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Inhibition of fibroblast growth factor receptor 2 attenuates proliferation and invasion of pancreatic cancer

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Key words

FGFR-2 IIIb, FGFR-2 IIIc, fibroblast growth factor receptor-2 (FGFR-2), pancreatic cancer, short hairpin RNA

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The alternative splicing of the extracellular domain of fibroblast growth factor receptor (FGFR)-2 generates the IIIb and IIIc isoforms. Expression of FGFR-2 IIIb correlates with vascular endothelial growth factor-A (VEGF-A) expression and venous invasion of pancreatic ductal adenocarcinoma (PDAC). By contrast, FGFR-2 IIIc expression correlates with faster development of liver metastasis after surgery, and increased proliferation rates and invasion of the cancer. In this study, we analyzed the expression and roles of total FGFR-2 (both isoforms) to determine the effectiveness of FGFR-2-targeting therapy for PDAC. Immunohistochemically, FGFR-2 was highly expressed in 25/48 (52.1%) PDAC cases, and correlated with advanced stage cancer. In FISH analysis, *FGFR2* was amplified in 3/7 PDAC cell lines. We stably transfected an FGFR-2 shRNA targeting the IIIb and IIIc isoforms into *FGFR2*-amplified PDAC cells. The proliferation rates, migration, and invasion of FGFR-2-shRNA-transfected cells were lower than those of control cells *in vitro*. In response to FGF-2, FGFR-2-shRNA-transfected cells showed decreased phosphorylation of ERK compared with control cells. The FGFR-2-shRNA-transfected cells also expressed lower levels of vascular endothelial growth factor-A than control cells, and formed smaller s.c. tumors in nude mice. These findings suggest that FGFR-2 is a therapeutic target for inhibition in PDAC.

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive human malignancy with an overall 5-year survival rate of 6%.[1] At the molecular level, a high percentage of PDACs overexpress a number of growth factors and their receptors, including the epidermal growth factor, epidermal growth factor receptor, transforming growth factor-α, all three transforming growth factor-β isoforms, fibroblast growth factor (FGF), and FGF receptors (FGFR).[2–4]

In humans, the FGF/FGFR family consists of 22 FGF genes (*FGF1* to *FGF23*), and four FGFR genes (*FGFR1* to *FGFR4*).[5,6] Recent studies have shown that gene amplification, abnormal activation, or single nucleotide polymorphisms of *FGFR2* play important roles in cancer progression.[7–10] Single nucleotide polymorphisms in intron 2 of *FGFR2* are associated with an increased risk of breast[11,12] and endometrial cancers;[13] amplification and overexpression of *FGFR2* is strongly associated with the poorly differentiated, diffuse type of gastric cancer, which has an unfavorable prognosis.[14]

An important feature of FGFR-2, and a mode of regulation of its functions, is the generation of structural variants of FGFR-2 by alternative gene splicing. The alternative splicing of the C-terminal half of the third Ig-like domain generates the IIIb and IIIc isoforms of FGFR-2. The appropriate tissue-specific expression of FGFR-2 IIIb or FGFR-2 IIIc, in conjunction with the presence of appropriate ligands, is crucial for maintenance of cellular homeostasis and function. Fibroblast growth factor-1, -3, -7, -10, and -22 bind to FGFR-2 IIIb with high affinity, whereas FGF-1, -2, -4, -6, -9, -17, and -18 bind to FGFR-2 IIIc with high affinity.[15,16]

FGF1, *FGF2*, *FGF5*, and *FGF7* are overexpressed in PDAC.[17–19] Concomitant expression of FGF-7/keratinocyte growth factor and its receptor, FGFR-2 IIIb/keratinocyte growth factor receptor, correlates with increased vascular endothelial growth factor-A (VEGF-A) expression, venous invasion, and poor prognosis.[3] Furthermore, FGFR-2 IIIc promotes the proliferation and migration of PDAC cells, and confers cancer stem cell-like features onto these cells.[4] These results indicate that both FGFR-2 IIIb and IIIc may be novel therapeutic targets for PDAC. However, the effect of FGFR-2-targeting therapy on *FGFR2*-amplified-PDAC has been unclear. In the present study, we inhibited the expression of both FGFR-2 IIIb and FGFR-2 IIIc isoforms in *FGFR2*-amplified PDAC cells to clarify the effectiveness of FGFR-2 targeting therapy for PDAC.

