer cells were plated in 96-well plates at a density of 4.0×10^3 cells/well for proliferation assay. Then for 7 days, every 24 h a batch of cells were stained with 20 μl sterile MTT dye (5 mg/ml; Sigma, USA) at 37°C for 4 h, then culture medium was removed and 150 μl of DMSO was added and thoroughly mixed in for 10 min. Spectrometric absorbance at 570 nm was measured by using a microplate reader. All groups were performed five times. To assess chemosensitivity to Gemcitabine, 1.0×10^4 untransfected or stably transfected cells were plated in 96-well plates and allowed to attach for 24 h, Cells were then placed in media containing different doses of Gemcitabine and incubated for another 96 h, and every 24 h a batch of cells were treated with MTT as described before. The cell growth inhibition rate was calculated as follows: 100% × (*A*570 untreated cells − *A*570 treated cells)/(*A*570 untreated cells).

Colony formation assay

Approximately 2.0×10^2 PANC-1 cells stably transfected with pS-sh, pS-nc and pS-vc parental vector (named as pS-sh-PANC-1, pS-nc-PANC-1 and pS-vc-PANC-1) were plated in 10-cm culture dishes, respectively. After 18 days, cells were fixed with methanol and stained with 0.1% crystal violet. Visible colonies were manually counted.

Flow cytometric analysis for cell cycle and apoptosis

A measure of 1.0×10^5 pS-sh-PANC-1, pS-nc-PANC-1, pS-vc-PANC-1 and untransfected PANC-1 cells were harvested with trypsinization at the 90% confluent stage, fixed with cold 70% ethanol, and stained by propidium iodide (PI) in PBS, then analyzed for PI fluorescence intensity by flow cytometry to assess celluar DNA content. The relative proportions of cells in the G1, S and G2/M phases of the cell cycle were determined from the flow cytometry data. The percentage of apoptotic cells was determined by the sub-G1 proportion. Apoptosis of stable transfectants was also measured with an Annexin V-fluorescein isothiocyanate apoptosis detection kit (Zymed, USA). Briefly, the PANC-1 cells were seeded in 100 ml flasks and incubated until there was approximately 90% confluence. After 72 h, the cells were harvested, washed with ice-cold PBS twice and resuspended in binding buffer. Annexin V-fluorescein isothiocyanate (0.5 μg/ml) and propidium iodide (0.6 μg/ml) were then added to an aliquot (1.0×10^6 cells) of this cell suspension. After 15-min incubation in the dark at room temperature, stained cells were immediately analyzed by FACSCalibur (BD, USA). All of the samples were assayed in triplicate, and the cell apoptosis rate was

calculated as follows: apoptosis rate = (apoptotic cell number/total cell number) × 100%.

Measurement for caspase-3 activity

pS-sh-PANC-1, pS-nc-PANC-1, pS-vc-PANC-1 and untransfected PANC-1 cells were plated and incubated until there was approximately 90% confluence, then for 72 h, every 24 h a batch of cells were harvested and 1.0×10^6 cells was washed with PBS, pelleted at low speed and resuspended in lysis buffer for 10 min at 4°C. Cell lysates were cleared by centrifugation and assayed for caspase-3 activity using a DEVDpNA peptide substrate and incubated for 6 h at 37°C. Caspase-3 activity was measured using Colorimetric Assay kits from R&D Systems (Minneapolis, MN, USA). The activities were quantified spectrophotometrically at a wavelength of 405 nm. Caspase 3 activity was calculated as the change in absorbance at 405 nm and divided by total protein concentration.

Tumorigenicity in mice xenograft model

The effect of Mcl-1 on tumorigenicity was assessed by subcutaneous injection of pS-sh-PANC-1, pS-nc-PANC-1 and pS-vc-PANC-1 cells and untransfected PANC-1 cells in athymic nude mice. Each aliquot of approximately 2.0×10^6 cells suspended in 100 μl of PBS containing 20% of Matrigel Growth Factor Reduced (Becton Dickinson Labware, Flanklin, NJ, USA) were injected subcutaneously into the flank of 8-week-old male BALB/c nude mice. For each group, six animals were used. Animals were kept under pathogen-free conditions. Tumor growth over a period of 4 weeks was monitored and measured. Each tumor volume in mm^3 was calculated by the following formula: $V = 0.4 \times D \times d^2$ (V, volume; D, longitudinal diameter; d, latitudinal diameter). Four weeks later, the mice were sacrificed and the tumors were separated and weighed. Animal experiments in this study were done in compliance with the Fourth Military Medical University of Medicine institutional guidelines.

