

## Pirfenidone Inhibits Pancreatic Cancer Desmoplasia by Regulating Stellate Cells

Shingo Kozono<sup>1</sup>, Kenoki Ohuchida<sup>1,2</sup>, Daiki Eguchi<sup>1</sup>, Naoki Ikenaga<sup>1</sup>, Kenji Fujiwara<sup>1</sup>, Lin Cui<sup>1</sup>, Kazuhiro Mizumoto<sup>1,3</sup>, and Masao Tanaka<sup>1</sup>

### Abstract

Pancreatic stellate cells (PSC), which are implicated in desmoplasia in pancreatic cancer, enhance the malignancy of cancer cells and confer resistance to established treatments. We investigated whether the antifibrotic agent pirfenidone can suppress desmoplasia and exert antitumor effects against pancreatic cancer. Primary PSCs were established from pancreatic cancer tissue obtained during surgery. *In vitro*, pirfenidone inhibited the proliferation, invasiveness, and migration of PSCs in a dose-dependent manner. Although supernatants of untreated PSCs increased the proliferation, invasiveness, and migration of pancreatic cancer cells (PCC), supernatants of pirfenidone-treated PSCs decreased these effects. Exposure to PCC supernatant increased the production of platelet-derived growth factor-A, hepatic growth factor, collagen type I, fibronectin, and periostin in PSCs, which was significantly reduced by pirfenidone. Mice were subcutaneously implanted with PCCs (SUIT-2 cells) and PSCs into the right flank and PCCs alone into the left flank. Oral administration of pirfenidone to these mice significantly reduced tumor growth of co-implanted PCCs and PSCs, but not of PCCs alone. Pirfenidone also decreased the proliferation of PSCs and the deposition of collagen type I and periostin in tumors. In mice with orthotopic tumors consisting of PCCs co-implanted with PSCs, pirfenidone suppressed tumor growth, reduced the number of peritoneal disseminated nodules, and reduced the incidence of liver metastasis. Pirfenidone in combination with gemcitabine more effectively suppressed orthotopic tumor growth compared with pirfenidone or gemcitabine alone. In conclusion, our findings indicate that pirfenidone is a promising antitumor agent for pancreatic cancer, owing to its suppression of desmoplasia through regulating PSCs. *Cancer Res*; 73(7); 2345–56. ©2013 AACR.

### Introduction

Pancreatic cancer is characterized by excessive desmoplasia, which contributes to the aggressive behaviors of pancreatic cancer and resistance to traditional therapies through tumor–stromal interactions (1). Pancreatic stellate cells (PSC) have been identified in the interlobular areas and in peri-acinar lesions of the pancreas and are responsible for generating extensive fibrosis in pancreatic diseases (2, 3). PSCs are activated by various stimuli and transform from quiescent cells to myofibroblast-like cells expressing  $\alpha$ -smooth muscle actin (SMA; ref. 4). In pancreatic cancer, PSCs are activated by tumor–stromal interactions, including by direct contact with

pancreatic cancer cells (PCC) and paracrine growth factors secreted from PCCs including TGF- $\beta$ 1, platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and VEGF (5–8). Activated PSCs also secrete various growth factors, including PDGF, bFGF, TGF- $\beta$ 1, and connective tissue growth factor (CTGF), to stimulate PCCs via paracrine pathways and also activate PSCs via autocrine pathways. Activated PSCs can also produce abundant extracellular matrix (ECM) and generate preferable microenvironments for PCCs. *In vivo* studies revealed that PCCs co-implanted with PSCs showed significant augmentation of tumor growth and very excessive desmoplasia (8–10). Excessive ECM deposition around tumor cells was reported to inhibit the distribution and penetration of anti-cancer drugs by decreasing tumor vascularity and constructing physical barriers (11, 12). These data suggest that desmoplasia consisting of PSCs and ECM provides a significant contribution to the malignant behaviors of cancer cells and resistance to established therapies, particularly for pancreatic cancer. Therefore, new strategies targeting desmoplasia are needed to improve the treatment paradigm of pancreatic cancer.

Pirfenidone [5-methyl-1-2-[1*H*]-pyridone; Shionogi & Co. Ltd.; MARNAC Inc.] is a pyridone compound with therapeutic potential for idiopathic pulmonary fibrosis (IPF). Some double blind, placebo-controlled phase II and III studies showed that pirfenidone has clinically meaningful effects and a favorable

**Authors' Affiliations:** Departments of <sup>1</sup>Surgery and Oncology and <sup>2</sup>Advanced Medical Initiatives, Graduate School of Medical Sciences, Kyushu University; and <sup>3</sup>Kyushu University Hospital Cancer Center, Fukuoka, Japan

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**Corresponding Authors:** Kazuhiro Mizumoto, Department of Surgery and Oncology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Fukuoka 812-8582, Japan. Phone: 81-92-6425440; Fax: 81-92-6425458; E-mail: mizumoto@med.kyushu-u.ac.jp; and Kenoki Ohuchida, kenoki@med.kyushu-u.ac.jp

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safety profile in patients with IPF (13, 14). IPF is a chronic, progressive, and often fatal inflammatory lung disease that results in the proliferation of fibroblasts and deposition of ECM in the interstitium and alveolar spaces of lung (15). In animal models of lung fibrosis, pirfenidone inhibited fibrosis and downregulated TGF- $\beta$ , PDGF, and collagen synthesis (16–18). In liver fibrosis models, pirfenidone suppressed the proliferation of hepatic stellate cells, TGF- $\beta$  mRNA expression, and the production of collagen type I (19, 20). These reports suggest that pirfenidone exerts antifibrotic effects by inhibiting fibroblasts and the production of TGF- $\beta$ , PDGF, and collagen type I. To date, however, no studies have analyzed the effects of pirfenidone on PSCs derived from pancreatic cancer tissues.

Therefore, the aim of this study was to evaluate whether pirfenidone could suppress desmoplasia in pancreatic cancer. We assessed the effects of pirfenidone on human PSCs and tumor–stromal interactions between PCCs and PSCs. We also investigated the effects of pirfenidone on pancreatic cancer xenografts co-implanted with PCCs and PSCs.

## Materials and Methods

### Cell isolation and culture conditions

We established human PSCs from pancreatic cancer surgical specimens using the outgrowth method described by Bachem and colleagues (3, 6), and cells were maintained as previously described (21). We confirmed that the PSCs exhibited a fibroblast-like morphology and were immunohistochemically positive for  $\alpha$ -SMA and glial fibrillary acidic protein (GFAP), markers of PSCs, and were negative for cytokeratin, an epithelial cell marker (Supplementary Fig. S1). All of the established PSCs were used between passages 3 and 8. The use of pancreatic cancer surgical specimens was approved by the Ethics Committee of Kyushu University (Fukuoka, Japan) and was conducted according to the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government and the Helsinki Declaration. We also used 2 human pancreatic cell lines in this study; SUIT-2 and MIA PaCa-2 cells [Japan Health Science Research Resources Bank (JCRB), Osaka, Japan]. Both cell lines were propagated and frozen immediately after arrival. The cells revived from the frozen stock were used within less than 3 months. Cell lines were regularly authenticated and matched short tandem repeat DNA profiles of the original cell lines by JCRB. The cells were maintained as previously described (21).