Materials and Methods

Materials. Histofine Simple Stain MAX PO kits and Peroxidase conjugated streptavidin were from Nichirei (Tokyo,

Japan); the goat polyclonal anti-human FGFR-2 (N-20), rabbit polyclonal anti-VEGF-A (A-20), and rabbit polyclonal anti-ERK1 (K-23) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); the mouse monoclonal anti-cytokeratin (CK) 19 was from Boehringer Mannheim (Mannheim, Germany); the guinea pig polyclonal anti-swine insulin antibody was from Dako (Santa Barbara, CA, USA); biotinylated anti-guinea pig IgG was from Vector Laboratories (Burlingame, CA, USA); the mouse monoclonal anti-human CD31 antibody was from AbD Serotec (Kidlington, UK); FuGene HD was from Roche Diagnostics (Mannheim, Germany); *Taq*Man Gene Expression Assays for FGFR-2 (Hs01552926_m1) and 18S rRNA (Hs99999901_s1) were from Applied Biosystems (Foster City, CA, USA); human serum was from Lonza (Walkersville, MD, USA); the Zenon rabbit IgG labeling kit (Z-25351) and rabbit IgG (ab37415) were from Abcam (Cambridge, UK); the rabbit monoclonal phospho-p44/42 MAPK (p-ERK) antibody was from Cell Signaling Technology (Danvers, MA, USA); the HRP-conjugated goat anti-rabbit IgG secondary antibodies were from American Qualex (San Clemente, CA); recombinant human basic fibroblast growth factor (bFGF, FGF-2) was from ReproCELL (Kanagawa, Japan); the 35-μm filters, 8-μm pore size cell culture inserts, and BioCoat Matrigel invasion chambers were from BD Bioscience (Franklin Lakes, NJ, USA); NanoCulture 96-well plates (NCP-L-MS) and NanoCulture medium (NCM-M) were from Scivax (Kanagawa, Japan).

Pancreatic ductal adenocarcinoma cell lines. Human PDAC cell lines, AsPC-1, PANC-1, MIAPaCa-2, PK-1, PK-8, and PK-45H were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan), and Capan-1 was purchased from ATCC (Manassas, VA, USA). The cells were grown in the RPMI-1640 medium containing 10% FBS at 37°C under a humidified 5% CO_2 atmosphere. Capan-1 cells were maintained in the same medium with 15% FBS. Human pancreatic duct epithelial cell lines HPDE4 and HPDE6 were kind gifts from Prof. Tsao.[20]

Patients and tissues. Tissue was obtained from 48 patients with invasive PDAC who received treatment at Nippon Medical School Hospital (Tokyo, Japan) from 1995 to 2005 (Table 1). This study was carried out in accordance with the principles embodied in the Declaration of Helsinki, 2008, and informed consent for the use of pancreatic tissues was obtained from each patient. Normal pancreatic tissues were obtained from Human Digestive Tissue Sets and Human Tissue Microarray 1 from Novagen (Darmstadt, Germany), and from surgical pancreatic tissues from ectopic spleen in pancreas (Nippon Medical School Hospital).

Immunohistochemistry. Paraffin-embedded sections were immunostained using Histofine Simple Stain MAX PO kits.[21] The tissue sections were incubated overnight with the appropriate antibodies (1:400 anti-FGFR-2; 1:50 anti-CD31; 1:100 anti-CK19; and 1:1000 anti-insulin). For the evaluation of positivity and the intensity of FGFR-2 immunostaining, the following scale was used: 0, no staining; 1+, mild staining; 2+, moderate staining; and 3+, intense staining. Thus, the positively stained proportion (%) in cancer cells was: 1+, 1–25%; 2+, 26–50%; 3+, 51–75%; and 4+, 76–100%. Each score was added, and those that were under the mean value (3.7) were classified into the low FGFR-2 group; those over the mean value were in the high FGFR-2 group. Two pathologists (T.I. and Y.M.) independently evaluated the staining results. To confirm the positive staining of FGFR-2, blocking peptides of FGFR-2 were pre-incubated with the anti-FGFR-2 antibody, and immunohistochemical staining was carried out (Fig. S1).

Table 1. Clinicopathologic features and fibroblast growth factor receptor (FGFR)-2 expression in pancreatic cancers (n = 48)

	FGFR-2		*P*-value
	Low (n = 23)	High (n = 25)	
Gender			
Female	7	12	0.2138
Male	16	13	
Age, years: 65.19 ± 1.488			
<65	10	11	0.9710
≥65	13	14	
UICC classification			
T – primary tumor			
T1	0	2	0.1881
T2	1	0	
T3	10	6	
T4	12	17	
N – regional lymph nodes			
N0	7	8	0.9070
N1	16	17	
M – distant metastasis			
M0	22	25	0.2921
M1	1	0	
Stage			
I	0	2	0.0475
II	1	0	
III	8	2	
IV	14	21	

Data represents mean ± SE. UICC, Union for International Cancer Control.

