

CD10⁺ Pancreatic Stellate Cells Enhance the Progression of Pancreatic Cancer

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BACKGROUND & AIMS: Pancreatic stellate cells (PSCs) promote the progression of pancreatic cancer by producing extracellular matrix and soluble factors. However, the functional heterogeneity of PSCs has not been identified until now. Detailed characterization of the PSCs in human pancreatic cancer would provide a set of potential targets for stroma-directed therapy. **METHODS:** We isolated PSCs from fresh pancreatic ductal adenocarcinoma tissue and sorted them by flow cytometry according to cell surface expression of CD10, which is a stromal prognostic marker for various tumors. We analyzed the functional differences between CD10⁺ PSCs and CD10⁻ PSCs. **RESULTS:** Immunohistochemical analysis showed that the frequency of CD10 expression by PSCs was markedly higher in tumor tissue than in normal tissue (33.7% vs 0%, respectively, $P = .028$). In pancreatic ductal adenocarcinoma, CD10 expression by PSCs was associated with positive nodal metastases ($P = .011$) and a shorter survival time ($P < .001$). In vitro coculture experiments showed that CD10⁺ PSCs promoted the invasiveness of pancreatic cancer cell lines, SUIT-2 and Panc-1 cells more intensively than CD10⁻ PSCs. CD10⁺ PSCs significantly increased the tumor growth and invasiveness of SUIT-2 cells in a murine co-transplantation model. CD10⁺ PSCs secreted higher levels of matrix metalloproteinase 3 than CD10⁻ PSCs, and knockdown of matrix metalloproteinase 3 in cocultured PSCs reduced the invasion of SUIT-2 and Panc-1 cells. **CONCLUSIONS:** CD10⁺ PSCs enhance the progression of pancreatic cancer cells. CD10⁺ PSCs may be a candidate for selective therapeutic targeting in the treatment of pancreatic cancer.

Keywords: CD10; Pancreatic Cancer; Pancreatic Stellate Cells.

Pancreatic cancer is characterized by excessive desmoplasia, which plays a crucial role in its aggressive behavior.¹ Pancreatic stellate cells (PSCs) have been identified as the principal source of the excessive extracellular matrix observed in chronic pancreatitis² and pancreatic adenocarcinoma.³ Like hepatic stellate cells, which are known to be the most important cell type for extracellular matrix production in hepatic fibrosis, PSCs store fat

droplets, containing vitamin A, within their cytoplasm.⁴ PSCs are transformed into the activated phenotype upon stimulation by various autocrine or paracrine factors. They express α -smooth muscle actin (α -SMA) and produce various extracellular matrix proteins.^{5,6} Soluble factors secreted by activated PSCs promote proliferation, migration, invasion, and survival against gemcitabine therapy of pancreatic cancer cells.⁷

Recently, research into cancer biology has focused on the concept of cancer stem cells (CSCs). CSCs comprise a very small population of cancer cells and have the ability to initiate and sustain tumor formation.⁸⁻¹⁰ CSCs have been isolated on the basis of their expression of cell surface markers such as CD24, CD44, and CD133.⁸⁻¹⁰ This concept may cause a shift in the paradigm of cancer therapy because treatments specifically targeting CSCs may be more effective for treating solid tumors. However, myofibroblasts and mesenchymal cells isolated from various human tissues also exhibit different phenotypes.^{11,12} The role of PSCs in the progression of pancreatic cancer has been discussed extensively, but the specific phenotypes of PSCs with different functions have not yet been investigated. Detailed characterization of human PSCs in pancreatic cancer would help to clarify the mechanism underlying the interaction between cancer cells and stromal cells and may provide a set of potential targets for stroma-directed therapy. Thus, we hypothesized that, similar to CSCs, PSCs have functional heterogeneity and are the leading cell population promoting the progression of pancreatic cancer.

CD10 is a 90–110 kilodalton, zinc-dependent, cell membrane-associated metalloproteinase commonly expressed in bone marrow lymphoid stem cells, pro-B lymphoblasts, and mature neutrophils. It is a marker for categorizing acute leukemias and for the subclassifica-

Abbreviations used in this paper: α -SMA, α -smooth muscle actin; CTGF, connective tissue growth factor; CSCs, cancer stem cells; MMP, matrix metalloproteinase; mRNA, messenger RNA; PSCs, pancreatic stellate cells; rRNA, ribosomal RNA; RT-PCR, reverse transcription polymerase chain reaction; siRNA, small interfering RNA.

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0016-5085/\$36.00

doi:10.1053/j.gastro.2010.05.084

tion of malignant lymphomas.¹³ It has been reported that CD10⁺ cells also exist in the stroma of gastric cancer,¹⁴ breast cancer,^{15,16} and colorectal cancer.¹⁷ Several immunohistochemical studies have shown that stromal CD10 expression is a prognostic marker in malignancies and is associated with biologic aggressiveness.^{14,16-18} However, these studies were limited to immunohistochemistry, and the biologic mechanism by which CD10⁺ stromal cells promote tumor progression has not been elucidated. Moreover, there have not been any reports on the expression of CD10 by stromal cells in pancreatic cancer. CD10⁺ stromal cells may represent one phenotype of the PSCs in pancreatic cancer and may contribute to the progression of pancreatic tumor cells.

The aim of this study was to identify the specific PSCs that promote the progression of cancer cells by focusing on the stromal stem cell marker CD10. We assessed the impact of CD10⁺ PSCs on pancreatic cancer progression and investigated the biologic mechanism by which CD10⁺ PSCs promote tumor progression.

Materials and Methods

Patients and Pancreatic Tissues

Pancreatic cancer tissues were obtained from 83 patients who underwent pancreatic resection for pancreatic cancer at our institution. The clinicopathologic characteristics of the patients are described in Supplementary Method 1 and Supplementary Table 1. We also obtained 10 normal pancreatic tissue samples from intact pancreases resected for bile duct cancer, or pancreatic tumors, as control tissues. The study was approved by the Ethics Committee of Kyushu University and conducted according to the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government and the Helsinki Declaration.

Cells and Culture Conditions

Human PSCs were isolated from fresh pancreatic cancer surgical specimens using the out-growth method.^{2,19} Primary cultures of PSCs derived from 12 patients with invasive pancreatic cancers were established in our laboratory. The PSCs cell type was confirmed by immunofluorescence staining for α -SMA and Vimentin and by morphology (stellate-like or spindle-shaped cells).^{7,19} Passage numbers 3 to 8 were used for the assays. In addition, 3 pancreatic cancer cell lines, SUIT-2, Panc-1 (Dr Iguchi, National Shikoku Cancer Center, Matsuyama, Japan), and SW1990 (American Type Culture Collection, Manassas, VA) were used. Cells were maintained as previously described.²⁰

Quantitative Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted from cultured cells using a High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) and DNase I (Roche Diagnostics)

treatment according to the manufacturer's instructions. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed using a QuantiTect SYBR Green Reverse Transcription-PCR kit (Qiagen, Tokyo, Japan) and a Chromo4 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). We designed specific primers for matrix metalloproteinase (MMP) 14, stromal cell-derived factor 1, hepatocyte growth factor, transforming growth factor β 1, basic fibroblast growth factor, vascular endothelial growth factor, and 18S ribosomal RNA using primer 3. The primers for CD10, MMP2, MMP3, MMP7, MMP9, and connective tissue growth factor (CTGF) were purchased from Takara Bio Inc (Tokyo, Japan). The sequences of these primers are shown in Supplementary Table 2. Detailed conditions and procedures for RT-PCR are described in Supplementary Method 2.

