The impact of cellular senescence and senescence-associated secretory phenotype in cancer-associated fibroblasts on the malignancy of pancreatic cancer

MASAYA HIGASHIGUCHI[1,2*], HIROTOMO MURAKAMI[1,2*], HIROFUMI AKITA[1,2], SHOGO KOBAYASHI[1], SHOKICHI TAKAHAMA[2], YOSHIFUMI IWAGAMI[1], DAISAKU YAMADA[1], YOSHITO TOMIMARU[1], TAKEHIRO NODA[1], KUNIHITO GOTOH[1], YUICHIRO DOKI[1], TAKUYA YAMAMOTO[2-4] and HIDETOSHI EGUCHI[1]

[1]Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, Osaka 565-0871;
[2]Laboratory of Immunosenescence, Center for Vaccine and Adjuvant Research, National Institutes of Biomedical Innovation, Health and Nutrition, Osaka 567-0085; [3]Department of Virology and Immunology, Graduate School of Medicine, Osaka University; [4]Laboratory of Aging and Immune Regulation, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka 565-0871, Japan

Abstract. Cancer-associated fibroblasts (CAFs) are implicated in the strong malignancy of pancreatic cancer (PC). Various CAF subtypes have different functions, and their heterogeneity likely influence the malignancy of PC. Meanwhile, it is known that senescent cells can create a tumor-promoting microenvironment by inducing a senescence-associated secretory phenotype (SASP). In the present study, the effects of individual differences in CAFs on PC malignancy were investigated with a focus on cellular senescence. First, primary cultures of CAFs from 8 PC patients were generated and co-cultured with PC cell lines. This co-culture assay showed that differences in CAFs induce differences in PC cell proliferation. It was further investigated which clinical factors affected the malignant potential of CAF and it was found that the difference of malignant potential of each CAF was marginally related to the age of original patients. Next, to verify the senescence of CAFs really affected the malignant potential of CAF, PCR array analysis of each CAF sample was performed and it was revealed that expression of genes about cellular senescence and SASP such as tumor protein p53, nuclear factor kappa B subunit 1, and IL6, are related to the malignant potential of CAFs impacting on PC proliferation. Finally, to elucidate the effect of p53-mediated cellular senescence of CAFs on malignant potential of PC, it was examined whether CAFs with the treatment of p53 inhibitor affected PC cell proliferation in co-culture assays. The treatment of CAFs with p53 inhibitor significantly suppressed PC cell proliferation. In addition, a comparison of the concentration of IL-6, a SASP cytokine, in the co-culture supernatant showed a significant decrease in the sample after p53 inhibitor treatment. In conclusion, the present results suggested that proliferation potential of PC may be related to p53-mediated cellular senescence and SASP of CAFs.

Correspondence to: Dr Shogo Kobayashi, Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
E-mail: skobayashi@gesurg.med.osaka-u.ac.jp

Professor Takuya Yamamoto, Laboratory of Immunosenescence, Center for Vaccine and Adjuvant Research, National Institutes of Biomedical Innovation, Health and Nutrition, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan
E-mail: yamamotot2@nibiohn.go.jp

*Contributed equally

Abbreviations: ACTA2, actin alpha 2; α-SMA, α-smooth muscle actin; ATM, ataxia telangiectasia mutated; CAF, cancer-associated fibroblasts; CDKN1A, cyclin-dependent kinase inhibitor 1A; CDKN2A, cyclin-dependent kinase inhibitor 2A; DMEM, Dulbecco's modified Eagle's medium; FAP, fibroblast activation protein; FBS, fetal bovine serum; FIR, fluorescence intensity ratio; GADPH, glyceraldehyde-3-phosphate dehydrogenase; KEGG, Kyoto encyclopedia of genes and genomes; NFKB1, nuclear factor kappa B subunit 1; PC, pancreatic cancer; PFT, pifithrin; SASP, senescence-associated secretory phenotype; TERT, telomerase reverse transcriptase; TP53, tumor protein p53; TSR, tumor size ratio; VIM, vimentin

Key words: pancreatic cancer, CAF, cellular senescence, SASP, tumor microenvironment

Introduction

Pancreatic cancer (PC) still has a poor prognosis. Even with the latest multimodality treatment, the 5-year relative survival rate is only 11%, which is substantially worse than for other gastrointestinal cancers (1). This is partly because PC tends to metastasize to lymph nodes or distant sites at an early stage due to its high capacity for invasion and migration. Therefore, to improve the prognosis, it is important to examine the cause of PC's high malignant potential.

Previous studies demonstrated that the tumor stromal components, which are abundant in PC, are involved in the malignant potential (2,3). In particular, fibroblasts are the main component of the stromal tissue-referred to as 'cancer-associated fibroblasts (CAFs)'-and are reportedly involved in tumor proliferation, invasion, and drug resistance through interactions with cancer cells via chemokines, cytokines and growth factors (4-6). Furthermore, previous studies reported that CAFs can be classified into subpopulations based on various functional characteristics, and their heterogeneity may be related to cancer malignancy (7,8). The heterogeneity of CAFs in PC was also reported to be closely related to its malignant potential (9,10), however, it remains unclear how CAFs affect PC malignancy.

In general, malignancy risk increases with aging (11,12), and numerous studies have focused on the relationship between cellular senescence and malignant tumors (12,13). Cellular senescence is the irreversible growth arrest of normal cells with proliferative potential-caused by telomere shortening, oncogene activation, and unrepairable DNA damage with carcinogenic risk, which can be induced by oxidative stress (14,15). This phenomenon was originally considered a mechanism to protect bodies from the carcinogenic stress that accumulates with aging (16,17). Furthermore, previous studies indicated that senescent cells could foster a tumor-promoting microenvironment by increasing the expressions of inflammation-related genes, such as inflammatory cytokines and chemokines, and, i.e., the senescence-associated secretory phenotype (SASP) (18-20).

Although several studies described the role of SASP in various type of cancers such as liver, colorectal and prostate cancer (21-23), it is not well understood how SASP derived from CAFs may affect the malignancy of PC. In the present study, the differences in malignant potential of CAFs derived from several primary PC tumors were investigated and it was clarified how the senescence of CAFs affected PC cells by examining the significance of SASP in CAFs.

