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Effect of inhibition of MEK pathway on 5-aza-deoxycytidine-suppressed pancreatic cancer cell proliferation

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ABSTRACT. Pancreatic adenocarcinoma is a lethal disease because it is inoperable at the time of diagnosis. Therefore, the search for new therapeutic approaches is critical. The abnormal expression of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK) pathway and alteration in epigenetic modification (DNA methylation and acetylation of histones) is a common feature in the majority of human pancreatic adenocarcinomas. Because DNA methyltransferase levels are regulated by the MEK pathway, we examined the effects of an MEK inhibitor, PD98059, on the action of DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-aza-dC), the epigenetic agent in the pancreatic cell line CFPAC1. Our results showed that PD98059 significantly potentiated the capability of 5-aza-dC to induce a cessation of cell proliferation concomitant with cell cycle arrest. We also observed an increase in tumor suppressor gene expression associated with the efficacy of treatment with PD98059 and 5-aza-dC. Further studies explored the molecular mechanisms by which

5-aza-dC induced the expression of $p21^{WAF1}$. We found that 5-aza-dC induced acetylation of histone H3 on the $p21^{WAF1}$ gene promoter and demethylation status on the $p21^{WAF1}$ gene promoter region. These effects were strikingly enhanced by the concomitant blockade of the MEK pathway. Furthermore, knockdown of $p21^{WAF1}$ by small interfering RNA rescued human pancreatic cancer cells from 5-aza-dC-mediated growth inhibition. Taken together, our results show that the MEK inhibitor enhanced the effects of 5-aza-dC in human pancreatic cancer cells. These results suggest that the MEK signal pathway may be a potential target for pancreatic cancer therapy.

Key words: Pancreatic cancer cell; MEK pathway; DNA methylation; Histone H3; $p21^{WAF1}$

INTRODUCTION

Pancreatic adenocarcinoma is a lethal disease and the 5th most common cause of cancer death worldwide (Pérez-Mancera et al., 2012). Mortality in this disease occurs mainly because the cancer is inoperable at the time of diagnosis and has a striking resistance to conventional treatments (Rittenhouse et al., 2011). The molecular basis of this aggressive clinical behavior remains to be completely elucidated. Epigenetic modifications, such as DNA methylation and acetylation and methylation of histones, modulate chromatin structure and regulate gene expression (Lomberk et al., 2008). Alteration in methylation status has been recognized as an epigenetic mechanism of selection during tumorigenesis in pancreatic cancer, and a correlation between methylation patterns of pancreatic adenocarcinomas and their clinicopathological features has been suggested (Omura and Goggins, 2009). In addition, the hypermethylation of multiple specific genes appears to be a way for the tumor to silence key genes, such as retinoblastoma protein 1, von Hippel-Lindau tumor suppressor, cyclin kinase inhibitors (p15, p16), estrogen receptors, and mutL homolog 1 (Jiao et al., 2007; Cavallari et al., 2009). Epigenetic drugs such as histone deacetylase inhibitors and DNA methyltransferase (DNMT) inhibitors can partially restore the distorted epigenetic picture by removing inactive markers (e.g.; DNA methylation) and inducing active markers (e.g.; histone acetylation), resulting in upregulation of the target genes (Espino et al., 2009).

Of all human malignancies, pancreatic cancer displays the highest frequency (70-90%) of somatic activating mutations in RAS genes (mainly KRAS) (Kennedy et al., 2011). RAS-regulated signaling pathways play an important role in the initiation and progression of the disease. The best understood of these pathways is the mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase (MEK) pathway, which consists of Raf-MEK-ERK kinases (Shin-Kang et al., 2011). This pathway has been the subject of intense scrutiny in attempts to understand its fundamental role in cancer cell biology and as a target for therapeutic intervention (Wallace et al., 2005). One of the key roles of the Raf-MEK-ERK pathway in a wide variety of mammalian cell types is the regulation of the cell division cycle. A number of pharmacologic agents, including PD098059, U0126, and CI-1040, have been described that inhibit MEK signaling in mammalian cells. These agents inhibit ERK activity accompanied by G1 cell cycle arrest. Cell cycle progression is regulated by interactions between cyclins and cyclin-dependent kinase inhibitors (CDKIs) (Gysin et al., 2005). CDKIs

include 2 families: CIP/KIP (e.g., $p21^{WAF1}$ and $p27^{KIP1}$) and INK4 (e.g., $p16^{INK4a}$). The increased expression of $p21^{WAF1}$, $p27^{KIP1}$, and $p16^{INK4a}$ may play a crucial role in the growth arrest induced in transformed cells (Gysin et al., 2005; Jiao et al., 2007).

In the present study, we used PD98059 to explore the role of the MEK pathway in the proliferation of human pancreatic cancer-derived cell lines *in vitro*. PD98059-induced cell G1 cycle arrest was invariably accompanied by the induced expression of CDKI $p21^{WAF1}$. Blockade of the MEK pathway strikingly potentiated the capability of 5-aza-2'-deoxycytidine (5-aza-dC) to mediate growth arrest of pancreatic cancer cells and upregulate $p21^{WAF1}$. The upregulation of $p21^{WAF1}$ may be involved in epigenetic modifications.

