1 *Anti-fibrotic agent pirfenidone suppresses proliferation of human*
2 *pancreatic cancer cells by inducing G0/G1 cell cycle arrest*

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11 Short Title: PFD suppresses cell proliferation of PCCs

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22 **Key Words**

23 Pirfenidone · Anti-proliferative effects · Pancreatic cancer cells · Fibroblasts · G0/G1 cell cycle arrest

1. Abstract

Background: Pirfenidone (PFD), which is an anti-fibrotic agent used for treatment of idiopathic pulmonary fibrosis (IPF), induces G0/G1 cell cycle arrest in fibroblasts. We hypothesized that PFD-induced G0/G1 cell cycle arrest might be achieved in other types of cells, including cancer cells. Here we investigated the effects of PFD on the proliferation of pancreatic cancer cells (PCCs) *in vitro*. ***Method:*** Human skin fibroblasts ASF-4-1 cells and human prostate stromal cells PrSC were used as fibroblasts. PANC-1, MIA PaCa-2 and BxPC-3 cells were used as human PCCs. Cell cycle and apoptosis were analyzed using flow cytometer. ***Results:*** First, we confirmed that PFD suppressed cell proliferation of ASF-4-1 cells and PrSC, and induced G0/G1 cell cycle arrest. Under these experimental conditions, PFD also suppressed cell proliferation and induced G0/G1 cell cycle arrest in all PCCs. In PFD-treated PCCs, expression of p21 was increased but that of CDK2 was not clearly decreased. Of note, PFD did not induce significant apoptosis among PCCs. ***Conclusions:*** These results demonstrated that the anti-fibrotic agent PFD might have anti-proliferative effects on PCCs by inducing G0/G1 cell cycle arrest. This suggests that PFD may target not only fibroblasts but also PCCs in the tumor microenvironment of pancreatic cancer.

Abbreviations

CAFs	cancer-associated fibroblasts
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
IPF	idiopathic pulmonary fibrosis
PCCs	pancreatic cancer cells
PFD	pirfenidone
PSCs	pancreatic stellate cells

2. Introduction

Pirfenidone (PFD), 5-methyl-1-phenyl-2-(1H)-pyridone, is an anti-fibrotic agent used for treatment of idiopathic pulmonary fibrosis (IPF) [1]. IPF is a chronic, progressive and often fatal lung disease that results in the proliferation of fibroblasts and deposition of extracellular matrix (ECM) in the interstitium and alveolar spaces of the lung. Early studies of PFD reported that it improved bleomycin-induced lung fibrosis in animal models [2, 3]. Several double blind, placebo-controlled phase III studies showed that PFD achieved a clinically meaningful effect and had a favorable safety profile in patients with IPF [1, 4]. On the basis of these data, PFD has been approved as a treatment for IPF in Japan, Europe and the United States since 2014. The most commonly reported side effects of PFD are gastrointestinal symptoms, including nausea, dyspepsia, anorexia and gastroesophageal reflux. These side effects are mild to moderate and reversible [1]. The safety of PFD was recently demonstrated in a study that pooled patients from the CAPACITY trials with several open-label trials; this large cohort demonstrated that long-term treatment with PFD was safe and well tolerated [1].

Efficacy of PFD on fibrosis has been well demonstrated. Specifically, PFD was shown to be effective in various *in vivo* models including a rat model of myocardial infarction [5, 6], a mouse model of pulmonary fibrosis [7], a mouse model of nonalcoholic steatohepatitis [8] and a rats model of renal fibrosis [9]. In agreement with *in vivo* model data, *in vitro* anti-proliferative effects of PFD were also confirmed with human leiomyoma cells [10], human Tenon fibroblasts [11] and rat cardiac fibroblasts [12]. Notably, Lin et al. have reported that PFD suppressed cell proliferation of human Tenon fibroblasts by inducing G0/G1 cell cycle arrest [11]. Other reports have demonstrated anti-proliferative effects of PFD in cancer-associated fibroblasts (CAFs) derived from human lung cancer patients [13] and myofibroblast-like pancreatic stellate cells (PSCs) derived from human pancreatic cancer patients [14].