Statistical analysis

Data are expressed as mean ± SE and all statistical analyses were performed by using SPSS10.0. Comparisons among all groups were performed with the one-way analysis of variance (ANOVA) test and Student Newman Keuls method. Differences were considered significant at $P < 0.05$.

Results

Construction of shRNAs expressing vectors from pSilencer4.1-CMVneo

Four designed shRNAs targeting Mcl-1 were cloned into the pSilencer4.1-CMVneo vector, and PANC-1 cells were transiently transfected with the recombinant vectors. The Mcl-1 expression was detected by RT-PCR, one vector expressing shRNA for Mcl-1 was the most valid for inhibition in PANC-1 cells (date not shown). This vector was used and named as pS-sh. Vector expressing negative control shRNA was constructed as the interference control, named as pS-nc, the pSilence4.1 blank vector was used as vector control, and named as pS-vc. All vectors were confirmed to be correct by DNA sequencing.

Isolation of colonies stably expressing shRNA targeting Mcl-1 gene

PANC-1, BxPC-3 and SW1900 cells were transfected with plasmid pS-sh, pS-nc and pS-vc, respectively. Several stably transfected PANC-1, BxPC-3 and SW1900 cell colones with pS-sh, pS-nc and pS-vc were obtained by G418 selection for 14 days. As shown in Fig. 2, the results of RT-QPCR (Fig. 2a) and Western blotting (Fig. 2b) analysis showed that the expression of Mcl-1 gene was downregulated in pS-sh transfected cells, and not inhibited in pS-nc, pS-vc transfected cells compared with untransfected cells. The inhibition rate of pS-sh-PANC-1, pS-sh-BxPC-3 and pS-sh-SW1900 was 69.3, 61.2 and 58.6% respectively (Table 1). The results indicated that the shRNA designed for Mcl-1 could effectively downregulate the expression of Mcl-1, especially in PANC-1cells.

Inhibition of cell proliferation and colony formation in vitro by Mcl-1 downregulation

The effect of inhibiting Mcl-1 gene expression on cell growth was determined by MTT assay. As shown in Fig. 3, Cell proliferation was inhibited notably in a time-dependent manner for pS-sh transfected cells in three different pancreatic cell lines, and the highest inhibitory rate was 58.1 ± 2.5, 50.0 ± 3.4, 42.8 ± 2.6% for pS-sh-PANC-1, pS-sh-BxPC-3, pS-sh-SW1900 cells respectively on day 4, higher than that of their controls ($P < 0.05$). There was no difference of inhibition between control (pS-nc and pS-vc) and untransfected cells ($P > 0.05$). PANC-1 cells transfected with pS-sh, pS-nc and pS-vc vector were plated in 10-cm culture dishes, respectively for colony formation assay, the result