MATERIAL AND METHODS

Cell cultures and treatments

Pancreatic cancer cell line CFPAC-1 cells (originating from liver metastasis) were cultured at 37°C in 5% CO_2 using RPMI 1640 medium (GIBCO, USA) supplemented with 10% fetal bovine serum, penicillin, streptomycin, and glutamine. Cells were grown to low density 24 h before treatment and switched to culture medium supplemented with 0-5 μM 5-aza-dC (Sigma, USA) diluted in acidic phosphate-buffered saline (PBS) or 5-75 μM MEK inhibitor PD98059 (Promega, USA) diluted in dimethyl sulfoxide. Cells were treated with PBS or dimethyl sulfoxide as a vehicle control according to the observations of Primeau et al. (2003).

Cell viability assay

Cells (5 x 10^5/mL) were cultured with various concentrations of 5-aza-dC or PD98059 for 1 or 2 days in 96-well plates. After culture, 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent (Sigma) was added and incubated for 3 h at 37°C before adding 20% sodium dodecyl sulfate to dissolve formazan crystals and measuring at the 570-nm wavelength using a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA, USA). All experiments were performed in triplicate and repeated at least 3 times.

Cell cycle profile

Cell cycle analysis was performed with flow cytometry on cells incubated with various concentrations of either 5-aza-dC or PD98059. Approximately 1 x 10^8 cells were trypsinized, washed twice with PBS, and fixed in ice-cold ethanol for 1 h. The samples were then concentrated via removal of ethanol and exposure to 1% (v/v) Triton X-100 (Sigma) and 100 μg/mL RNase A (Sigma) for 10 min at 37°C. Cellular DNA was stained with propidium iodide. Cell cycle distributions were determined using a flow cytometer (Model FACSCALIBAR, USA). Data analysis was performed using the MultiCycle software package (Phoenix, USA).

Inhibition of $p21^{WAF1}$ using small interfering RNA (siRNA)

Control siRNA and siRNA against $p21^{WAF1}$ were purchased from Sigma. Briefly, CFPAC-1 cells were seeded in 12-well plates in medium containing 10% serum at a density that

would allow cells to reach 50% confluence on the day of transfection. The transient transfection was performed using 100 nM siRNA duplex and then complexed with transfection reagent in serum- and antibiotic-free culture fluid for 5 h at 37°C. A day after the transfection, the medium was replaced with normal growth medium, and 1 μM 5-aza-dC or 25 μM PD98059 was added for another 48 h of incubation. All experiments were repeated at least 3 times. The transfection efficiency was determined with fluorescein-conjugated nonspecific siRNA-transfected cells using a fluorescence microscope (Olympus, Japan). Subsequently, the cells were prepared for protein extraction and fluorescence-activated cell sorting analysis. Cells were incubated for 5 min at room temperature with lysis buffer (K_2HPO_4, Triton), and lysates were assayed for luciferase activity. Luciferase activity was quantitated with luciferase assays (Promega, USA) using a luminometer.

RNA extraction and real-time polymerase chain reaction (PCR)

Total RNA was extracted using TRIZOL (Gibco, USA) according to the manufacturer protocol. Real-time PCR was carried out to measure the levels of $p21^{WAF1}$ using SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) as described. The primer sequences were as follows: $p21^{WAF1}$ forward, 5'-CTGGAGACTCTCAGGGTCGAA-3', reverse 5'-GGATT AGGGCTTCCTCTTGGA-3', $p16^{INK4a}$ forward, 5'-CATAGATGCCGCGGAAGGT-3', reverse 5'-CAGAGCCTCTCTGGTTCTTTCAA-3', $p27^{KIP1}$ forward 5'-GCAGTGTCCAGGGATGAG GA-3', reverse 5'-TCTGTTCTGTTGGCCCTTTTGT-3'. Sequences were provided by Shenyou (Shanghai, China). The results were expressed as the ratio of copies of target genes to β-actin. Relative quantitation data were obtained using the comparative cycle threshold (Ct) method with an ABI PRISM 7700 Sequence Detection System (software version 1.6, ABI, America) according to the manufacturer protocol. The relative changes in gene expression were calculated using the following formula: fold change in gene expression, $2^{-\Delta\Delta Ct} = 2 - [^{\Delta}Ct$ (drug-treated samples) $- {}^{\Delta}Ct$ (untreated control)], where $^{\Delta}Ct = Ct$ (detected genes) - Ct (β-actin) and Ct represents threshold cycle number (Gasparino et al., 2012).

Western blot analysis

Protein concentration was measured using a Bio-Rad protein assay (Bio-Rad, America) and bovine serum albumin (Sigma) to generate a standard curve. In all, 20 mg proteins was fractionated by electrophoresis in 8 or 15% sodium dodecyl sulfate-polyacrylamide 1-mm gels and electroblotted onto nitrocellulose membranes. Blocking buffers were either 5% non-fat dry milk or 1.5% bovine serum albumin plus 0.05% Tween 20 in PBS. The primary antibodies were all from Upstate (USA) and included ERK1/2, phospho-ERK1/2 (Cell Signaling Technology, America); $p21^{WAF1}$ (Calbiochem, Germany), $p16^{INK4a}$, $p27^{WAF1}$ (Santa Cruz Biotechnology, USA); and β-actin (Sigma). Secondary antibodies (anti-rabbit anti-mouse) conjugated with horseradish peroxidase were from Santa Cruz Biotechnology. Blots were visualized using enhanced chemiluminescence (Amersham Biosciences, UK) and BioMax films (Kodak, Japan). The antibody of β-actin was used as a control for protein input. The western blot analysis was repeated a minimum of 3 times.

