the combination of erlotinib and gemcitabine provided a small, yet statistically significant survival benefit (31). However, the efficacy of EGFR TKI in pancreatic cancer trials has not met expectations, not as it has in a proportion of patients with non-small cell lung cancer (NSCLC) (30). This is likely due to differences in the presence of activating mutations within EGFR, which are associated with prolonged responses in NSCLC (32). Many studies have identified activating EGFR gene mutations in only a small number of cases in PDAC patients (33,34). However, Tan *et al* demonstrated that higher genetic amplification of the *EGFR* region of chromosome 7 is associated with better clinical responses to erlotinib treatment in advanced NSCLC patients (35). Therefore, there is a possibility that quantitative analysis of *EGFR* mRNA expression levels could be helpful in predicting sensitivity to erlotinib in PDAC patients. However, further investigations incorporating larger patient numbers are required to evaluate the usefulness of this approach.

In conclusion, we demonstrate that quantitative analysis of *EGFR* mRNA expression using FFPE tissue samples is useful for predicting the prognosis of PDAC patients receiving gemcitabine-based AC. In addition, quantitative analysis of *EGFR* mRNA in neoplastic cells microdissected from EUS-FNA specimens is useful for determining treatment for patients with PDAC, even when the tumor is unresectable. Thus, quantitative analysis of genes associated with sensitivity to cytotoxic agents could be a potent tool for individualized chemotherapy.

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Insig2 is overexpressed in pancreatic cancer and its expression is induced by hypoxia

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A hypoxic microenvironment is a characteristic feature of pancreatic cancer, and induces the expressions of various genes involved in malignant behaviors. Insulin-induced gene 2 (*Insig2*) has recently been shown to be correlated with cellular invasion in colon cancer. However, there have been no reports regarding its expression in pancreatic cancer. In this study, we evaluated *Insig2* mRNA expression and the biological function of *Insig2* in pancreatic cancer. We measured *Insig2* mRNA expression in cultured pancreatic cancer cell lines and invasive ductal carcinoma (IDC) cells, normal pancreatic epithelial cells, and pancreatic intraepithelial neoplasia cells obtained by laser-capture microdissection. We also investigated the effects of *Insig2*-targeting siRNAs on the cell proliferation and cell invasion of pancreatic cancer cell lines. All pancreatic cancer cell lines expressed *Insig2* mRNA. The PANC-1 and MIA PaCa-2 pancreatic cancer cell lines showed >2-fold higher *Insig2* mRNA expression levels under hypoxic conditions (1% O_2) than under normoxic conditions (21% O_2). Cell proliferation was significantly decreased in SUIT-2 cells and cell invasion was significantly decreased in SUIT-2, Capan-2, and CFPAC-1 cells after transfection of the *Insig2*-targeting siRNAs. In analyses of microdissected cells, cells from IDC tissues expressed significantly higher levels of *Insig2* mRNA than normal pancreatic cells ($P < 0.001$) and pancreatic intraepithelial neoplasia cells ($P = 0.082$). In analyses of IDC cells, the levels of *Insig2* mRNA expression were significantly higher in late-stage patients than in early-stage patients. The present data suggest that *Insig2* is associated with the malignant potential of pancreatic cancer under hypoxic conditions. (*Cancer Sci* 2011; 102: 1137–1143)

Pancreatic cancer is the fourth leading cause of cancer-related death in Western Europe and has the lowest survival rate of any solid tumor.[1,2] Owing to a lack of early detection methods and the absence of effective biomarkers, pancreatic cancer patients are usually diagnosed at late stages and have a 5-year survival rate of <5%. The first-line agent gemcitabine produces some clinical benefits in the advanced setting but yields limited disease control, with <15% of patients being progression-free at 6 months after diagnosis.[3,4] Recently, advances in our understanding of the genetics and epigenetics of pancreatic cancer have revealed that alterations of several tumor-related genes, including *K-ras*, *p53*, *MMP*, *HGF*, and *EGFR*,[5–10] could underlie the aggressiveness of this neoplasm and its resistance to conventional therapies.[11] There is a great need to understand the biological mechanisms that contribute to pancreatic cancer development and progression. Therefore, the identification of effective markers for pancreatic neoplasms to more effectively detect pancreatic cancer and its precursors may contribute to the discovery of new approaches to treat this fatal disease.

Pancreatic cancer is characterized by intratumoral hypoxia, which is involved in early and aggressive local invasion and metastatic potential. Hypoxia-inducible factor (HIF)-1 is the major transcriptional activator of hypoxia-responsive genes, and intratumoral hypoxia is associated with an increased risk of metastasis.[12] In this study, we carried out microarray analyses using the PDAC cell line under normoxic conditions (21% O_2) and hypoxic conditions (1% O_2), and our expression profiling identified several genes that were aberrantly expressed in PDAC cells under hypoxic conditions. Some of these genes, namely *PFKFB4*, *ADM*, *ANKRD37*, *ENO2*, *HIG2*, *ANGPTL4*, *SPAG4*, and *UPK1A*, have been reported to be differentially expressed in pancreatic cancer or other cancers.[13–15]

Insulin-induced gene 2 (*Insig2*) was also upregulated in this expression profiling, and was recently reported to be a biomarker for colon cancer.[16] The *Insig2* gene encodes closely related endoplasmic reticulum proteins that regulate the proteolytic activation of sterol regulatory element-binding proteins, comprising transcription factors that activate the synthesis of cholesterol and fatty acids in animal cells.[17,18] *Insig2* is correlated with cellular invasion in colon cancer and has a univariate negative prognostic capacity to discriminate human colon cancer survivorship. Overexpression of *Insig2* appears to suppress chemotherapeutic drug treatment-induced expression of Bcl2-associated X protein (Bax) expression and activation in human colorectal cancer cells.[19,20] *Insig2* was also found to localize to the mitochondria/heavy membrane fraction and to associate with conformationally altered Bax in HeLa cells.[21] Moreover, *Insig2* alters the expressions of several additional apoptosis genes located in mitochondria, further supporting its newly described functional role in regulating mitochondria-mediated apoptosis in human colorectal cancer cells.[16] These data suggest that *Insig2* plays an important role in carcinogenesis or cancer progression. However, there are no reports regarding the involvement of *Insig2* in pancreatic cancer.

In the present study, we analyzed *Insig2* expression in cultured pancreatic cancer cell lines exposed to normoxic and hypoxic conditions and in invasive ductal carcinoma (IDC) cells, normal pancreatic epithelial cells, and pancreatic intraepithelial neoplasia (PanIN) cells obtained by laser-capture microdissection. We also investigated the biological function of *Insig2* by examining the effects of *Insig2*-targeting siRNAs on the cell proliferation and cell invasion of pancreatic cancer cell lines. Our data suggest that *Insig2* is associated with carcinogenesis and malignant behaviors in pancreatic cancer.

Materials and Methods

Cell lines. The following 15 pancreatic cancer cell lines were used in this study: human pancreatic cancer cell lines SUIT-2, AsPC-1, BxPC-3, PANC-1, KP-1N, KP-2, and KP-3 (generously donated by Dr. H. Iguchi, National Shikoku Cancer

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Center, Matsuyama, Japan); MIA PaCa-2 (Japanese Cancer Resource Bank, Tokyo, Japan); Capan-1, Capan-2, CFPAC-1, H48N, Hs 766T, and SW1990 (American Type Culture Collection, Manassas, VA, USA); and NOR-P1 (established by Dr. N. Sato in our laboratory).[22] A human pancreatic ductal epithelial (HPDE) cell line (HPDE6-E6E7 clone6) immortalized by transduction with the *E6/E7* genes of human papillomavirus 16 (kindly provided by Dr. Ming-Sound Tsao, University of Toronto, Toronto, Canada) was also used. The cells were maintained as described previously.[23,24]

Clinical samples. Invasive ductal carcinoma cells from 29 patients, PanIN cells from nine patients, and normal pancreatic ductal epithelial cells from 32 patients were selectively isolated using a laser microdissection and pressure catapulting system (PALM Microlaser Technologies, Bernried, Germany) in accordance with the manufacturer's protocols. All the tissues were taken from surgically resected specimens at the Department of Surgery and Oncology, Kyushu University Hospital (Fukuoka, Japan) and its affiliated hospitals from December 2005 to November 2008. All the tumors were staged according to the TNM classification system of the International Union against Cancer.[25] The histologic grading of the tumors and the diagnosis of PanIN lesions were carried out according to the classification system of the World Health Organization.[26] Other pathological variables (lymphatic invasion, vascular invasion, and perineural invasion) were assessed using the classification system of the Japan Pancreas Society.[27] This study was carried out in accordance with the principles embodied in the Declaration of Helsinki. The study was also approved by the Ethics Committee of Kyushu University and carried out according to the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government.[28]

Quantitative assessment of *Insig2* mRNA levels by one-step real-time qRT-PCR. Total RNA was extracted from pellets of cultured cells using a High Pure RNA Kit (Roche Diagnostics, Mannheim, Germany) with DNase I treatment (Roche Diagnostics) according to the manufacturer's instructions. We designed specific primers (*Insig2*: forward, 5′-TCA CAC TGG CTG CAC TAT CC-3′ and reverse, 5′-ACA GTT GCC AAG AAG GCA AT-3′; *18S rRNA*: forward, 5′-GTA ACC CGT TGA ACC CCA TT-3′ and reverse, 5′-CCA TCC AAT CGG TAG TAG CG-3′), and carried out BLAST searches to ensure the specificity of each primer. The extracts were analyzed by qRT-PCR using a QuantiTect SYBR Green RT-PCR Kit (Qiagen, Tokyo, Japan) and a Chrom4 real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Each reaction mixture was initially incubated at 50°C for 30 min to allow reverse transcription, in which first-strand cDNA was synthesized by priming the total RNA with the same gene-specific primer (reverse). The PCR was initiated by incubation at 95°C for 15 min to activate the polymerase, followed by 40 cycles of 94°C for 15 s, 58°C for 30 s, and 72°C for 30 s. Each primer set used in this study produced a single prominent band of the expected size after electrophoresis. Each sample was analyzed twice, and any samples showing more than 10% deviation in the qRT-PCR values were tested a third time. The level of mRNA expression in each sample was calculated by reference to a standard curve generated using total RNA from the PANC-1 human pancreatic cancer cell line. The expression of *Insig2* mRNA was normalized by the expression of *18S rRNA* mRNA. A cut-off point for *Insig2* mRNA expression was selected by searching for the cut-off point yielding the smallest log-rank *P*-value, and the expression was divided to high and low level groups.

Microdissection-based quantitative analysis of *Insig2* mRNA. Frozen tissue samples were cut into 5-μm thick sections. One section from each sample was stained with H&E for histologic examination. Similar numbers of cells were isolated from sections of IDC lesions, PanIN lesions, and normal ductal epithelium. More than 500 cells were obtained from each IDC section, whereas 3–10 sections were required to isolate sufficient normal ductal epithelial cells and PanIN cells owing to the lower numbers of these cells per section. After the microdissection, total RNA was extracted from the selected cells and subjected to qRT-PCR for quantification of *Insig2* mRNA expression.[29,30]

Microarray analysis. We carried out microarray analyses using MIA PaCa-2 cells cultured under normoxic conditions (21% O_2) and hypoxic conditions (1% O_2) for 24 h. The qualities of the RNA samples were evaluated using a 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) as described previously.[31] We used a HumanWG-6 Expression BeadChip (Illumina, San Diego, CA, USA) for these analyses. The data were analyzed using BeadStudio software version 3.2.3 (Illumina). All of the microarray data were deposited in CIBEX under the accession number CBX147 (http://cibex.nig.ac.jp/index.jsp).