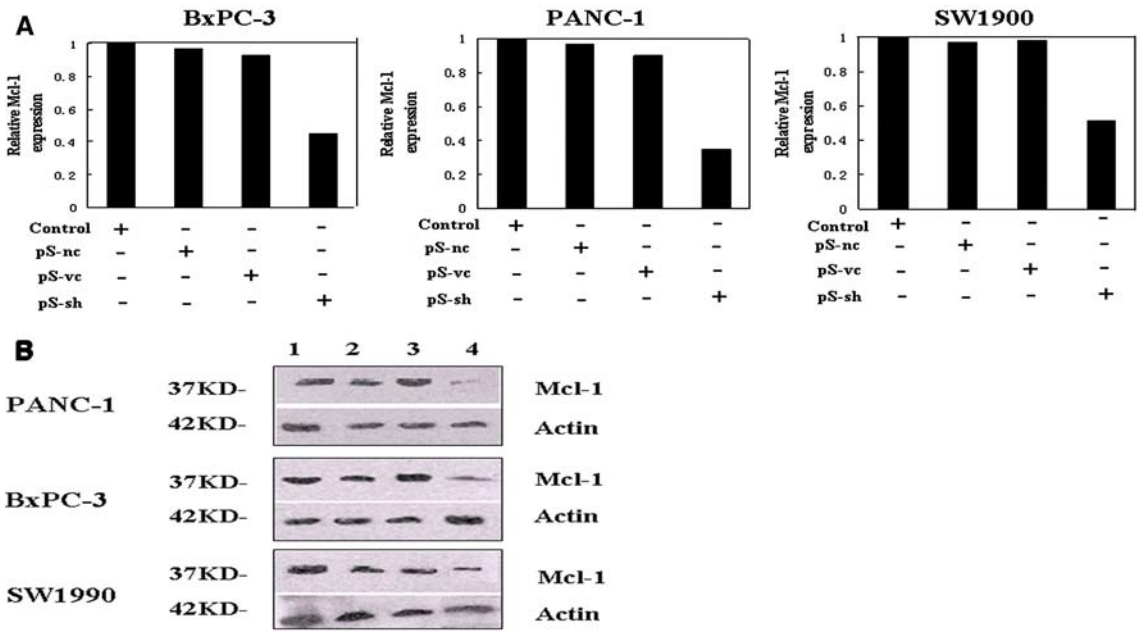

Fig. 2 Inhibiting Mcl-1 expression in pancreatic cancer cells by RNA interference. **a** mRNA expression of Mcl-1 detected by quantitative real-time RT-PCR (qRT-PCR). Expression of Mcl-1 was normalized to actin in each sample. Relative Mcl-1 expression was calculated as described in the "Materials and methods". The expression of untransfected cells (control) was looked as 1, and the stably transfected cells (pS-sh, pS-nc, pS-vc) relative Mcl-1 was calculated contrast to the control. **b** Protein expression of Mcl-1 detected by Western blotting. Densitometric analysis was performed using the Labworks Image Acquisition. *1* untransfected cells (control); *2* pS-nc stably transfected cells; *3* pS-vc stably transfected cells; *4* pS-sh. stably transfected cells

indicated that the number of colonies of pS-sh-PANC-1 cells were much less than that of pS-nc-PANC-1, pS-vc-PANC-1 and untransfected cells ($P < 0.01$) (Fig. 4).

Table 1 Inhibition rates of Western blotting in stably transfected cells

Cell line	$\bar{x} \pm s$[a]	Inhibition rate '(%)[b]
PANC-1		
PANC-1	0.9325 ± 0.0533	0
pS-nc-PANC-1	0.8449 ± 0.0486	9.39
pS-vc-PANC-1	0.8633 ± 0.0545	7.42
pS-sh-PANC-1	0.2863 ± 0.0052**	69.3**
BxPC-3		
BxPC-3	1.0816 ± 0.0533	0
pS-nc-BxPC-3	0.9571 ± 0.0512	11.51
pS-vc-BxPC-3	0.9635 ± 0.0632	10.92
pS-sh-BxPC-3	0.4197 ± 0.0038**	61.2**
SW1900		
SW1900	0.7889 ± 0.0533	0
pS-nc-SW1900	0.6917 ± 0.0533	12.32
pS-vc-SW1900	0.7126 ± 0.0564	9.67
pS-sh-SW1900	0.3266 ± 0.0214*	58.6**

** $P < 0.001$ versuscontrol group

[a] Integral absorption ratio (Mcl-1/β-actin) from Western blotting analysis, experiments were repeated three times

[b] Inhibition rate was determined as described in "Materials and methods"

These results show that downregulation of Mcl-1 gene significantly inhibit the cell proliferation and colony formation.