Methylation status of the $p21^{WAF1}$ promoter region

Cells were incubated in growth medium supplemented with 0-5 μM 5-aza-dC or 25 μM

PD98059 for 48 h. Methylation patterns in the CpG island of these genes were assessed with chemical bisulfite modification as described previously (Almeida et al., 2009). We tested the presence of methylcytosine in the CpG site inside in the promoter region of $p21^{WAF1}$ using PCR associated with the methylation-sensitive restriction enzyme *Hpa*II (New England Biolabs, UK). The primers were designed without CpG dinucleotides to enable both methylated and unmethylated alleles to be amplified (forward primers 5'-GAGTGTAGGGTGTAGGGAGATTG-3' and reverse primers 5'-TCTGGCAGGCAAGGATTTAC-3'). The amplification conditions were 25 cycles of 94°C for 45 s, 60°C for 40 s, and 72°C for 60 s, with a final extension of 5 min at 72°C. The PCR reaction product was directly loaded onto 3% agarose gels and electrophoresed.

Chromatin immunoprecipitation assay

CFPAC-1 cells (5 x 10^5/mL) were cultured with 0-5 μM 5-aza-dC or 25 μM PD98059 for 24 h. Formaldehyde was added to the cells to a final concentration of 1% and the cells were incubated at 37°C for 10 min. The cells were collected and subjected to a chromatin immunoprecipitation kit (Upstate Biotechnology) according to the manufacturer protocol. Antibodies used in the immunoprecipitations were purchased from Upstate Biotechnology and they were recognized as acetylated histone H3. Immunoprecipitated DNA was eluted in a total volume of 50 μL, and 2 μL were used for PCR. The primers for the $p21^{WAF1}$ promoter were as follows: forward, 5'-CGTGGTGGTGGTGAGCTAGA-3', reverse, 5'-CTGTCTGCACCTTCGCTCC T-3'. Real-time PCR was carried out using SYBR Green PCR Master Mix (Applied Biosystems). The amplified DNA was electrophoresed in a 2% agarose gel and visualized after staining with ethidium bromide. Enrichment was calculated as the ratio between the net intensity of each bound sample divided by the input (cross-linked DNA/protein complexes) and the vehicle control sample divided by the input [(bound/input)/(control/input)].

Statistical analysis

Each growth inhibition or flow cytometry experiment was repeated 3-4 times and the average data has been presented. Values of growth inhibition studies were expressed as a percentage of untreated controls. The difference between 2 groups under multiple conditions was analyzed with one-way analysis of variance. All data are reported as means ± standard error. Data were analyzed using StatView, and a P value of <0.05 indicated statistically significant difference.

RESULTS

Effect of blockade of MEK pathway on the growth delay induced by 5-aza-dC treatment

We examined the growth inhibition response of CFPAC-1 pancreatic cancer cells exposed to either the DNMT inhibitor 5-aza-dC (0.5, 1, 3, and 5 μM) or the MEK inhibitor PD98059 (5, 25, 50, and 75 μM) for 1 or 2 days. Cell growth was significantly inhibited by treatment with 3 μM 5-aza-dC for 24 h and 1 μM 5-aza-dC for 48 h compared with that in the control cells. We used various concentrations of PD98059 and found that 25 μM PD98059 significantly enhanced 5-aza-dC-induced growth inhibition of CFPAC-1 cells, although the

effect of PD98059 was not dose dependent. PD98059 significantly potentiated the capability of 5-aza-dC to inhibit the proliferation of CFPAC-1 at 48 h (Figure 1). Therefore, 25 μM PD98059 was used for further experiments.