Transfection of *Insig2*-targeting siRNAs. Pancreatic cancer cells were transfected with Insig2-si1 (sense, 5′′-cuauguucguucuugguuatt-3′; antisense, 5′-uaaccaagaacgaacauagtt-3′) and Insig2-si2 (sense, 5′-cucacacuggcugcacuautt-3′; antisense, 5′-auagugcagccagugugagtt-3′) siRNAs (Qiagen) by electroporation using a Nucleofector System (Amaxa Biosystems, KölnCologne, Germany) according to the manufacturer's instructions. To verify the specificity of the *Insig2* knockdown effects, we used negative control siRNAs (Qiagen).

Cell proliferation assay. Cell proliferation was evaluated by measuring the fluorescence intensity of propidium iodide (PI) as described previously.[32] Pancreatic cancer cells were transfected with the *Insig2*-targeting siRNAs and negative control siRNAs, then seeded in 90-mm dishes at 1×10^6 cells/dish. At 24 h after the transfection, viable cells were plated in 24-well tissue culture plates (Becton Dickinson Labware, Bedford, MA, USA) at 2×10^4 cells/well and cultured for the indicated hours. Cell proliferation was then evaluated by the PI assay. All experiments were carried out in triplicate wells and repeated at least three times.

Invasion assay. Using SUIT-2, Capan-2, and CFPAC-1 cells, the invasion of pancreatic cancer cells was evaluated by the number of cells invading Matrigel-coated Transwell chambers (Becton Dickinson Labware). Briefly, Transwell inserts with 8-μm pores were coated with Matrigel (20 μg/well for all cells; Becton Dickinson) and reconstituted with DMEM supplemented with 10% FBS for 2 h before the experiment. Cells (2×10^5 cells/mL) were seeded into the upper chambers in 250 μL DMEM supplemented with 10% FBS. The same medium (750 μL) was placed in the lower wells. Thereafter, all cells were incubated for 4848 h. Cells that had degraded the Matrigel and invaded to the lower surface of the Matrigel-coated membranes were fixed with 70% ethanol, stained with H&E, and counted in five random fields at ×400 magnification under a light microscope. The results were expressed as the average number of invasive cells per field.

Statistical analysis. Comparison between two groups was done using Student's *t*-test. Survival analyses undertaken using Kaplan–Meier analyses and curves were compared using the log-rank test. Values are expressed as the mean ± SD. All experiments were repeated twice. Statistical significance was defined as $P < 0.05$. All statistical analyses were carried out using JMP 8.01 software (SAS Institute, Cary, NC, USA).

Results

mRNA expression profiling of pancreatic cancer cell lines under hypoxic conditions. We used the MIA PaCa-2 cell line for the microarray analyses, because MIA PaCa-2 cells showed the greatest morphological changes under hypoxic conditions

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compared with normoxic conditions in our experiments. We defined a difference as significant when there was a change of >5-fold between the hypoxic and normoxic conditions and found that 23 genes were significantly upregulated under hypoxic conditions compared with normoxic conditions. The 23 highest ranking genes are shown in Table 1. Among the genes identified, *PFKFB4* and *ALDOC* were significantly upregulated, as previously reported,[13,33] and we therefore considered that the conditions used in this experiment were appropriate.

Among the upregulated genes, we focused on *Insig2*, because it has been reported to be a novel biomarker for colon cancer[16] and its expression is correlated with the invasiveness of colon cancer.[16]

***Insig2* mRNA expression levels in cultured pancreatic cancer cell lines.** We investigated the levels of *Insig2* mRNA expression in cultures of 15 different pancreatic cancer cell lines and HPDE cells. As shown in Figure 1(a), all 15 pancreatic cancer cell lines and the HPDE cells as well as two primary cultures of fibroblasts isolated from pancreatic cancer specimens expressed *Insig2* mRNA. Compared with HPDE cells, SUIT-2 and KP-2 cells expressed higher levels of *Insig2* mRNA, and the other 13 pancreatic cancer cell lines expressed lower levels of *Insig2* mRNA.

Next, we confirmed the effects of hypoxia on *Insig2* mRNA expression in the pancreatic cancer cell lines MIA PaCa-2 and PANC-1, because these two cell lines have already been shown to induce HIF-1 protein expression under hypoxic conditions[34] and hypoxia increases the amount of *Insig2* in a response mediated by HIF-1.[35] As shown in Figure 1(b), PANC-1 and MIA PaCa-2 cells showed 2.3-fold and 5.2-fold higher levels of *Insig2* mRNA expression under hypoxic conditions (1% O_2) than under normoxic conditions (21% O_2), respectively. We also investigated the mRNA expression levels of three other genes, *ANGPTL4*, *ADM*, and *HIG2*, in MIA PaCa-2 cells under hypoxic conditions to confirm the accuracy of the microarray data, and found that all three mRNAs were expressed at significantly higher levels under hypoxic conditions than under normoxic conditions (Fig. 1c).

Inhibition of *Insig2* expression decreases the proliferation of SUIT-2 pancreatic cancer cells. To investigate the effects of *Insig2*-targeting siRNAs, we first measured the levels of *Insig2* mRNA expression in pancreatic cancer cells transfected with the *Insig2*-targeting or negative control siRNAs using SUIT-2, Capan-2, and CFPAC-1 cells. SUIT-2, Capan-2, and CFPAC-1 cells showed significantly lower levels of *Insig2* mRNA at 48 h after transfection with the *Insig2*-targeting siRNAs than the control cells transfected with the negative control siRNAs (Fig. 2a, $P < 0.01$).

Next, we carried out PI assays using SUIT-2 cells, which expressed the highest level of *Insig2* mRNA (Fig. 1a) and examined the effects of *Insig2* knockdown on cell proliferation. SUIT-2 cells transfected with the *Insig2*-targeting siRNAs showed significantly decreased cell proliferation compared with the control cells at 120 h after transfection (Fig. 2b, $P < 0.001$). No significant changes in the proliferation of CFPAC-1 and Capan-2 cells, which had moderate levels of *Insig2* expression, were observed after transfection of the *Insig2*-targeting siRNAs (data not shown).

Inhibition of *Insig2* decreases the invasion of pancreatic cancer cells. Next, we investigated the effects of *Insig2* knockdown on cancer cell invasion, which is an important function for malignant progression and metastasis, using SUIT-2, Capan-2, and CFPAC-1 cells. Capan-2 and CFPAC-1 cells, which expressed

Table 1. Twenty-three highest ranking genes that were significantly upregulated under hypoxic conditions compared with normoxic conditions in microarray analyses

Overexpression rank	Gene symbol	Gene function	Average difference between normoxia *vs* hypoxia
1	*PFKFB4*	Resonse to hypoxia via a hypoxia-induced factor (HIF)-1-dependent mechanism	22.17
2	*ADM*	Relate invasiveness in pancreatic cancer	16.11
3	*ANKRD37*	HIF-1 target gene	15.81
4	*PPFIA4*	Implicated in trafficking of LAR subfamily PTPases and AMPA-type glutamate receptors	12.68
5	*FER1L4*	Mus musculus fer-1-like 4	10.83
6	*SPAG4*	Association with the germline nuclei	10.23
7	*ALDOC*	Bind HIF-1, and functioning as a hypoxia response element	9.61
8	*ANG*	Member of the angioprotein family	8.61
9	*RNASE4*	Member of the RNase A gene superfamily	8.14
10	*UPK1A*	Urothelium-specific markers of terminal urothelial cytodifferentiation	7.99
11	*ENO2*	Cytosolic glycolytic enzyme	7.78
12	*HIG2*	Canonical Wnt signaling, both as target and activator,and relate invasiveness in renal cell carcinoma (RCC)	7.72
13	*C20orf46*	Transmembrane protein	7.62
14	*INSIG2*	Inhibits Bax-mediated apoptosis	6.66
15	*TLE6*	Transcriptional repressor and are recruited by transcription factors containing an eh1 or WRPW/Y domain	6.44
16	*PDK3*	Induced HIF-1 and leading to inhibition of mitochondrial respiration	6.27
17	*CCNG2*	T-cell cycle progression and activation	6.01
18	*BNIP3L*	Promotes autophagy and apoptosis	5.81
19	*NDRG1*	N-myc downstream regulated gene	5.26
20	*SCAND2*	Zinc finger protein	5.21
21	*P4HA1*	HIF-1 target gene	5.12
22	*ANGPTL4*	HIF-1 target gene	5.09
23	*ANKZF1*	Encode zinc finger domain-containing protein1	5.04

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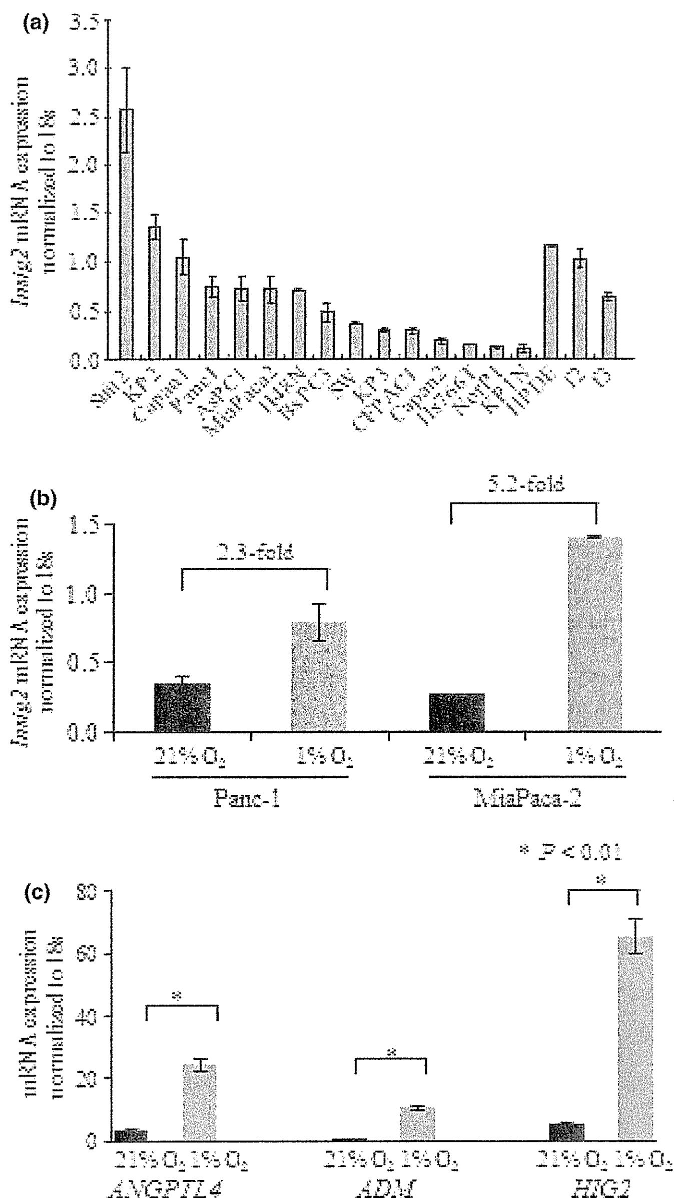

Fig. 1. *Insig2* mRNA expression levels in cell lines. (a) *Insig2* mRNA expression levels in 15 pancreatic cancer cell lines. The expression of *Insig2* mRNA was normalized by the expression of *18S rRNA* mRNA. All 15 pancreatic cancer cell lines and human pancreatic ductal epithelial (HPDE) cells, as well as two primary cultures of fibroblasts isolated from pancreatic cancer tissues (f2, f3), express *Insig2* mRNA. Compared with HPDE cells, SUIT-2 and KP-2 cells express higher levels of *Insig2* mRNA, whereas the other 13 pancreatic cancer cell lines express lower levels. (b) Comparisons of *Insig2* mRNA expression levels under normoxic (21% O_2) and hypoxic (1% O_2) conditions in two pancreatic cancer cell lines. The *Insig2* mRNA expression levels in PANC-1 and MIA PaCa-2 cells are 2.3-fold and 5.2-fold higher under hypoxic conditions than under normoxic conditions, respectively. (c) Comparisons of the *ANGPTL4*, *ADM*, and *HIG2* mRNA expression levels under normoxic (21% O_2) and hypoxic (1% O_2) conditions in the MIA PaCa-2 cell line. The *ANGPTL4*, *ADM*, and *HIG2* mRNA expression levels in MIA PaCa-2 cells are all significantly higher under hypoxic conditions than under normoxic conditions.