Apoptosis induction by downregulating Mcl-1 expression

The profile of cell cycle distribution and apoptosis in stably transfected PNAC-1 cells were analyzed by flow cytometry. The cell cycle assay indicated that inhibition of Mcl-1 gene expression induced the significant changes of the proportions in the G1, S and G2/M phases. The percentage of the sub-G1 proportion increased notably in pS-sh-PANC-1 (41.6%), while there were no significant changes among pS-nc-PANC-1 (5.7%), pS-vc-PANC-1 (6.1%) and untransfected PANC-1 cells (3.1%) (Fig. 5). Stable transfected PNAC-1 cells were stained by Annexin V and PI and analysised by flow cytometry. Cell apoptosis analysis indicated that the apoptosis rate of pS-sh-PANC-1 markedly increased to 43.8% compared with those of pS-nc-PANC-1, pS-vc-PANC-1 and PANC-1 control cells ($P < 0.01$, respectively), while there were no obvious differences in apoptosis rates among the pS-vc-PANC-1, pS-nc-PANC-1 and PANC-1 control cells ($P > 0.05$; Fig. 6a), which was 7.1, 5.4 and 3.6%, respectively. We detected the PARP cleavage which is the evidence for apoptosis, and there was an increase in PARP cleavage in pS-sh-PANC-1, but not in pS-nc-PANC-1,

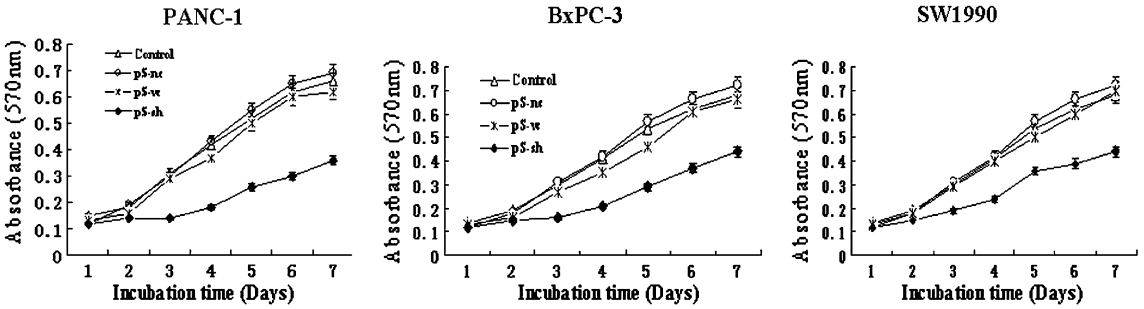

Fig. 3 Cell proliferation detected by MTT. The data in each time point are averaged values from six replicates. Cell proliferation was inhibited notably in a time-dependent manner, and the highest inhibitory rates were 58.1 ± 2.5, 50.0 ± 3.4, 42.8 ± 2.6% for pS-sh-PANC-1, pS-sh-BxPC-3, pS-sh-SW1900 cells, respectively on day 4

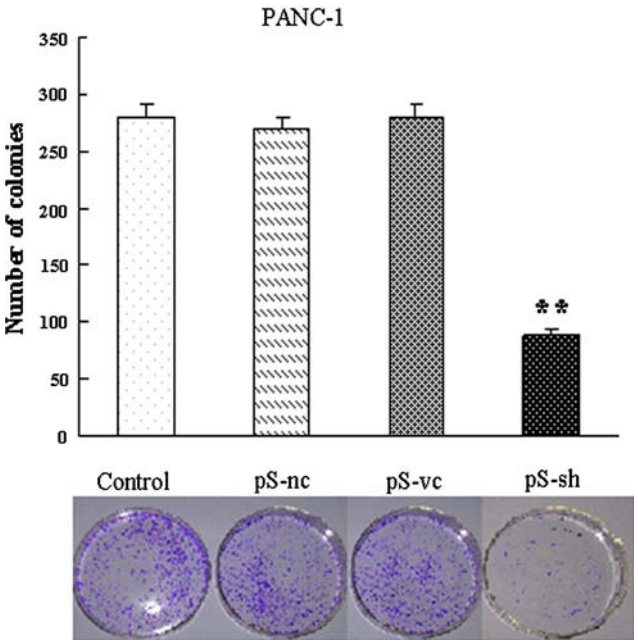

Fig. 4 Results of colony formation assay. The pS-sh-PANC-1 showed much less colonies than pS-nc-PANC-1, pS-vc-PANC-1 and untransfected cells. These experiments were performed four times. ** $P < 0.01$

pS-vc-PANC-1 and unintransfected PANC-1 cells (Fig. 6b). Results of caspase-3 activity assay showed that caspase-3 activity significantly increased in pS-sh-PANC-1 cells at 48 and 72 h ($P < 0.05$ and $P < 0.01$ Fig. 6c). All these results showed that the RNAi-mediated inhibition of Mcl-1 expression could lead to accelerate the apoptosis of human pancreatic carcinoma cells.