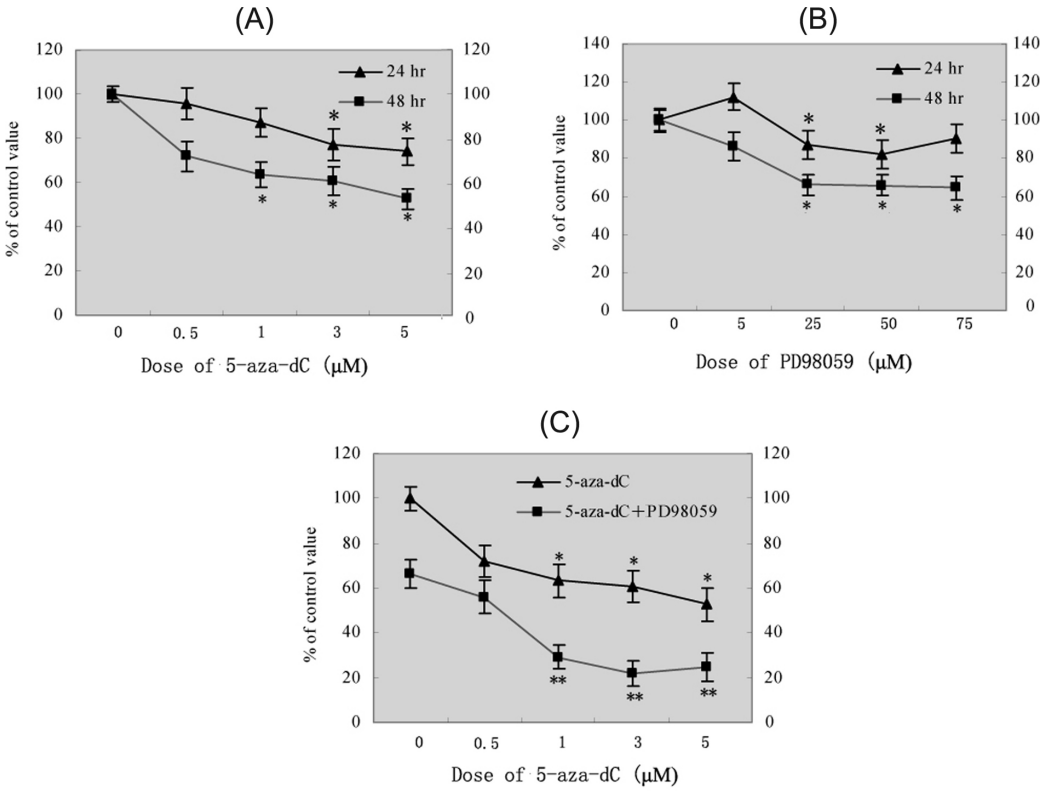

Figure 1. PD98059 potentiates the cell growth suppression induced by 5-aza-deoxycytidine (5-aza-dC) in pancreatic cancer cell line CFPAC-1 cells. CFPAC-1 cells were cultured on a 96-well plate until sub-confluent growth. Viable cells were measured by MTT assay. Two independent time and dose courses were performed in triplicate. Cells were treated with (**A**) 5-aza-dC (0.5-5 μM), or (**B**) PD98059 (5-75 μM), and (**C**) the combination of both for 48 h. Results are reported as means ± SD of three experiments performed in triplicate; and the control cells were treated with phosphate-buffered saline (PBS) and/or dimethyl sulfoxide (DMSO). *P < 0.05, **P < 0.01 compared with the control.

Effect of inhibition of MEK pathway on the cell cycle arrest induced by 5-aza-dC treatment

To study further the influence of the MEK pathway inhibitor on cell cycle arrest induced by 5-aza-dC, we evaluated the effects of 25 μM PD98059 and 1 μM 5-aza-dC on cell cycle distribution of CFPAC-1 at 48 h. As shown in Table 1, cell cycle analysis using propidium iodide staining showed a delay in the progression, with an increased proportion of cells in the G2/M and S phases when cells were exposed to 5-aza-dC alone. These cells accumulated in the G0/G1 phase of the cell cycle when cells were exposed to PD98059 alone. The percentage of cells in the G2/M and S phase of the cell cycle were further increased when cells were

exposed to PD98059 and 5-aza-dC at these same concentrations compared with that when treated with 5-aza-dC alone or in control cells.

Table 1. Cell cycle distribution: percentage of cells involved in the three phases of cell cycle as measured by propidium iodide staining in treated and control samples.

Treatment group	G1/G0	S	G2/M
Control	60.32 ± 3.71	27.93 ± 2.81	11.78 ± 1.54
5-aza-dC	45.54 ± 3.42*	32.82 ± 2.36*	22.67 ± 2.41**
PD98059	69.52 ± 4.25**	20.38 ± 2.41	10.13 ± 1.24
5-aza-dC + PD98059	40.81 ± 3.32**	32.23 ± 2.21*	25.45 ± 2.37**
$p21^{WAF1}$siRNA + 5-aza-dC	55.83 ± 3.64	26.38 ± 2.53	18.44 ± 1.56
$p21^{WAF1}$siRNA + PD98059	60.35 ± 3.83	22.32 ± 2.87	17.23 ± 1.98
$p21^{WAF1}$siRNA + 5-aza-dC+PD98059	52.32 ± 3.21	30.32 ± 3.01*	16.97 ± 2.03

The assay was carried out in triplicate after the treatment with 1 μM 5-aza-deoxycytidine (5-aza-dC), 25 μM PD98059, siRNA alone or combination, the control group treatment with PBS, DMSO, and/or siRNA, and analyzed by flow cytometry. *P < 0.05, **P < 0.001.

Effects of 5-aza-dC and PD98059 with cell cycle-related proteins

Cells were incubated with the MEK signaling inhibitor, and cell lysates were analyzed for ERK phosphorylation via western blot analysis. Total ERK1/2 protein levels did not decrease in the 5-aza-dC- and PD98059-treated lysates compared with those in the vehicle controls. However, the active form of ERK1/2, phospho-ERK1/2, was decreased in the presence of PD98059. PD08059 treatment decreased the phosphorylation of ERK, but exposure to 5-aza-dC alone failed to decreased levels of phospho-ERK1/2 (Figure 2). Because both 5-aza-dC and the MEK signal pathway can affect the cell cycle, we next explored whether 5-aza-dC or PD98059 modulate the expression of a panel of key components of the cell cycle machinery.

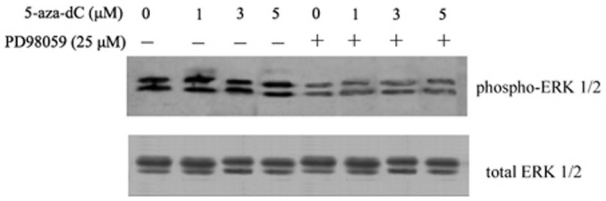

Figure 2. MEK inhibitors downregulate the phosphorylation of ERK. Cells were lysed, and equal amounts of protein were analyzed by Western blot analysis using antibodies against phospho-ERK1/2 or total ERK1/2. The data shown are representative of three replicate experiments. Cells were treated for 48 h with 5-aza-deoxycytidine (5-aza-dC) (0-5 μM) and/or PD98059 (25 μM).