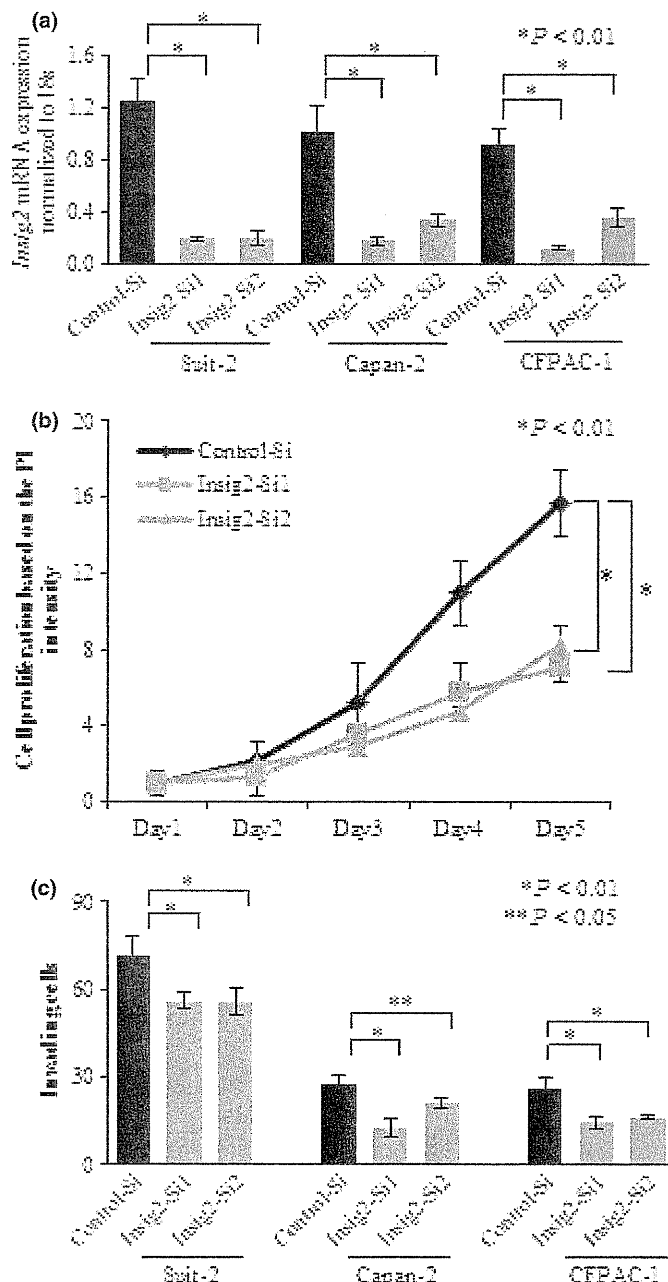

Fig. 2. Knockdown of *Insig2* expression in pancreatic cancer cell lines by *Insig2*-targeting siRNAs and the effects on cell proliferation and invasion. (a) *Insig2* expression is significantly suppressed by the *Insig2*-targeting siRNAs in SUIT-2, Capan-2, and CFPAC-1 cells. (b) Decreased proliferation is observed in SUIT-2 cells transfected with the *Insig2*-targeting siRNAs compared with the control cells at 120 h after transfection (*$P < 0.01$, Student's *t*-test). (c) Decreased invasion is observed in SUIT-2, Capan-2, and CFPAC-1 cells transfected seen with the *Insig2*-targeting siRNAs compared with the control cells at 48 h after seeding (*$P < 0.01$, Student's *t*-test). The data are the means ± SD of triplicate measurements.

relatively moderate levels of *Insig2*, and SUIT-2 cells, which expressed the highest level of *Insig2*, showed significantly decreased cell invasion at 48 h after transfection with the *Insig2*-targeting siRNAs compared with the control cells (Fig. 2c, $P < 0.01$ and $P < 0.05$), Although there were no changes in the proliferation of SUIT-2, Capan-2, and CFPAC-1 cells at 48 h after transfection (Fig. 2b for SUIT-2 cells; data not shown for Capan-2 and CFPAC-1 cells).

Quantitative analyses of *Insig2* mRNA expression in microdissected IDC, PanIN, and normal ductal epithelial cells. Among the 32 normal epithelial samples, 15 were normal epithelial samples from pancreatic cancer cases. We obtained the tissues from sites near the tumor stumps, which were pathologically diagnosed as being free of carcinoma cells. The

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remaining 17 samples were normal epithelial lesions from patients with pancreatic cystic disease, intraductal papillary mucinous neoplasm, pancreatic endocrine tumor and other cancers (cholangiocarcinoma and carcinoma of the ampulla of Vater). Representative images of the dissected cells from pancreatic normal ductal epithelial, PanIN-1A, and IDC lesions are shown in Figure 3(a). As shown in Figure 3(b), the levels of *Insig2* mRNA expression were significantly higher in IDC cells and PanIN cells than in normal ductal epithelial cells ($P < 0.001$, normal ductal epithelial cells versus IDC cells; $P = 0.019$, normal ductal epithelial cells versus PanIN cells). Although the median *Insig2* mRNA expression level was higher in IDC cells than in PanIN cells, the difference was not significant ($P = 0.082$).

Relationships between *Insig2* mRNA expression and clinicopathological factors. The *Insig2* mRNA expression levels were significantly higher in patients with lymph node metastasis than in those without lymph node metastasis ($P = 0.003$; Fig. 4a). The levels of *Insig2* mRNA expression were significantly higher in stages IIB–IV ($n = 20$) than in stages I–IIA ($n = 9$) according to the UICC classification ($P = 0.0321$; Fig. 4b). Regarding recurrence after surgical resection, among 29 pancreatic cancer tissues of microdissected samples, 12 cases showed recurrence and 17 cases remained free from recurrence. Among the 12 cases with recurrence, nine were distant metastatic and peritoneal dissemination cases and three were local recurrence cases. The *Insig2* mRNA expression levels were significantly higher in patients with distant metastasis (lung, liver) and peritoneal dissemination than in those with local recurrence ($P = 0.0124$; Fig. 4c).

In the analysis of 32 cases with normal ducts, there was a tendency toward higher levels of *Insig2* mRNA expression in the normal ducts of the pancreatic cancer cases than in those of the pancreatic cystic disease, intraductal papillary mucinous neoplasm, and other cancer cases ($P = 0.062$; Fig. 4d).

In the analysis of the 29 pancreatic cancer cases, one case dropped out from our follow up. Among the remaining 28 cases, there was a tendency for a poor prognosis in the high *Insig2* group ($n = 15$) compared with the low *Insig2* group ($n = 13$) (Fig. 4e). However, the difference was not significant ($P = 0.3011$), possibly owing to the small number of samples in the present study.

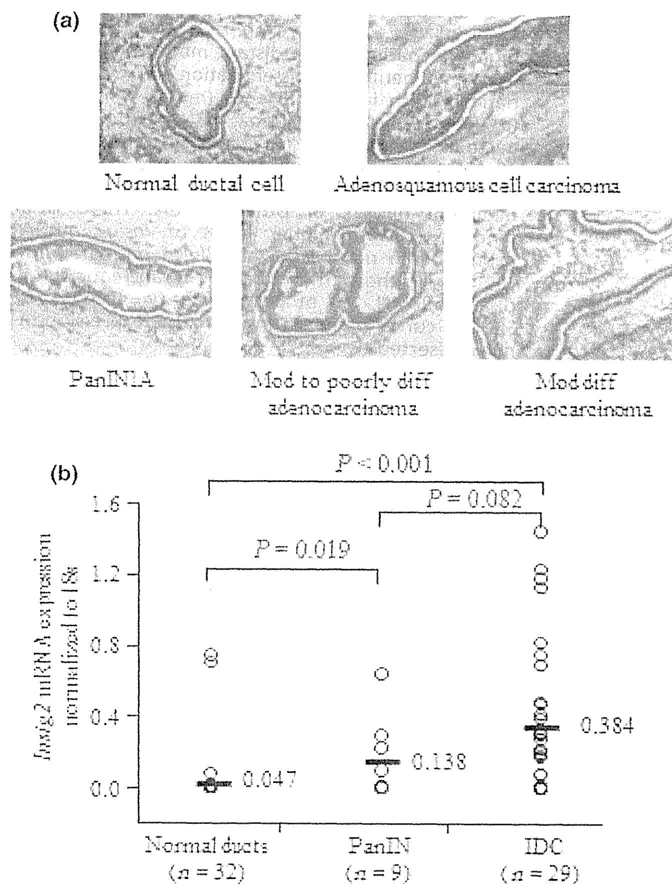

Fig. 3. *Insig2* mRNA expression levels in microdissected cells. (a) Representative micrographs of cells in pancreatic normal ductal epithelia, pancreatic intraepithelial neoplasia (PanIN) lesions, and invasive ductal carcinoma (IDC) lesions stained with 1% toluidine blue. Diff, differentiated; mod, moderately. (b) Relative levels of *Insig2* mRNA expression normalized by the levels of *18S rRNA* mRNA expression in microdissected normal pancreatic ductal, PanIN, and IDC cells. The center horizontal lines represent the median values. The levels of *Insig2* mRNA expression are significantly higher in IDC cells and PanIN cells than in normal ductal epithelial cells ($P < 0.001$, normal ductal epithelial cells versus IDC cells; $P = 0.019$, normal ductal epithelial cells versus PanIN cells). Although the levels of *Insig2* mRNA expression in IDC cells tend to be higher than those in PanIN cells, the difference is not significant ($P = 0.082$).

Discussion

In the present study, we found that *Insig2* mRNA was expressed in all pancreatic cancer cell lines examined and was correlated with the malignant behaviors of pancreatic cancer in analyses involving microdissection and tissues. This is the first report regarding the involvement of *Insig2* in pancreatic cancer under hypoxic conditions.

Insig2 was reported to serve as a novel biomarker for colorectal cancer and to act as a potential tumor promoter with multiple biological functions.[16] In the present study, the *Insig2* mRNA expression levels were significantly higher in pancreatic cancer cells than in normal cells in analyses of microdissected cells, although cultured pancreatic cancer cells showed low levels of *Insig2* mRNA expression. These findings seem to be inconsistent. However, under hypoxic conditions, which are characteristic features of pancreatic cancer tissues, the levels of *Insig2* mRNA expression were significantly upregulated in both Panc1 and MIA PaCa-2 cells. These findings could support the results of analyses with pancreatic cancer cells isolated from pancreatic cancer tissues, which are possibly under hypoxia.

We also found that *Insig2* knockdown decreased the cell proliferation and invasion of SUIT-2 cells, which expressed a high level of *Insig2*, suggesting that *Insig2* has a functional role in the proliferation and invasion of pancreatic cancer cells with high levels of *Insig2*. Capan-2 and CFPAC-1 cells showed significantly decreased cell invasion after *Insig2* knockdown but no changes in their cell proliferation, suggesting that *Insig2* may have a functional role in the invasion but not the proliferation of pancreatic cancer cells with moderate levels of *Insig2*. We also found that the levels of *Insig2* mRNA expression were significantly higher in cells from late-stage tumors than in cells from early-stage tumors, and were significantly higher in cells from tumors with lymph node metastasis than in cells from tumors without lymph node metastasis. Furthermore, in the analyses of the recurrence patterns after surgery, the levels of *Insig2* mRNA expression were significantly higher in cells from tumors with distant metastatic recurrences than in cells from tumors with local recurrences. All these findings suggest that *Insig2* may have potential roles in pancreatic cancer progression.