Downregulation of Mcl-1 expression inhibits tumorigenicity in vivo

We wondered whether the inhibition of Mcl-1 expression in pancreatic cancer cells would affect their ability to develop tumors in nude mice. To address this question, we established stable transfectants of pS-sh-PANC-1, pS-nc-PANC-1 and pS-vc-PANC-1 cells. Next, we injected the aliquot of 2×10^6 above cells and untransfected PANC-1 cells s.c. into each group of six athymic nude mice, respectively and monitored their tumor growth. As shown in Fig. 7, the growth of tumors formed from the pS-sh-PANC-1 xenografts mice was significantly inhibited compared with tumors formed from other control xenografts mice (Fig. 7a). At 28 days after inoculation, the average tumor weight of the mice was decreased by 66.2% in pS-sh-PANC-1 xenografts mice compared with the tumor weight formed in control xenografts mice (Fig. 7b). These results indicated that RNAi-mediated downragulation of Mcl-1 expression exerted a strong antitumorigenic effect in vivo on pancreatic cancer cells.

Increased chemosensitivity to Gemcitabine in pancreatic cell lines by inhibiting Mcl-1

Because Mcl-1 contributes to apoptosis resistance and is identified to play significant roles in the resistance to chemotherapy in many cancers [13–16], we next explored whether downregulation of Mcl-1 mediated by RNAi affect chemosensitivity in pancreatic carcinoma cell line to Gemcitabine. First, we determined the chemosensitivity to Gemcitabine in different pancreatic cell lines. The cells proliferations were assayed by MTT in three different pancreatic cell lines with different doses of Gemcitabine (0.01, 0.1, 1, 10 μg/ml). There was obvious inhibition with the Gemcitabine concentration of 1 and 10 μg/ml, but not 0.1 and 0.01 μg/ml, and the inhibitory rate of PANC-1 was much lower than that of the BxPC-3 and SW1900 ($P < 0.05$ Fig. 8a–c). Following, we analysised the proliferation in stably transfected PANC-1 cells (pS-sh, pS-nc and pS-vc) with or without 0.1 μg/ml Gemcitabine. The inhibitory rate of pS-sh group (48.1% day 3) was higher than that of other control groups (pS-nc 25.3%, pS-vc

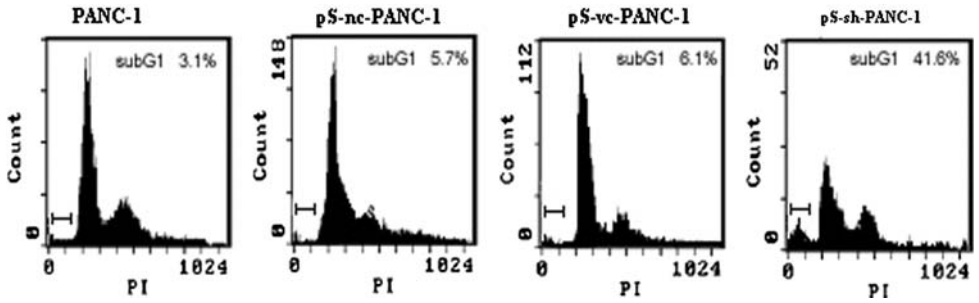

Fig. 5 Changes of cell cycle detected by FCM. Different stably transfected PANC-1 cells and untransfected PANC-1 cells were harvested at the 90% confluent stage, stained with propidium iodide, and analyzed for DNA content by flow cytometry. The percentage of cells with sub-G1 DNA content of pS-sh-PANC-1 significantly increased compared with that of the pS-nc-PANC-1, pS-vc-PANC-1 and PANC-1 ($P < 0.01$)