### Pirfenidone and gemcitabine preparation and treatment

For *in vitro* studies, pirfenidone was purchased from Sigma-Aldrich. The powder was dissolved in sterile water at 60°C to a concentration of 10 mg/mL and frozen at –20°C until use. For *in vivo* studies, we purchased Pirespa tablets from Shionogi & Co. and gemcitabine from Eli Lilly & Company. The tablets were crushed using a pestle and mortar, and dissolved in sterile water to a pirfenidone concentration of 200 mg/mL. Pirfenidone was orally administered to nude mice (BALB/c *nu/nu*; Kyudo Co.) using a flexible gastric tube (KN-349, Natsume Sisakusyo Co. Ltd.) at a dose of 500 mg/kg. Gemcitabine was dissolved in PBS and intravenously injected into nude mice at a dose of 40 mg/kg.

### Production of conditioned media of PCCs and PSCs

We produced conditioned media of PSCs and PCCs using serum-free Dulbecco's Modified Eagle's Media (DMEM) to exclude the effects of growth factors present in serum for the following experiments. Confluent (70%) SUIT-2 cells (SUIT-2-SN) and 100% confluent PSCs (PSC-SN) were cultured in serum-free DMEM for 24 or 48 hours, respectively. In experiments designed to analyze the effects of pirfenidone on PSCs, 100% confluent PSCs were cultured in serum-free DMEM containing 0.3 mg/mL pirfenidone for 48 hours (pirfenidone-treated PSC-SN). The resulting conditioned media were used to stimulate PCCs and PSCs. Proteins secreted into the supernatant were also examined. The conditioned media were centrifuged for 10 minutes at 1,000 rpm after collection and stored at –80°C until use.

### Cell proliferation assay

Cell proliferation was evaluated by measuring the fluorescence intensity of propidium iodide (PI), as previously described (22). PSCs and PCCs were seeded in 24-well tissue culture plates (Becton Dickinson Labware) at a density of  $1 \times 10^4$  or  $2 \times 10^4$  cells/well, respectively. The cells were then cultured in DMEM containing 10% FBS for 24 hours. After confirmation of cellular adhesion to the plates, the medium was replaced with fresh DMEM containing 10% FBS plus pirfenidone at concentrations of 0, 0.1, 0.3, 0.5, or 1.0 mg/mL. To analyze cell proliferation following stimulation with conditioned medium, the medium was replaced with SUIT-2-SN, PSC-SN, or pirfenidone-treated PSC-SN containing 1% FBS plus 0 or 0.3 mg/mL pirfenidone. Cell proliferation was evaluated after culture for 72 hours using PI assays. A separate well containing the same medium and the same concentration of pirfenidone was used to provide a baseline PI signal as a control. The difference in intensity between each sample well and the control well was calculated.

### Matrigel invasion and migration assays

Cell invasion was measured by counting the number of cells that invaded through Matrigel-coated Transwell chambers with 8- $\mu$ m pores (BD Biosciences). The Transwell inserts were coated with 20  $\mu$ g/well Matrigel (BD Biosciences). PSCs ( $3 \times 10^4$  cells) and PCCs ( $5 \times 10^4$  cells) were resuspended in 250  $\mu$ L of DMEM containing 10% FBS plus pirfenidone at concentrations of 0, 0.1, or 0.3 mg/mL and were placed in the upper chamber. The upper chamber was then placed in a 24-well culture dish containing 750  $\mu$ L of the same medium with the same concentration of pirfenidone as used in the upper chamber. The migration of PSCs and PCCs was assessed using uncoated Transwell inserts. Separate batches of cells were cultured with SUIT-2-SN, PSC-SN, or pirfenidone-treated PSC-SN containing 2% FBS plus 0 or 0.3 mg/mL pirfenidone to assess the effects of PSC and PCC supernatant stimulation on migration and invasion. After incubation for 48 hours for invasion or 24 hours for migration, the invaded and migrated cells were fixed with 70% ethanol, stained with hematoxylin and eosin (H&E), and counted in 5 random fields at a magnification of  $\times 100$  under a light microscope. The results are expressed as the mean number of invaded

cells per field. Each experiment was carried out in triplicate wells and repeated 3 times.

#### Quantitative real-time reverse transcription-PCR

Quantitative real-time reverse transcription (qRT)-PCR was carried out as previously described (23). We designed specific primers for  $\alpha$ -SMA, periostin, FGF-2, and 18S rRNA using Primer 3 software (<http://primer3.sourceforge.net/>). The primers used for collagen type I, fibronectin, TGF- $\beta$ 1, PDGF-A, PDGF-B, CTGF, hepatocyte growth factor (HGF), VEGF, and 18S rRNA were purchased from Takara Bio Inc. The primer sequences are listed in Supplementary Table S1.

#### Western blotting analysis

Protein was extracted from PSCs using PRO-PREP (iNtRON biotechnology) according to the manufacturer's instructions. The supernatants were concentrated using an Amicon Ultra-10 filter unit (Millipore) at 4,000 rpm for 60 minutes, and Halt Protease and Phosphatase Inhibitor Single-Use Cocktail (Thermo Scientific) was added to each sample. The supernatants were stored at  $-80^{\circ}\text{C}$  until use. Cell lysate protein (20  $\mu\text{g}$ ) and supernatant (30  $\mu\text{g}$ ) were fractionated on 4% to 15% Mini-PROTEAN TGX Precast Gel (Bio-Rad Laboratories) and transferred to Trans-Blot Turbo Mini PVDF Transfer Packs (Bio-Rad Laboratories) using a Trans-Blot Turbo Transfer Starter System (Bio-Rad Laboratories). The membrane was incubated overnight at  $4^{\circ}\text{C}$  with anti-TGF- $\beta$ 1 (sc-146; 1:200), anti-PDGF-A (sc-128; 1:200), anti-PDGF-B (sc-127; 1:200), anti-CTGF (sc-101586; 1:200), anti-HGF (sc-13087; 1:200), anti-VEGF (sc-152; 1:200), anti-FGF-2 (sc-79; 1:200), anti-collagen type I (sc-8783; 1:1,000), anti-fibronectin (sc-6952; 1:1,000), anti-periostin (sc-67233; 1:1,000) antibodies (Santa Cruz Biotechnology); anti- $\alpha$ -SMA (1:500; DAKO); or anti- $\alpha$ -tubulin (05-829; 1:1,000; Millipore) antibodies and then probed with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). Immunoblots were detected by enhanced chemiluminescence with a ChemiDoc XRS System (Bio-Rad Laboratories), and the density of each band was measured using Quantity One software (Bio-Rad Laboratories).

#### In vivo experiments

To analyze the effects of pirfenidone on PSCs *in vivo*, SUIT-2 cells ( $1 \times 10^6$ ) suspended in 100  $\mu\text{L}$  DMEM and PSCs ( $1 \times 10^6$ ) and SUIT-2 cells ( $1 \times 10^6$ ) in 100  $\mu\text{L}$  DMEM were subcutaneously transplanted into the left limb and the right limb of 6-week-old female nude mice, respectively. One week after implantation, we randomly divided the mice into 2 groups (15 mice/group) and orally administered them with either 500 mg/kg pirfenidone or sterile water as a control daily for 35 days. We also analyzed the effects of pirfenidone alone or in combination with gemcitabine on orthotopic implantation models. Nude mice were orthotopically implanted with SUIT-2 cells ( $5 \times 10^5$ ) and PSCs ( $5 \times 10^5$ ) in 50  $\mu\text{L}$  DMEM into the tail of the pancreas. One week after implantation, the mice were randomized into 4 groups: control group, pirfenidone group, gemcitabine group, and pirfenidone plus gemcitabine group. We orally

administered 500 mg/kg pirfenidone or sterile water as a control daily and injected gemcitabine at a dose of 40 mg/kg or PBS as a control via the tail vein once weekly for 35 days. Twelve mice were used in each group. The mice were sacrificed on day 35, and all orthotopic tumors were excised and weighed. Tumor volume was calculated using the following formula:  $\pi/6 \times (L \times W \times W)$ , where  $L$  = the largest tumor diameter and  $W$  = the smallest tumor diameter. The presence of peritoneal dissemination was evaluated by counting the number of nodules larger than 1 mm in size disseminated within the peritoneum. The presence of liver metastasis was evaluated by counting the number of nodules larger than 1 mm in size on the surface of the liver. All mouse experiments were approved by the Ethics Committee of Kyushu University.