Pancreatic cancer exhibits a very poor prognosis and poses unsolved problems in cancer treatment. Gemcitabine is often considered a standard drug for pancreatic cancer and is used as monotherapy or

73 in combination with other drugs [15, 16]. However, only 25–30% of patients respond to such
74 chemotherapeutic treatments [17, 18]. Pancreatic cancer is characterized by a very high content of
75 stroma. Active PSCs induced by their interaction with pancreatic cancer cells (PCCs), differentiate into
76 αSMA positive myofibroblast-like cells that produce high amounts of ECM proteins [19, 20]. The tumor
77 stroma or tumor microenvironment comprises various cell types, including CAFs, immune cells,
78 endothelial cells, mesenchymal cells, and multiple ECM components [21, 22]. Such a complex
79 heterogeneous environment forms a physical barrier against the delivery of cytotoxic anti-cancer drugs,
80 molecularly targeted biologics or nanomedicines to the tumoral milieu [13]. Consequently, the
81 heterogeneous environment diminishes the tumor-targeting performance and anti-tumor efficacy of
82 anti-tumor drugs. These findings highlight the need for novel agents that can improve therapeutic
83 outcome with less toxicity and fewer serious adverse events than gemcitabine monotherapy.

84 Recently, Kozono et al. reported that PFD showed not only anti-proliferative effects for PSCs but
85 also inhibition of invasiveness and migration of PSCs [14]. Moreover, their data demonstrated that PFD
86 suppressed orthotopic tumor growth consisting of PCCs co-implanted with PSCs. These data strengthen
87 the idea that targeting tumor stroma may improve cancer treatment for pancreatic cancer as compared
88 with gemcitabine monotherapy. Current evidence obtained *in vitro* indicates that PFD exerts its
89 pharmacokinetic effect by modulating TNFα and TGFβ pathways, decreasing collagen synthesis and
90 inhibiting differentiation of fibroblasts into myofibroblasts [23]. However, the effects of PFD on cancer
91 cells are not well understood. In this study, we investigated the anti-proliferative effects and the
92 mechanisms of action of PFD on PCCs *in vitro*.

93

94 ## 3. Materials and Methods

95

96 ***Drug***

97 PFD was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). PFD was dissolved in
98 DMSO (Nacalai Tesque, Inc., Kyoto, Japan) at a concentration of 0.1 to 0.5 g/mL and frozen at -20℃
99 until use.

100

101 ***Cell Culture***

102 Human skin fibroblasts (ASF-4-1 cells) were obtained from the Japanese Collection of Research
103 Bioresources Cell Bank (National Institutes of Biomedical Innovation, Health and Nutrition, Osaka,
104 Japan). Normal human prostate stromal cells (PrSC) were purchased from Lonza Group Ltd. Inc. (Basel,
105 Switzerland). Human pancreatic epithelioid carcinoma cell lines (PANC-1 and MIA PaCa-2) and the
106 human primary pancreatic adenocarcinoma BxPC-3 cell line were purchased from Public Health
107 England (Salisbury, UK). ASF-4-1 cells were cultured in Eagle's minimal essential medium (Sigma-Aldrich,
108 Inc., St Louis, MO, USA). PANC-1 and BxPC-3 cells were cultured in RPMI-1640 medium (Sigma-Aldrich,
109 Inc., St Louis, MO, USA), and MIA PaCa-2 cells were cultured in Dulbecco's Modified Eagle's Medium
110 (Sigma-Aldrich, Inc., St Louis, MO, USA). Each medium was supplemented with 10% fetal bovine serum
111 (FBS; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and antibiotics (100 U/mL penicillin and
112 100 µg/mL streptomycin; Nacalai Tesque, Inc., Kyoto, Japan). PrSC was cultured in a complete
113 commercial medium (SCBM Bullet Kit, Lonza Group Ltd., Walkersville, MD, USA). All cells were cultured
114 in a humidified atmosphere of 5% CO_2 at 37ºC.