Immunohistochemical Procedures and Evaluation

Immunohistochemistry was performed using a Histofine SAB-PO kit (Nichirei, Tokyo, Japan). Sections were incubated with a mouse monoclonal anti-CD10 (56C6; 1:100; Novocastra, Newcastle Upon Tyne, UK) or α -SMA antibodies (1:50; DAKO, Glostrup, Denmark) overnight at 4°C. Cytoplasmic and membrane immunoreactivity was detected in the stromal and carcinoma cells. We identified and counted the stromal cells based on cell morphology (spindle-shaped cells) in at least 20 fields per section at 200-fold magnification. Their identities were confirmed by staining for α -SMA. The stromal expression of CD10 was determined to be positive when >5% of the stromal cells around the neoplastic tubules or glands were stained because CD10 was difficult to detect and the intensity was uniform. The stromal cells around normal pancreatic ducts were also evaluated in normal pancreatic tissues. In carcinoma cells, the staining intensity of CD10 was scored as 0, no staining; 1, weak; 2, moderate; and 3, strong. The expression of CD10 in carcinoma cells was defined as positive when >30% of carcinoma cells were scored as 2 or 3. All slides were evaluated independently by 3 investigators without any knowledge of the clinical features of each case.

Flow Cytometry Analysis

Cultured cells were obtained from subconfluent monolayer cultures, suspended in 1% fetal bovin serum/phosphate-buffered saline solution at 1×10^6 cells/100 μ L and incubated with 20 μ L of phycoerythrin-conjugated anti-CD10 antibody (eBioscience Inc, San Diego, CA) on ice for 40 minutes. Cellular expression of α -SMA was examined using phycoerythrin-conjugated anti- α -SMA antibody (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Nonspecific mouse immunoglobulin (Ig) G (Miltenyi Biotec, Auburn, CA) was used as a negative control. Labeled cells were

analyzed using an EPICS ALTRA flow cytometer (Beckman Coulter, Fullerton, CA).

Laser Scanning Confocal Microscopy for Immunofluorescence Staining of α -SMA and CD10

PSCs were plated on Glass Bottom Dishes (Matsunami, Osaka, Japan) at a density of 1×10^5 and incubated for 24 hours. PSCs were then fixed with methanol, blocked with 10% normal goat serum in phosphate-buffered saline and incubated with rabbit anti- α -SMA (1:100; Epitomics, Burlingame, CA) and mouse anti-CD10 antibodies (56C6; 1:100; Novocastra) overnight at 4°C. The cells were then incubated for 1 hour with Alexa 488-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR) and Alexa 546-conjugated anti-rabbit IgG (Molecular Probes). Nuclear DNA was counterstained with 4',6-diamidino-2-phenylindole (0.05 μ g/mL). A laser-scanning confocal fluorescent microscope (A1R; Nikon, Tokyo, Japan) was used for immunofluorescence microphotography. Images were managed using NIS-Elements software (Nikon).

Matrigel Invasion and Migration Assays

The invasiveness of pancreatic cancer cells was assessed based on the number of cells invading through Matrigel-coated transwell chambers (BD Biosciences, Franklin Lakes, NJ) as previously described.²⁰ Detailed procedures are described in Supplementary Method 3. The migration of pancreatic cancer cells was assessed using non-Matrigel-coated transwell inserts.

Propidium Iodide Assay

Cell proliferation was evaluated by measuring the fluorescence intensity of propidium iodide as previously described.²¹ Detailed procedures are described in Supplementary Method 4.

In Vitro Coculture System

In vitro coculture was performed using either 6- or 24-well transwell cell culture systems (Becton Dickinson Labware, Bedford, MA) as previously described.²² Detailed procedures are described in Supplementary Method 5.