#### Immunohistochemistry

*In vivo* tumor tissues were immunostained as previously described (23) with following primary antibodies: mouse monoclonal anti- $\alpha$ -SMA (1:500; DAKO), rabbit polyclonal anti-periostin (sc-67233; 1:500; Santa Cruz Biotechnology), mouse monoclonal anti-cytokeratin 19 (sc-376126; 1:100), and rabbit polyclonal anti-proliferating cell nuclear antigen (PCNA; ab2426; 1:500; abcam). The  $\alpha$ -SMA-positive cells were counted in 3 fields at a magnification of  $\times 100$  under a light microscope. The PCNA index (%) was calculated as (PCNA-positive tumor cells/all tumor cells)  $\times 100$  in 3 fields at a magnification of  $\times 100$  under a light microscope.

#### Sirius red staining and measurements

Sections were stained using a Picosirius Red Staining Kit (Polysciences Inc.) according to manufacturer's instructions. The Sirius red-stained area was measured using Adobe Photoshop CS (Adobe Systems Incorporated) by selecting stained fibers in three fields at a magnification of  $\times 100$  under a light microscope.

#### Statistical analysis

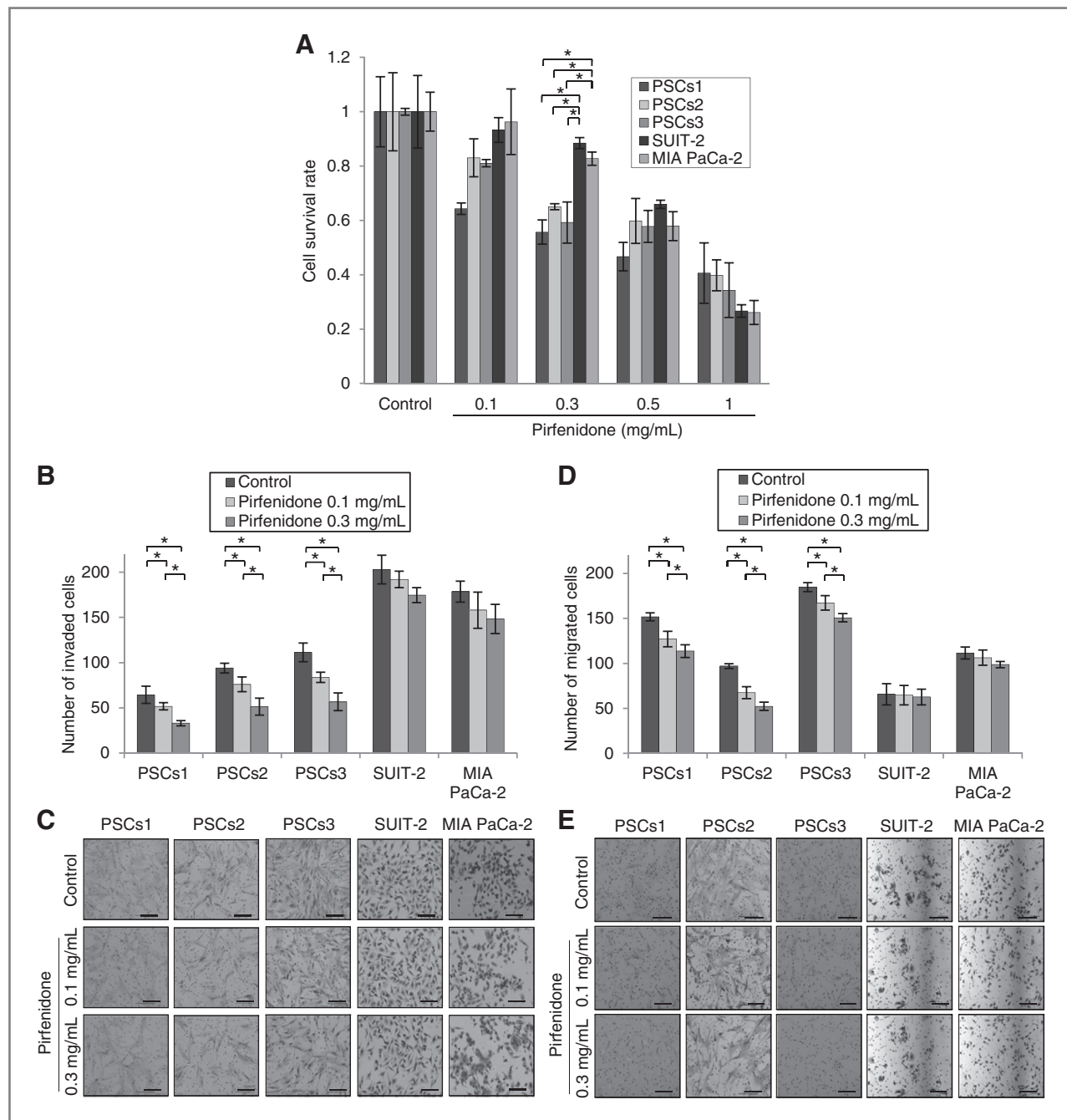
Results are expressed as means  $\pm$  SD. Comparisons between groups were evaluated by one-way ANOVA test followed by the Tukey-Kramer multiple comparisons test. Values of  $P < 0.05$  were considered statistically significant in all analyses. All statistical analyses were conducted using JMP 8.01 software (SAS Institute).

## Results

### Pirfenidone decreases the proliferation, invasion, and migration of PSCs

Pirfenidone inhibited the proliferation of PSCs in a dose-dependent manner ( $P < 0.05$ , Fig. 1A). Although pirfenidone also inhibited PCCs, SUIT-2 cells, and MIA PaCa-2 cells in a dose-dependent manner, low concentrations of pirfenidone, particularly 0.3 mg/mL, showed greater inhibitory activity on PSCs than on PCCs. Pirfenidone significantly decreased the invasion and migration of PSCs at concentrations of 0.1 and 0.3 mg/mL but did not affect PCCs ( $P < 0.05$ , Fig. 1B-E). These data indicate that PSCs are more