Fluorescence *in situ* hybridization analysis. The FISH analysis was carried out as previously described.[22] The probes were generated from appropriate clones from a library of human genomic clones (GSP Laboratory, Kawasaki, Japan). Ten microliters of probe was heated for 5 min at 73–75°C. The slides with cultured PDAC cells were placed in a denaturant solution (70% formamide/2× SSC) at 73–75°C, denatured for 5 min, and dehydrated in 70 and 100% ethanol for 1 min each at room temperature (RT). Denatured probes were applied, and the cells were covered with a coverglass and placed at 45–50°C. The slides were then sealed with rubber cement, and placed in a humidified box overnight at 37°C. Stringent washing was carried out using 2× SSC/0.3% NP-40 at RT and 0.4× SSC/0.3% NP-40 at 73°C for 2 min, and then with 2× SSC at RT for 1 min. The signals were observed using fluorescence microscopy, and were evaluated by independent observers (Y.M. and T.S.). A positive result for copy number gain is determined as follows: *FGFR2/CEN10p* ratio > 3.0.

Construction of expression vector for FGFR-2-shRNA. Expression vectors for human FGFR-2-shRNA were constructed as previously described.[21] The sense target sequence for FGFR-2 (NM_000141.4; 5′- GAG GCT ACA AGG TAC GAA A -3′), the hairpin loop, and the antisense target sequences were synthesized and inserted into the pBAsi-hU6 Neo DNA vector. Likewise, the scrambled sequence (5′-TCT TAA TCG CGT ATA AGG C-3′) was used to construct the sham vectors that served as negative controls. Transfections were carried out using the FuGENE HD transfection reagent.

Flow cytometry. Anti-FGFR-2 antibody was labeled with allophycocyanin, using a Zenon rabbit IgG labeling kit.[21] Cells

(5×10^5 per 25 μL) were incubated with 1 μg allophycocyanin-labeled anti-FGFR-2 antibody for 60 min; 1 μg propidium iodide was added to label dead cells. Expression of FGFR-2 was analyzed using a FACSAria II flow cytometer (BD Bioscience). Isotype-matched rabbit IgG was used as a negative control.

Immunofluorescent analysis. The cells were fixed with 4% paraformaldehyde, and were incubated with a goat polyclonal anti-FGFR-2 antibody (1:100) or a rabbit anti-VEGF-A antibody (1:200) at 4°C overnight. The cells were washed with PBS, then incubated with an Alexa 488-labeled anti-goat IgG or anti-rabbit IgG antibody (1:1000) with or without Alexa 568-labeled phalloidin (1:50) for 60 min. Fluorescent images were observed under a Digital Eclipse C1 TE2000-E confocal microscope (Nikon Insteck, Kanagawa, Japan), and analyzed using control software EZ-C1 (Nikon Insteck). Confocal settings, including the laser power and detector sensitivity, remained unchanged during the acquisition of all images. The images of 3-D culture were collected at 0.5-μm intervals with a laser to form a stack in the Z-axis, and were used to generate a 3-D image, using Volocity (Improvision, Coventry, UK). Total intensity of VEGF-A was measured by MetaMorph software 7.6 (Universal Imaging, Marlow, UK).

***In vitro* cell proliferation.** To monitor cell proliferation, nonradioactive cell proliferation assays were carried out. Cells were plated at a density of 1×10^4 cells per flask in the RPMI-1640 medium supplemented with 10% FBS. After 72 h, cell numbers per flask were counted. The analysis was carried out in triplicate.

Cell signaling pathway analysis of FGFR-2 shRNA transfected PDAC cells. Cells (2.5×10^5) were seeded in a 60-mm dish, and grown in RPMI-1640 medium supplemented with 10% FBS for 24 h. The cells were then washed with serum-free medium, and cultured with the same medium for 24 h, and recombinant human FGF-2 (100 ng/mL) and heparin (1 μg/mL) were added to the plates. Protein extraction was carried out according to the M-Per Mammalian Protein Extraction reagent protocol. The cleared protein lysates were subjected to SDS-PAGE under reducing conditions, and the separated proteins were transferred to Immobilon P transfer membranes, which were then incubated overnight at 4°C with the rabbit monoclonal anti-p-ERK (1:1000) and rabbit polyclonal anti-ERK antibodies (1:1000). The blot was visualized by enhanced chemiluminescence.