Materials and methods

Primary culture of CAFs. Tissue samples of human PC were collected with the approval (approval no. 18138-4) of the Human Ethics Review Committee of the Graduate School of Medicine of Osaka University (Osaka, Japan) and the National Institutes of Biomedical Innovation, Health and Nutrition (approval no. 201-01; Osaka, Japan). Written informed consent for sample use was obtained from all patients before surgery. All tissue samples were collected at Osaka University Hospital (Suita, Japan) from June 2019 to March 2020. Human pancreatic stellate cells, the reported origin cells of CAFs, were cultured from the tissue samples as previously described (24,25). Briefly, the cancerous portion was cut from the tissue samples, and chopped into small pieces. These tissue pieces were washed with phosphate-buffered saline (PBS) containing 5% penicillin-streptomycin (Sigma-Aldrich; Merck KGaA), and then three pieces were sown in each well of a six-well plate (Corning, Inc.). The samples were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS) for 2 days at 37°C, and thereafter in DMEM containing 10% FBS. Gradual proliferation of only spindle-shaped cells was observed as previously reported

(Fig. S1), which was considered to be CAFs based on their cell morphology. This outgrowth method, which was previously reported by Bachem *et al* (24), was already well-established method for the CAF primary culture. It was indicated in the aforementioned study that α-smooth muscle actin (α-SMA), vimentin (VIM), and desmin were expressed in all primary cultured cells and immunocytochemistry of primary cultured cells was also performed in previous studies and it has been already confirmed that primary culture cells were true CAFs and there was no contamination of cancer cells (24,25). On the 14th day of culture, these CAFs were cryopreserved for use in further experiments. In the present study, primary CAFs were used without immortalization. This is because, to immortalize primary cultured cells, viral vector or gene induction such as telomerase reverse transcriptase (TERT) had to be used, but it is considered that such immortalized cells were very different from originally primary cultured cells. Therefore, primary cultured cells were established with great care and cell damage was minimized by using a programmed freezer when storing cells and accutase when passaging CAFs. In addition, all assays were performed using primary culture CAFs within three passages. This is due to the fact that culture time and passages could cause changes in cell morphology and gene expression in CAFs.

Primary culture of CAFs was performed using tissue samples collected from 8 PC patients. The clinical information for these 8 patients before the initiation of treatment are presented in Table SI. The median age was 67 years (range 53-77 years), 3 were male, and the median tumor diameter was 25.1 mm (range 9.5-34.3 mm). Of the 8 patients, 6 underwent preoperative chemotherapy (details presented in Table SII). The mean age of the male and female groups was 65.6 and 65.7 years, respectively, with no significant difference (P=0.993). The tumor diameters measured by contrast-enhanced CT before and after chemotherapy, and the calculated tumor size ratio (TSR) for each case: TSR=(Tumor size after neoadjuvant chemotherapy)/(Tumor size before neoadjuvant chemotherapy) are also included in Table SII. The macroscopic and microscopic pathology findings for each case are demonstrated in Fig. S2. The following procedure was used for H&E staining: First, tissues were fixed with neutral formalin 10% at room temperature for 2 days, embedded in paraffin, and manually sectioned with a microtome to obtain 3-μm thick paraffin sections. The sections were dewaxed with xylene and stained with hematoxylin for 6 min and eosin for 2 min and 30 sec at room temperature. Tissue-Tek®Eosin and Tissue-Tek® Mayer hematoxylin for Prisma (both from Sakura Finetek USA, Inc.) were used as staining solutions, and staining was performed on a Tissue-Tek®DRS TM 2000 (Sakura Finetek USA, Inc.).

Co-culture assay. The co-culture assay was performed in a non-contact manner, using a Transwell with 0.4-μm pores (Corning, Inc.). First, CAFs (1.0x10^4 per well) were plated on the top layer and incubated overnight in DMEM containing 10% FBS. Once the CAFs had settled in the Transwell, PSN-1 cells (a human PC cell line, p53 mutant) were plated at 1.0x10^5 per well on the lower layer of a 24-well culture plate (Corning, Inc.). PSN-1 cells were obtained from the American Type Culture Collection. After an additional 48 h of co-culture in

DMEM containing 0.5% FBS, the Transwell was removed and PSN-1 cell proliferation was evaluated.

Proliferation assay. PSN-1 cell proliferation was evaluated by fluorescence staining using Hoechst 33342 (Thermo Fisher Scientific, Inc.). Briefly, after removing the Transwell and the culture medium, diluted Hoechst 33342 was added and allowed to react for 10 min. Then the PSN-1 cells were washed three times with PBS, and the fluorescence intensity was measured using the EnSpire Multimode Plate Reader (PerkinElmer, Inc.). An assay was performed to compare the effects of CAFs from 8 different patients on the proliferation of PSN-1 cells. The fluorescence intensity ratio (FIR) was calculated based on the fluorescence intensity of the canonical CAFs (#1) showing the lowest fluorescence intensity: FIR (#X)=Fluorescence intensity (#X)/Fluorescence intensity (#1).

Reverse transcription-quantitative (RT-q) PCR array. RT-qPCR arrays were performed using two kits: QIAGEN RT^2 Profiler™ PCR Array 'Human Cellular Senescence' (GeneGlobe ID-PAHS-050Z) and 'Human Cancer Inflammation and Immunity Crosstalk' (GeneGlobe ID-PAHS-181Z) following the supplier's instructions (http://www.sabiosciences.com). After CAFs were settled on the plate, they were incubated at 37°C for 1 day under serum-free conditions, and then incubated at 37°C for 12 h with or without serum stimulation (2% human AB serum), respectively, before mRNA extraction was performed. RNA was extracted from CAFs of $1.0x10^4$ cells under both serum unstimulated and serum stimulated conditions using the RNeasy Micro kit (Qiagen GmbH), following the supplier's protocol. At the same time, gene expression of actin alpha 2 (ACTA2: alias α-SMA) and fibroblast activation protein (FAP) were also examined for CAFs. The following primers were used for gene expression analysis of human ACTA2 and FAP: ACTA2 forward, 5'-GTGTTGCCCCTG AAGAGCAT-3' and reverse, 5'-GCTGGGACATTGAAA GTCTCA-3'; FAP forward, 5'-TCTAAGGAAAGAAAGGTG CCAA-3' and reverse, 5'-GATCAGTGCGTCCATCATGAA G-3'. Expression level was calculated using the $2^{-\Delta\Delta Cq}$ method based on the expression level of GAPDH, the housekeeping gene. Additionally, the results were analyzed by the $2^{-\Delta\Delta Cq}$ method, using the sample without serum stimulation as a reference (26). Gene expression levels were evaluated as the ratio of expression with serum stimulation to expression without serum stimulation: RNA level (fold change)=(Expression level with serum stimulation)/(Expression level without serum stimulation).

Pathway enrichment analysis. Pathway enrichment analysis was performed using two databases: Kyoto Encyclopedia of Genes and Genomes (KEGG: http://www.genome.ad.jp/kegg) and Reactome (https://reactome.org/). Enriched pathways were identified according to the cut-off value of a false discovery rate (FDR) <0.05.

Inhibition of p53 expression in CAFs. First, CAFs were plated and incubated overnight in DMEM containing 10% FBS. Once the CAFs had settled in the plate, the medium was changed and CAFs were cultured in DMEM containing 10% FBS for 5 days with the p53 inhibitor pifithrin-alpha (PFT-alpha)

(Sigma-Aldrich; Merck KGaA), or with the same amount of DMSO as a control. A PFT-alpha concentration of 10 μM was used based on previous a previous study (27). After 5 days of incubation, the cells were thoroughly washed and seeded in the top layer of Transwell, and then co-cultured with PSN-1 cells for 2 days, as in the aforementioned co-culture assay, to assess proliferation of the PSN-1 cells. Notably, viability of CAFs at the start of co-culture was measured using Guava® Muse cell analyzer (Luminex) as described in the instructions to confirm that no problems with CAF viability had occurred after 5 days of incubation. This assay was conducted using CAFs from three patients, #2, #7 and #8. The experiments of #2, #7, and #8 were performed simultaneously on the same plate. Data for a control, a sample without CAF is shared.