In our analyses of normal duct cells, the normal ducts associated with pancreatic cancer had a tendency to express higher levels of *Insig2* mRNA than those associated with non-pancreatic cancer

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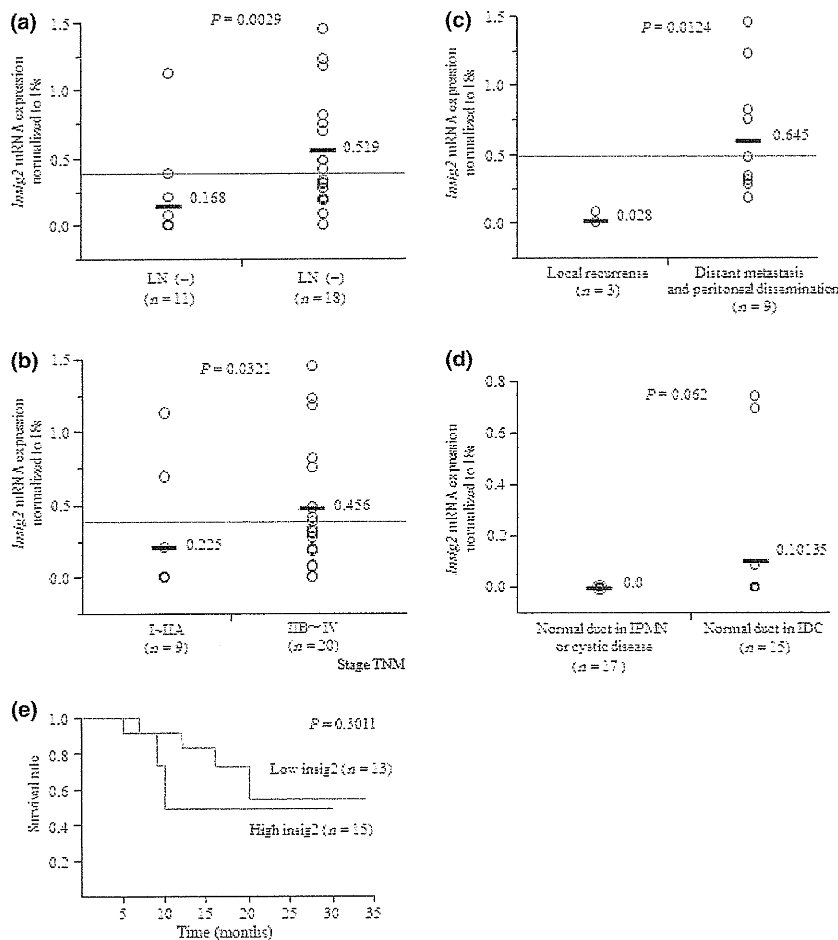

Fig. 4. Relationships between *Insig2* mRNA expression levels in microdissected cells and clinicopathological factors. (a) The levels of *Insig2* mRNA expression are significantly higher in patients with lymph node metastasis (n = 18) than in those without lymph node metastasis (n = 11) (P = 0.003). (b) According to the UICC classification, the levels of *Insig2* mRNA expression are significantly higher in stage IIB–IV cases (n = 20) than in stage I–IIA cases (n = 9) (P = 0.0321). (c) The levels of *Insig2* mRNA expression are significantly higher in patients with distant metastasis (lung or liver) and peritoneal dissemination (n = 9) than in patients with local recurrence (n = 3) (P = 0.0124). (d) Relative levels of *Insig2* mRNA expression in microdissected normal pancreatic ductal cells from 15 cases with IDC and 17 cases with pancreatic cystic disease, intraductal papillary mucinous neoplasm (IPMN), or other cancers. There is a tendency toward higher levels of *Insig2* mRNA expression in normal ductal cells of patients with IDC than in those of patients with pancreatic cystic disease, IPMN, or other cancers (P = 0.062). (e) Survival curves based on *Insig2* mRNA expression in microdissected tissues.

tissues, such as pancreatic cystic disease, intraductal papillary mucinous neoplasm, endocrine tumors, and other cancers, suggesting that *Insig2* was overexpressed in the precancerous lesions of pancreatic cancer, although the cells appeared to be morphologically normal epithelial cells.

In conclusion, our present data showed that *Insig2* is overexpressed in pancreatic cancer under hypoxic conditions and is correlated with the malignant behaviors of pancreatic cancer. These data suggest that *Insig2* is a possible marker for pancreatic cancer diagnosis, evaluation of malignant behaviors, and prediction of prognosis.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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Transforming Growth Factor β1 Contributes to the Invasiveness of Pancreatic Ductal Adenocarcinoma Cells Through the Regulation of CD24 Expression

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Objectives: The aim of this study was to investigate the role of CD24 in the invasiveness of pancreatic ductal adenocarcinoma (PDAC).

Methods: We used 2 human PDAC cell lines containing large numbers of CD24-positive ($CD24^+$) cells (>65%; AsPC-1 cells) or few $CD24^+$ cells (<20%; CFPAC-1 cells). Invasiveness was estimated using the Matrigel invasion assay. The role of CD24 in invasiveness was evaluated using small interference RNA against *CD24* mRNA.

Results: The invasive ability of $CD24^+$ cells collected by cell sorter was higher than that of CD24-negative ($CD24^-$) cells. On the other hand, silencing of CD24 decreased the invasive ability of $CD24^+$ cells. Importantly, considerable amount of $CD24^+$ cells was converted to $CD24^-$ cells within 24 hours under in vitro culture condition. Transforming growth factor β1 significantly inhibited this conversion and consequently maintained the high invasiveness of $CD24^+$ cells.

Conclusions: Our data show that CD24 contributes to the invasive ability of PDAC and also suggest that transforming growth factor β1 may contribute to the invasiveness of PDAC by suppressing the conversion from $CD24^+$ cells to $CD24^-$ cells at the tumor site.

Key Words: pancreatic cancer, CD24, invasion, TGF-β1, RNAi, cell sorter

(*Pancreas* 2011;40: 1034–1042)

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal cancers worldwide.[1] This is because PDAC often goes undetected during the early stage and is particularly aggressive, with highly invasive and metastatic abilities. In addition, most PDACs are resistant to conventional chemotherapeutic agents such as 5-fluorouracil, gemcitabine, and taxanes. Thus, it is essential to identify new therapeutic targets that can prolong the survival time of patients with incurable PDAC.

CD24 is a mucin-like cell surface protein with a short, heavily glycosylated protein core comprising 27 amino acids and is attached to the cell membrane by a glycosylphosphatidylinositol anchor. CD24 is thought to act as an adhesion molecule[2,3] and is a ligand for the adhesion receptor P-selectin expressed on activated endothelial cells and platelets.[4] Importantly, it has been shown that CD24 is overexpressed in many types of cancers, including renal cell carcinoma, small cell lung carcinoma, hepatocellular carcinoma, ovarian cancer, gastric cancer, breast cancer, and PDAC.[5–11] Based on these findings, it seems likely that CD24 may support the rolling of tumor cells on endothelial monolayers, or aid the formation of tumor thrombi with activated platelets, and contribute to enhanced tumor cell invasiveness and metastasis.[12] In fact, several investigators have reported that CD24 is a predictor for poor prognosis of various malignancies.[5,9,10,13–16] It has also been reported that CD24 is a potential target for early intervention in the prevention and treatment of colorectal cancer and PDAC.[17] Furthermore, CD24 is a candidate molecule for detecting cancer stem cells in PDAC.[18] Indeed, Li et al[18] reported that a highly tumorigenic subpopulation of PDACs expresses the cell surface markers CD44, CD24, and epithelial-specific antigen. However, despite these important observations, the biological significance of CD24 remains unclear.

Therefore, in this study, we focused on the contribution of CD24 to the invasiveness of PDAC. For this purpose, we used CD24-negative ($CD24^-$) and CD24-positive ($CD24^+$) cells separated from parental PDAC cell lines. We conclude that $CD24^+$ cells have a higher invasive ability than that of $CD24^-$ cells. We also found that transforming growth factor β1 (TGF-β1) may regulate the expression of CD24 at the tumor site.

MATERIALS AND METHODS

Specimens and Immunohistochemistry

Twelve human pancreatic adenocarcinoma specimens were collected from patients who underwent surgery at the Department of Surgery and Oncology, Kyushu University, Fukuoka, Japan, between 2007 and 2008. All patients gave written informed consent before surgery and were enrolled in the present study. Immunohistochemistry was performed as previously described.[13] Briefly, pancreas tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were deparaffinized in xylene and ethanol. Heat-mediated antigen retrieval was achieved using a pressure cooker with 0.01 M citrate for 5 minutes, and samples were left at room temperature for 20 minutes. Sections were then immersed in phosphate-buffered saline (PBS). Endogenous peroxidase activity was blocked using 3% H_2O_2 in methanol for 30 minutes at room temperature. Samples were blocked with 10% goat serum in PBS and incubated overnight at 4°C with CD24 primary antibody (SC-10741; Santa Cruz Biotechnology, Santa Cruz, Calif) diluted 1:50. Goat immunoglobulin G was used as a negative control. After 3 washes with PBS, the sections were incubated with peroxidase-conjugated goat anti–rabbit antibodies for 1 hour and incubated with DAB substrate for 5 minutes. Counterstaining was

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performed with hematoxylin followed by dehydration with ethanol and Histosolve. Slides were mounted with Permount (Santa Cruz Biotechnology) and visualized by Axio Imager (Carl Zeiss, Göttingen, Jena, Germany).

Cell Culture, Reagents, and Antibodies

Two human PDAC cell lines (AsPC-1 and CFPAC-1) were purchased from American Type Culture Collection (Manassas, Va) and maintained in RPMI 1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal calf serum (Life Technologies, Grand Island, NY) and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) at 37°C, in 5% CO_2. Transforming growth factor β1 was purchased from R&D Systems (Minneapolis, Minn). Mouse anti–CD24 (sc-10741), used for immunofluorescence, was purchased from Santa Cruz Biotechnology.

Flow Cytometry

Cells were stained with phycoerythrin-conjugated anti-human CD24 antibodies (Beckman Coulter, Fullerton, Calif) for 20 minutes at room temperature. Single-color flow cytometry was performed using a FACS Calibur M (Becton Dickinson Immunocytometry Systems, San Jose, Calif), and data were analyzed with CELL Quest software (BD Biosciences, San Jose, Calif). The expression level was determined as the median fluorescence intensity on $CD24^-$ gated cells.

Cell Sorting

Cells (1×10^6 cells/mL) were incubated in prewarmed PBS supplemented with 2% heat-inactivated fetal calf serum containing freshly added phycoerythrin-conjugated anti–human CD24 antibody for 20 minutes at room temperature. At the end of the incubation, cells were spun down at 4°C and resuspended in ice-cold PBS. Samples were analyzed and collected using an EPICS ALTRA system (Beckman Coulter).