115

116

117 ***Cell viability assay***

118 Fibroblasts (ASF-4-1 cells and PrSC) and PCCs (PANC-1, MIA PaCa-2 and BxPC-3 cells) were seeded
119 onto 12-well culture plates at a density of 1 x 10^4 fibroblasts per well or 3 x 10^4 PCCs per well. After 24
120 h, cells were treated with PFD (0.1 to 0.5 mg/mL) or vehicle (0.1% DMSO) and incubated for 72 h. Viable
121 cells were measured using a hemocytometer.

122

123 ***Cell-cycle analysis***

124 Fibroblasts (ASF-4-1 cells and PrSC) and PCCs (PANC-1, MIA PaCa-2 and BxPC-3) were seeded onto
125 90-mm culture dishes at a density of 3 x 10^5 fibroblasts per dish or 5 x 10^5 PCCs per dish. After 24 h,
126 cells were treated with PFD (0.3 mg/mL) or vehicle (0.1 % DMSO) for 24 h. The cells were isolated and
127 the phosphatidyl inositol (PI) stained using a Cycle TEST PLUS DNA Reagent kit (Becton Dickinson, San
128 Jose, CA, USA) following the protocol. The cells were incubated with trypsin solution for 10 min
129 followed by trypsin inhibitor and RNase solution for 10 min. Finally, the cells were further treated with
130 PI staining on ice for 10 min. A total number of 2 x 10^4 cells was subject to the cell cycle analysis using
131 a BD FACS Canto II flow cytometer (Becton Dickinson). The fractions of cells in the G0/G1, S, M phases
132 were calculated using a BD FACSDiva™ software (Becton Dickinson), and then the number of gated
133 cells in each phase was presented as %.

134

135 ***Western blot analysis***

136 Fibroblasts (ASF-4-1 cells and PrSC) and PCCs (PANC-1, MIA PaCa-2 and BxPC-3) were seeded onto
137 60-mm culture dishes at a density of 5 x 10^5 cells per dish. After 24 h, cells were treated with PFD (0.3
138 mg/mL) or vehicle (0.1% DMSO) for 24-48 h. The cells were washed with ice-cold PBS and then lysed
139 with the CelLytic™ M kit (Sigma-Aldrich, Inc., St Louis, MO, USA) that included a protease inhibitor
140 cocktail (Nacalai Tesque, Inc., Kyoto, Japan). The lysates were incubated for 15 min on ice, and the
141 lysates were centrifuged at 10,000g for 10 min, after which the supernatants were collected. The

protein concentrations were measured using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). For Western blot analysis, protein samples were boiled for 3 min in Lane Marker Sample Buffers (Thermo Fisher Scientific, Waltham, MA, USA) and separated by gel electrophoresis (NuPAGEß Bis-Tris Precast Gel, Thermo Fisher Scientific, Waltham, MA, USA). The gel was transferred to an Immobilon membrane (Merck Millipore, Darmstadt, Deutschland) by a tank transfer method. The membranes were incubated with primary antibodies overnight at 4°C. Anti-p21, anti-CDK2, and anti-ß-actin (Cell Signaling Technology, Danvers, MA, USA) were used at dilutions of 1:1000, 1:1000, and 1:10000, respectively. After washing 3 times in Tris-buffered saline/Tween 20 (TBS-T), the membranes were incubated with secondary antibody conjugated with horseradish peroxidase (HRP) for 1 h at room temperature and after washing 3 times in TBS-T. The specific protein bands were detected with the LAS-4000 mini (Fuji Photo Film, Tokyo, Japan) using an Immobilon™ Western Chemiluminescent HRP Substrate (Merck Millipore, Darmstadt, Deutschland). Protein levels were compared using ß-actin as a loading control.

Detection of apoptosis

PCCs (PANC-1, MIA PaCa-2 and BxPC-3) were seeded onto 90-mm dishes at a density of 5 x 10^6 cells per dish. After 24 h, cells were treated with 0.3 mg/mL PFD or vehicle (0.1% DMSO) for 24 h. Cells were trypsinized and collected, and then cells were stained with Annexin V-fluorescein isothiocyanate (PE) and propidium iodide (PI) simultaneously using the PE Annexin V Apoptosis Detection kit I (Becton Dickinson, San Jose, CA, USA). The cell suspensions were analyzed with a BD FACS Canto II flow cytometer (Becton Dickinson, San Jose, CA, USA) to determine the percentage of early apoptotic cells (PE stained cells; lower right hand quadrant) and late apoptotic cells (PI stained cells; upper right hand quadrant).