IL-6 quantification by ELISA. The IL-6 concentration was analyzed in the co-culture supernatant using an enzyme-linked immunosorbent assay (ELISA) kit (cat. no 3460-1A-6; Mabtech, Inc.), following the manufacturer's instructions. Co-culture supernatants were collected at the end of the 2-day co-culture in the aforementioned experiment. When performing the analysis, the co-culture supernatant was diluted 10-fold. This assay was also conducted using CAFs from three patients, #2, #7 and #8.

Statistical analysis. Numerical data are presented as mean ± standard deviation. Statistical analysis between two groups was performed using a two-tailed Student's t-test (unpaired t-test). Statistical analysis among three groups or more was performed using Dunnett's t-test, with the test level α=0.05. Correlation analysis was performed using Pearson's correlation coefficient. P<0.05 was considered to indicate a statistically significant difference. JMP software (JMP® Pro 16.2.0, SAS Institute, Inc.) was used as the software for these analyses.

Results

Differences in CAFs induce differences in PC cell proliferation. To examine whether differences in CAFs affected PC cell proliferation, 8 CAF samples were primarily cultured and co-cultured with PSN-1 cells. The PSN-1 showed different proliferation ability when cultured with each CAFs from different patients (Fig. 1). In particular, the mean fluorescence intensity of #8 was significantly higher than that of #1. The fluorescence intensity rate (FIR) of #8 was ~1.4 (P<0.001), indicating that differences in CAFs could affect the proliferation ability of PC cells.

The clinical impact of CAF's malignant potential. It was examined how the results of co-culture assays were associated with the original clinical data of patients. The correlation between FIR in co-culture assays and five clinical data is demonstrated in Fig. 2. Unfortunately, tumor size, serum CA19-9 level, and SUV max before the initiation of treatment were not significantly associated with the FIR (Fig. 2A) and this may be partially because sample size (only 8 patients) was too small and these factors depended on the time of diagnosis. There was also no significant association between sex and co-culture assays results. Fluorescence intensity (mean ± SD)
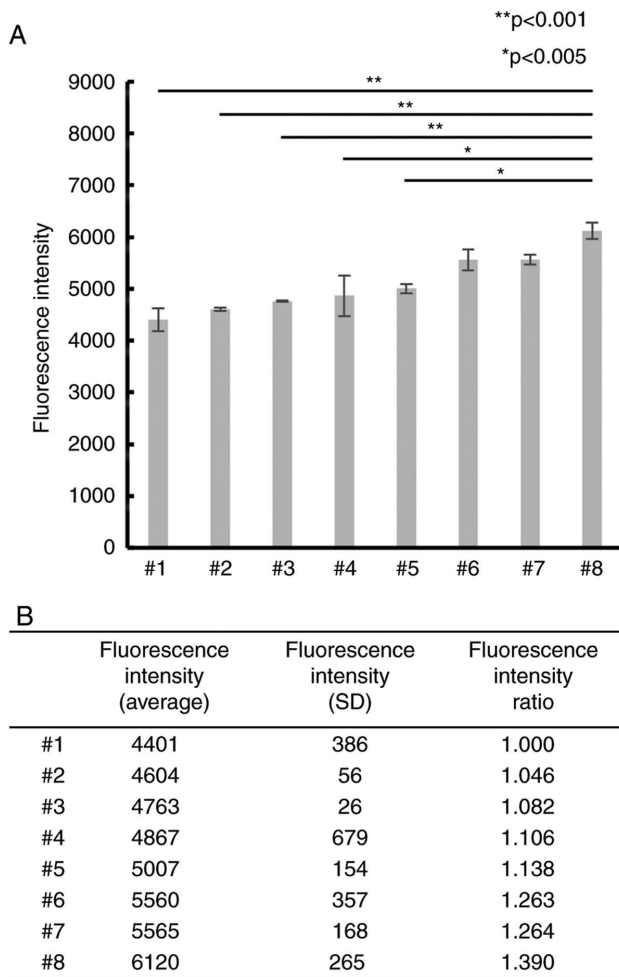
Figure 1. Differences in CAFs induce differences in PC cell proliferation. (A) Graphical representation of the fluorescence intensity of PC cells co-cultured with each CAF sample. Values represent the mean ± SD from three independent experiments. (B) Tabular representation of the same data. Fluorescence Intensity Ratio (#X)=Fluorescence Intensity (#X)/Fluorescence Intensity (#1). *P<0.005 and **P<0.001. CAF, cancer-associated fibroblasts; PC, pancreatic cancer.

in the co-culture experiment was 5,139.4±682.6 for females and 5,063.4±536.8 for males (P=0.779).

Thus, next we examined the correlation between TSR and FIR in six patients who received preoperative treatment to clarify the clinical impact of CAFs on the effect of preoperative treatment. As revealed in Fig. 2B, the FIR was significantly correlated with the TSR (R^2=0.821, P=0.013), indicating that the efficacy of chemotherapy may be determined by the malignant potential of CAFs. Notably, further examination of the relationship between FIR and the patients' original clinical data revealed that patient's age was marginally related to the FIR (P=0.051), as shown in Fig. 2C, raising the suspicion that it was possible that the senescence of CAFs may be related to its malignant potential to grow cancer cells.

Gene expression profiling related to cellular senescence and SASP in CAFs. To elucidate the senescence of CAFs really affected the malignant potential of CAFs, a PCR array was performed among the 8 CAFs by using the 'Human Cellular Senescence' PCR array kit (GeneGlobe ID-PAHS-050Z QUIAGEN) and 'Human Cancer Inflammation and Immunity

Crosstalk' PCR array kit (GeneGlobe ID-PAHS-181Z QUIAGEN). First, the expression of the CAF markers, ACTA-2 and FAP, was examined simultaneously with the analysis using these kits. Fortunately, the genes analyzed in these kits included genes known to be marker of CAF, VIM. Therefore, the expression of these genes in 8 primary CAFs was investigated and it was found that the expression of these 3 genes was positive in all CAFs (Fig. S3). By contrast, the expression of TERT, which is expressed in 85-90% of cancer tissues and is also known to be expressed in PC (28,29), was extremely low, suggesting that the cells in primary culture in the present study were CAFs without cancer cells. The gene expression of CAFs in relation to cancer cell growth was subsequently examined by investigating the relationship between the data of each gene expression analyzed by RT-qPCR and the results of the co-culture assay. Unfortunately, each gene expression with and without serum stimulation did not significantly correlate with the results of the co-culture assay. Then, the fold-change of RNA level of each gene was calculated using the $2^{-\Delta\Delta Cq}$ method and the relationship between fold-change of RNA level and co-culture assay data was analyzed (Table SIII and Fig. 3). The top 30 genes that exhibited a significant correlation between the fold-change of RNA level and FIR are presented in Fig. 3A. Table SIII shows the results for all 162 genes. The plot of absolute value of the correlation coefficient versus the P-value for each gene showed that 38 genes were significantly correlated with the FIR (Fig. 3B). These 38 genes included cellular senescence pathway-associated genes [such as tumor protein p53 (TP53), cyclin-dependent kinase inhibitor 1A (CDKN1A)/p21 and cyclin-dependent kinase inhibitor 2A (CDKN2A)/p16] and cytokines and chemokines known as SASP factors (such as IL1B, IL6, and CXCL12). Next, to clarify which pathway was related to the malignant potential of CAFs, pathway analysis for the top 30 genes was performed by using the KEGG and Reactome databases. The cellular senescence pathway was significantly enriched in both the KEGG and Reactome databases (Fig. 3C). Furthermore, Reactome pathway enrichment analysis revealed that the 'SASP' pathway showed the 6th strongest relationship with malignancy-related genes in CAFs.