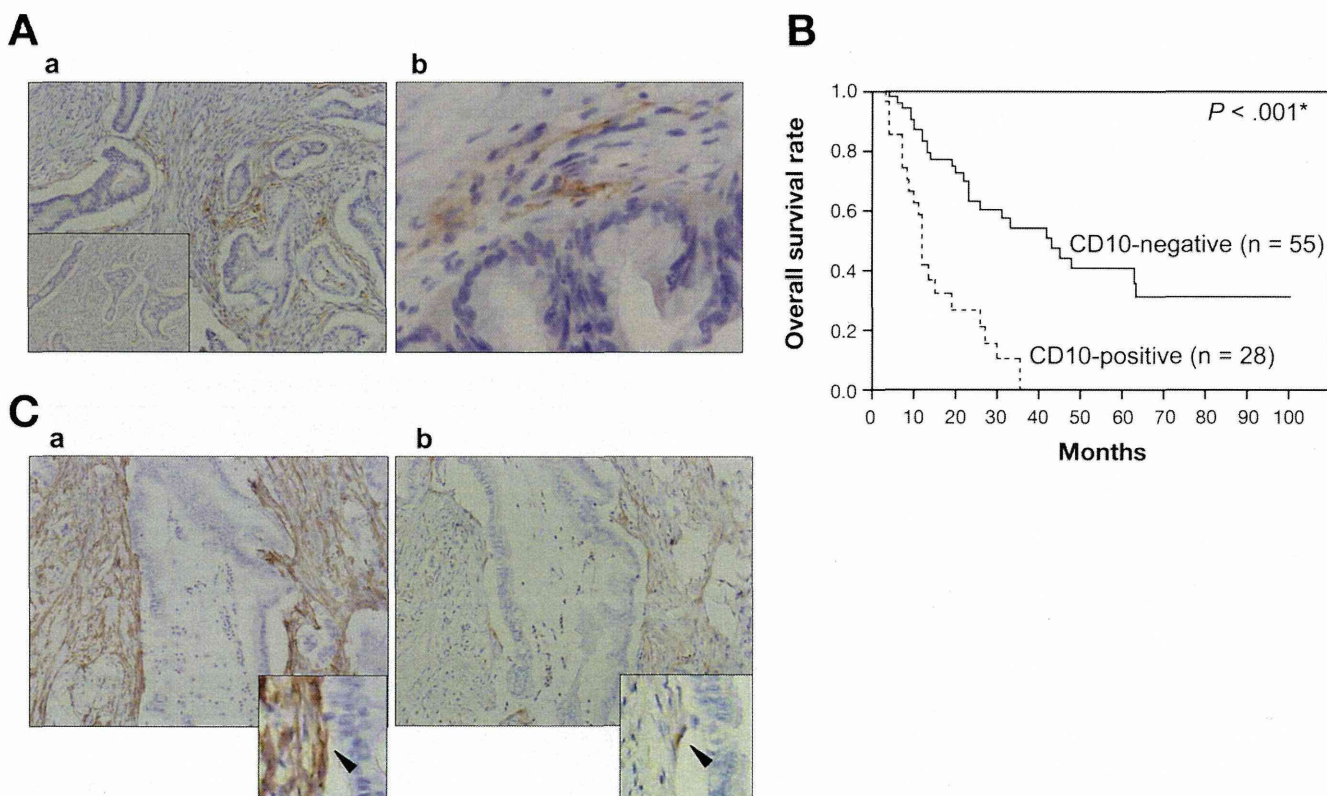


Figure 1. Characterization of CD10 expression by stromal cells in pancreatic cancer. (A) Immunohistochemistry of CD10 in pancreatic cancer. (A-a) CD10⁺ stromal cells appear adjacent to and surrounding the tumor cells. Negative control is shown in the *inset*. No CD10 expression is detectable in normal pancreatic tissues. (A-b) In some cases, both CD10⁺ and CD10⁻ stromal cells resided around tumor cells. (Original magnification: a, $\times 100$; b, $\times 200$) (B) Kaplan-Meier survival analysis of CD10 expression in the stroma of pancreatic ductal adenocarcinomas. CD10 positive is defined as $>5\%$ of stromal cells staining for CD10. Stromal CD10 expression was associated with shorter patient survival times ($P < .001$). (C) Distribution of α -SMA⁺ and CD10⁺ stromal cells. α -SMA (a) and CD10 (b) were stained in serial sections of pancreatic ductal adenocarcinoma. CD10 was expressed in areas with strong α -SMA expression. *Arrowheads* in the *insets* indicate α -SMA⁺ and CD10⁺ cells in the serial sections. (Immunohistochemistry of α -SMA and CD10; original magnification: $\times 100$, *insets*: $\times 200$)

Silencing of CD10 and MMP3 by Small Interfering RNA

PSCs (90% confluent) were transfected with CD10-1 (sense, 5'-gguugaauuucacaaaugatt-3'; antisense, 5'-ucauuugugaaauucaaccag-3') and CD10-2 (sense, 5'-gugugguguggaaccuauatt-3'; antisense, 5'-uauagguuccacaccacact-3') small interfering RNA (siRNA) (Qiagen) or MMP3-1 (sense, 5'-gaagagucuuccaauccuatt-3'; antisense, 5'-uaggauuggaagacucuucacat-3') and MMP3-2 (sense, 5'-cgccugucucaagaugauatt-3'; antisense, 5'-uaucaucuugagacaggcgga-3'), siRNA (Qiagen) by electroporation using a Nucleofector System (Amaxa Biosystems, Köln, Germany) according to the manufacturer's instructions. To verify the specificity of the knockdown effects, we used a control siRNA (Qiagen). PSCs were used in the subsequent experiments 24–96 hours after transfection.

Western Blotting Analysis

PSCs (1×10^6) were transfected with siRNA and incubated for 24 hours. The medium was changed to serum-free Dulbecco's modified Eagle medium, and cells were cultured for a further 72 hours. The supernatants were concentrated using an Amicon Ultra-15 filter unit (Millipore, Billerica, MA) at 3500 rpm for 30 minutes, and PSCs were lysed in PRO-PREP (iNtRON Biotechnology, Seongnam, Korea). Supernatant and cell lysate proteins (15 μ g) were fractionated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was incubated overnight at 4°C with anti-CD10 (56C6; 1:100; Novocastra), anti-MMP2 (sc-10736; Santa Cruz Biotechnology, Santa Cruz, CA; 1:200), anti-MMP-3 (sc-6839; 1:200), anti-MMP7 (sc-80205; 1:200), anti-MMP9 (EP1254; 1:1000; Millipore), anti-MMP14 (sc-12367-R; 1:200), anti-collagen type 1 (sc-8783; 1:200), anti-fibronectin (sc-6952; 1:200), or anti- β -actin (sc-1616; 1:2000) antibodies and then probed with secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology). Immunoblots were detected by enhanced chemiluminescence with ChemiDoc XRS (Bio-Rad Laboratories).

In Vivo Experiments

Sorted PSCs (1×10^6) and SUIT-2 cells (3.5×10^6) were suspended in 100 μ L Dulbecco's modified Eagle medium and subcutaneously cotransplanted into the limbs of 5-week-old female nude mice (BALB/c nu/nu; Kyudo Co, Saga, Japan). Fifteen and 16 mice were used for the CD10⁺ PSC group and CD10⁻ PSC group, respectively. SUIT-2 cells alone (3.5×10^6) were transplanted into 17 additional mice. Forty days after implantation, the tumors were resected. The tumor volume was calculated using the following formula: $\pi/6 \times (L \times W \times W)$, where L represents the largest tumor diameter and W represents the smallest tumor diameter. Also, sorted PSCs (1×10^6) and SUIT-2 cells (1×10^6) were implanted

into the pancreas of 5-week-old female nude mice. Four mice were used in each group. Two weeks after implantation, tumors were resected along with the surrounding tissue. Tissues were fixed in 10% neutral formalin, embedded in paraffin, and sections stained with H&E. To semiquantitate the invasiveness of the implanted pancreatic cancer, we based the invasion score on histologic observations as previously reported²⁰: score 0, invasion was undetectable, and the tumor was surrounded by a capsule; score 1, invasion was undetectable, but the tumor was not surrounded by a capsule; score 2, invasion was partial; score 3, invasion was extensive, and normal pancreatic and tumor regions could not be distinguished.

Statistical Analysis

A χ^2 test was used to analyze the correlation between stromal CD10 expression and clinicopathologic characteristics seen in the immunohistochemical study. Survival analysis undertaken using Kaplan-Meier analysis and curves were compared using the log-rank test. For the in vitro experiments, values are expressed as means \pm standard deviation. Comparison between 2 groups was done using the Student *t* test. All experiments were repeated twice. Statistical significance was defined as *P* < .05. All statistical analyses were performed using JMP 7.01 software (SAS Institute, Cary, NC).

Results

Correlation Between Stromal CD10 Expression and Clinicopathologic Characteristics

To evaluate the correlation between stromal CD10 expression and the clinicopathologic factors of

Table 1. Relationship Between Stromal CD10 Expression and Various Clinicopathologic Factors

Characteristics	CD10 positive, n = 28, (%)	CD10 negative, n = 55 (%)	P value
Age, y			.582
≥ 65	14 (50.0)	31 (56.4)	
<64	14 (50.0)	24 (43.6)	
pT category			.583
pT1/pT2	2 (7.1)	6 (10.9)	
pT3/pT4	26 (92.9)	49 (89.1)	
pN category			.011
pN0	4 (14.3)	23 (41.8)	
pN1	24 (85.7)	32 (58.2)	
UICC stage			.102
I	0 (0.0)	6 (10.9)	
II	26 (92.9)	48 (87.3)	
III/IV	2 (7.1)	1 (1.8)	
Histologic grade			.005
G1/G2	12 (42.9)	41 (74.6)	
G3	16 (57.1)	14 (25.5)	
Pathologic margin			.084
Negative	15 (53.6)	40 (72.7)	
Positive	13 (46.4)	15 (27.3)	