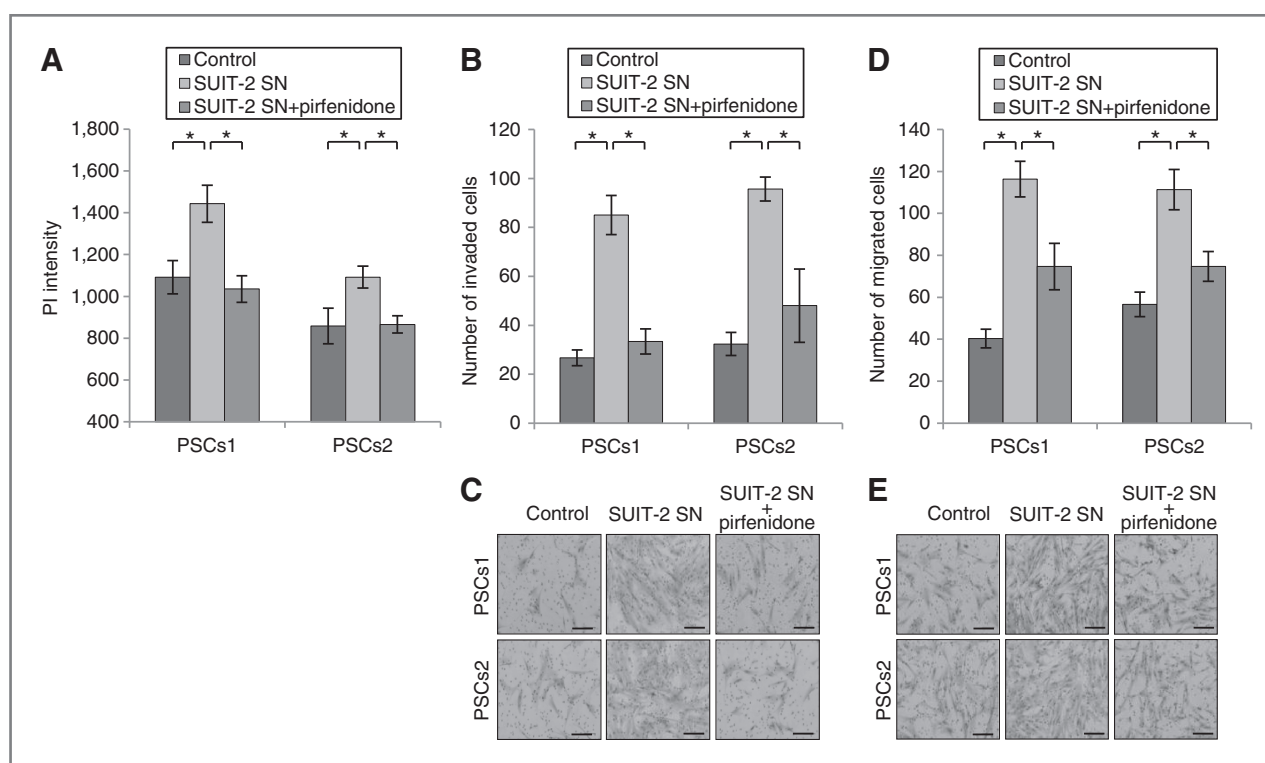


**Figure 1.** A, effects of pirfenidone on the proliferation of PSCs (PSC1-3 cells) and PCCs (SUIT-2 and MIA PaCa-2 cells). Pirfenidone dose dependently inhibited the proliferation of PSCs and PCCs. Low concentrations of pirfenidone, particularly 0.3 mg/mL, showed stronger inhibitory effects on PSCs than PCCs. \*,  $P < 0.05$  for PSCs versus PCCs treated with 0.3 mg/mL pirfenidone. B, pirfenidone dose dependently decreased the invasiveness of PSCs but not of SUIT-2 or MIA PaCa-2 cells. C, representative photomicrographs of invading PSCs and PCCs. D, pirfenidone dose dependently decreased the migration of PSCs but not of SUIT-2 or MIA PaCa-2 cells. E, representative photomicrographs of migrating PSCs and PCCs. \*,  $P < 0.05$ . H&E; original magnification,  $\times 100$ ; scale bars, 200  $\mu$ m.

strongly affected by pirfenidone than are PCCs and suggest that pirfenidone exerts antitumor effects on pancreatic cancer by inhibiting the interaction between PSCs and PCCs. To investigate the specific effects of pirfenidone on PSCs, we used 0.3 mg/mL pirfenidone in the following *in vitro* experiments.

#### Pirfenidone suppresses PSCs by disrupting tumor-stromal interactions

It was reported that the biologic activities of PSCs, such as proliferation and invasion, are potentiated by tumor-stromal interactions. Therefore, we investigated the effects of pirfenidone on the tumor-stromal interactions of PSCs using SUIT-2-



**Figure 2.** Effects of pirfenidone on PSCs stimulated with SUI-2-SN. A, exposure to SUI-2-SN enhanced the proliferation of PSCs compared with control cells. Pirfenidone also suppressed the effects of SUI-2-SN on PSC proliferation. B and D, PSC invasiveness (B) and migration (D) were significantly increased by SUI-2-SN and were suppressed by pirfenidone. C and E, representative photomicrographs of invading (C) and migrating (E) PSCs. H&E; original magnification,  $\times 100$ ; scale bars, 200  $\mu\text{m}$ . \*,  $P < 0.05$ .

SN. Stimulation with SUI-2-SN significantly increased the proliferation ( $P < 0.05$ , Fig. 2A), invasiveness ( $P < 0.05$ , Fig. 2B and C), and migration ( $P < 0.05$ , Fig. 2D and E) of PSCs. Pirfenidone significantly decreased these effects of SUI-2-SN on the proliferation, invasiveness, and migration of PSCs (all  $P < 0.05$ ). These results suggest that pirfenidone suppresses PSCs by disrupting the tumor–stromal interactions.

#### Pirfenidone inhibits the stimulatory effects of PSC supernatants on PCCs

To determine the effects of pirfenidone on the tumor–stromal interactions between PSCs and PCCs, we prepared PSC supernatant (PSC-SN), PSC-SN supplemented with pirfenidone (PSC-SN + pirfenidone), and pirfenidone-treated PSC-SN. PSC-SN increased the proliferation of PCCs (SUI-2 cells and MIA PaCa-2 cells,  $P < 0.05$ , Fig. 3A). In contrast, pirfenidone-treated PSC-SN did not enhance the proliferation of PCCs compared with PSC-SN and PSC-SN + pirfenidone. Similarly, pirfenidone-treated PSC-SN decreased the ability of PSCs to enhance the invasiveness and migration of PCCs ( $P < 0.05$ , Fig. 3B–E). These findings suggest that pirfenidone may suppress the production of factors involved in tumor–stromal interactions in PSC-SN.