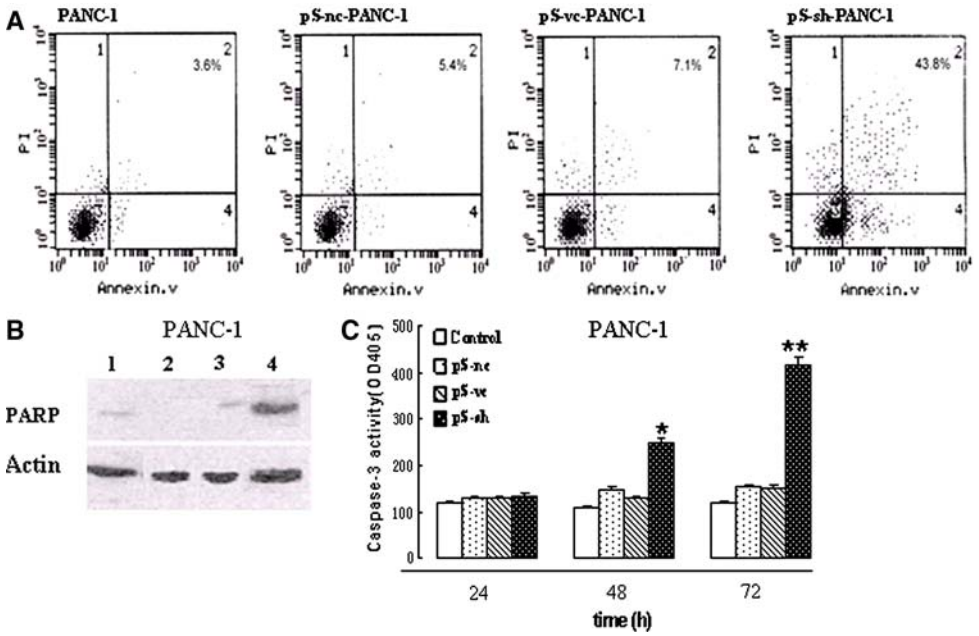

Fig. 6 Effects of apoptosis in stably transfected PANC-1 cells. **a** Apoptosis detection by FCM, the apoptotic rate of pS-sh-PANC-1 obviously increased (43.8%, $P < 0.01$); while there were no significant differences of cell apoptosis among pS-nc-PANC-1, pS-vc-PANC-1 and untransfected cells ($P > 0.05$). **b** Western blotting. Stably transfected PANC-1 and untransfected cells were harvested after incubation for 72 h; cells were lysed and subjected to western blotting with an antibody that recognizes the cleaved form of PARP (89 kDa). *1* untransfected PANC-1 cells; *2* pS-nc-PANC-1; *3* pS-vc-PANC-1; *4* pS-sh-PANC-1. **c** Caspase activation. Cells were incubated for 72 h, every 24 h, a banch of cells were lysised and cell lysates were assayed for caspase-3 activity as described in "Materials and methods". * $P < 0.05$ and ** $P < 0.01$, compared with controls

21.4%, untransfected PANC-1 19.1%) without Gemcitabine, however, there was a notable increase of inhibitory rate in pS-sh group with Gemcitabine (pS-nc + G, 80.3% day 3), not only higher than that of other control groups (pS-nc + G 35.2%, pS-vc + G 32.6%, PANC-1 + G 24.2%), but also higher than that of the pS-vc group without Gemcitabine ($P < 0.05$) (Fig. 8d, e). Compared with the inhibitory rates of pS-sh groups with or without Gemcitabine and unintransfected PANC-1 group, the inhibitory rate of pS-sh group with Gemcitabine (pS-sh + G) was higher than that of pS-sh groups and untransfected PANC-1 group (G) ($P < 0.05$) (Fig. 8f). These results indicated that the downregulation of Mcl-1 by RNAi could effectively enhance the chemosesentivity to Gemcitabine in pancreatic carcinoma cells.

Discussion

Defects of apoptosis contribute to tumorigenesis and chemotherapy resistance of pancreatic carcinoma cells. Stabilization of mitochondrial integrity is a key mechanism for both the survival of a malignant cell and for its resistance to chemotherapy [28, 29]. The Bcl-2 family is a