UICC, International Union Against Cancer

pancreatic ductal adenocarcinoma, immunohistochemistry for CD10 was performed. CD10⁺ stromal cells appeared in the close vicinity of the tumor cells and surrounded the neoplastic tubules (Figure 1A-a). In some cases, both CD10⁺ and CD10⁻ stromal cells resided around the tumor cells (Figure 1A-b). Positive CD10 expression in stromal cells was found in 33.7% (28/83) of pancreatic ductal adenocarcinomas, whereas there was no expression of CD10 in stromal cells of the 10 normal pancreatic tissue samples ($P = .028$). G3 grade tumors ($P = .005$) and nodal metastasis ($P = .011$) were observed more frequently in the CD10-positive group than in the CD10-negative group (Table 1). Interestingly, the pathologic invasiveness of cancer cells in stromal CD10-positive cases tended to reach the resection margin, although the difference did not reach statistical significance ($P = .084$). These results suggest that stromal CD10 expres-

sion is associated with tumor progression, including nodal metastasis and local invasion of cancer cells.

Stromal CD10 Expression Independently Indicates Shorter Patient Survival Times

Stromal CD10 expression was associated with shorter patient survival times (Figure 1B). The median survival times for CD10-positive cases and CD10-negative cases were 12 and 43 months, respectively. Next, we performed a multivariate survival analysis based on the Cox proportional hazard model on all parameters found to be significant by univariate analysis, including stromal CD10 positivity, pN1, International Union Against Cancer stages III/IV, G3 tumor, and pathologic margin positivity (data not shown). Stromal CD10 expression was an independent poor prognostic marker in pancreatic cancer patients, with a relative risk of 2.586 (Supplementary

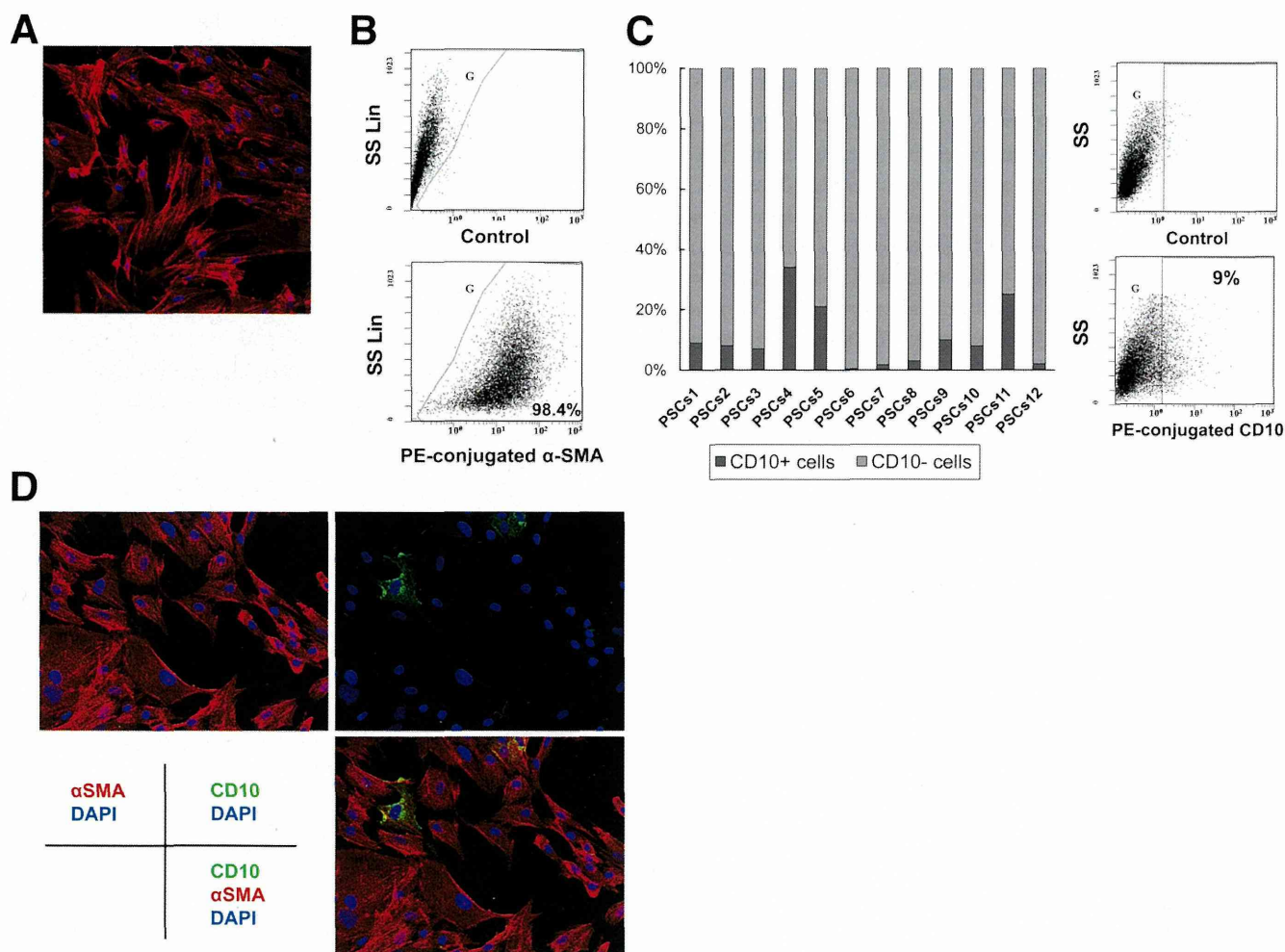


Figure 2. Expression analysis of α -SMA and CD10 in human PSCs isolated from pancreatic ductal adenocarcinoma. (A) Representative microphotograph of immunofluorescence staining of α -SMA in PSCs. PSCs were stellate-like or spindle-shaped and expressed α -SMA (original magnification: $\times 200$). (B) Using flow cytometry, we confirmed that isolated PSCs expressed α -SMA. (C) The percentage of CD10⁺ PSCs in human PSCs, as evaluated by flow cytometry, ranged between 0.4% and 34% of CD10⁺ PSCs. Representative flow cytometry image of CD10 in activated PSCs (right). (D) Laser-scanning confocal microscopy of human PSCs stained with anti- α -SMA (red) and anti-CD10 (green) antibodies. 4',6-Diamidino-2-phenylindole (DAPI) (blue) was used for nuclear staining. CD10 was expressed in some of the PSCs expressing α -SMA. (original magnification: $\times 200$)

Table 3). However, positive CD10 expression in carcinoma cells was found in 31.3% (26/83) of pancreatic ductal adenocarcinomas (Supplementary Figure 1A), but the expression of CD10 in carcinoma cells was not associated with stromal CD10 expression, clinicopathologic factors (Supplementary Table 4), or patient survival (Supplementary Figure 1B).