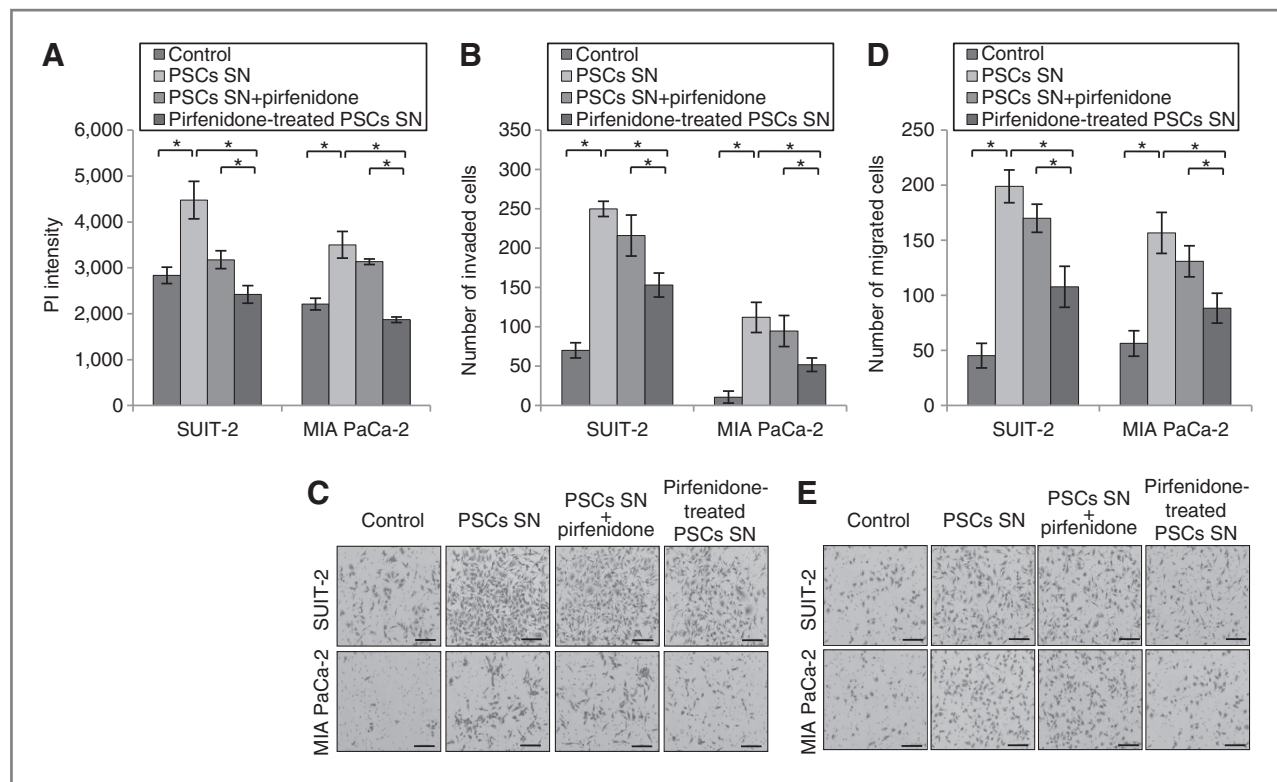
#### Pirfenidone decreases the mRNA and protein expression of genes involved in tumor–stromal interactions in PSCs

To assess the mechanism by which pirfenidone suppresses tumor–stromal interactions, we determined the expression of

the major factors involved in these interactions by qRT-PCR and Western blotting of whole-cell lysates and PSC-SN. PSCs derived from pancreatic cancer tissues secreted PDGF-A, HGF, FGF-2, CTGF, and ECM proteins including collagen type I, fibronectin, and periostin. Treatment with pirfenidone significantly reduced the protein and mRNA expression of PDGF-A, HGF, and periostin in PSCs (Fig. 4). Next, we analyzed the effects of SUI-2-SN and pirfenidone on PSC protein production and tumor–stromal interactions. Exposure to SUI-2-SN considerably enhanced the synthesis and secretion of PDGF-A, HGF, collagen type I, fibronectin, and periostin, which were inhibited by pirfenidone. Pirfenidone also decreased mRNA, but not protein,  $\alpha$ -SMA expression in PSCs. These findings indicate that pirfenidone disrupts tumor–stromal interactions by suppressing the synthesis and secretion of factors involved in these interactions, particularly PDGF-A, HGF, periostin, collagen type I, and fibronectin.

#### Pirfenidone inhibits subcutaneous tumor formation *in vivo* in mice co-transplanted with SUI-2 cells and PSCs

To examine the effects of pirfenidone on PCCs and PSCs *in vivo*, we transplanted nude mice with SUI-2 cells and PSCs into the right flank and SUI-2 cells alone into the left flank (Fig. 5A). One week after implantation, we started oral administration of pirfenidone daily for 5 weeks, whereas a control group was administered with sterile water. In the control group, tumor growth was much greater with co-implantation

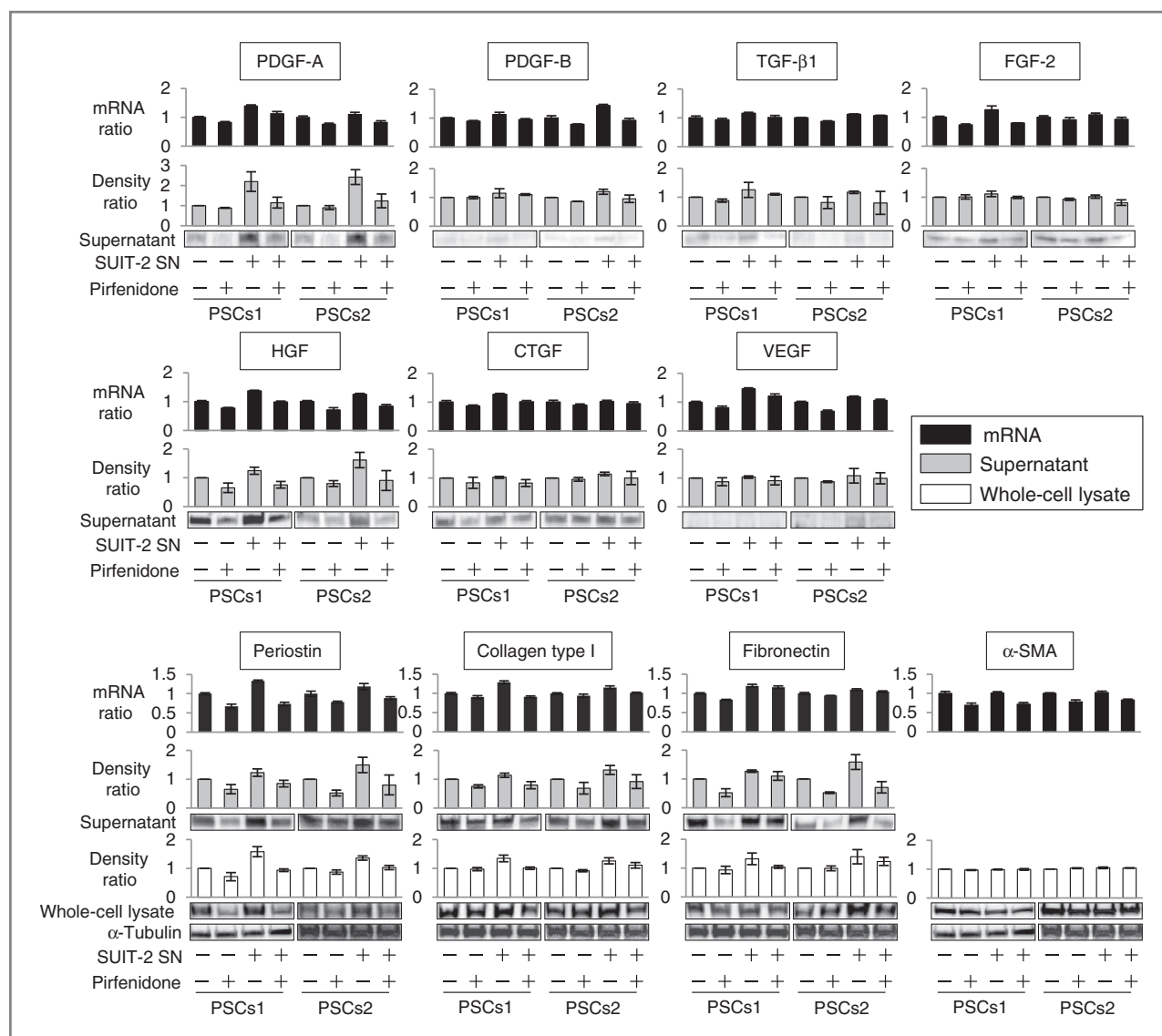


**Figure 3.** Effects of pirfenidone on the tumor–stromal interactions between PSCs and PCCs. A, PSC-SN increased the proliferation of SUIT-2 and MIA PaCa-2 cells compared with control cells. Pirfenidone-treated PSC-SN (0.3 mg/mL pirfenidone) was unable to stimulate PCC proliferation. B and D, PSC-SN significantly enhanced invasion (B) and migration (D) of SUIT-2 and MIA PaCa-2 cells. Pirfenidone-treated PSC-SN reduced the activation of PCCs compared with PSC-SN and PSC-SN + pirfenidone (0.3 mg/mL pirfenidone). \*,  $P < 0.05$ . C and E, representative photomicrographs of invading (C) and migrating (E) PCCs (SUIT-2 and MIA aCa-2 cells). H&E; original magnification,  $\times 100$ ; scale bars, 200  $\mu\text{m}$ .