The cell cycle inhibitor $p21^{WAF1}$ is a well-characterized target of 5-aza-dC and PD98059. We analyzed the expression of $p21^{WAF1}$ and other CDKIs, $p16^{INK4a}$ and $p27^{WAF1}$, in CFPAC-1 cells using western blot analysis. 5-Aza-dC (1, 3, or 5 μM) or PD98059 (25 μM) alone induced $p21^{WAF1}$ expression, and the combination of both dramatically increased levels of $p21^{WAF1}$. Real-time PCR results showed the same tendency (Figure 3). Inactivation of the $p16^{INK4a}$ gene is a frequent event in pancreatic cancer owing to either

mutation or epigenetic silencing by DNA methylation. The status of $p16^{INK4a}$ in various pancreatic cancer cell lines has already been assayed and has shown promoter hypermethylation. As expected, we found that the expression of $p16^{INK4a}$ protein was upregulated in CFPAC1 when treated with 5-aza-dC, whereas the upregulation of $p16^{INK4a}$ was not significant compared with that after treatment with 5-aza-dC alone or in combination with PD98059. PD98059 slightly induced $p16^{INK4a}$ expression (see Figure 3). Further experiments confirmed that PD98059 induced expression of $p27^{KIP1}$, but 5-aza-dC failed to induce expression of $p27^{KIP1}$ (see Figure 3). These results agree with those of the real-time PCRPCR data YES, Please help to make the revise and show that 5-aza-dC and PD98059 upregulate transcription and translation of the $p16^{INK4a}$ and $p27^{WAF1}$ genes (see Figure 3B and C). Therefore, we hypothesize that $p21^{WAF1}$ plays an important role in 5-aza-dC- and PD98059-mediated growth inhibition of pancreatic cancer cells.

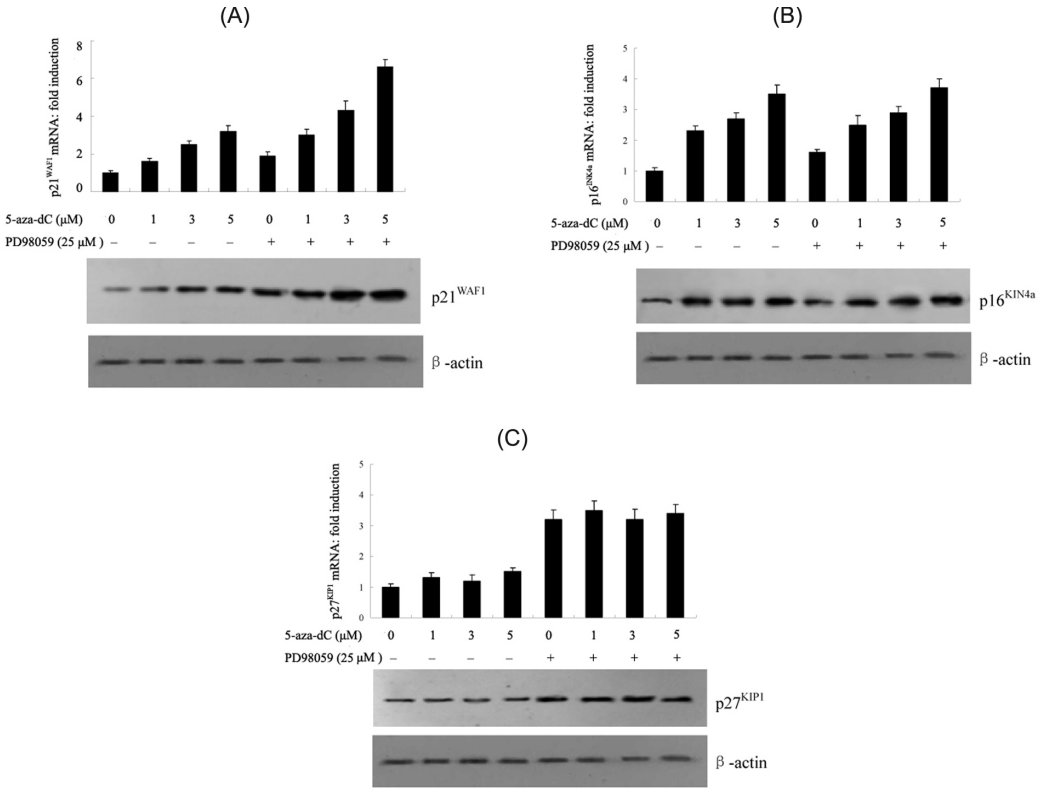

Figure 3. Induction of tumor supressor genes in pancreatic cancer cells after 5-aza-deoxycytidine (5-aza-dC) and PD98059 treatment. CFPAC-1 cell lines were treated for 48 h. The concentration was determined in a separate experiment (Material and Methods). The total RNA was isolated from the various dose treatment. The histogram shows the fold induction of mRNA measured by real-time PCR analysis from the same dish of cells with Western blot. The data are representative of three replicate experiments. **A.** Induction of $p21^{WAF1}$ mRNA and protein, the fold induction was calculated by taking the ratio of $p21^{WAF1}$ mRNA abundance in the 5-aza-dC and/or PD98059-treated cells relative to the PBS and/or DMSO control-treated cells. **B.** Induction of $p16^{INK4a}$ mRNA and protein expression. **C.** Induction of $p27^{KIP1}$ mRNA and protein expression.