Stromal Cells Expressing α -SMA Around Cancer Cells Partially Expressed CD10

To identify CD10⁺ stromal cells, we performed immunohistochemistry for CD10 and α -SMA, which is one of the markers for activated PSCs, on serial sections of pancreatic ductal adenocarcinoma. We found that α -SMA was expressed in almost all of the stromal cells around cancer cells and neoplastic tubules (Figure 1C-a). CD10 was expressed in areas with strong α -SMA expression, suggesting that activated PSCs also express CD10 (Figure 1C-b). These findings indicate that CD10⁺ stromal cells are a subpopulation of activated PSCs.

Analysis of CD10 Expression in Human PSCs

We established in vitro cultures of PSCs from pancreatic ductal adenocarcinoma to confirm the results of our immunohistochemical analysis, ie, that PSCs consisted of both CD10⁺ and CD10⁻ cells. Twenty PSC cultures (PSCs 1–12) were isolated from 12 fresh surgical specimens of human pancreatic adenocarcinoma, and their identity was confirmed by immunohistochemical staining for Vimentin and α -SMA (Figure 2A). Established PSCs were stellate-like or spindle-shaped, and $93.7\% \pm 7.1\%$ of them expressed α -SMA, indicating that they were of the activated phenotype (Figure 2B). Flow cytometry and immunofluorescence staining of cultured PSCs for α -SMA and CD10 showed that activated PSCs contained varying amounts (0.4%–34%) of CD10⁺ PSCs (Figure 2C and D). PSCs were found to consist of different populations of cells, indicating that, similar to cancer cells, PSCs are heterogeneous.

CD10⁺ PSCs Enhance the Invasiveness of Pancreatic Cancer Cells More Intensively Than CD10⁻ PSCs

To investigate the functional differences between CD10⁺ and CD10⁻ PSCs, we sorted CD10⁺ PSCs and CD10⁻ PSCs from primary cultures of parental PSCs (Supplementary Figure 2). The level of CD10 messenger RNA (mRNA) in these 2 populations was consistent with the level of CD10 cell surface protein expression. We investigated the effects of these 2 populations on the invasiveness of Panc-1 and SUI-2 cells using invasion assays in a coculture system. CD10⁺ PSCs in PSCs1–5 promoted the invasiveness of Panc-1 cells and SUI-2 cells more intensively than CD10⁻ PSCs (Figure 3). The migration of Panc-1 cells and SUI-2 cells was promoted by both CD10⁺ and CD10⁻ PSCs to the same extent (Supplementary Figure 3). These data suggest that

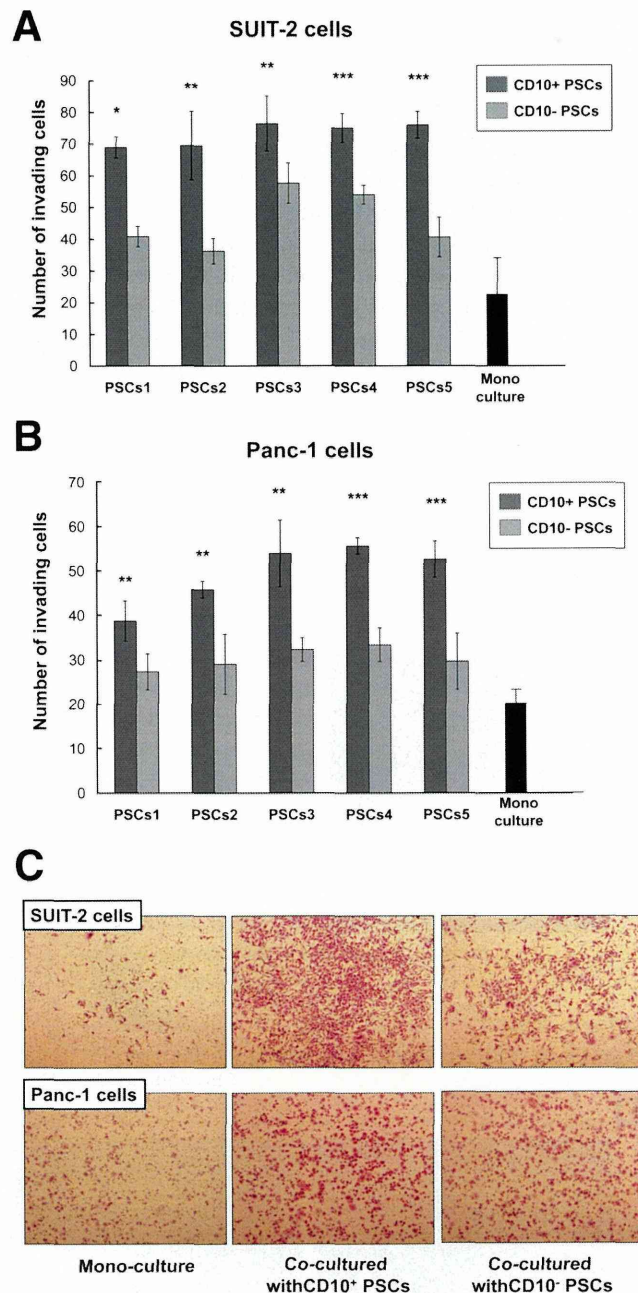


Figure 3. Effects of CD10⁺ PSCs (PSCs 1–5) on the invasive potential of pancreatic cancer. CD10⁺ PSCs promoted the invasiveness of SUI-2 (A) and Panc-1 (B) cells compared with CD10⁻ cells (* $P < .001$; ** $P < .05$; *** $P < .01$). (C) Representative photomicrographs of invading SUI-2 and Panc-1 cells cocultured with CD10⁺ and CD10⁻ PSCs (H&E original magnification, $\times 40$).

CD10⁺ PSCs promote the invasiveness of cancer cells, which may be the reason that stromal CD10-positive carcinomas tend to be more invasive than CD10-negative carcinomas, as found by immunohistochemistry.

There Is no Difference in Fibrogenic Capacity Between CD10⁺ and CD10⁻ PSCs

The ability to proliferate and synthesize extracellular matrix, as represented by collagen I and fibronectin