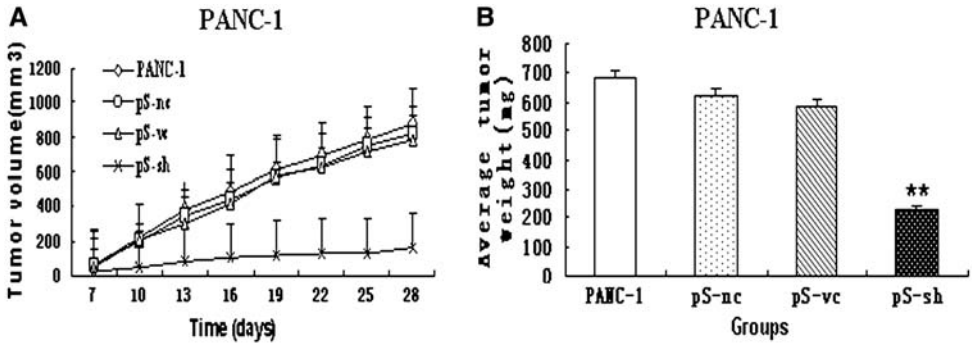

Fig. 7 Effects of the downregulation of Mcl-1 expression on the formation of tumors in vivo Tumor growth in the mice injected with different stably transfected PANC-1 cells and untransfected cells. **a** The tumor volume was measured every 3 day from day 7 after inoculation. **b** Average tumor weight at day 28. The average tumor weight of pS-sh-PANC-1 was notably higher than that of the untransfected cells. ** $P < 0.01$

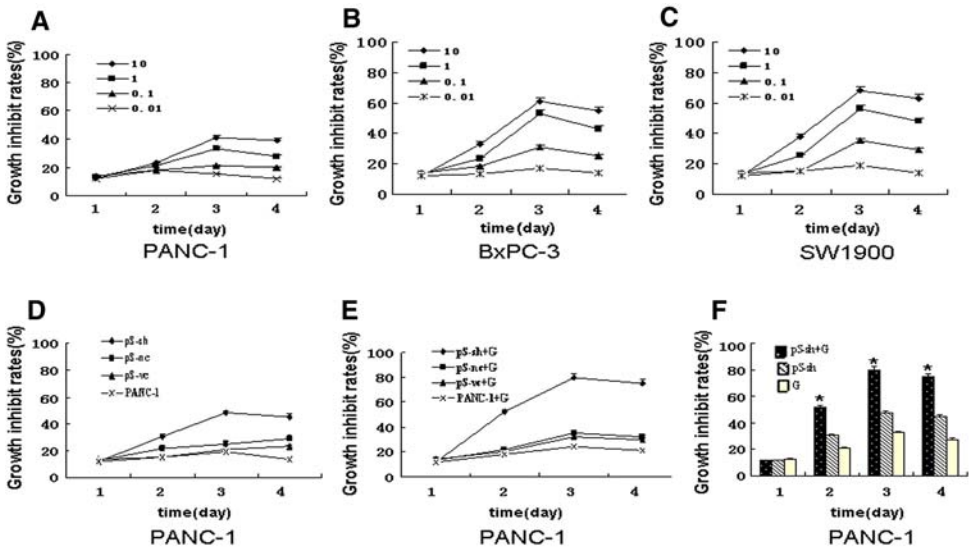

Fig. 8 Result of chemosensitivity to Gemcitabine by growth inhibit rates Cells were incubated for 96 h with or without different dose of Gemcitabine, every 24 h a batch of cells were treated with MTT assay, the growth inhibit rates were calculated. **a** Effects of different doses of Gemcitabine (μg/ml) on growth of pancreatic cancer cell PANC-1. **b** Effects of different doses of Gemcitabine (μg/ml) on growth of pancreatic cancer cell BxPC-3. **c** Effects of different doses of Gemcitabine (μg/ml) on growth of pancreatic cancer cell SW1900. **d** The growth inhibition rates of pS-sh-PANC-1, pS-nc-PANC-1, pS-vc-PANC-1 and untransfected PANC-1 cells. **e** The growth inhibition rates of pS-sh-PANC-1, pS-nc-PANC-1, pS-vc-PANC-1 and untransfected PANC-1 cells incubated with 0.1 μg/ml of Gemcitabine. **f** The growth inhibition rates of pS-sh-PANC-1 (pS-sh), pS-sh-PANC-1 incubated with 0.1 μg/ml of Gemcitabine (pS-sh + G), untransfected PANC-1 cells incubated with 0.1 μg/ml of Gemcitabine (**g**). * $P < 0.05$