Silencing p21^{WAF1} with siRNA rescue of CFPAC-1 cells from 5-aza-dC-induced growth arrest

To address the important role of p21^{WAF1} in 5-aza-dC- and PD98059-mediated growth inhibition of pancreatic cancer cells, we downregulated p21^{WAF1} via transient transfection with siRNA. After 24 h of transfection, the cells were exposed to either 1 μM 5-aza-dC or 25 μM PD98059 for 48 h and subjected to western blot and flow cytometry to quantify the levels of p21^{WAF1} and cell cycle progression, respectively. The transfection of control siRNA had little or no effect on p21^{WAF1} expression, whereas p21^{WAF1} siRNA led to a striking reduction in p21^{WAF1} expression. The siRNA against p21^{WAF1} almost completely blocked 5-aza-dC- and PD98059-induced expression of p21^{WAF1} (Figure 4). Depletion of p21^{WAF1} increased cell viability compared with that observed with control siRNA (see Figure 4). The p21^{WAF1} siRNA reduced the percentage of 5-aza-dC-treated cells that were arrested in G2/M with a concomitant increase in the percentage of cells transiting through S phase (see Table 1). These results suggested that the induction of p21^{WAF1} was critical in 5-aza-dC-mediated growth inhibition of pancreatic cancer cells.

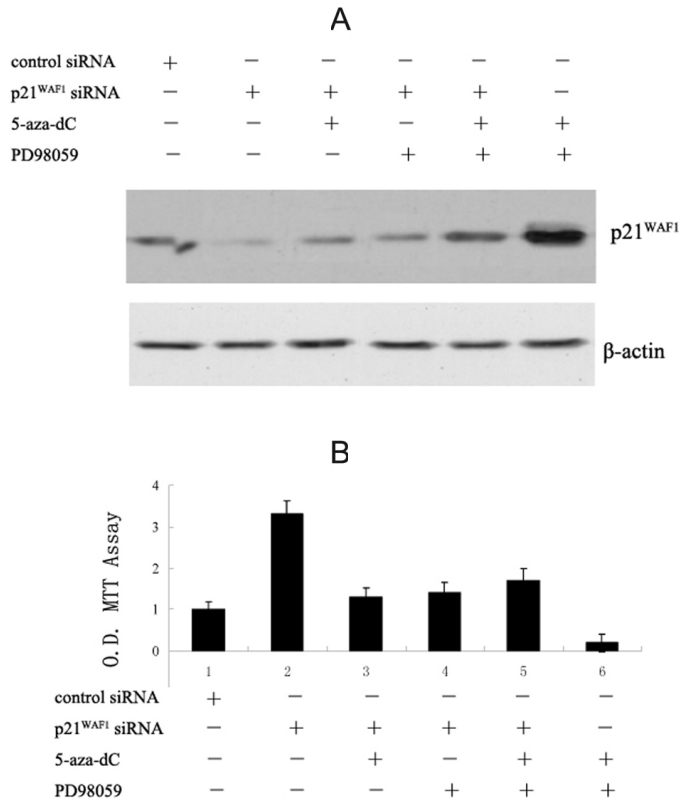

Figure 4. Depletion effect of p21^{WAF1} by siRNA potentiates the effects of 5-aza-deoxycytidine (5-aza-dC) in CFPAC-1 cells. The cells were transiently transfected either with control or p21^{WAF1} siRNA. After 24 h, these cells were exposed to either 5-aza-dC (1 μM) and/or PD98059 (25 μM) for 48 h and subjected to Western blot. The cell viability was quantified by MTT. **A.** siRNA almost completely blocked 5-aza-dC-induced expression of p21^{WAF1}. **B.** Cell viability was dramatically restored even expoured to 5-aza-dC.

Methylation status and acetylation of histone H3 induced by 5-aza-dC and PD98059

To investigate the mechanism of the p21^{WAF1} response to combined 0-5 μM 5-aza-dC and 25 μM PD98059 exposure for 48 h, we examined the epigenetic alteration of p21^{WAF1}. The promoter region of p21^{WAF1} contains palindromic sequences recognized by signal transducers and activators of transcription 1 (Chen et al., 2000). We analyzed the status of promoter methylation for p21^{WAF1} in CFPAC-1 cells by bisulfite-modified PCR of the modified DNA samples. The results are shown in Figure 5. The CpG island of the p21^{WAF1} promoter was methylated in CFPAC-1, which was reverted by 5-aza-dC but not by PD98059. These results suggested that 5-aza-dC-induced messenger RNA expression of the p21^{WAF1} gene might be by a methylation-dependent mechanism. One study has suggested that covalent chromatin changes could regulate the expression of p21^{WAF1} (Zheng et al., 2006). Chromatin immunoprecipitation analysis was performed using acetylated histone H3 antibodies as described in the materials and methods. Either 5-aza-dC or PD98059 treatment enriched acetylated histone H3 close to the p21^{WAF1} transcription start sites in CFPAC-1. The combination of 1 μM 5-aza-dC and 25 μM PD98059 for 48 h strikingly induced acetylation of histone H3 around the p21^{WAF1} gene (Figure 6) consistent with the results of western blot analysis.

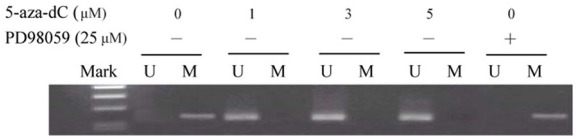

Figure 5. 5-aza-deoxycytidine (5-aza-dC) induced demethylation of the p21^{WAF1} promoters. Methylation-specific PCR (MSP) was performed on DNA from CFPAC-1 cells with primers designed to specifically detect methylated and unmethylated promoter regions. U = unmethylated-MSP; M = methylated-MSP. The data are representative of three replicate MSP experiments. Cells were treated for 48 h with 0-5 μM 5-aza-dC and 25 μM PD98059.