Expression of cellular senescence- and SASP-related genes in CAFs correlates with clinical data related to PC malignant potential. As revealed in Fig. 4, for the 8 CAF samples, the relationship between FIR and the RNA levels of genes (selected from the top 30 genes) was analyzed. Ataxia telangiectasia mutated (ATM), TP53, CDKN1A/p21, and CDKN2A/p16 were related to the p53/p21 pathway or p16/RB pathway, both of which are known as cellular senescence pathways (30-32) (Fig. 4A). SASP-related genes, such as nuclear factor kappa B subunit 1 (NFKB1), IL6, and CXCL12 are revealed in Fig. 4B. NFKB1, which is reportedly essential for SASP induction (33,34), showed a strong correlation with FIR (R^2=0.703, P=0.009). Additionally, the expression of these genes without and with serum stimulation, respectively, is shown in Fig. S4. Notably, most of these genes were significantly related to the TSR (Fig. 5A and B) and to the serum CA19-9 level at the time of surgery which is related to postoperative prognosis (Fig. S5). These findings indicated that these genes may be associated with the malignant potential of CAFs.
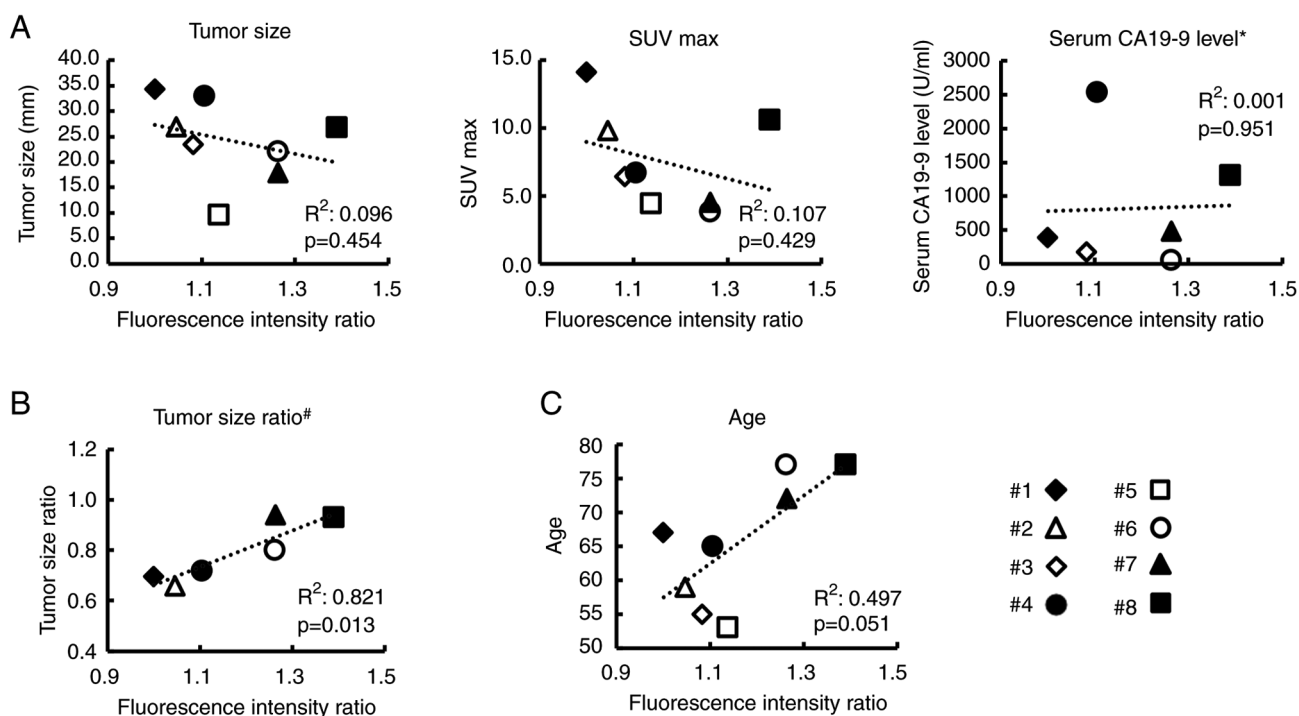
Figure 2. Correlation between FIR in co-culture assays and clinical data before treatment. Graph shows the FIR on the horizontal axis, and clinical data on the vertical axis. (A) Data regarding tumor size, serum CA19-9 level, and SUV max of fluorodeoxyglucose-positron emission tomography. (B) Data regarding tumor size ratio. (C) Data regarding age of patients. The square of the correlation coefficient and the P-value are shown. *N=6 (excluding the two Lewis antigen-negative patients). #N=6 (only for patients who received preoperative chemotherapy). Each symbol legend appears at the right below. FIR, fluorescence intensity ratio; CA19-9, carbohydrate antigen.

Suppression of p53 expression in CAFs inhibits PC cell proliferation in co-culture assays. Based on the present results, focus was next addressed on the p53-mediated cellular senescence of CAFs. It was examined how CAFs treatment with the p53 inhibitor PFT-alpha altered the PC cell proliferation in co-culture assays. After 5 days of exposure to PFT-alpha, each CAF samples retained its spindle-like shape (Fig. S6) and exhibited no decrease of viability (all >90%). Upon co-culture of PSN-1 cells with DMSO-treated CAFs, a significant increase of fluorescence intensity compared with PSN-1 cells cultured alone was observed. This indicated that DMSO-treated CAFs also enhanced PSN-1 proliferation similar to untreated native CAFs. By contrast, PSN-1 co-cultured with PFT-alpha-treated CAFs showed significantly reduced fluorescence intensity compared with PSN-1 with DMSO-treated CAFs (Fig. 6A). This trend was observed in all 3 different CAFs. To further examine why the p53 inhibitor affected proliferative potential, the IL-6 concentration in the co-culture supernatant was measured. As revealed in Fig. 6B, IL-6 level significantly increased in PSN-1 cells with DMSO-treated CAFs compared with PSN-1 alone. By contrast, IL-6 level in PSN-1 co-cultured with PFT-alpha-treated CAFs was significantly reduced almost to the level of PSN-1 alone, indicating that PFT-alpha could suppress IL-6 secretion from CAFs by inhibiting p53-mediated cellular senescence, resulting in inhibition of PSN-1 proliferation.