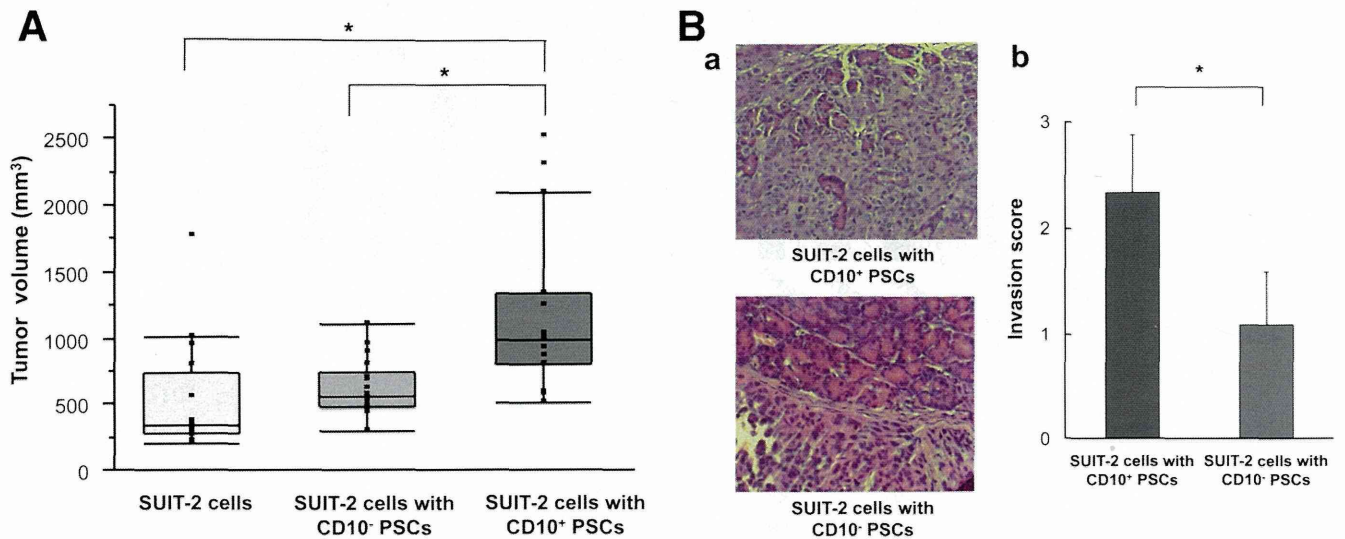


Figure 4. (A) Effects of CD10⁺ PSCs on the in vivo tumor growth of pancreatic cancer. CD10⁺ PSCs significantly enhanced the tumor growth of SUIT-2 cells more than CD10⁻ PSCs (**P* < .01). Seventeen, 16, and 15 mice were used for SUIT-2 alone, CD10⁻ PSCs, and CD10⁺ PSCs group, respectively. (B) Effects of CD10⁺ PSCs on the invasion of pancreatic cancers in in vivo orthotopic models. Suit-2 cells and sorted PSCs were mixed and implanted into the pancreases of mice. Four mice were used in each group. Fourteen days after implantation, tumors were resected along with the surrounding tissues, stained with H&E (×100; a) and evaluated for invasiveness (b; **P* < .05).

secretion, was the same in CD10⁺ and CD10⁻ PSCs (Supplementary Figure 4A). Also, CD10⁺ PSCs did not particularly influence the proliferation and collagen I and fibronectin expression by parental PSCs compared with CD10⁻ PSCs (Supplementary Figure 4B). These findings indicate CD10 expression by PSCs is not associated with fibrogenic capacity.

CD10⁺ PSCs Enhance Tumor Growth and Invasion in Pancreatic Cancer Models In Vivo

To evaluate the effects of CD10⁺ and CD10⁻ PSCs on in vivo tumor growth, we cotransplanted SUIT-2

cells with CD10⁺ or CD10⁻ PSCs into nude mice. CD10⁺ PSCs significantly enhanced the growth of SUIT-2 cells compared with CD10⁻ PSCs (Figure 4A). The tumor volume of cotransplanted SUIT-2 cells and CD10⁻ PSCs was similar to that of SUIT-2 cells alone. We also evaluated the effect of CD10⁺ PSCs on the invasion of pancreatic cancers in in vivo orthotopic models. Tumors derived from Suit-2 cells and CD10⁺ PSCs invaded into normal pancreatic tissues without forming a capsule, whereas the tumor cells derived from Suit-2 cells and CD10⁻ PSCs were encapsulated by layers of stromal cells (Figure 4B-a). The invasive score in the CD10⁺ PSCs

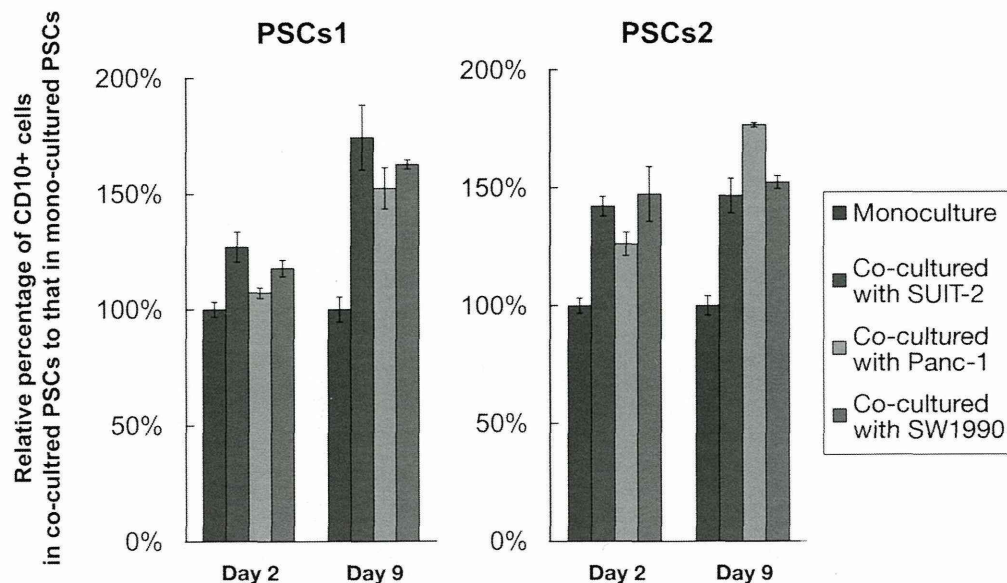
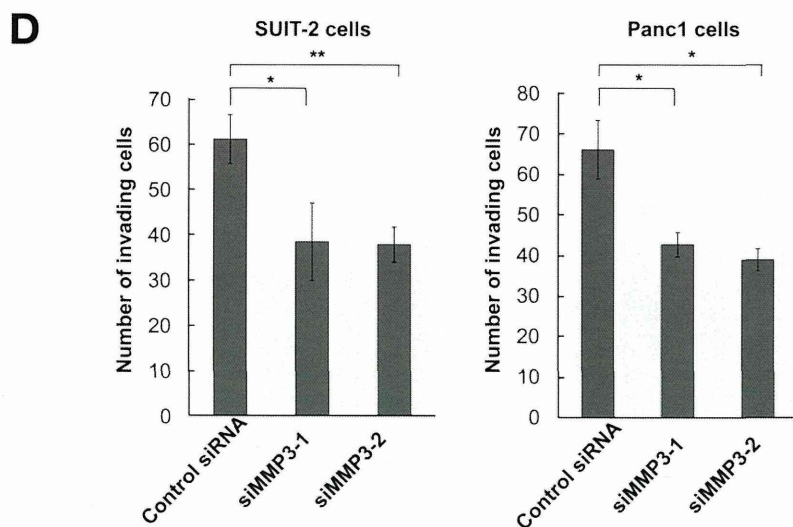
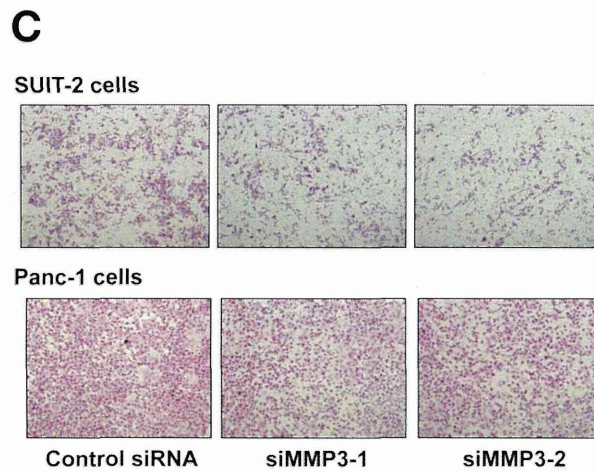
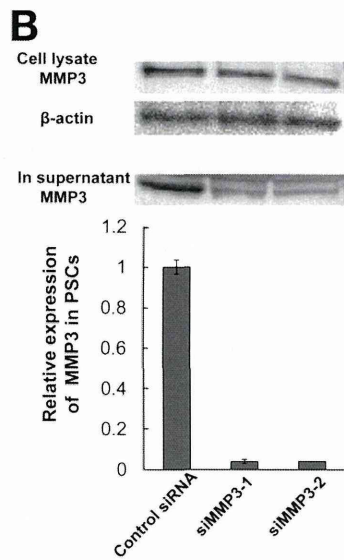
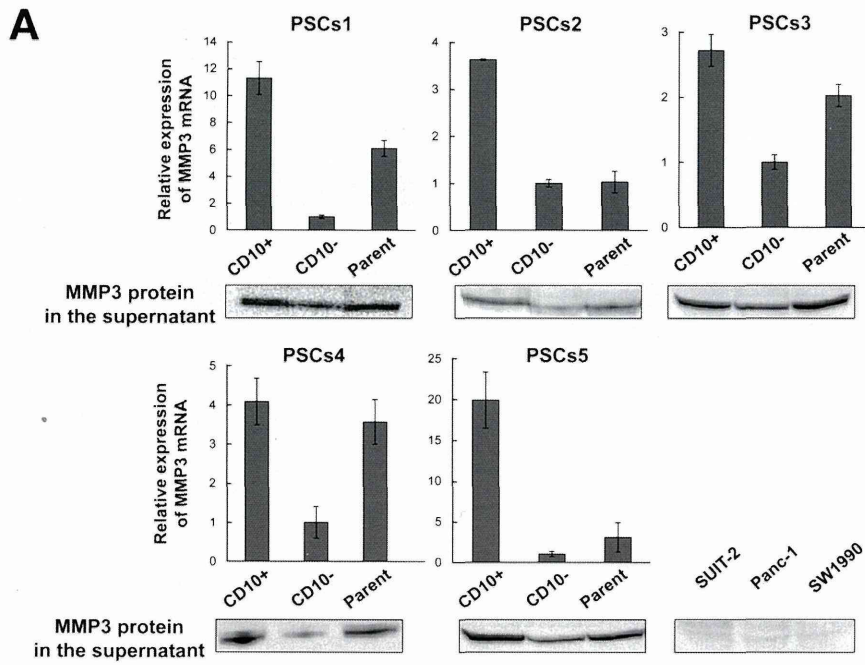