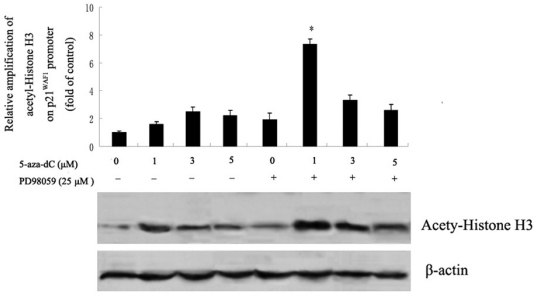

Figure 6. PD98059 enhances 5-aza-deoxycytidine (5-aza-dC)-induced acetylation of histone H3. CFPAC-1 cells were exposed to 5-azadC (1-5 μM) and/or PD98059 (25μM). After 48 h, cells were harvested and subjected to Western blot and chromatin immunoprecipitation (ChIP) assay. The Figure is representative of the two experiments performed independently. **A.** Nuclear extracts were prepared and subjected to Western blot analysis to measure the level of acetyl-histone H3 and β-actin. **B.** Acetylation of histone H3 in the p21^{WAF1} promoter was analyzed by ChIP assay. Real-time PCR were employed to quantify acetylated DNA precisely. The amplified sequences were normalized to those from input (the cross-linked DNA/protein complexes which were not immunoprecipitated with anti-acetylated histone H3 antibody). Results are reported as means ± SD of three experiments performed in triplicate; *P < 0.05, compared with the control.

DISCUSSION

Previous studies have convincingly proven the role of aberrant methylation and histone acetylation in the initiation and progression of several tumor types, including pancreatic adenocarcinoma (Omura and Goggins, 2009; Hong et al., 2012). The MEK pathway plays an important role in the regulation of cell division, and the current study as well as others suggest that an increase of MEK pathway activity may be responsible for the increased DNA methyltransferase enzyme activity and promoter hypermethylation of tumor suppressor genes. Hypermethylation is frequently associated with the silencing of genes, which may represent a selective advantage for growth and survival in the affected cells (Sato et al., 2008; Wang et al., 2009; An et al., 2011). The MEK pathway also affects covalent histone modification such as phosphorylation, acetylation, and methylation in localized promoter regions, which are markers for chromatin packing and transcription (Espino et al., 2009).

Pancreatic adenocarcinoma is characterized clinically by resistance to all current chemotherapy treatment (Takayama et al., 2008). Therefore, the aim of this study was to assess the efficacy of 5-aza-dC treatment in inhibiting cell growth and the effect of PD98059 enhancement of 5-aza-dC sensitization in pancreatic cancer models. We sought to identify the molecular mechanisms behind differential effects by analyzing alterations in tumor suppressor gene expression profiles induced by 5-aza-dC and PD98059 treatment in cell models.

Several clinical trials of 5-aza-dC administration conducted in diverse tumor types have shown various degrees of efficacy, particularly in solid tumors (Mostovich et al., 2011). Control of cell cycle progression is considered a potentially effective strategy for tumor growth, and the molecular analysis of common malignancies in humans have revealed that cell cycle regulators are frequently mutated (Kennedy et al., 2011). The results of the present study revealed strong inhibition of CFPAC-1 cell proliferation associated with arrest or delay in S or G2/M phase after treatment with 5-aza-dC. The predominantly cytostatic effects of 5-aza-dC have also been observed for other pancreatic cancer cell lines, such as PaCa44 and Panc-1, although the MiaPaCa2 cell line is relatively unresponsive (Missiaglia et al., 2005). The effects differ among cell lines; therefore, we selected the relatively responsive cell line CFPAC-1 in our study.

Inhibition of MEK/ERK signaling accumulates cells in the G0/G1 phase of the cell cycle (Wallace et al., 2005). For example, sorafenib, a selective inhibitor of B-Raf/MEK kinase, induces accumulation of malignant peripheral nerve sheath tumors and liposarcoma cells in G0/G1 in association with dephosphorylation of the retinoblastoma protein, which plays an important role in the progression of cells into the S phase of the cell cycle (Ambrosini et al., 2008). Inhibition of MEK by PD98059 also induces G0/G1 cell cycle arrest in CFPAC-1 cells. Interestingly, 5-aza-dC-induced growth inhibition and cell cycle arrest of CFPAC-1 cells were strikingly blunted when the MEK pathway was blocked by PD98059.

The data presented here suggest disruption of the uncontrolled cell cycle progression of these cells. One mechanism for PD98059-induced cell cycle arrest is through induced expression of $p21^{WAF1}$ and $p27^{KIP1}$. The mechanism of 5-aza-dC-induced cell cycle arrest is through $p16^{INK4a}$ and $p21^{WAF1}$, leading to inhibition of cyclin/cyclin-dependent kinase 2 activity. The CDKIs $p16^{INK4a}$, $p21^{WAF1}$, and $p27^{KIP1}$ bind with and inhibit the activity of CDK-cyclin complexes and, thus, regulate both G0/G1 and G2/M arrest transitions (Kim et al., 2007; Pal-Bhadra et al., 2012). The combination of 5-aza-dC and PD98059 induced striking expression of $p21^{WAF1}$.