of SUIT-2 cells and PSCs compared with SUIT-2 cells alone ( $P < 0.05$ , Fig. 5A and B). The tumors consisting of SUIT-2 cells co-implanted with PSCs showed marked desmoplasia with larger Sirius red–positive areas and more  $\alpha$ -SMA–positive cells than the tumors consisting of SUIT-2 cells alone ( $P < 0.05$ , Fig. 5C–E; Supplementary Fig. S2). Treatment with pirfenidone significantly suppressed the growth of tumors consisting of SUIT-2 cells co-implanted with PSCs compared with that in the control group ( $P < 0.05$ , Fig. 5B). Pirfenidone also reduced the Sirius red–positive area and the number of  $\alpha$ -SMA–positive cells in tumors consisting of SUIT-2 cells co-implanted with PSCs ( $P < 0.05$ , Fig. 5C–E). In contrast, tumor growth of SUIT-2 cells alone was not significantly different between the pirfenidone-treated and control groups. Pirfenidone significantly reduced the PCNA index in tumors consisting of SUIT-2 cells co-implanted with PSCs ( $P < 0.05$ , Fig. 5F). However, the PCNA index was not significantly different between the control group and the pirfenidone group in tumors consisting of SUIT-2 cells alone ( $P = 0.98$ , Fig. 5F). Periostin expression was only detected immunohistochemically in the control tumors consisting of SUIT-2 cells co-implanted with PSCs (Fig. 5C). These findings suggest that, at the concentration used in this experiment, pirfenidone indirectly, rather than directly, targets pancreatic cancer cells (i.e., SUIT-2 cells) by suppressing the growth/proliferation of PSCs and by suppressing ECM deposition.

#### The inhibitory effects of pirfenidone on the growth and metastasis of tumors consisting of SUIT-2 cells co-implanted with PSCs are enhanced by combination treatment with gemcitabine

We next investigated the effects of pirfenidone alone or in combination with gemcitabine on growth and metastasis of orthotopic tumors in mice. The administration of pirfenidone and gemcitabine was started 1 week after tumor implantation. Pirfenidone significantly inhibited the growth of orthotopic tumors consisting of SUIT-2 cells co-implanted with PSCs, as observed in mice with subcutaneous tumors ( $P < 0.05$ , Fig. 6A and B; Supplementary Fig. S3B). Although gemcitabine alone inhibited the growth of orthotopic tumors consisting of SUIT-2 cells co-implanted with PSCs, the suppressive effects were significantly greater when gemcitabine was administered in combination with pirfenidone ( $P < 0.05$ ). Immunohistologic studies showed that pirfenidone significantly suppressed the desmoplastic reactions in orthotopic tumors whereas gemcitabine did not ( $P < 0.05$ , Fig. 6C and D; Supplementary Fig. S3A). Although the orthotopic mice showed evidence of peritoneal dissemination and liver metastasis, pirfenidone decreased the number of peritoneal disseminated nodules and the incidence of liver metastasis. These suppressive effects of pirfenidone were significantly enhanced by combination with gemcitabine ( $P < 0.05$ , Fig. 6E and F; Supplementary Fig. S3C; Table 1).



**Figure 4.** Effects of pirfenidone on gene and protein expression levels of growth factors and ECM proteins associated with tumor-stromal interactions. PSCs were untreated or treated with pirfenidone, SUIT-2-SN, or SUIT-2-SN + pirfenidone. mRNA expression levels were normalized to 18S rRNA expression and are presented as the fold change in gene expression relative to control PSCs. Protein expression of growth factors was analyzed by Western blotting of the supernatants of PSCs. Western blotting of ECM proteins and  $\alpha$ -SMA was conducted using whole-cell lysates and the supernatant of PSCs.  $\alpha$ -Tubulin was used as a control to confirm equal protein loading in each well for analysis of cell lysates. The densitometric data are presented as the fold change relative to control PSCs.

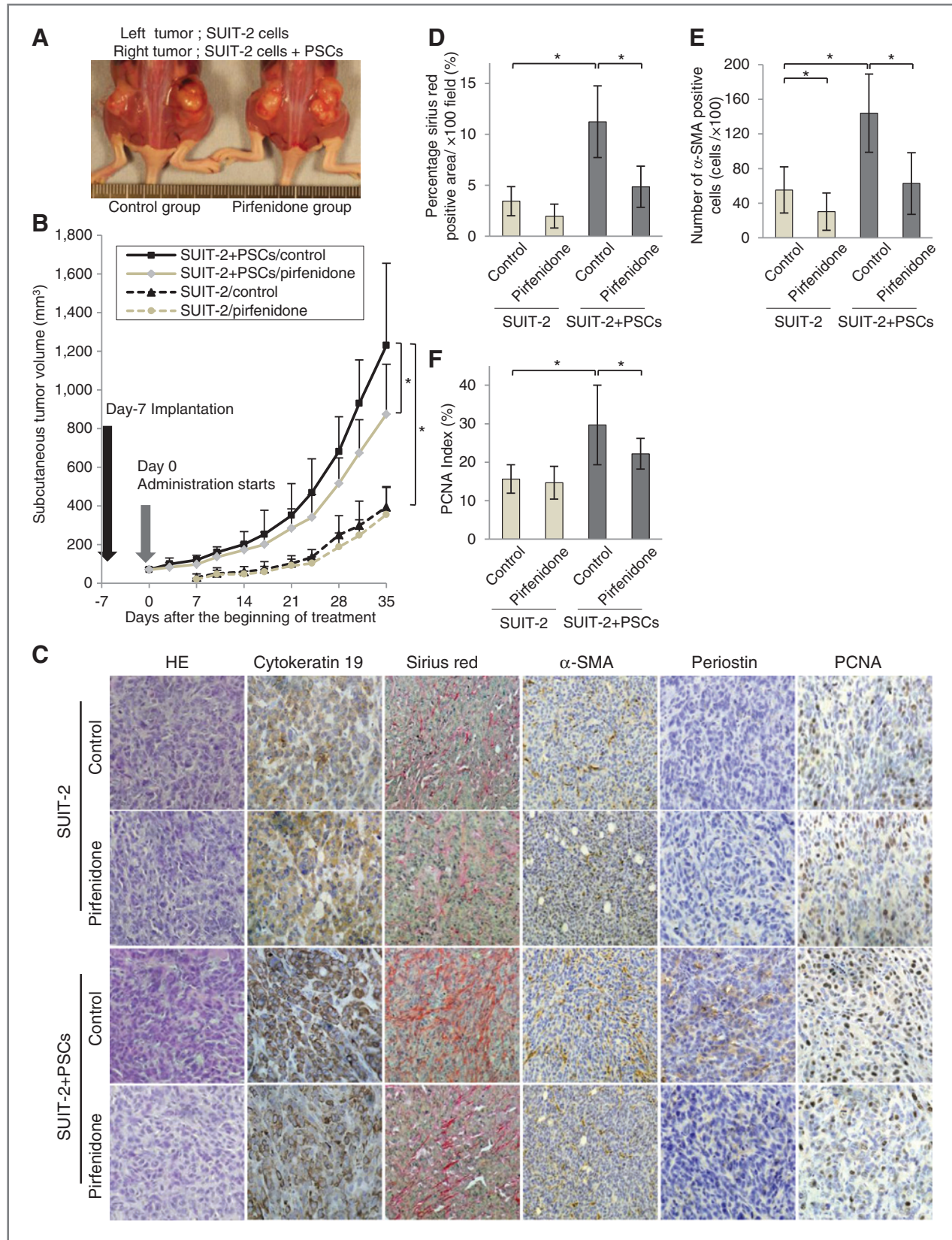
These findings suggest that pirfenidone can suppress tumor growth and metastasis of orthotopic tumors consisting of SUIT-2 cells co-implanted with PSCs and that combination treatment with gemcitabine has synergistic effects on these tumors.