Matrigel Invasion Assay

Using Transwell inserts (Corning, Lowell, Mass), the upper surface of a filter (pore size, 8.0 μm; BD Biosciences, Heidelberg, Germany) was coated with 100 μL of basement membrane Matrigel (BD Biosciences), at a concentration of 2 mg/mL, and incubated at 4°C for 3 hours. The thickness of Matrigel was about 1 mm. Then, 0.9×10^5 cells suspended in RPMI 1640 were added to the upper chamber and treated with or without TGF-β1 (2 ng/mL) for 24 hours at 37°C in 5% CO_2. Supernatant of Tokyo Metropolitan Institute of Gerontology, human fetal lung fibroblasts cell line (purchased from Cell Bank, Osaka, Japan) was added to the lower chamber with or without TGF-β1 (2 ng/mL).

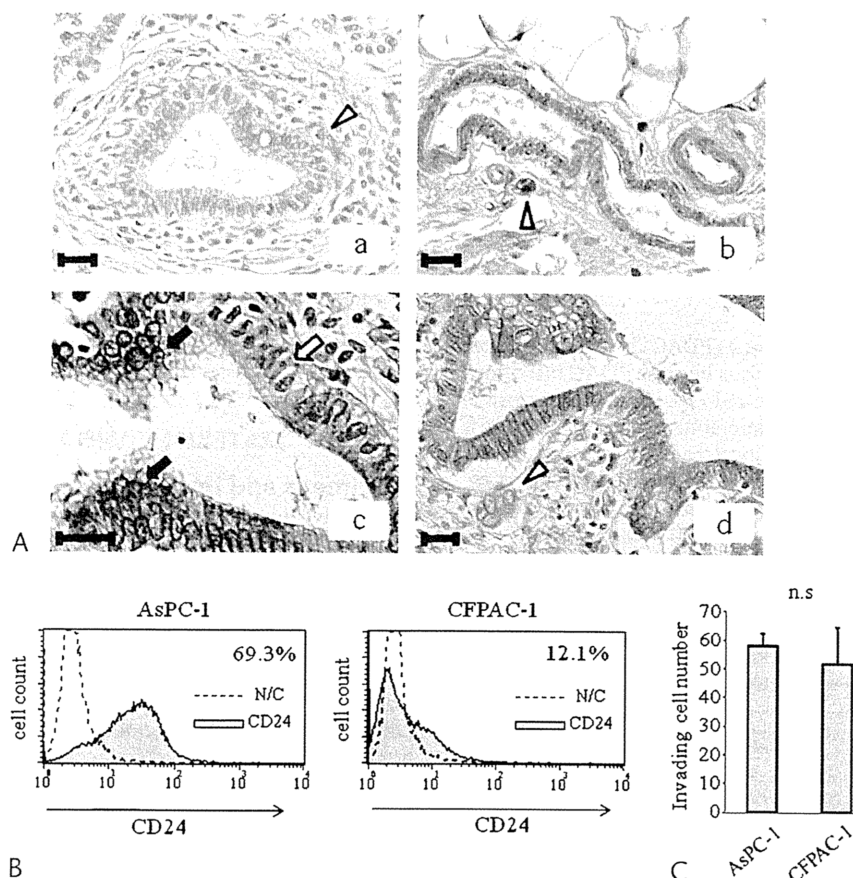

FIGURE 1. Heterogenous expression of CD24 in PDAC specimens. A, Representative photographs of immunohistochemistry for CD24 in PDAC specimens. Low CD24 expression (a). High CD24 expression (b). CD24 expression is heterogeneous within the same specimens (c) (large closed arrows indicate the high expression, and large open arrow indicates the low expression of CD24). High CD24 expression (d). Large open arrowheads show the stroma-invading PDAC cells (a, b, d). Magnification ×400; scale bars = 50 μm (a, b, d). Magnification ×640; scale bar = 50 μm (c). CD24 positive cells were stained brown with DAB. B, CD24 expression in PDAC cell lines assessed using flow cytometry. AsPC-1 cells contain large numbers of $CD24^+$ cells (69.3%), whereas CFPAC-1 cells contain relatively few $CD24^+$ cells (12.1%). C, Results of Matrigel invasion assay showing similar invasive abilities of both cell lines.

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After incubation, the filter was fixed with 70% ethanol and stained with Diff-Quik reagent (International Reagents, Kobe, Japan). Cells that had migrated from the upper to the lower side of the filter were counted under a light microscope at a magnification of 100×. Tumor cell invasiveness was defined as the total number of cells migrating across the membrane. Each experiment was carried out in triplicate wells.

Semiquantitative Reverse Transcription–Polymerase Chain Reaction and Real-Time Reverse Transcription–Polymerase Chain Reaction

RNA (1 μg) was treated with DNase and reverse transcribed using the Quantitect Reverse Transcription Kit (Qiagen, Valencia, Calif). Reactions were run on a DNA Engine Opticon 2 System (Bio-Rad, Hercules, Calif) using SYBR Premix Ex Taq II (Takara Bio Inc, Otsu, Japan). The primer sequences were *CD24*, forward 5′-TGAAGAACATGTGAGAGGTTTGAC-3′, reverse 5′-GAAAACTGAATCTCCATTCCACAA-3′; *E-cadherin*, forward 5′-CCTTCCTCCCAATACATCTCCC-3′, reverse 5′-TCTCCGCCTCCTTCTTCATC-3′; and *β-actin*, forward 5′-TTGTTACAGGAAGTCCCTTGCC-3′, reverse 5′-ATGCTATCACCTCCCCTGTGTG-3′. The amount of each target gene in a given sample was normalized to the level of β-actin in that sample.

RNA Interference

siRNA for CD24 (ON-TARGETplus SMART pool, J-187156) and negative control siRNA (ON-TARGETplus siCONTROL nontargeting pool, D-001810) were purchased from Dharmacon RNA Technologies (Chicago, Ill). Cells were transfected with 50 nM of CD24 siRNA, using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, Calif) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were harvested and were used for experiments.

Statistical Analysis

Student *t* test was used for statistical analysis. $P < 0.05$ was considered statistically significant.

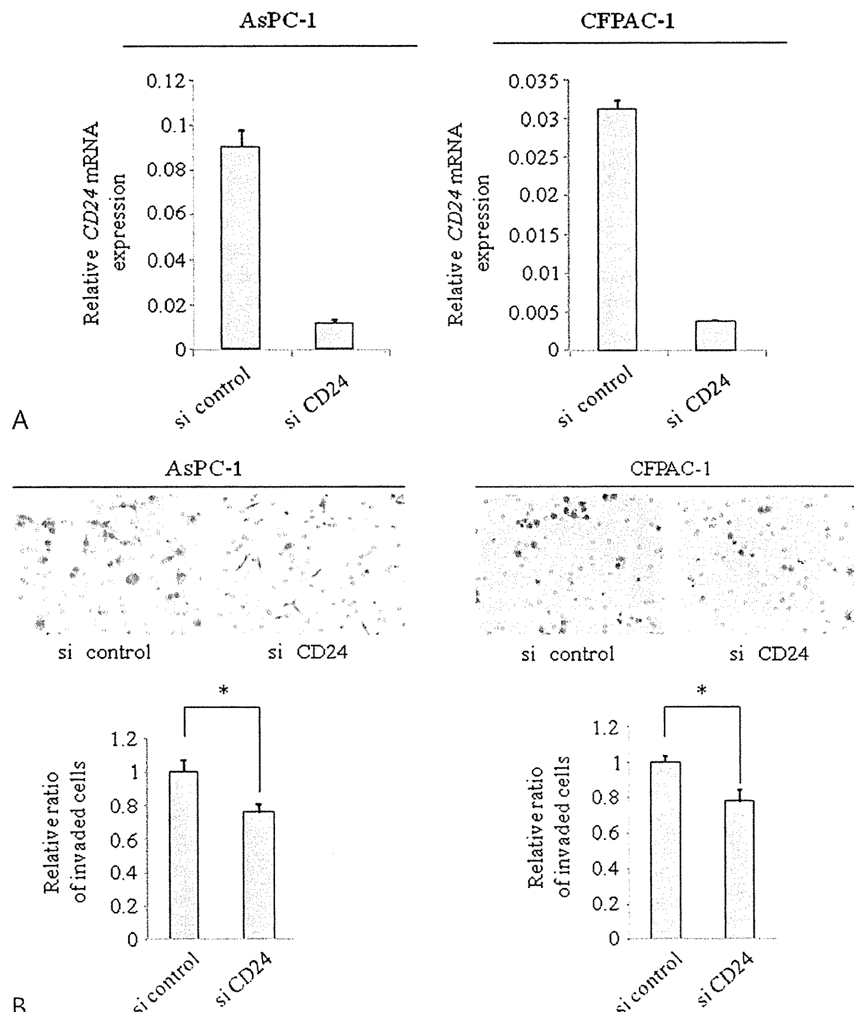

FIGURE 2. Silencing of CD24 reduces the invasiveness of PDAC cells. A, Cells were transfected with siRNA against *CD24* or control siRNA for 24 hours. The relative expression of *CD24* mRNA to that of *β-actin* mRNA was assessed by real-time reverse transcription–polymerase chain reaction. *CD24* siRNA reduced *CD24* mRNA expression by 80% or greater. B, The invasiveness of siRNA-transfected cells was assessed by Matrigel invasion assay. Representative photomicrographs of the Matrigel invasion assay are shown in the upper panels. Results in the lower panels are expressed as means ± SD. *$P < 0.05$.

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RESULTS

Heterogeneous Expression of CD24 in PDAC Specimens

It has been shown that CD24 is frequently expressed in PDAC.[11] Therefore, we first examined the expression and expression pattern of CD24 in 12 surgically resected PDAC specimens. Although CD24 was expressed in all 12 specimens, the degree of CD24 expression differed between each specimen (Fig. 1A, a and b). In addition, its expression is heterogeneous within the same specimen (Fig. 1A, c). Large closed arrows indicate the high expression, and the large open arrow indicates the low expression of CD24. Interestingly, PDAC cells invading into the stroma also express CD24 (Fig. 1A, a, b, and d). Large open arrowheads show the stroma-invading PDAC cells. To investigate whether CD24 contributes to the invasiveness of PDAC in conditions similar to the physiologic state, we selected 2 human PDAC cell lines containing large numbers of $CD24^+$ cells (AsPC-1; 69.3%) or relatively few $CD24^+$ cells (CFPAC-1; 12.1%) (Fig. 1B). Since CD24 was proposed to contribute to invasiveness,[12] we anticipated that the invasive ability of AsPC1 would be higher than that of CFPAC-1. Surprisingly, the invasive ability of both cells was very similar (Fig. 1C).

Silencing of CD24 Reduces the Invasiveness of PDAC Cells

To examine if CD24 contributes to the invasive ability, we silenced *CD24* at the mRNA level. Transfection of siRNA targeting for *CD24* significantly reduced the expression of *CD24* at mRNA level (Fig. 2A) and protein level (see Graphs, Supplemental Digital Content 1, http://links.lww.com/MPA/A60, which show the decreased CD24 expression by flow cytometry). When transfected cells were cultured for 24 or 48 hours in vitro, siRNA transfection did not affect the proliferation of PDAC cells (see Graphs, Supplemental Digital Content 2, http://links.lww.com/MPA/A69, which show that knocking down CD24 with siRNA does not affect the proliferation or viability of PDAC cells). However, silencing of CD24 significantly decreased the invasive ability of both cell lines (Fig. 2B). These data suggest that CD24 plays important roles in the invasiveness of these PDAC cells.

$CD24^+$ Cells Are Highly Invasive Cells

To clarify the contribution of CD24 to the invasiveness of PDAC cells, we separately collected $CD24^+$ and $CD24^-$ cells using a cell sorter. The purity of $CD24^+$ AsPC-1 and $CD24^+$ CFPAC-1 cells was 98% and 90%, respectively (Fig. 3A). $CD24^-$ AsPC-1 and $CD24^-$ CFPAC-1 cells' purity was more than 95% each. There was no significant difference in proliferation in both 2 cell lines during 24-hour assay time (see Graphs, Supplemental Digital Content 3, http://links.lww.com/MPA/A70, which show the similar increasing ability of $CD24^+$ cells and $CD24^-$ cells in both cell lines). The invasive ability of $CD24^+$ cells was significantly higher than that of $CD24^-$ cells in both cell lines (Fig. 3B). Taken together, our data strongly indicate that CD24 is involved in the invasiveness of PDAC cells.