Previously published data have suggested that reduced levels of $p27^{KIP1}$ expression are associated with poor prognosis in certain types of cancer (Culhaci et al., 2005). 5-Aza-dC

elicited no expression of $p27^{KIP1}$. Regulation of $p27^{KIP1}$ expression reportedly involves alterations in gene transcription, control of messenger RNA translation, and posttranslational regulation of protein stability (Missiaglia et al., 2005; Wang et al., 2008). The precise mechanisms underlying these observations are unknown. Consequently, MEK inhibitors may tend to have more of a cytostatic, as opposed to a cytotoxic, effect when used as single agents. However, inhibition of MEK might also prime the pancreatic cancer cell for apoptosis in response to concomitant radiotherapy or chemotherapy.

Previous studies on the effects of 5-aza-dC on gene expression profiles in colon and prostate cancer cell lines have already observed growth inhibition. Furthermore, inhibition of DNMT1 activity using a conditional mutation in a mouse model has shown a similar response at the gene expression profile level, indicating that the activation of this pathway is associated with the specific effect of 5-aza-dC on DNMT1 activity rather than a secondary cytotoxic outcome (Jackson-Grusby et al., 2001). In addition, a recent study has shown that the epigenetic silencing of multiple genes is involved in cellular immortalization, which is then reverted by treatment with 5-aza-dC, suggesting that this alteration may be associated with an early event in tumorigenesis (Guo et al., 2012). The tumor suppressor genes $p16^{INK4a}$ and p53, which are critically involved in pancreatic cancer tumorigenesis, are also implicated in the response to 5-aza-dC treatment (Ma et al., 2010; Yu et al., 2012). The analysis of the expression of $p16^{INK4a}$ protein confirmed its upregulation after 5-aza-dC treatment in CFPAC-1. However, the S and G2/M delay together with results of previous studies that have demonstrated $p16^{INK4a}$-independent growth inhibition of tumor cells induced by 5-aza-dC (Ma et al., 2010), suggesting that its effect is not limited to the re-expression of $p16^{INK4a}$.

Hence, we speculate that upregulation of $p21^{WAF1}$ may be responsible for cell cycle arrest or delay after treatment with 5-aza-dC and PD98059. Our study and others found hypermethylation of the Sis-inducible element (SIE)-1, SIE-1 site in the $p21^{WAF}$ promoter region in CFPAC1 cell lines, which was partially reverted by 5-aza-dC treatment and coupled with increased $p21^{WAF}$ expression at both the transcriptional and the protein levels (Missiaglia et al., 2005) Although the $p21^{WAF1}$ protein is frequently responsible for G1 arrest, substantial evidence supports another role in S/G2 transition. $p21^{WAF1}$ is thought to induce G2 arrest by interacting with the Cdc2-cyclinB1 complex together with proliferating cell nuclear antigen (PCNA). Furthermore, $p21^{WAF1}$ inhibits the interaction of PCNA and DNA polymerase delta, which is essential for DNA replication and repair and consequently inhibits DNA synthesis (Tan and Porter, 2009). Downregulation of DNMT1, which competes with $p21^{WAF1}$ for the same binding site on PCNA (Chuang et al., 1997; Tan and Porter, 2009), may further enhance the inhibitory effect of $p21^{WAF1}$ on both DNA synthesis and cell growth.

Other investigators have found that the exposure of colon carcinoma HCT116 cells or NB4 cells to 5-aza-dC induces G2/M cell-cycle arrest - a hallmark of DNA double-strand breaks - by monitoring the formation of g-H2AX foci and performing comet assay (Scott et al., 2006). The present study found an interesting biological function of 5-aza-dC using a low dose of 5-aza-dC (1 μM), which induced acetylation of histone H3. However, higher doses of this compound potently induced DNA demethylation in CFPAC-1 cells, and PD90859 enhanced the effects of 5-aza-dC through acetylation of histone H3 on the $p21^{WAF1}$ promoter region as well as through demethylation of the tumor suppressor gene promoter. Generally, 5-aza-dC is incorporated into DNA during replication, resulting in sequestration of DNMT1 and leading to replication-dependent global demethylation and gene reactivation (Chuang et al., 1997). Thus, 5-aza-dC might have biphasic effects.

Of particular interest, PD98059 enhanced the capability of 5-aza-dC to induce acetylation of histone H3 and DNA demethylation in CFPAC-1 cells, resulting in marked upregula-

tion of p21^{WAF1}. We found that inhibition of MEK by PD98059 modestly decreased acetylated histone H3 in CFPAC-1 cells. The inhibition of histone H3 acetylation by this MEK inhibitor may cause chromatin relaxation, leading to susceptibility to 5-aza-dC-induced expression of the p21^{WAF1} gene. Knockdown of p21^{WAF1} with siRNA protected CFPAC-1 cells from 5-aza-dC-induced cell growth delay, suggesting that p21^{WAF1} played a critical role in 5-aza-dC-induced growth inhibition in pancreatic cancer cells. In addition, these observations suggested that marked induction of the acetylated forms of histone H3 and p21^{WAF1} after exposure to 5-aza-dC may predict the responsiveness of pancreatic cells to this class of agent.

In a word, 5-aza-dC induced growth arrest and upregulation of p21^{WAF1} in CFPAC-1 cells via epigenetic mechanisms. Concomitant blockade of MEK signaling potentiated the action of 5-aza-dC in CFPAC-1 cells. The combination of a demethylation agent and a MEK inhibitor may open a new avenue for the treatment of individuals with pancreatic cancer.

Conflicts of interest

The authors declare no conflicts of interest.

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