167 ***Statistical analysis***

168 The results were expressed as means ± SD. Differences between 2 groups were determined using

169 Student's *t* test. Values of $p < 0.05$ were considered statistically significant.

4. Results

Effects of PFD on the proliferation of fibroblasts and PCCs

The proliferation of fibroblasts (ASF-4-1 cells and PrSC) was significantly suppressed by PFD treatment over 72 h (Figure 1). In this experimental condition, the proliferation of PCCs (PANC-1, MIA PaCa-2 and BxPC-3) was also significantly suppressed by PFD treatment over 72 h (Figure 1). Interestingly, the anti-proliferative effects of PFD on MIA PaCa-2 cells was comparatively lower than that on PANC-1 and BxPC-3 cells.

Effects of PFD on cell cycle progression of fibroblasts and PCCs

PFD induced G0/G1 cell cycle arrest in PCCs (PANC-1, MIA PaCa-2 and BxPC-3) as well as in fibroblasts (ASF-4-1 cells and PrSC) (Table 1). PFD-Induced G0/G1 cell cycle arrest in MIA PaCa-2 cells was modest although significant. To determine the molecular mechanisms by which PFD-induced G0/G1 cell cycle arrest in PCCs, we checked the expression of cell cycle regulatory proteins by Western blot. Compared with vehicle, expression of CDK2 was not clearly decreased in all PFD-treated cells including fibroblasts and PCCs (Figure 2). In contrast, expression of p21 was increased in PFD-treated PrSC, PANC-1, MIA PaCa-2 and BxPC-3 cells.

Effects of PFD on apoptosis in PCCs

In most PFD-treated PCCs (PANC-1, MIA PaCa-2 and BxPC-3), positive staining with PE and PI was not observed. As shown in Figure 3, PFD did not induce apoptosis among PCCs examined in this study.

5. Discussion

The major finding in this study was that the anti-fibrotic agent PFD showed anti-proliferative effects against PCCs as well as fibroblasts. In addition, we found that the anti-proliferative effects were due, at least in part, to induction of G0/G1 cell cycle arrest in PCCs.

Drug repositioning, which is the discovery of new indications for existing drugs that are outside their original indications, is an attractive mode of therapeutic discovery. For example, tricyclic antidepressants [24], and the anti-fungal agent itraconazole [25] reportedly suppress tumor-stromal interactions in various cancers, including small cell lung cancer and neuroendocrine cancer. In addition, tranilast, used for treatment of allergic rhinitis [26] and naftopidil, used for benign prostatic hyperplasia [27], reportedly possess anti-proliferative effects on human prostate cancer cells. Moreover, the anti-inflammatory agent aspirin [28], bazedoxifene (for treatment of osteoporosis) [29] and metformin (for treatment of type 2 diabetes) [30] have been explored as emerging chemoprevention and therapeutic agents in the treatment of pancreatic cancer via their anti-proliferative effects. Interestingly, PFD was reported to have anti-proliferative effects on a variety of cells, including human Tenon fibroblasts [11], rat cardiac fibroblasts [12], human leiomyoma cells [10], lung fibroblasts [7] and PSCs [14]. Therefore, we hypothesized that PFD might have anti-proliferative effects on PCCs.