Effect of Conversion of $CD24^+$ Cells to $CD24^-$ Cells on Invasiveness

To further confirm the contribution of CD24 to invasiveness, we silenced CD24 expression in $CD24^+$ cells using siRNA targeting *CD24*. Twenty-four hours after transfection, flow cytometry analysis was performed. Surprisingly, the percentage of $CD24^+$ cells in control siRNA-transfecting cells was significantly decreased compared with that in the first sorted cells (Fig. 4A). This suggests that a considerable number of $CD24^+$ cells are converted to $CD24^-$ cells within 24 hours by the in vitro culture condition. Transfection with control siRNA reduced the percentage of $CD24^+$ AsPC-1 cells or CFPAC-1 cells by 70% or 80% (Fig. 4A), meaning 30% or 20% of the control

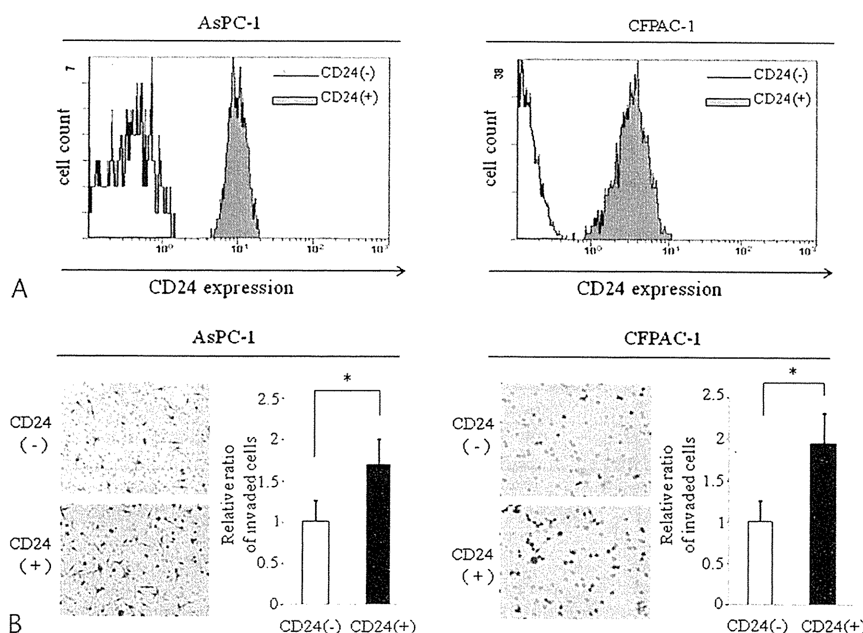

FIGURE 3. $CD24^+$ cells are highly invasive cells. A, $CD24^+$ and $CD24^-$ cells were separated and collected by a cell sorter. The purity of $CD24^+$ and $CD24^-$ cells is 98% or greater and 95% or greater, respectively. B, Invasiveness of the sorted cells was assessed by the Matrigel invasion assay. Representative photomicrographs of the Matrigel invasion assay. Results are expressed as means ± SD. $^*P < 0.05$.

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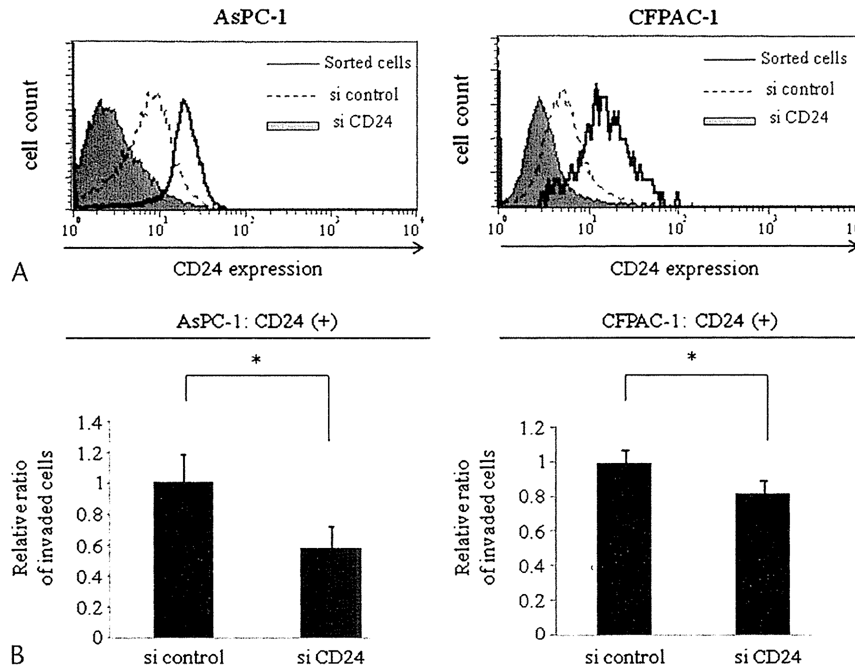

FIGURE 4. CD24$^+$ cells can be converted to CD24$^-$ cells. A, CD24$^+$ cells were collected by cell sorting and transfected with siRNA against *CD24* or control siRNA for 24 hours. CD24 expression was then examined by flow cytometry. The percentage of CD24$^+$ cells of control siRNA-transfected cells has decreased compared with that of the initially sorted cells. This is more evident in CFPAC-1 cells. B, The invasiveness of siRNA-transfected cells was assessed by the Matrigel invasion assay. Results are expressed as means ± SD. *$P < 0.05$.

siRNA-transfected cells expressed CD24, respectively. Therefore, the percentage of CD24$^+$ cells of control siRNA-transfected cells is still higher than that of CD24 siRNA-transfected cells. As a result, the invasive ability of CD24-silenced cells was significantly decreased compared with that of control siRNA-transfected cells (Fig. 4B). These data indicate that CD24$^+$ cells have a higher invasive ability than CD24$^-$ cells and also suggest that some CD24$^+$ cells can be converted to CD24$^-$ cells.

TGF-β1 Suppresses the Conversion From CD24$^+$ Cells to CD24$^-$ Cells

When CD24$^+$ cells were used as target cells, it was believed that CD24$^+$ cells were converted to CD24$^-$ cells. We know that TGF-β1 increases the invasiveness of cancer cells, including PDAC. Therefore, we hypothesized that TGF-β1 increases tumor cell invasiveness by suppressing the conversion from CD24^{++} to CD24$^-$ cells. To test this hypothesis, PDAC cells were cultured for 24 hours with TGF-β1 (0–10 ng/mL), and the percentage of CD24$^+$ cells was assessed by flow cytometry. In this experiment, 82% of AsPC-1 cells and 19% of CFPAC-1 cells, respectively, were CD24$^+$. In both cell lines, TGF-β1 at concentrations greater than 2 ng/mL significantly increased the percentage of CD24$^+$ cells (Fig. 5A). To confirm this effect of TGF-β1 on CD24 expression, we used CD24$^+$ cells collected by a cell sorter and incubated these cells with 2 ng/mL TGF-β1 for 24 hours in the following experiments. Consistent with the findings presented in Figure 5A with unsorted cells, the percentage of CD24$^+$ cells of AsPC-1 cells or CFPAC-1 cells in the presence of TGF-β1 remained higher than that in the absence of TGF-β1 (Fig. 5B). Of note, the conversion from CD24$^-$ cells to CD24$^+$ cells could not be detected (data not shown). These data suggest that TGF-β1 suppresses the conversion of CD24$^+$ cells into CD24$^-$ cells.

TGF-β1 Increases the Invasive Ability by Suppressing the Conversion From CD24$^+$ Cells to CD24$^-$ Cells

Our data suggest that TGF-β1 suppresses the conversion from CD24$^+$ cells to CD24$^-$ cells. Therefore, by maintaining CD24$^+$ expression, TGF-β1 should maintain or enhance the invasiveness of these cells. As expected, TGF-β1 increased the invasiveness of both cell lines (Fig. 6A). The increased invasiveness was significantly decreased by silencing of CD24, even in the presence of TGF-β1. To determine the contribution of CD24 to TGF-β1–induced increase of invasive ability, we compared the effect of TGF-β1 on invasiveness after silencing CD24 (Fig. 6B). The decrease in invasiveness in AsPC-1 cells or CFPAC-1 cells was greater in the presence of TGF-β1 (46% or 75%) than in the absence of TGF-β1 (25% or 31%), respectively. This indicates that CD24 plays a crucial role in the enhanced invasiveness induced by TGF-β1. Collectively, these data suggest that TGF-β1 enhances invasiveness by suppressing the conversion of CD24$^+$ cells to CD24$^-$ cells in PDAC cells.

To further confirm that CD24 contributes to enhanced invasiveness caused by TGF-β1, we used CD24$^+$ cells and CD24$^-$ cells collected by cell sorter and treated them with 2 ng/mL of TGF-β1 for 24 hours. Transforming growth factor β1 did not affect the invasiveness of CD24$^-$ AsPC-1 cells or CFPAC1 cells (Fig. 7A, left). On the other hand, TGF-β1 increased the invasiveness of control siRNA-transfected CD24$^+$ cells but did not affect the invasiveness of CD24-silenced CD24$^+$ cells (Fig. 7A, right). These data indicate that TGF-β1 does not affect the invasiveness of CD24$^-$ cells and that CD24 provides a significant contribution to TGF-β1–induced invasiveness of CD24$^+$ cells.

Because CD24$^+$ cells have a high invasive ability and can be converted to CD24$^-$ cells, and TGF-β1 can suppress the conversion from CD24$^+$ cells to CD24$^-$ cells, TGF-β1 maintains the

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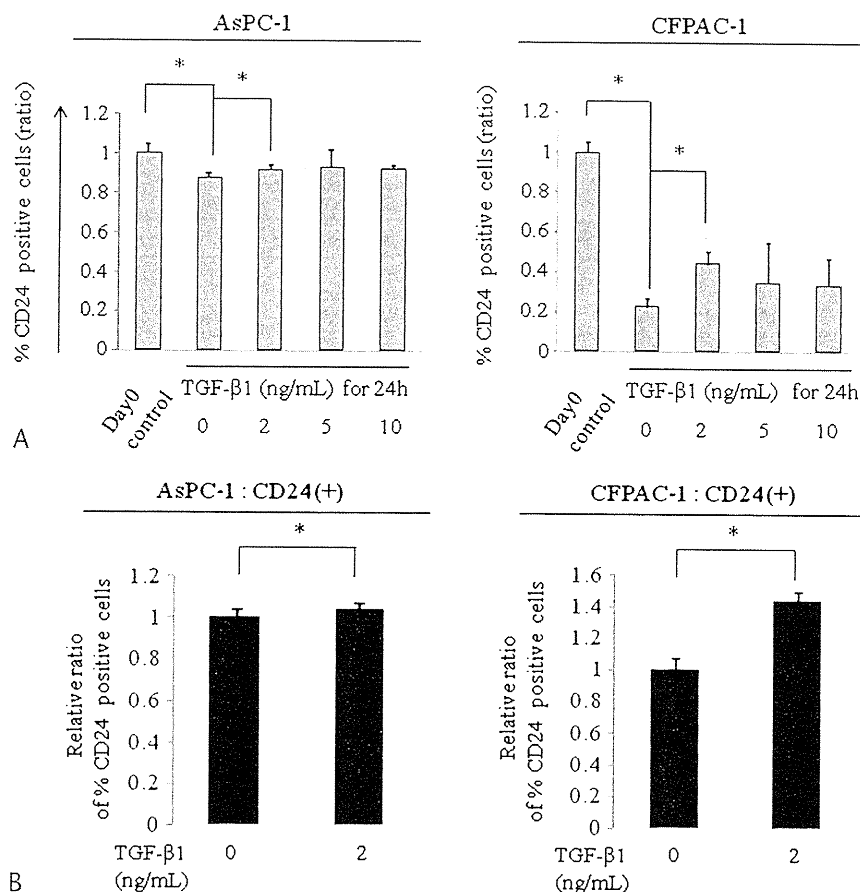

FIGURE 5. Transforming growth factor β1 suppresses the conversion of CD24+ cells to CD24− cells. A, PDAC cells were incubated with TGF-β1 (2–10 ng/mL) for 24 hours, and CD24 expression was examined by flow cytometry. Transforming growth factor β1 increased the percentage of CD24+ cells both in AsPC-1 cells and CFPAC-1 cells. Results are expressed as means ± SD. *$P < 0.05$. B, CD24+ cells were collected by cell sorting and incubated without or with 2 ng/mL TGF-β1 for 24 hours. CD24 expression was examined by flow cytometry. Results are expressed as means ± SD. *$P < 0.05$.

highly invasive ability of CD24+ cells by suppressing this conversion (Fig. 7B).