well-established family of proteins that has a significant impact on mitochondrial integrity by influencing the permeability of the mitochondrial membrane. Bcl-2 family members can be subdivided into anti- and pro-apoptotic proteins. Mcl-1 is an anti-apoptotic member of the Bcl-2 family, which has been demonstrated, to be highly expressed in various human tumor specimens, including pancreatic carcinoma [30, 31]. Mcl-1 expression predicts response to anti-cancer treatment in chronic lymphocytic leukemia or patients with metastasized colorectal cancer. Downregulation of Mcl-1 leads to sensitization of tumor cells to different treatment regimens in vitro, as shown in late-stage prostate cancer, malignant melanoma, ALL and CLL [18–20]. Direct targeting of Mcl-1 by antisense oligonucleotides has already been shown to sensitize the HCC cell line and lung carcinoma cell lines to cisplatin induced apoptosis [32, 33].

Gemcitabine has been shown to ameliorate disease-related symptoms and to extend median survival for patients with advanced pancreatic carcinoma. It is considered the standard of care as first-line treatment for pancreatic cancer [34]. Gemcitabine may be more effective as adjuvant therapy after complete surgical resection of pancreatic cancer and may increase survival by as much as 6 months in patients receiving surgery [35]. Gemcitabine is a deoxycytidine analogue. Intracellular phosphorylation of Gemcitabine

produces diphosphate and triphosphate molecular forms capable of acting as fraudulent bases in DNA and also capable of inhibiting DNA synthesis—dependent ribonucleotide reductase [36]. Studies indicated that resistance to Gemcitabine was dependent on mitochondria-mediated apoptosis [37, 38], but other mediators of gemcitabine-mediated apoptosis have been described [39, 40]. The precise mechanism has not been well known. Overexpression of anti-apoptotic bcl-2 members has been shown to be the main reason for failure of Gemcitabine treatment in pancreatic carcinoma [41], so downregulation of bcl-2 members by specific siRNA in pancreatic cancer cells may lower the apoptotic threshold and enhance the chemosensitivity to Gemcitabine.

In this study, we applied specific inhibition of Mcl-1 expression by RNAi to investigate the role of the anti-apoptotic Bcl-2 family member Mcl-1 for the sensitivity of three different pancreatic cancer cell lines towards chemotherapy. We established cell lines stably expressing shRNAs through G418 selection. MTT and colony formation assay showed that pS-sh against Mcl-1 inhibited cell proliferation and colony formation contrast with the pS-nc and pS-vc controls. The cell cycle and apoptosis analysis indicated that downregulation of Mcl-1 induced notable apoptosis. The PARP cleavage and caspase-3 activity were detected, and the results indicated that he PARP cleavage and caspase3 activity significantly increased in pS-sh-PANC-1, coincided with results of Yang et al, which believed that proteasome inhibitors induced increasing caspase-3 activity and cell apoptosis by down-regulating of Mcl-1 via the mitochondrial apoptotic pathway [38]. Our results indicated that the apotosis induced by inhibition of Mcl-1 in pancreatic cells may be mediated through the mitochondrial apoptotic pathway.

We injected the athymic nude mice with PANC-1 cells stably expressing shRNA targeting Mcl-1 and monitored the tumor growth. The tumorigenicity was significantly inhibited by down-regulating expression of Mcl-1 in vivo. Inhibition to tumorigenicity may be associated with the low expression level of Mcl-1 detected by RT-PCR in tumor tissue (data not shown). Moreover, we found that the PANC-1 cell line was not sensitive to Gemcitabine compared with BxPC3 and SW1900 as previous report [42], so we analysed the effects on chemosensitivity of PANC-1 cell by inhibition of Mcl-1 combined with Gemcitabine. Co-incubation with low concentration of Gemcitabine (0.1 μg/ml), which was ineffective when applied alone, lead to a synergistic increase in inhibition of the proliferation in the PANC-1 cells, the results showed that downregulation of Mcl-1 could markedly lower the apoptotic threshold and enhance chemosensitivity to Gemcitabine.

Taken together, our study supports the concept that Mcl-1 is an important survival factor for pancreatic carcinoma. Targeting of Mcl-1 via RNA interference is a potential therapeutic strategy to render pancreatic carcinoma cells more sensitive to chemotherapy and molecularly targeted treatment regimens. The molecular mechanisms that mediate the pro-apoptotic effect of Mcl-1 downregulation in pancreatic cells remain elusive and are subject to further studies.

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