Figure 5. Relative percentage of CD10⁺ PSCs in the PSCs cocultured with pancreatic cancer cells to that in monocultured PSCs. PSCs were cocultured with SUIT-2, Panc-1, and SW1990 for the indicated days, and then the percentage of CD10⁺ PSCs was assessed by flow cytometry.



group was significantly higher than that in the CD10⁻ PSC group (Figure 4B-b).

The Number of CD10⁺ PSCs Is Increased Through Cancer-Stromal Interactions

Because CD10⁺ PSCs were mainly observed around carcinoma cells in pancreatic cancer, we investigated the effect of cancer cells on the PSCs' phenotype using a coculture system. The percentage of CD10⁺ PSCs cocultured with SUI-2, Panc-1, and SW1990 cells increased in a time-dependent manner compared with monocultured PSCs (Figure 5). These findings suggest that the CD10⁺ phenotype in cancer-associated PSCs increases through cancer-stromal interactions.

CD10⁺ PSCs Secrete High Levels of MMP3

CD10, which is a cell surface metalloproteinase that cleaves small peptides, is reported to have a broad range of substrates, including bradykinin, endothelin, and the oxidized chain of insulin.²³ Therefore, we investigated whether the CD10 molecule itself was involved in the invasiveness of pancreatic cancer cells. Transfection of CD10-1 siRNA (siCD10-1) and CD10-2 siRNA (siCD10-2) decreased CD10 mRNA expression in PSCs, resulting in decreased levels of CD10 protein in PSCs cell lysates (Supplementary Figure 5A). The CD10 protein was not detected in the supernatant of PSCs transfected with control siRNA, siCD10-1, or siCD10-2, indicating that the CD10 molecule was not secreted by PSCs. In vitro invasion assays showed no difference in the invasiveness of cancer cells between PSCs with, or without, CD10 (Supplementary Figure 5B). These data suggest that the CD10 molecule does not work in a paracrine manner and that CD10⁺ PSCs promote the invasion of cancer cells via means other than the CD10 molecule. Next, we performed mRNA expression analysis of the candidate molecules involved in the progression of pancreatic ductal adenocarcinoma¹ in CD10⁺ and CD10⁻ PSCs by quantitative RT-PCR. The expression levels of MMP2, 7, 9, and 14; stromal cell-derived factor 1; hepatocyte growth factor; transforming growth factor β 1; basic fibroblast growth factor; vascular endothelial growth factor; and CTGF were similar in both CD10⁺ and CD10⁻ PSCs (Supplementary Figure 6A). Western blot analysis showed that only MMP3 expression was higher in the supernatant of the CD10⁺ PSCs than in the supernatant of the CD10⁻ PSCs (Figure 6A, Supplementary Figure 6B). SUI-2 and Panc-1 cells did not secrete

MMP3 into the supernatant. These results indicate that soluble MMP3 is abundantly secreted by CD10⁺ PSCs and may account for the impact of CD10⁺ PSCs on tumor progression. MMP3 expression in PSCs was not suppressed by the knockdown of CD10, suggesting that a direct association between CD10 and MMP3 is unlikely (Supplementary Figure 7). CD10 is considered to be a marker of PSCs that enhances the progression of pancreatic cancer.

PSCs Enhance the Invasion of Pancreatic Cancer Cells by Secreting MMP3

To investigate whether MMP3 secreted by PSCs was involved in the invasion of pancreatic cancer cells, RNA interference technology was used to knock down MMP3 mRNA in PSCs. Transient transfection of MMP3-1 siRNA (siMMP3-1) and MMP3-2 siRNA (siMMP3-2) decreased MMP3 mRNA expression to less than 10% of the control siRNA level. Western blot analysis confirmed that MMP3 secretion by PSCs decreased after MMP3 knock down (Figure 6B). In vitro invasion assays using cocultured cells showed that the invasion of SUI-2 and Panc-1 cells decreased when MMP3 secretion by PSCs was reduced (Figure 6C and D).