The G1/S transition is a vital event in cell cycle progression. Here, we showed that PFD induced G0/G1 cell cycle arrest in not only fibroblasts but also PCCs. These responses were accompanied by increased p21. Our data suggest that accumulation of p21 could be a direct result of PFD treatment. Thus, our data suggests that change of p21 levels could be a direct result of PFD treatment. Although upregulation of p21 levels is widely believed to be p53 dependent, functional mutation of p53 is reported among all PCCs examined in this study [31, 32], Therefore, our results showed that PFD upregulated p21 levels without requiring functional p53 in PCCs. The several previous studies revealed that p53-independent mechanism for p21 upregulation may contribute to the cell cycle response [33-35]. Further studies are required to verify the existence of a p53-independent signaling pathway in this

217 context. Of note, our results showed that PFD did not induce apoptosis among all PCCs (PANC-1, MIA
218 PaCa-2 and BxPC-3). These findings support the safety of PFD use in clinical trials. In addition to PFD,
219 there are other nontoxic compounds that suppress cancer cell proliferation. For example, Hori et al.
220 reported that naftopidil suppressed cell proliferation of human prostate cancer cells and human
221 prostate fibroblasts by inducing G0/G1 cell cycle arrest but not apoptosis [36]. In addition, Wang et al.
222 reported that metformin induced G0/G1 phase cell cycle arrest in myeloma cells by targeting the
223 AMPK/mTORC1 and mTORC2 pathways, whereas no significant apoptosis was observed [37].

224 In PANC-1 and BxPC-3 cells, PFD remarkably suppressed cell proliferation and induced G0/G1 cell
225 cycle arrest, but this was not the case in MIA PaCa-2 cells. This result suggests the importance of the
226 choice of cancer cell lines. As is common in epithelial tumors, carcinogenesis develops through
227 accumulation of mutations and genetic lesions leading to activation of oncogenes and inactivation of
228 tumor suppressor genes. To investigate these molecular events, understanding of the genetic
229 background of cancer cell lines is essential. Pancreatic cancer cell lines commonly possess altered genes
230 (*KRAS, TP53, CDKN2A* and *SMAD4*) [38]. Such a genetic background likely contributes to the disparate
231 response to anti-proliferative agents like PFD. In the future, we will assess the genetic background of
232 cell lines to seek correlations with the cells' sensitivity to PFD exposure.

233 In summary, we demonstrated that the anti-fibrotic agent PFD might have anti-proliferative effects
234 on PCCs by inducing G0/G1 cell cycle arrest. This suggests that PFD may target not only fibroblasts but
235 also PCCs in the tumor microenvironment of pancreatic cancer.

236

8. Statements

8. 1. Acknowledgement

None.

8. 2. Statement of Ethics

The authors have no ethical conflicts to disclose.

8. 3. Disclosure Statement

The authors have no conflicts of interest to declare.

8. 4. Funding Sources

None.

8. 5. Author Contributions

Conception and design of study: Kenichiro Ishii, Yoshifumi Hirokawa, Masatoshi Watanabe; acquisition of data: Eri Usugi, Kazuki Kanayama, Chise Matsuda; analysis and interpretation of data: Katsunori Uchida, Taizo Shiraishi