Discussion

In recent years, a deeper understanding of the interactions between stromal and cancer cells in the senescent microenvironment has been achieved. Yang *et al* (35) reported elevated activity of β-galactosidase in stromal fibroblasts, which resulted in senescent fibroblasts with high p53 expression and promoting tumor proliferation and migration potential in colorectal cancer. Yang *et al* (36) indicated the RAS-activated Gro-1 strongly induces senescence of stromal fibroblasts and those senescent fibroblasts enhance the cancer cell proliferation in ovarian cancer. In addition to the senescence of fibroblasts, SASP such as IL-6 and TNF-α induced by senescent CAFs was also reported to promote cancer cell proliferation and invasion potential in prostate and breast cancer (37,38). Nevertheless, the significance of cellular senescence of stromal cells in tumor microenvironment remains unclear due to the apparent complexity of the relationship between senescent stroma and cancer cells. Notably, these interactions seem to differ depending on the type of cancer, malignant potential of cancer cell itself and disease stage. Thus, well designed research is considered to be necessary to elucidate this topic.

In the present study, primary CAFs were examined and it was found that differences in the expression of cellular senescence and SASP-related genes in CAFs affected the PC cell proliferation. The advantage of the present study was that not primary PC cells but established cancer cell lines were used to examine the malignant potential of CAFs. Although the gene expression of CAFs may be influenced by cancer cells, the present study was able to assess the true malignancy of each CAF by conducting experiments using primary cultures of CAFs without cancer cells and unified cancer cell lines. In the current results, the same cancer cell grew differently by

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|	Gene	R	R²	p	Categoty
1	**MICB**	0.938095281	0.880022756	0.000565882	Antigen Presentation
2	**ACKR3**	0.911771748	0.83132772	0.001605362	Chemokine Receptors
3	**TP53**	0.855896848	0.732559415	0.006695788	Senescence Pathway
4	**HIF1A**	0.85280849	0.727282321	0.007118194	Transcription Factors
5	**IL1B**	0.850132162	0.722724693	0.007497691	Interleukins
6	**IL6**	0.845538446	0.714935264	0.008178683	Interleukins
7	**NFKB1**	0.838676256	0.703377862	0.00926727	Transcription Factors
8	**MYC**	0.836306342	0.699408297	0.009663496	p53 Signaling
9	**CDKN2C**	0.830251019	0.689316754	0.010724235	p53 Signaling
10	**IL15**	0.829568771	0.688184346	0.010848161	Interleukins
11	GLB1	0.824188066	0.679285968	0.011857377	Senescence Response Genes
12	CXCL12	0.823260292	0.677757509	0.012037159	Chemokines
13	SPP1	0.811644297	0.658766464	0.014435029	Cytokines
14	MIF	0.808598866	0.653832125	0.015109592	Immunosuppressive Factors
15	PLAU	0.808330263	0.653397815	0.015170019	p53 Signaling
16	CDKN2A	0.802135456	0.643421289	0.016605998	Senescence Pathway
17	HLA-A	0.798764291	0.638024393	0.017421928	Antigen Presentation
18	ATM	0.779150488	0.607075482	0.022666051	Senescence Pathway
19	CDKN2D	0.777332893	0.604246426	0.023196008	Senescence Pathway
20	PCNA	0.771897162	0.595825228	0.024826536	DNA Damage & Repair
21	COL1A1	0.771719151	0.595550448	0.024881097	Cell Adhesion Molecules
22	KITLG	0.771570434	0.595320934	0.024926736	Cytokines
23	IGFBP7	0.768686405	0.59087879	0.025822067	Insulin Growth Factor (IGF) Related
24	COL3A1	0.767723511	0.58939939	0.026125354	Cell Adhesion Molecules
25	CREG1	0.757631345	0.574005256	0.029436976	p53 Signaling
26	STAT3	0.757391898	0.573642487	0.02951852	Transcription Factors
27	CDKN1A	0.750921621	0.563883281	0.031774779	Senescence Pathway
28	FN1	0.748399409	0.560101675	0.032682079	Cytoskeleton Regulators
29	TGFB1	0.744110563	0.553700531	0.034260986	Growth Factors & Receptors
30	BCL2L1	0.743064191	0.552144392	0.034653138	Anti-Apoptotic
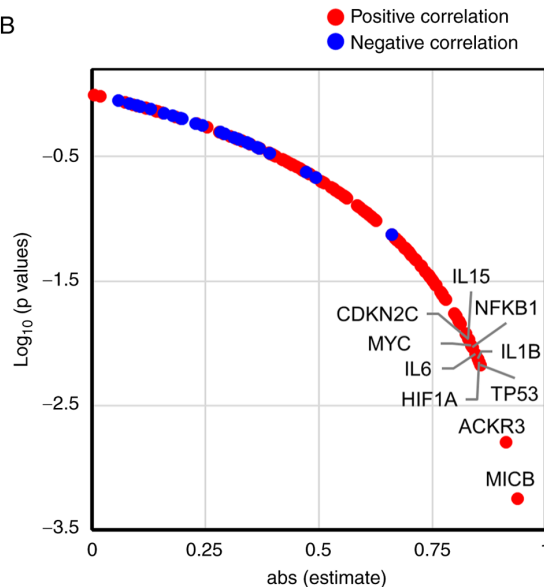
Figure 3. Continued.