Discussion

In this study, we identified a subpopulation of PSCs within human pancreatic cancer that enhances its progression. We found that CD10 was expressed by some α -SMA-positive myofibroblasts in vivo and in vitro, suggesting that cancer-associated PSCs contained a subpopulation of cells with the CD10⁺ phenotype. Li et al⁸ reported that pancreatic cancer cells with the CD44⁺CD24⁺ESA⁺ phenotype showed a 100-fold increase in tumorigenic potential compared with nontumorigenic cancer cells. Hermann et al²⁴ showed that pancreatic cancer cells with the CD133⁺ phenotype were exclusively tumorigenic and resistant to chemotherapy and that a subpopulation of CD133⁺CXCR4⁺ cells was essential for tumor metastasis. These subpopulations of malignant cells, called CSCs, are considered to be responsible for tumor regrowth. Interestingly, like cancer cells, PSCs have functional heterogeneity. We found that, compared with CD10⁻ PSCs, CD10⁺ PSCs had the capacity to promote the invasiveness of cancer cells and were responsible for subcutaneous tumor growth. The 2 phenotypes had no morphologic differences and had the

Figure 6. (A) Expression of MMP3 in PSCs. CD10⁺ PSCs showed higher expression of MMP3 mRNA than CD10⁻ PSCs. The MMP3 protein concentration was higher in the supernatants of CD10⁺ PSCs than in the supernatants of CD10⁻ PSCs. (B–D) Effects of MMP3 secreted from PSCs on the invasive potential of pancreatic cancer cells. (B) Transfection of PSCs with siMMP3-1 and siMMP3-2 resulted in knockdown of MMP3 mRNA expression to less than 10% of the control siRNA level at 72 hours (*lower panel*). MMP3 secretion from PSCs dramatically decreased after MMP3 knockdown, whereas levels of MMP3 protein in the cytoplasm of PSCs did not change (*upper panel*). (C) Representative photomicrographs of invading SUI-2 and Panc-1 cells cocultured with PSCs transfected with siMMP3-1 and siMMP3-2 (H&E original magnification, \times 40). (D) The invasion of SUI-2 and Panc-1 cells was attenuated by decreasing the MMP3 secretion of PSCs (* P < .05; ** P < .001).

same capacity for fibrogenesis, which is one of the key characteristics of PSCs. The expression levels of growth factors involved in cancer-stromal interactions were also equal in both phenotypes. Only MMP3 expression was higher in CD10⁺ PSCs.

CD10 was originally considered to be one of the stromal stem cell markers and has been reported to be associated with malignant behavior in several solid tumors.^{14,16,17} Makretsov et al¹⁶ showed that stromal CD10 expression in invasive carcinoma of the breast correlated with a poor prognosis. Ogawa et al¹⁷ showed that the expression of CD10 in stromal cells of colorectal carcinomas was more frequently detected in invasive tumors than in noninvasive tumors. Interestingly, according to their report, CD10 expression by more than 10% of the stromal cells was detected only in the invasive growth front of invasive colorectal carcinomas, suggesting that CD10⁺ stromal cells are directly associated with invasion. We are the first to show that stromal CD10 expression is associated with a poor prognosis in pancreatic cancer. Co-culture and co-implantation models showed that CD10⁺ stromal cells promoted invasion and tumor growth to a higher extent than CD10⁻ PSCs, which strongly supports the observations made in the previous reports. In addition, we found that, within cancer-associated PSCs, the CD10⁺ phenotype was increased through cancer-stromal interactions, indicating that pancreatic cancer cells might create a tumor-supportive microenvironment by changing the phenotype of the surrounding PSCs.

The CD10 knockdown experiments showed that the CD10 molecule itself did not play a role in enhancing the invasiveness of pancreatic cancer. However, CD10 could be a promising target for the treatment of pancreatic cancer because CD10-expressing PSCs contribute to the progression of pancreatic cancer. Pan et al²⁵ showed that CD10 molecules expressed by tumor cells are capable of cleaving the peptide prodrug of doxorubicin, resulting in the selective generation and uptake of doxorubicin at the tumor site. CD10 is selectively overexpressed by stromal cells in invasive pancreatic tumors but also in colon, gastric, and breast cancer.¹⁴⁻¹⁷ Using the capacity of the CD10 molecule to cleave peptides, or targeting the CD10⁺ stromal cells within tumors, would provide a novel and selective cancer therapy, especially effective for aggressive malignant tumors. However, the present study also showed that CD10⁻ PSCs promoted the invasion and migration of cancer cells, whereas CD10⁺ PSCs enhanced cancer progression. These findings indicate that CD10⁺ PSCs are not the only factor mediating PSCs-induced cancer cell invasion. In our in vivo xenograft model, CD10⁻ PSCs did not affect the growth of tumors resulting from co-transplantation of CD10⁻ PSCs and SUIT-2 cells because they were equal to that seen in tumors derived from SUIT-2 cells alone. Whether selective therapies targeting the CD10⁺ PSCs will be suffi-

ciently effective or whether all PSCs' populations should be targeted in the treatment of pancreatic cancer, still requires further investigation.

In this study, we have shown that soluble MMP3 is abundantly secreted by CD10⁺ PSCs and contributes to the invasiveness of the tumor cells. MMP3 is a key enzyme for tumor invasion and metastasis via the destruction of basement membranes and the proteolysis of the extracellular matrix.²⁶ MMP3 degrades proteoglycans, collagens, laminins, elastin, fibrin, and fibronectin and can activate other MMPs.²⁷ Additionally, it has been shown that MMP3 induces epithelial-mesenchymal transition.²⁸ Recently, a novel function of MMP3 as a trans-regulator of CTGF, an important factor for pancreatic tumor growth,²⁹ was reported.³⁰ These observations indicate that CD10⁺ PSCs may promote the invasiveness of tumor cells by secretion of MMP3, which degrades the extracellular matrix and induces epithelial-mesenchymal transition of tumor cells and enhances tumor growth. The results obtained from this study have significant implications for the treatment of pancreatic cancer. It may be possible to reduce the progression of cancer cells by targeting CD10⁺ PSCs, which would decrease MMP3 secretion and diminish the cancer-stromal interactions. The combination of stromal-directed therapy with conventional chemotherapy targeting cancer cells is a promising new therapeutic strategy.

In conclusion, PSCs have functional heterogeneity and influence the progression of pancreatic cancer. Although CD10⁺ PSCs comprise only a small population of cancer-associated PSCs, their existence is correlated with tumor aggressiveness and a poor prognosis. CD10⁺ PSCs promote the invasiveness of pancreatic cancer cells and enhance the growth of pancreatic cancer compared with CD10⁻ PSCs. CD10⁺ PSCs abundantly secrete MMP3, which may contribute to the progression of pancreatic cancer. Thus, CD10⁺ PSCs may be a candidate for selective therapeutic targeting in the treatment of pancreatic cancer.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2010.05.084.

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Received October 22, 2009. Accepted May 27, 2010.

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Acknowledgment

The authors thank the Research Support Center, Graduate School of Medical Sciences, Kyushu University for technical support.

Conflicts of interest

The authors disclose no conflicts.

Funding

Supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labor and Welfare of Japan and by grants from The Takeda Science Foundation, Pancreas Research Foundation of Japan, Naito Foundation, and Nakajima Foundation.