## Discussion

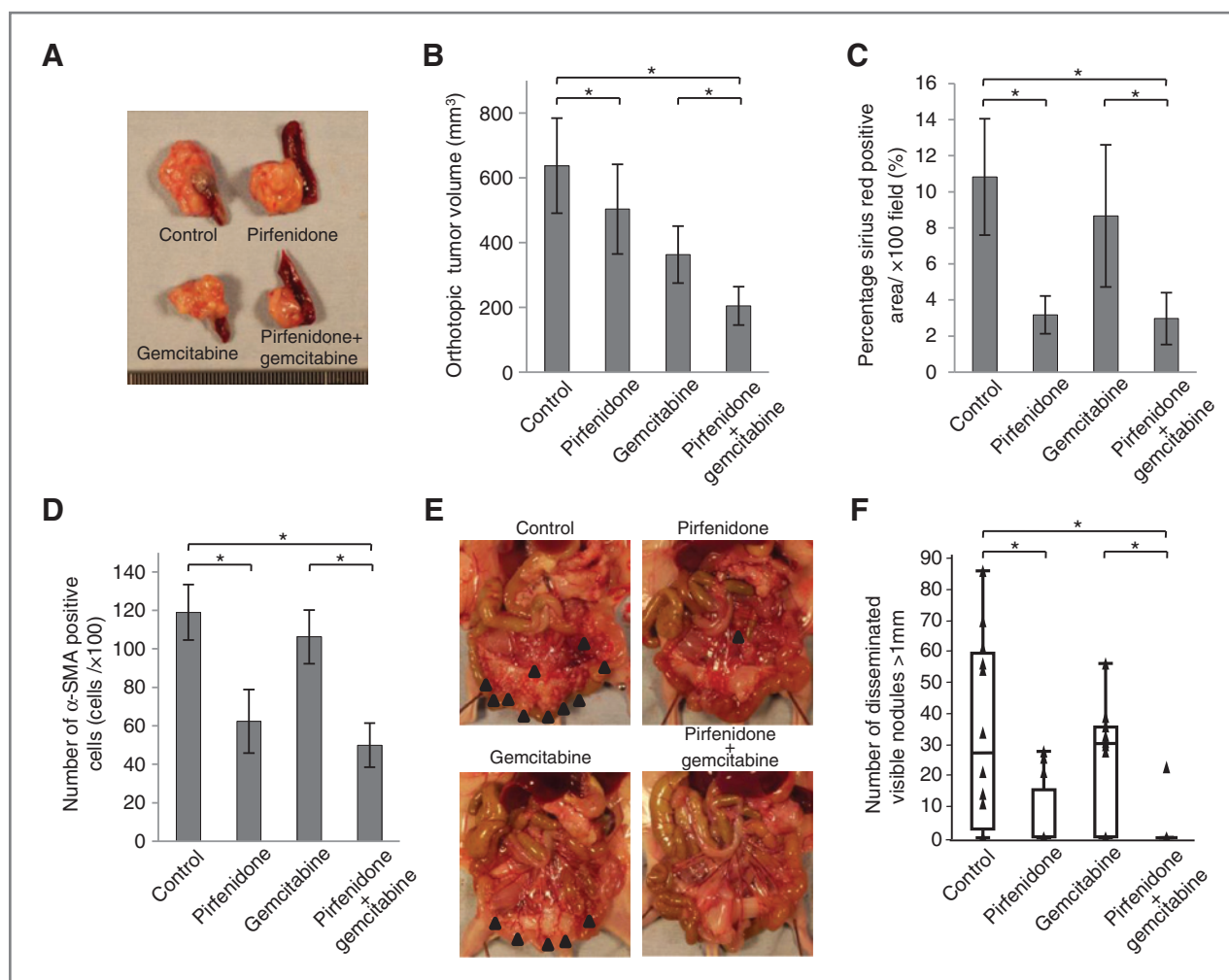
We showed that PSCs increased the biologic activities and augmented the production of PDGF-A, HGF, collagen type I, fibronectin, and periostin through tumor-stromal interactions with PCCs. In addition, we found that pirfenidone strongly suppressed the activities of PSCs by disrupt-

ing the tumor-stromal interactions and significantly inhibited the production of these factors. These factors play critical roles in the tumor-stromal interactions in pancreatic cancer. PDGF was reported to induce the proliferation and migration of PSCs through paracrine and autocrine pathways (24–27). We previously reported that irradiated stromal fibroblasts increased the invasiveness of PCCs through the HGF-c-Met pathway (21). Ide and colleagues (28) reported that HGF secreted from fibroblasts under hypoxic conditions increased the invasiveness of PCCs. ECM components, including collagen type I, fibronectin, and periostin, were reported to augment the proliferation









**Figure 6.** Antitumor effects of pirfenidone and/or gemcitabine on orthotopic pancreatic cancer mouse models. SUIT-2 cells and PSCs were co-implanted into the tail of the pancreas of nude mice ( $n = 12$  mice/group). Starting 1 week after orthotopic implantation of SUIT-2 cells and PSCs, mice were intravenously injected with gemcitabine weekly and orally administered with pirfenidone daily for 5 weeks. A, representative photographs showing the effects of gemcitabine in combination with pirfenidone. B, pirfenidone in combination with gemcitabine significantly suppressed the growth of tumors consisting of SUIT-2 cells co-implanted with PSCs. The Sirius red–positive area (C) and the number of  $\alpha$ -SMA–positive cells (D) were decreased in mice treated with pirfenidone alone but not in mice treated with gemcitabine alone. E, representative photographs showing metastasis in the peritoneal cavity of mice after the indicated treatments (arrowhead). F, pirfenidone decreased the number of disseminated visible nodules larger than 1 mm in size, and this suppressive effect was enhanced by combination treatment with gemcitabine. \*,  $P < 0.05$ .

and migration of PCCs and enhance their resistance to anti-cancer drugs (29–33). Consistent with these reports, PSC-SN greatly increased the malignant potential of PCCs. Meanwhile pirfenidone-treated PSC-SN decreased the ability of PSCs to enhance the invasiveness and migration of PCCs in

our study. The antitumor effects of pirfenidone may involve suppression of the biologic activation of PSCs and attenuation of the tumor–stromal interactions by suppressing the production of PDGF-A, HGF, collagen type I, fibronectin, and periostin.