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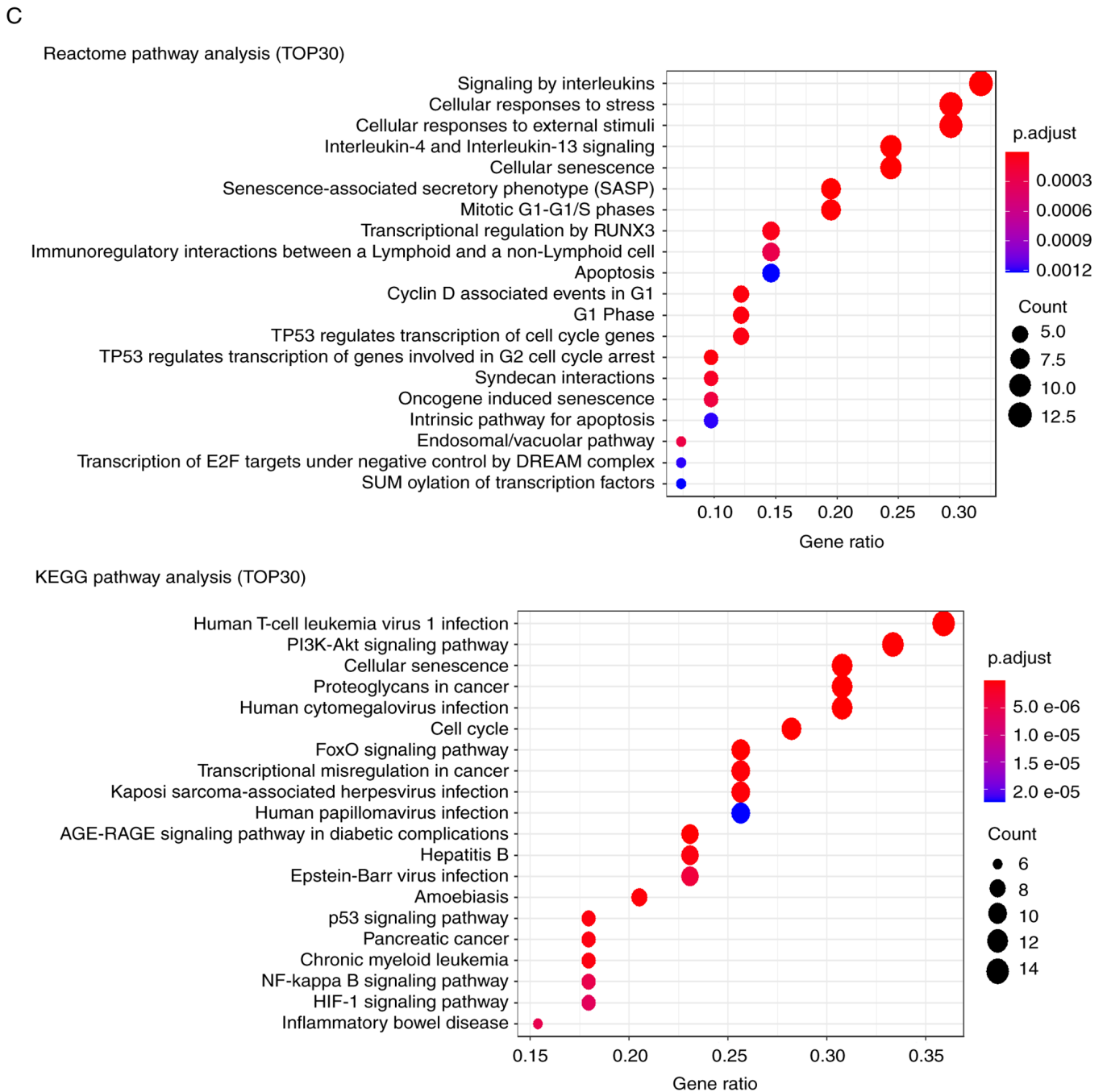
Figure 3. Expression of cellular senescence- and senescence-associated secretory phenotype-related genes in CAFs is related to PC cell proliferation. (A) Top 30 genes expressed by CAFs, for which the RNA level (fold-change) strongly correlates with the FIR in co-culture assays. (B) Graphical representation of the correlation between the RNA level (fold-change) of each gene and FIR. The P-value is shown on the vertical axis, and the absolute value of the correlation coefficient on the horizontal axis. Red plot: positive correlation; Blue plot: negative correlation. (C) Pathway analysis for the top 30 genes using the KEGG and Reactome databases. Text on the left indicates the enriched pathway. The ball size indicates the number of the genes enriched, and the color indicates the level of enrichment. CAF, cancer-associated fibroblasts; PC, pancreatic cancer; FIR, fluorescence intensity ratio; KEGG, Kyoto Encyclopedia of Genes and Genomes.

co-culture with different primary CAFs, indicating directly that different primary CAFs had different malignant potential. In addition, the malignant potential of each CAFs was significantly related to original age of the patient, thus it was considered that the malignant potential could be relevant to the senescence of CAFs and SASP. Actually, Toste *et al* (39) reported that gemcitabine-treated CAFs showed upregulated SASP cytokines, which exacerbated PC proliferation, metastasis and resistance to treatment. The present study also

indicated that increased expression of cellular senescence and SASP-related genes in CAFs was correlated with tumor cell proliferation and the tumor size ratio (i.e., chemotherapy resistance). If the effects of CAF reported in the present study are similarly observed in experiments using other PC cell lines, it would be more certain that cellular senescence and SASP in CAFs increase the malignant potential of PC. It was actually examined whether the same effect could be obtained by co-culturing CAFs with other PC cell lines such
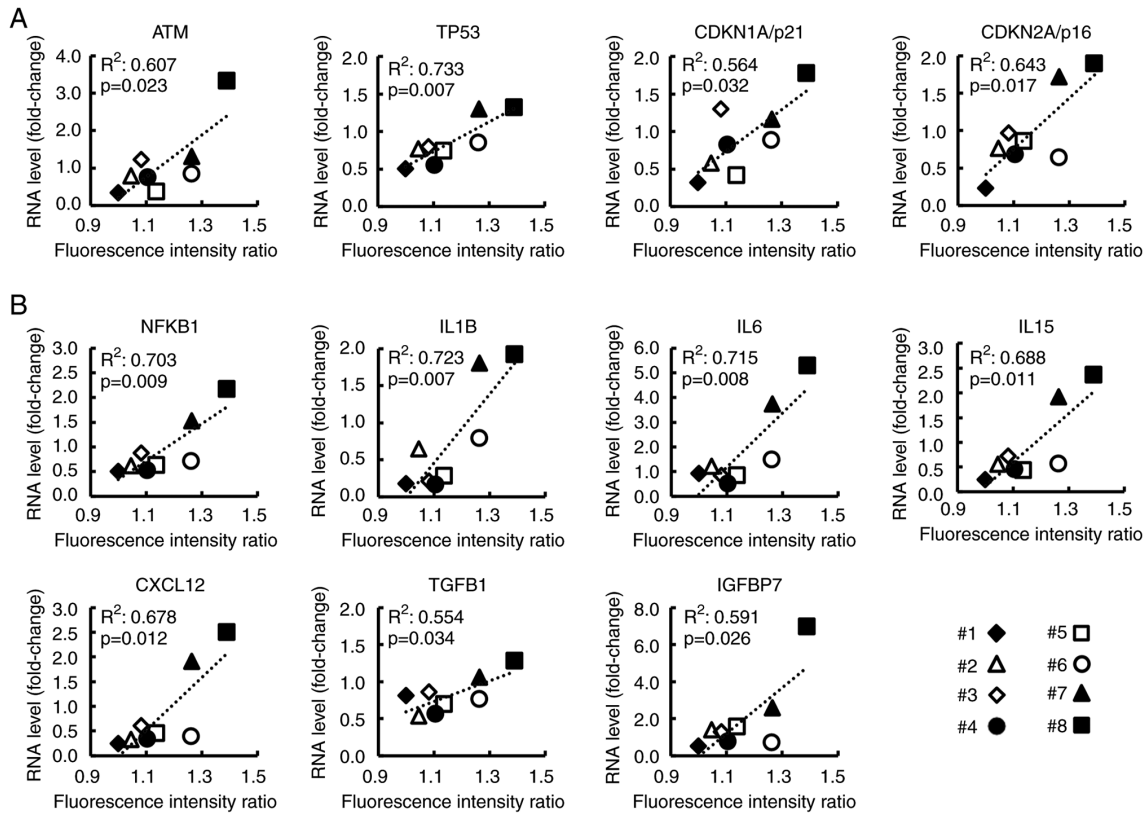
Figure 4. Correlation between FIR in co-culture assays and RNA level of cellular senescence- and SASP-related genes. (A) Data regarding four genes associated with the p53/p21 and p16/RB pathways. (B) Data regarding seven genes associated with SASP. Graph shows FIR on the horizontal axis, and RNA level (fold-change) of each gene on the vertical axis. The square of the correlation coefficient and the P-value are shown. Each symbol legend appears at the right below. FIR, fluorescence intensity ratio; SASP, senescence-associated secretory phenotype.
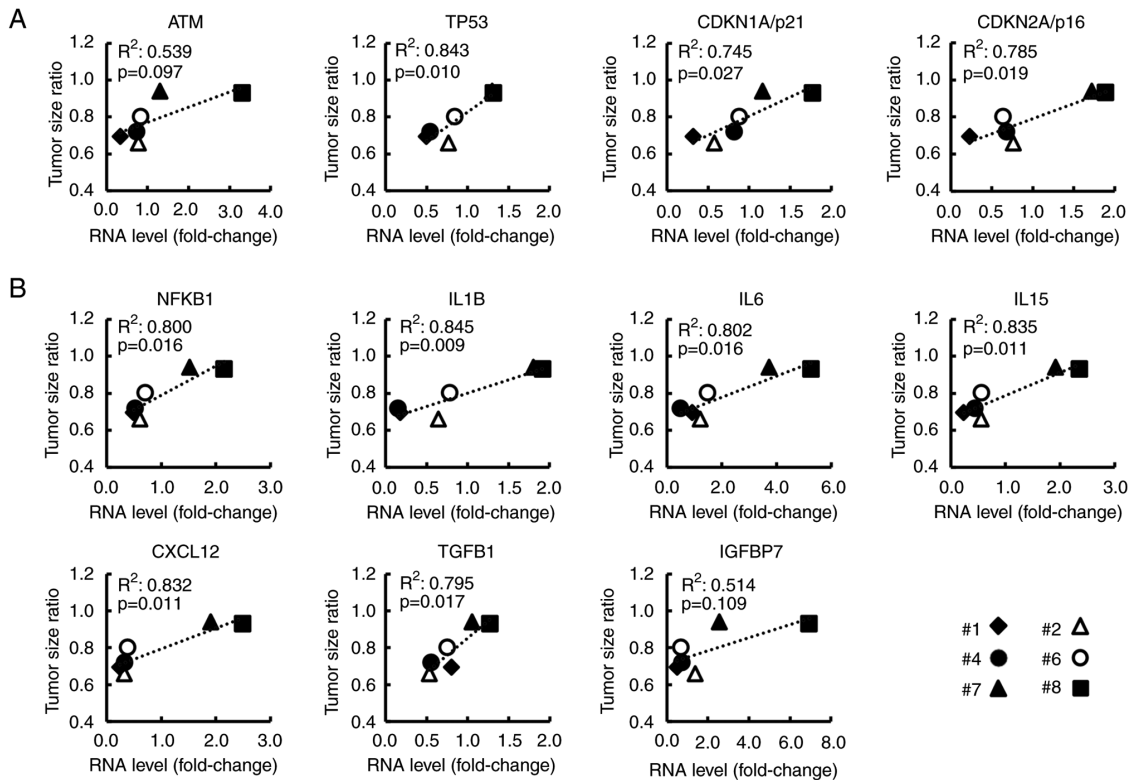