DISCUSSION

In this study, we showed that CD24+ cells show greater invasive ability than CD24− cells in PDAC cell lines. However, many CD24+ cells spontaneously convert to CD24− cells in vitro. We also revealed, for the first time, that TGF-β1 suppresses the conversion from CD24+ cells to CD24− cells and thus maintains the invasiveness of PDAC cells.

CD24 is overexpressed in various malignancies, including PDAC,[5–11] and is considered to be associated with tumor proliferation, invasion, and metastasis. Baumann et al[19] show that CD24 expression can be sufficient to promote metastasis in vivo by altering the adhesive properties of tumor cells by activating integrins. However, the influence of CD24 expression on invasiveness is inconsistent, with some studies showing positive roles, whereas others showed negative roles of CD24. A positive role of CD24 has been demonstrated in colorectal and pancreatic cancer, glioma, glioblastoma, and breast cancer, in vitro and in vivo.[17,19–24] On the other hand, a negative influence of CD24 has been reported only in breast cancer.[25,26] These previous studies often used CD24-expressing clones or CD24-silenced cells as target cells. Consistent with other studies, our immunohistochemical analysis of PDAC tissues revealed that the expression of CD24 is quite heterogeneous.[11] Therefore, to analyze the contribution of CD24 to invasiveness in a more physiological situation, we studied 2 PDAC cell lines, one containing a large number of CD24+ cells (AsPC-1) and the other containing relatively few CD24+ cells (CFPAC-1). Because CD24 was proposed to contribute to invasiveness,[12] we expected that the invasive ability of AsPC-1 would be higher than that of CFPAC-1. But the results were not so (Fig. 1C). We think the reason why invasive ability of AsPC-1 (CD24 >65%) was at the same level as CFPAC-1 (CD24 <15%) is because of the difference of the ratio of CD24 participation in each cell line. E-cadherin also contributes to the invasiveness.[27] For example, when the expression of CD24 was knocked down with CD24 siRNA in AsPC-1 cells, the expression of *E-cadherin* mRNA became higher, but on the other hand, *E-cadherin* mRNA level in CFPAC-1 tranfected with CD24 siRNA had no change (see Graphs, Supplemental Digital Content 4, http://links.lww.com/MPA/A71, which show that CD24 knock down increased the *E-cadherin* mRNA only in AsPC-1 cells). It shows that E-cadherin also contributes to the invasiveness in AsPC-1. Silencing of CD24 by siRNA significantly decreased the invasiveness of both cell lines. Notably, siRNA transfection did not affect cell viability or cell proliferation during the experimental period (see Graphs, Supplemental Digital Content 2, http://links.lww.com/MPA/A69, which show

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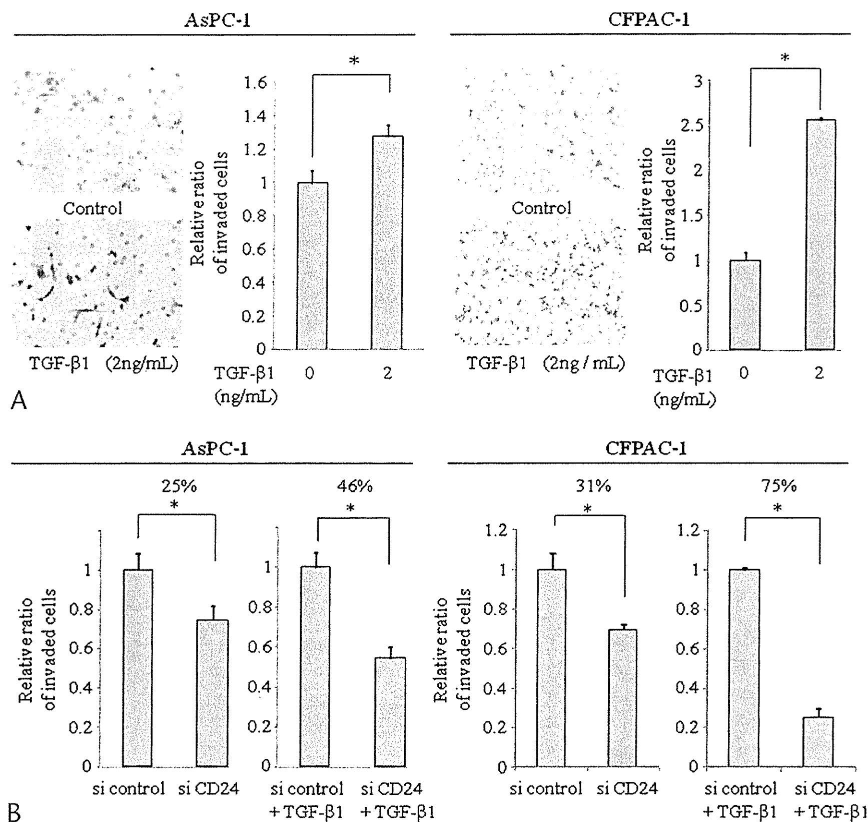

FIGURE 6. Transforming growth factor β1 increases the invasiveness and CD24 contributes to TGF-β1–induced increased invasiveness. A, Matrigel invasion assay of PDAC cells cultured without or with TGF-β1 (2 ng/mL). Representative photomicrographs of the Matrigel invasion assay. Transforming growth factor β1 increased the invasive ability in both cell lines. Results are expressed as means ± SD. *$P < 0.05$. B, Effect of CD24 silencing on TGF-β1–induced invasiveness. The role of CD24 in TGF-β1–induced invasiveness was estimated based on the difference in invasiveness caused by silencing CD24 between the presence and absence of TGF-β1. Because TGF-β1 enhances invasiveness, the greater decrease in invasiveness in the presence of TGF-β1 means CD24 is involved in TGF-β1–induced invasiveness. Results are expressed as means ± SD. *Relative decrease in invasiveness.

that knocking down CD24 with siRNA does not affect the proliferation or viability of PDAC cells). In addition, $CD24^+$ cells collected by cell sorting were more invasive than $CD24^-$ cells in both cell lines. To further confirm the positive contribution of CD24 to invasiveness, we silenced CD24 in highly purified $CD24^+$ cells. As expected, silencing of CD24 decreased the invasiveness of both cell lines, but the decreasing rate of AsPC-1 cells was much greater than that of CFPAC-1 cells. To investigate why silencing of CD24 provided different decreasing rate between these 2 cell lines, we performed flow cytometry. Flow cytometry showed that half of the control siRNA-transfected $CD24^+$ AsPC-1 cells and most of the control siRNA-transfected $CD24^+$ CFPAC-1 cells transformed into $CD24^-$ cells. This means that most of the $CD24^+$ CFPAC-1 cells were converted to $CD24^-$ cells during the 24-hour culture for control siRNA transfection and consequently decreased the invasiveness of these cells even if there was still significant difference. Meyer et al[28] reported that CD24 expression is dynamically regulated in vitro in breast cancer cells. They showed that noninvasive, epithelial-like $CD44^+$ $CD24^+$ cells can give rise to invasive, mesenchymal $CD44^+$ $CD24^-$ progeny. However, in the PDAC cell lines used in the present study, conversion from $CD24^-$ cells to $CD24^+$ cells was not detected during culture. Based on these findings, we conclude that CD24 contributes to the increased invasiveness of these PDAC cell lines, and we believe that $CD24^+$ cells, particularly highly purified $CD24^+$ cells, spontaneously convert to $CD24^-$ cells in vitro. This conversion from $CD24^+$ cells to $CD24^-$ cells may be partially supported by the evidence that CD24 is a surface marker of pluripotent cancer stem cells in PDAC.[18] However, the molecular mechanisms involved in the conversion of $CD24^+$ cells to $CD24^-$ cells are unclear.

Accordingly, we hypothesized that TGF-β1 affects the interconversion between $CD24^+$ cells and $CD24^-$ cells. Indeed, in CFPAC-1 cells, we found that TGF-β1 significantly suppressed the conversion from $CD24^+$ cells to $CD24^-$ cells and consequently maintained the invasiveness of $CD24^+$ cells. This effect of TGF-β1 on the increased invasiveness was remarkably abolished by silencing CD24, suggesting that CD24 has an important role in the increased invasiveness induced by TGF-β1 in CFPAC-1 cells. On the other hand, the effect of TGF-β1 on the increased invasiveness of AsPC-1 cells was only partially reduced by silencing CD24, suggesting that CD24 only partially contributes to the increased invasiveness induced by TGF-β1. In the present study, we could not confirm that TGF-β1 promotes the conversion from $CD24^-$ cells to $CD24^+$ cells. Therefore, it seems unlikely that TGF-β1 modifies CD24 expression by regulating CD24 transcription. However, we cannot exclude the possibility that TGF-β1 enhances the translocation of CD24 from the cytoplasm to cell membrane or suppresses the translocation of CD24 from the membrane to the cytoplasm.[29,30]

Consistent with other studies, our data also reveal that CD24 plays an important role in the invasiveness of PDAC cells.