**Figure 5.** Antitumor effects of pirfenidone on subcutaneous tumor formation *in vivo*. SUIT-2 cells plus PSCs (right flank) or SUIT-2 cells alone (left flank) were subcutaneously implanted into nude mice ( $n = 15$  mice/group). From 1 week after implantation, the mice were orally administered with pirfenidone daily for 5 weeks. A, representative micrographs after 5 weeks of treatment. B, time course of tumor growth. C, histologic evaluation of desmoplastic activity. Periostin was expressed in tumors consisting of SUIT-2 cells co-implanted with PSCs in the control group but not in pirfenidone-treated tumors. D, Sirius red staining was more intense in tumors consisting of SUIT-2 cells co-implanted with PSCs than in tumors consisting of SUIT-2 cells alone. Pirfenidone significantly decreased the Sirius red–positive area in tumors consisting of SUIT-2 cells co-implanted with PSCs. E, the number of  $\alpha$ -SMA–positive cells was higher in tumors consisting of SUIT-2 cells co-implanted with PSCs than in tumors consisting of SUIT-2 cells in the control group. Pirfenidone decreased the number of  $\alpha$ -SMA–positive cells in tumors consisting of SUIT-2 cells co-implanted with PSCs. The  $\alpha$ -SMA–positive cells in tumors consisting of SUIT-2 cells alone probably originated from the host, and their number was decreased by pirfenidone. F, the PCNA index (%) was decreased by pirfenidone in tumors consisting of SUIT-2 cells co-implanted with PSCs but not in tumors consisting of SUIT-2 cells alone. \*,  $P < 0.05$ .

**Table 1.** Effects of pirfenidone and/or gemcitabine on orthotopic mouse models of pancreatic cancer

Group	Incidence of macroscopic tumors			Tumor weight (mean $\pm$ SD), g	Body weight (mean $\pm$ SD), g
	Pancreatic tumor	Peritoneal dissemination	Liver metastasis		
Control	12/12	9/12	6/12	0.706 $\pm$ 0.169	22.07 $\pm$ 1.16
Pirfenidone	12/12	3/12	1/12	0.575 $\pm$ 0.134	21.55 $\pm$ 1.36
Gemcitabine	12/12	8/12	3/12	0.401 $\pm$ 0.054	21.08 $\pm$ 1.34
Pirfenidone plus gemcitabine	12/12	1/12	0/12	0.261 $\pm$ 0.046	21.14 $\pm$ 1.40

For the first time, we found that pirfenidone significantly suppressed the production of periostin by PSCs. Periostin, originally called osteoblast-specific factor 2, is a 93.3-kDa secreted protein involved in the recruitment and attachment of osteoblast precursors in the periosteum (34, 35). Periostin is predominantly expressed in collagen-rich fibrous connective tissues in several organs, where it regulates collagen fibrillogenesis (36). Recent studies have revealed that periostin is overexpressed in the stroma of several types of cancer including ovarian cancer, non-small cell lung cancer, colon cancer, prostate cancer, and pancreatic cancer (37). Periostin promoted tumor growth and metastasis and was associated with poor prognosis of patients with these cancers. Erkan and colleagues (33) reported that once PSCs are activated by PCCs, the PSCs remained active via an autocrine periostin loop, and produced excessive ECM creating a tumor-supportive microenvironment. Recently, periostin was reported to maintain cancer stem cells by increasing Wnt signaling and facilitate metastatic colonization (38). Zhu and colleagues reported that a neutralizing monoclonal antibody to periostin could inhibit ovarian tumor growth and metastasis in mice (39). These findings indicate that periostin provides a significant contribution to fibrogenesis and the malignant behaviors of cancer cells. These findings also suggest that the antitumor effects of pirfenidone are partly mediated by inhibition of periostin production. Therefore, inhibition of periostin production may be new mechanism explaining the antifibrotic effects of pirfenidone.

In this study, PSCs significantly increased the growth of a pancreatic cancer xenograft and induced excessive desmoplasia in tumor tissues, as previously reported (6, 8–10). We revealed that oral administration of pirfenidone significantly suppressed *in vivo* tumor growth, but only when SUIT-2 cells were co-implanted with PSCs. Pirfenidone did not affect tumor growth of SUIT-2 cells alone. Histologically, the tumors excised from pirfenidone-treated nude mice showed a considerable reduction in the number of  $\alpha$ -SMA-positive cells (i.e., PSCs), Sirius red-positive area, and periostin expression. These findings suggest that the antitumor effects of pirfenidone *in vivo* include suppressed PSC proliferation and the production of stromal components. These effects of pirfenidone might contribute to the decreased rates of peritoneal dissemination and liver metastasis of orthotopic tumors consisting of SUIT-2 cells co-implanted with PSCs by suppressing the tumor-stromal interactions. Extensive deposition of ECM components forms a barrier that prevents the penetration of antitumor drugs and

generates a hypovascular microenvironment that limits drug delivery (11). Therefore, researchers have started to focus on pharmacologic agents that can suppress stromal formation. Olive and colleagues (12) reported that a hedgehog signaling inhibitor improved the delivery of gemcitabine to PCCs by decreasing fibrotic deposition and increasing intratumoral density in Kras<sup>LSL.G12D/+</sup>, p53<sup>R172H/+</sup>, PdxCre<sup>tg/+</sup> mice, which are engineered to spontaneously generate pancreatic cancers resembling those in humans. Although our orthotopic pancreatic cancer mice models did not show strong resistance to gemcitabine, the combination of pirfenidone and gemcitabine significantly suppressed the growth of SUIT-2 cells co-implanted with PSCs compared with treatment with gemcitabine alone. Histologic examination of the orthotopic tumors revealed that pirfenidone, but not gemcitabine, decreased desmoplastic reactions, as observed in subcutaneous tumors. These results suggest that co-administration of gemcitabine, which targets cancer cells, and pirfenidone, which targets desmoplastic activity, have synergistic antitumor effects. Therefore, this combination may represent an ideal treatment strategy for pancreatic cancer associated with excessive desmoplasia.

In conclusion, the present study revealed that pirfenidone inhibits the biologic activity of PSCs. Pirfenidone also inhibited the production of PDGF-A, HGF, collagen type I, fibronectin, and periostin, factors that play important roles in tumor-stromal interactions in pancreatic cancer. Pirfenidone and gemcitabine had synergistic antitumor effects on pancreatic cancer xenografts. Therefore, combining pirfenidone with traditional anticancer drugs such as gemcitabine may offer a promising treatment strategy for pancreatic cancer.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Authors' Contributions

**Conception and design:** S. Kozono, K. Ohuchida, D. Eguchi, K. Mizumoto  
**Development of methodology:** D. Eguchi, N. Ikenaga, L. Cui  
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** S. Kozono, N. Ikenaga  
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** S. Kozono, K. Ohuchida, N. Ikenaga, K. Fujiwara  
**Writing, review, and/or revision of the manuscript:** S. Kozono, K. Ohuchida, N. Ikenaga, K. Mizumoto, M. Tanaka  
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** K. Fujiwara, K. Mizumoto  
**Study supervision:** K. Ohuchida, M. Tanaka

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## Pirfenidone Inhibits Pancreatic Cancer Desmoplasia by Regulating Stellate Cells

Shingo Kozono, Kenoki Ohuchida, Daiki Eguchi, et al.

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