Figure 5. Correlation between TSR and RNA level of cellular senescence- and SASP-related genes. (A) Data regarding four genes associated with the p53/p21 and p16/RB pathways. (B) Data regarding seven genes associated with SASP. Graph shows RNA level (fold-change) of each gene on the horizontal axis, and TSR on the vertical axis. The square of the correlation coefficient and the P-value are shown. Data were obtained for the six patients who received preoperative chemotherapy. Each the symbol legend appears at the right below. TSR, tumor size ratio; SASP, senescence-associated secretory phenotype.
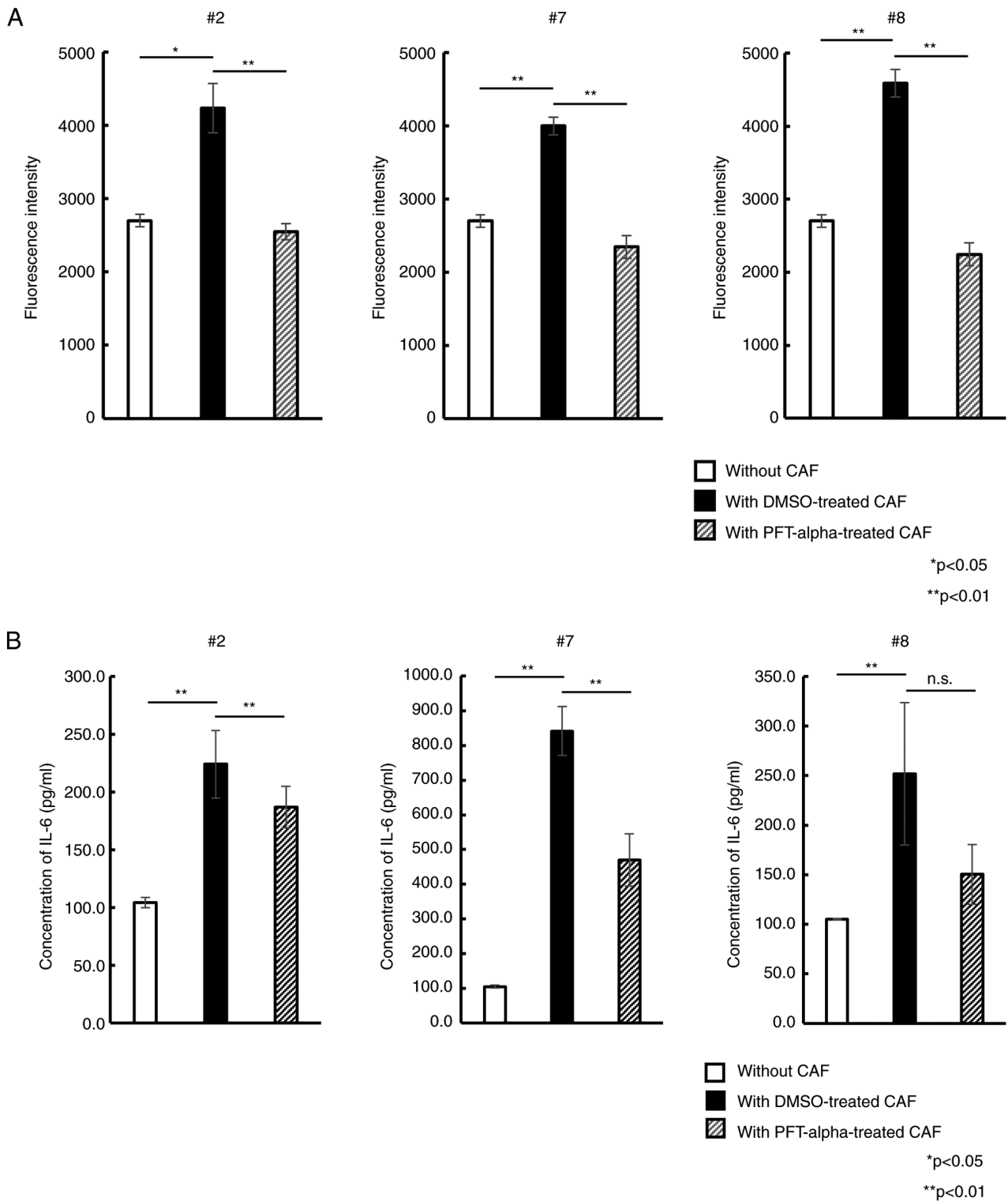
Figure 6. Treatment of CAFs with PFT-alpha reduces PC cell proliferation and the IL-6 concentration in the co-culture supernatant. (A) Fluorescence intensity of PC cell lines when co-cultured with DMSO-treated CAFs or PFT-alpha-treated CAFs. (B) IL-6 concentration (pg/ml) in the co-culture supernatant following co-culture with DMSO-treated CAFs or PFT-alpha-treated CAFs. The graphs show data for 3 cases, #2, #7 and #8, respectively. [*]P<0.05 and [**]P<0.01. CAF, cancer-associated fibroblast; PFT-alpha, pifithrin-alpha; PC, pancreatic cancer.