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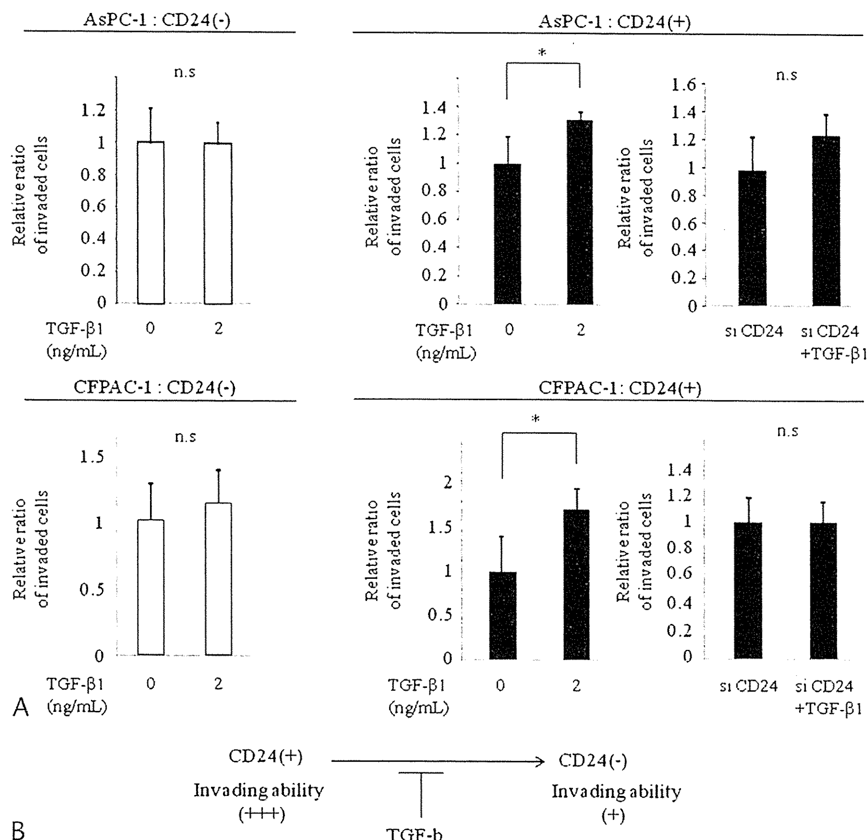

FIGURE 7. Transforming growth factor β1 increases PDAC cell invasiveness by suppressing the conversion from $CD24^+$ cells to $CD24^-$ cells. A, To confirm the role of CD24 in TGF-β1–induced invasiveness, we collected $CD24^-$ and $CD24^+$ cells using a cell sorter. Transforming growth factor β1 did not affect the invasiveness of $CD24^-$ cells (white columns). In both cell lines, silencing of CD24 completely abolished TGF-β1–induced invasiveness. Results are expressed as mean ± SD. $P < 0.05$. B, A proposed model for the effect of TGF-β1 on invasiveness. We believe that TGF-β1 suppresses the conversion of $CD24^+$ to $CD24^-$ cells and thus maintains the invasiveness of PDAC cells.

This means that CD24 is not only a surrogate marker for highly invasive or metastatic cancers, but also that CD24 might offer a therapeutic target for PDAC. Importantly, our data indicate for the first time that TGF-β1 inhibits the conversion from $CD24^+$ cells with high invasive ability to $CD24^-$ cells with low invasive ability. This effect of TGF-β1 seems to be cell-dependent, and we know that TGF-β1 is overexpressed in various types of human cancers, including PDAC.[31,32] Overall, this newly identified effect of TGF-β1 on CD24 expression suggests that TGF-β1 produced by tumors maintains the invasiveness of PDAC cells at the tumor site. Therefore, a combination of silencing of CD24 and inhibition of TGF-β1 signaling may offer a novel therapeutic approach for highly invasive and metastatic PDAC.

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Predicting the chemosensitivity of pancreatic cancer cells by quantifying the expression levels of genes associated with the metabolism of gemcitabine and 5-fluorouracil

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Abstract. Gemcitabine (GEM) is the standard treatment for advanced/metastatic pancreatic cancer. However, there is a substantial subset of patients in whom the efficacy of GEM, when used as a single agent, is inadequate. Recently, the 5-fluorouracil (5-FU) prodrugs capecitabine and S-1 have been used as an alternative, either alone or in combination with GEM. The aim of the present study was to investigate the expression pattern of genes that render pancreatic cancer cells sensitive to GEM and 5-FU, and to identify markers for individualized chemotherapy, even in patients who have developed resistance. We investigated the correlation between the expression of genes associated with the metabolism of GEM and 5-FU, and sensitivity to these drugs in 15 human pancreatic cancer cell lines. We also established GEM- and 5-FU-resistant pancreatic cancer cell lines to investigate changes in the expression levels of these genes and the effects of one drug on cells resistant to the other. We found no correlation between pancreatic cancer cell sensitivity to either GEM- or 5-FU. GEM-resistant cells did not become resistant to 5-FU and *vice versa*. High expression of *RRM1* (P=0.048) and *TS* x *DPD* (P=0.035) correlated significantly with sensitivity to GEM and 5-FU, respectively. 5-FU-resistant cells expressed significantly higher levels of *TP* than parental cells (P<0.05). In conclusion, pancreatic cancer cells showed no cross-resistance to GEM and 5-FU. Quantitative analyses of *RRM1*, *TP*, *DPD* and *TS* mRNA levels in pancreatic cancer cells may be useful for predicting their sensitivity to GEM and 5-FU.

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Key words: gemcitabine, 5-fluorouracil, reverse transcription-polymerase chain reaction, chemosensitivity, pancreatic cancer

Introduction

The prognosis for patients with pancreatic cancer is extremely poor. The tumor is extremely aggressive and early detection is difficult due to the lack of early disease-specific signs and symptoms. Only 10-20% of patients with pancreatic cancer are candidates for curative resection (1,2) and, even if surgery is performed, the post-operative 5-year survival rate is only 15-25% due to the high incidence of postoperative recurrence (1,3,4). Gemcitabine (difluorodeoxycitidine, dFdC; GEM) is the standard treatment for advanced/metastatic pancreatic cancer based on a landmark trial comparing its effects with those of fluorouracil (FU) (5). However, the clinical benefit of GEM as a single agent is inadequate, as indicated by the median survival time of only 5.7-7.2 months and a low objective response rate (5-9). Thus, there is a pressing need to develop new treatment strategies. Recently, a phase III trial for advanced pancreatic cancer showed a significant increase in both overall survival (OS) and progression-free survival (PFS) after treatment with erlotinib plus GEM compared with GEM alone. Although these results were statistically significant, the absolute benefit of OS was modest (only 2 weeks) (9).

GEM is a deoxycytidine analog that has significant single-agent activity against a number of malignancies, including pancreatic cancer (10,11). GEM is transported into cells via the human equilibrative nucleoside transporter-1 (hENT1) (12) and must be phosphorylated by deoxycytidine kinase (dCK) to be activated. The phosphorylated forms of GEM inhibit DNA synthesis through its incorporation into DNA leading to masked chain termination, and by inhibiting the enzyme ribonucleotide reductase (RR) (13,14). In addition, the deoxyribonucleotide and ribonucleotide pools, both essential for DNA repair, are seriously depleted by the phosphorylated forms (15). Conversely, GEM is inactivated by cytidine deaminase (CDA) (16). We, and other investigators, showed that the expressions of hENT1, dCK, the RR subunits M1 (RRM1) and M2 (RRM2), and the genes that encode them were, at least partially, correlated with sensitivity to GEM (17-25).

Recently, the orally administered fluoropyrimidine prodrugs, capecitabine and S-1, have been used as alternative

Table I. Primer sequences and product sizes.

Primer	Forward sequence 5'-3'	Reverse sequence 5'-3'	Product size
hENT1	gcaaaggagaggagccaagag	gggctgagagttggagactg	65
dCK	gctgcagggaagtcaacattt	ttcaggaaccacttcccaatc	69
RRM1	actaagcaccctgactatgctatcc	cttcctcacatcactgaacacttt	88
RRM2	ggctcaagaaacgaggactg	tcaggcaagcaaaatcacag	93
CDA	tcaaagggtgcaacatagaaaatg	cggtccgttcagcacagat	61
TP	cctgcggacggaatcct	gctgtgatgagtggcaggct	71
DPD	aggacgcaaggagggtttg	gtccgccgagtccttactga	84
OPRT	tcctgggcagatctagtaaatgg	tgctcctcagccattctaacc	156
TS	gcctcggtgtgcctttca	cccgtgatgtgcgcaat	67
18S rRNA	gtaacccgttgaaccccatt	ccatccaatcggtagtagcg	151

or additional agents for advanced pancreatic cancer. A recent phase III clinical trial for advanced pancreatic cancer showed that treatment with GEM plus capecitabine led to a significant increase in PFS and a tendency to prolonged OS compared with GEM alone (7). Capecitabine is metabolized to 5-FU via a three-step enzymatic process, the final step being catalyzed by thymidine phosphorylase (TP) (26). Meanwhile, a late phase II study using S-1 to treat metastatic pancreatic cancer showed promising results, with a 37.5% response rate and a median OS of 9.2 months (27). S-1 consists of tegafur (FT; a prodrug of 5-FU) and two biochemical modulators, 5-chloro-2,4-dihydroxypyridine (CDHP) and potassium oxonate (Oxo) (28), which improve the tumor-selective toxicity of 5-FU. CDHP is a competitive inhibitor of dihydropyrimidine dehydrogenase (DPD), which rapidly catabolizes 5-FU and maintains efficacious 5-FU concentrations in the plasma and tumor tissues (29). Oxo decreases phosphorylation of 5-FU within the gastrointestinal tract by competitively inhibiting orotate phosphoribosyltransferase (OPRT), thereby reducing the serious gastrointestinal toxicity associated with 5-FU (30). Finally, in both agents, 5-FU interacts with its pharmacological target, thymidylate synthase (TS) and inhibits DNA synthesis and repair. Increasing evidence suggests that the expression levels of TP, DPD, OPRT and TS (along with the genes that encode them) predict sensitivity to 5-FU or its prodrugs (26,29-32).

Although GEM and 5-FU prodrugs are effective against advanced pancreatic cancer when used as single agents, there is a substantial subset of patients in whom the efficacy is limited or inadequate. Also, few studies have investigated whether these agents are effective in patients who have developed resistance to other agents. Recent studies show that altered gene expression can, at least in part, explain the efficacy of cytotoxic agents (19,33). Therefore, in the present study, we investigated the correlation between the expression of genes associated with the metabolism of GEM and 5-FU and cancer cell sensitivity to the drugs using 15 human pancreatic cancer cell lines. Furthermore, we established pancreatic cancer cell lines that are resistant to each agent to investigate the effects of one drug on pancreatic cancer cell lines that were resistant to the other. We also analyzed the expression levels of genes related to the transport and metabolism of GEM and 5-FU to clarify the underlying mechanisms involved in drug-resistance.

Materials and methods

Cell lines and establishment of GEM or 5-FU-resistant cells. The following 15 human pancreatic cancer cell lines were used in this study: BxPC-3, Capan-1, Capan-2, CFPAC1, Hs766T, SW1990 (American Type Culture Collection, Manassas, Virginia, USA), AsPC-1, H48N, KP-1N, KP-2, KP-3, Panc-1, SUIT-2 (generously provided by Dr H. Iguchi (National Shikoku Cancer Center, Matsuyama, Japan), MIA PaCa-2 (Japanese Cancer Resources Bank, Tokyo, Japan) and NOR-P1; established in our laboratory (34). Cells were maintained as previously described (35). Cells resistant to GEM (Wako, Osaka, Japan) or 5-FU (Kyowa Hakko Kogyo, Tokyo, Japan) were generated by exposure to gradually increasing concentrations of each drug as previously described (23). The final concentrations of GEM and 5-FU were 200 nM and 2 μM, respectively. Both agents were dissolved in phosphate-buffered saline and added to the culture medium [Dulbecco's modified Eagle's medium, DMEM; Sigma Chemical Co., St. Louis, MO, USA; supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/ml) and penicillin (100 U/ml)].

Propidium iodide (PI) assay. To calculate the 50% inhibitory concentration (IC_{50}) for each cell line when exposed to GEM or 5-FU, cells were seeded in 24-well plates (Becton-Dickinson Labware, Bedford, MA, USA) at a density of $2x10^4$ per well, using cell numbers previously counted using a particle distribution analyzer (CDA 500; Sysmex, Kobe, Japan). Several different concentrations of GEM or 5-FU were added to the cells 24 h after seeding. Cell populations were evaluated by measuring the fluorescence intensity of PI after a further incubation for 72 h, as previously described (19,23).

Quantitative one-step real-time reverse transcription-polymerase chain reaction (qRT-PCR). Total RNA was extracted from cultured cells using a High Pure RNA isolation kit (Roche, Mannheim, Germany) with DNase (Roche) treatment according to the manufacturer's instructions. qRT-PCR was performed for 40 cycles of 15 sec at 95˚C and 1 min at 55˚C using a Chromo4 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) and a