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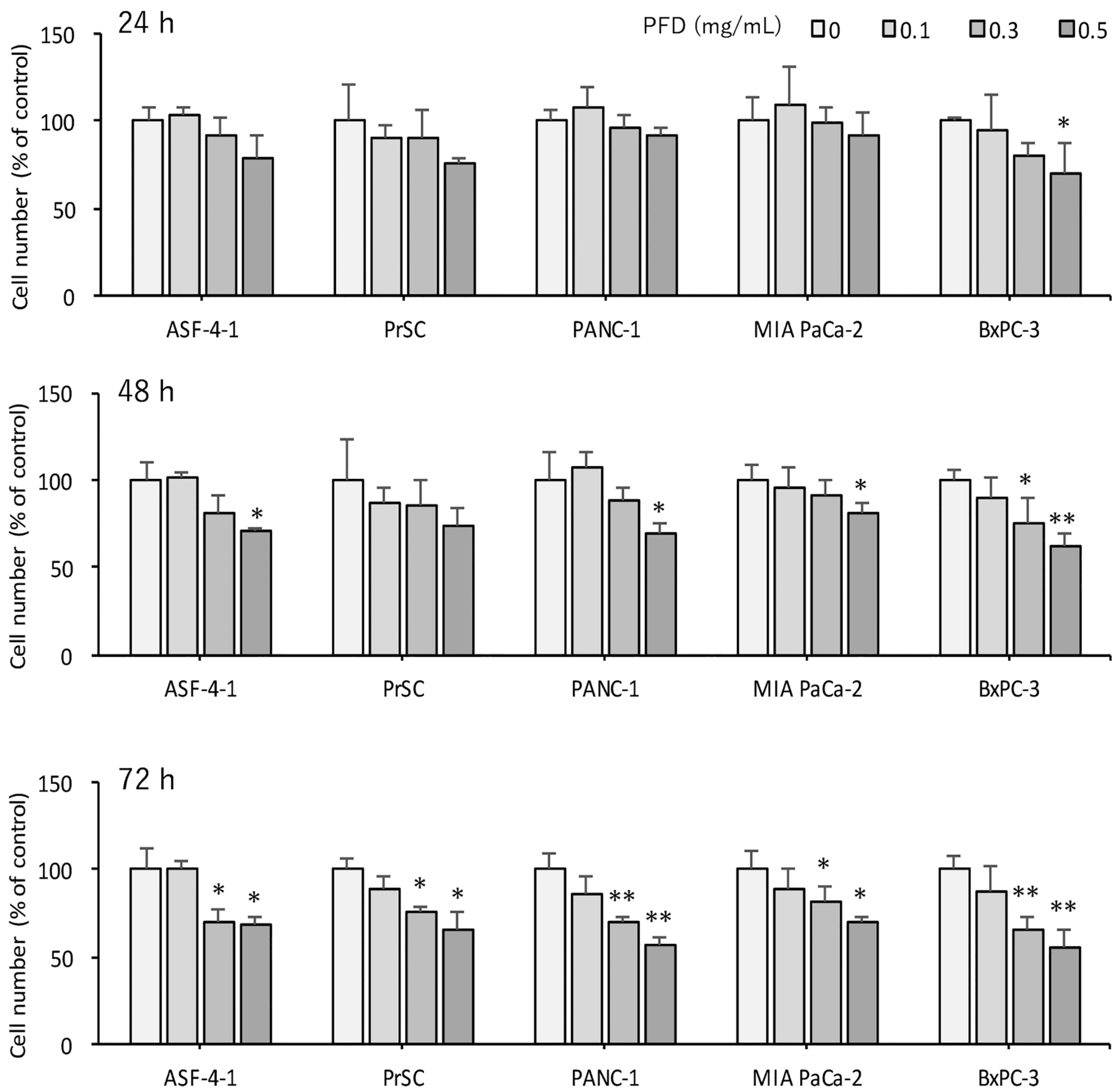
371 **10. Figure Legends**



372

373 **Fig. 1.** Effects of PFD on the proliferation of fibroblasts and PCCs

374 Fibroblasts (ASF-4-1 cells and PrSC) and PCCs (PANC-1, MIA PaCa-2 and BxPC-3) were treated with PFD.

375 Viable cells were counted using a hemocytometer. Values that represent the mean percentage ± SD of

376 viable cells are shown. *, $p < 0.05$; **, $p < 0.01$ versus vehicle-treated control.

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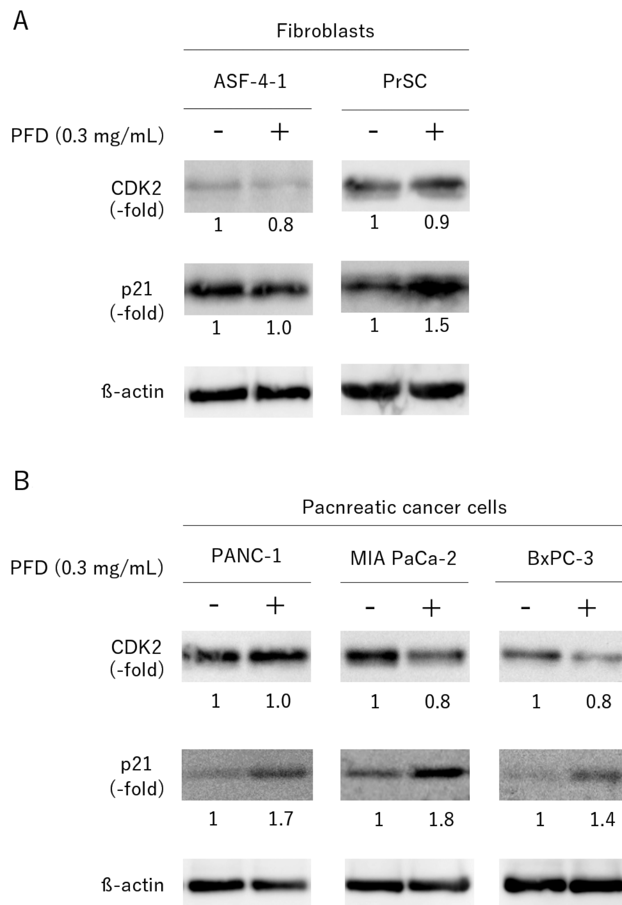