as MiaPaCa-2, Panc1, TYPK1 and SUIT2. However, in the limited opportunity to use primary CAFs, it was not possible to obtain the similar results as satisfactorily as the results with PSN-1. This is possibly due to the fact that different cell types have somewhat different rates of proliferation and cell sizes; under the experimental conditions of the present study,

significant differences in the effects of each CAF as observed with PSN-1 were not observed with the other cell lines.

Notably, what was important for the cancer cell proliferation was not the expression levels of senescence and SASP-related genes in the presence or absence of serum stimulation, but the increase of the expression ratio of the genes with versus without

serum stimulation. Serum stimulation is known to promote cell division and cause telomere shortening and replicative senescence (40,41). Although two types of fibroblast senescence, replicative senescence and stress-induced premature senescence, are known, they are considered to be different phenomena, as reported by Dierick *et al* (42). Therefore, in the present study, senescence was not induced by stress factors such as irradiation or H_2O_2, but experiments were conducted by inducing senescence via cell culture in serum-containing medium. Therefore, the senescence changes were not stress-related but development-related under serum stimulation. In fact, based on RT-qPCR analysis, the gene expression of p53/p21-related and p16/Rb-related cellular senescence pathways including ATM, p53, p21, and p16/Rb were altered by serum stimulation, suggesting that cellular senescence is induced by serum stimulation. Therefore, it may be suggested that CAFs with higher rates of increased expression of these genes due to replicative senescence more aggressively promote SASP, which in turn promotes cancer proliferation. Meanwhile, 6 of the 8 patients in the present study had received preoperative chemotherapy containing gemcitabine. As aforementioned, since gemcitabine is reported to induce cellular senescence in CAFs (38), it was desirable to study only the sample without preoperative treatment to exclude this effect. However, neoadjuvant chemotherapy has been increasingly performed in PC, even when resectable PC, and samples without preoperative treatment were not readily available. The use of EUS-FNA samples collected prior to neoadjuvant therapy was also considered, but the volume of samples was markedly smaller than that from resected specimens, and it was considered difficult to culture sufficient quantities of fibroblasts for the experiment. If more samples without neoadjuvant chemotherapy are collected, the analysis would reflect *in vivo* phenomena in an improved way.

Based on our co-culture assays and PCR array results, a validation experiment focusing on TP53 was further conducted, among 11 senescence- and SASP-related genes that are considered to be related to cancer cell proliferation. Focus was addressed on TP53 because p53, the nuclear protein encoded by TP53 and one of the best-known tumor suppressor genes, plays a vital role in the induction of both cellular senescence and SASP. Additionally, the specific inhibitor of p53, PFT-alpha, reportedly alters post-translational modification patterns and differentially inhibits p53 target genes (43), such that it was relatively easy to optimize the experimental conditions (27). The results of the present study indicated that treatment of CAFs with 10 μM PFT-α attenuated the ability of CAFs to promote PC cell proliferation and this may be related to cellular senescence via the p53/p21 pathway in CAFs. Notably, in the present experiment, the supernatant medium containing PFT-alpha was completely washed out before initiating co-culturing; therefore, there was no direct effect of PFT-alpha on the cancer cells. Moreover, it was verified that PFT-alpha up to at least 10 μM did not affect the proliferation ability of cancer cells. Nevertheless, it remains unclear whether inhibition of the p53/p21 pathway by PFT-alpha directly affects secretion of the SASP factor. Further investigations are needed to address this point. Additionally, it is also understandable that numerous other SASP besides IL-6 secreted from senescent CAFs were related to cell proliferation potential of cancer cells and it has to be examined which cytokine was dominant in future research. Meanwhile, it is understandable

that cellular senescence was induced not only by p53 but by other significant molecules, such as p16, thus experiments were also conducted using RRD-251, an inhibitor of the RB gene in the senescence pathway, although this result was not included because the data are very preliminary. Similar to the experiment with PFT-α, the proliferation potential of cancer cells was reduced by co-culture with RRD-251 treated CAFs, in which the senescence of CAFs was recovered by the inhibition of RB. Unfortunately, due to a lack of sample stock, these experiments were performed in only two other CAFs, but it is considered by the authors that it does provide some support for the importance of the p53/p21 cellular senescence pathway in CAFs.

The present study revealed that cellular senescence of CAFs and expression of SASP-related genes are involved in the malignant potential of PC by analyzing CAFs alone, but other parameters in addition to the age of patients may also influence the malignant potential of CAFs, such as the characteristics of the original cancer, and this requires further investigation. It is also very important to examine whether this cellular senescence of CAF is a change unique to CAFs or it also occurs in normal fibroblasts. The authors are also interested in the differences between normal fibroblasts and CAFs and primary culture of normal fibroblasts from normal pancreatic tissue in resected specimens has been attempted. However, normal fibroblasts were difficult to culture and the quality of the cells was unfortunately inadequate for the assay. The results may indicate that CAFs may have been affected in some way by the original cancer cells, thus in the next study it will be attempted to perform further assays to determine the differences between the original fibroblasts and CAFs. It is also considered that future study is needed to examine the effects of the cancer microenvironment on CAFs in terms of induction of cellular senescence.

In summary, the present data provided the first evidence, to the best of our knowledge, that p53-mediated cellular senescence and SASP of CAFs affect the malignant potential of PC cells. Thus, controlling cellular senescence and SASP in CAFs may be a new strategy for PC treatment.

Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MH and HM designed the present study. ST helped to analyze data. HA, SK, ST, YI, DY, YT, TN, KG, YD, TY and HE helped to design the study. SK and TY confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Tissue samples of human PC were collected with the approval (approval no. 18138-4) of the Human Ethics Review Committee of the Graduate School of Medicine of Osaka University (Osaka, Japan) and the National Institutes of Biomedical Innovation, Health and Nutrition, (approval no. 201-01; Osaka, Japan). Written informed consent for sample use was obtained from all patients before surgery.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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