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380 **Fig. 2.** Effects of PFD on cell cycle progression in fibroblasts and PCCs

381 A: Fibroblasts (ASF-4-1 cells and PrSC) and B: PCCs (PANC-1, MIA PaCa-2 and BxPC-3) were treated with

382 PFD. Expression of cell cycle regulatory proteins was determined using Western blotting analysis.

383 Protein levels were compared using ß-actin as a loading control.

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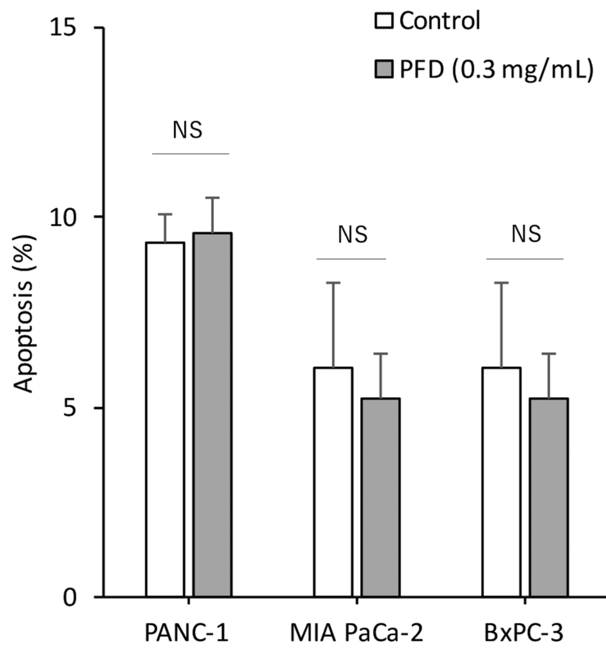

386

387 **Fig. 3.** Effects of PFD on apoptosis in PCCs

388 PCCs (PANC-1, MIA PaCa-2 and BxPC-3) were treated with PFD. Apoptosis was assessed through flow
389 cytometric analysis of Annexin/7AAD staining. Values represent the mean percentages ± SD of
390 apoptosis are shown. NS, not significant.

391

Table 1. Effects of PFD on cycle progression in fibroblasts and PCCs

			Phase (%)		
	Cell	PFD (mg/mL)	G0/G1	S	M
Fibroblasts	ASF-4-1	0	55.0±1.2	12.4±1.8	31.4±1.1
		0.3	62.1±0.9**	8.7±0.1*	27.6±0.5**
	PrSC	0	64.1±2.1	21.2±1.1	13.2±0.7
		0.3	74.2±4.1*	13.9±2.8*	10.5±1.2*
Pancreatic cancer cells	PANC-1	0	48.3±0.7	30.7±0.9	16.9±0.5
		0.3	56.8±2.0**	22.9±1.0**	15.6±0.9
	MIA PaCa-2	0	61.0±0.6	14.4±0.5	21.8±0.8
		0.3	65.8±0.4**	11.9±0.4**	19.7±0.8*
	BxPC-3	0	47.0±1.6	27.9±0.6	22.7±1.0
		0.3	60.0±1.0**	24.3±0.7**	13.8±0.9**

*, p <0.05; **, p < 0.01 versus vehicle-treated control.