Cell migration and invasion assays. Cell migration was assessed using modified Boyden chambers with uncoated inserts (8-μm pores), as previously described.[23] After 6 h, cells were stained using the Diff-Quick staining kit, and counted in five high-power fields (20× objective). Cell invasion assays were carried out using modified Boyden chambers in which the inner surfaces of the inserts were coated with Matrigel. All assays were carried out in triplicate.

Three-dimensional culture. Recently, 3D culture systems have been designed to mimic *in vivo* environments, and several materials have been developed.[24–28] Nanoculture plates, which have a specific microsquare pattern on the bottom, allow the formation of spheroids for PDAC cells,[24] and permit the staining and observation of the cells without removing them from the plates. Therefore, we analyzed expression of FGFR-2 using these 3-D culture plates. PANC-1 cells were suspended in 0.1 mL NanoCulture medium containing 10% FBS, and plated on NanoCulture plates (1×10^4 cells/0.1 mL) for 3-D cell culture. After 72 h, the cells were fixed with 4% paraformaldehyde, and stained by immunofluorescence, as described above.

Heterotopic implantation of FGFR-2-shRNA-transfected PANC-1 cells. To assess the effect of reduced expression of FGFR-2 on *in vivo* tumorigenicity, 1×10^6 cells/animal ($n = 6$ per cell line) were s.c. injected into 6-week-old, male, athymic mice (BALB/cA Jcl-*nu/nu*; CLEA Japan, Tokyo, Japan). Tumor volume was calculated using the formula: volume = $a \times b^2 \times 0.5$, where a is the longest diameter and b is the shortest.[29] The animals were monitored for 5 weeks. The experimental protocol was approved by the Animal Ethics Committee of Nippon Medical School.

Statistical analysis. Results are shown as mean ± SE, and the data between different groups were compared using the Student's *t*-test. The χ^2 and Fisher's exact tests were used to analyze the correlation between FGFR-2 expression and clinicopathological features. Cumulative survival rates were calculated using the Kaplan–Meier method, and the significance of differences in survival rate was analyzed by the log–rank test. The data between multiple groups were compared using one-way ANOVA. $P < 0.05$ was considered significant in all analyses. Computations were carried out using the StatView J version 5.0 software package (SAS Institute, Cary, NC, USA).

Results

Immunohistochemical analysis of FGFR-2 in PDAC tissues. To investigate FGFR-2 expression in PDAC, immunohistochemical analysis of PDAC tissue samples was carried out. There was strong FGFR-2 immunoreactivity in the cancer cells of 25/48 (52.1%) PDAC samples (Fig. 1a, Table 1). Moderate FGFR-2 immunoreactivity was also evident in the fibroblasts adjacent to the cancer cells (Fig. 1a, arrows). In normal pancreatic tissues, FGFR-2 weakly localized in islet cells, as well as in endothelial cells and a few smooth muscle cells of large-sized vessels (Fig. 2). Clinicopathologically, FGFR-2 expression in the cancer cells correlated with advanced stage cancer (Table 1). The overall 2-year survival rate for all 48 cases of PDAC was 16.2%, and the overall survival rates of the FGFR-2-high group and FGFR-2-low group were not statistically significant ($P = 0.46$; Fig. 1b).

Gene amplification of *FGFR2* in PDAC cell lines. By FISH analysis, it was determined that *FGFR2* is amplified in 3/7

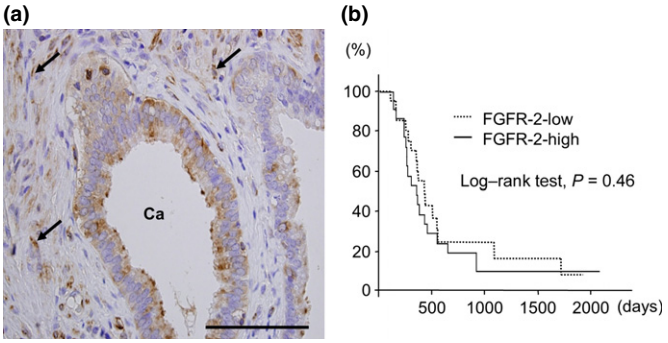

Fig. 1. Patients with low fibroblast growth factor receptor (FGFR)-2 expression in pancreatic ductal adenocarcinoma (PDAC) may survive longer than those with high expression. (a) A representative immunohistological section is shown, stained for FGFR-2 with counterstaining. FGFR-2 staining is strong in the cell membrane and cytoplasm of PDAC cells (Ca), as well as in adjacent stromal fibroblasts (arrows). Bar = 100 μm. (b) Survival curve of PDAC patients, stratified by FGFR-2 expression level. The survival rates of the FGFR-2-high group and FGFR-2-low group were not statistically significantly different ($P = 0.46$).

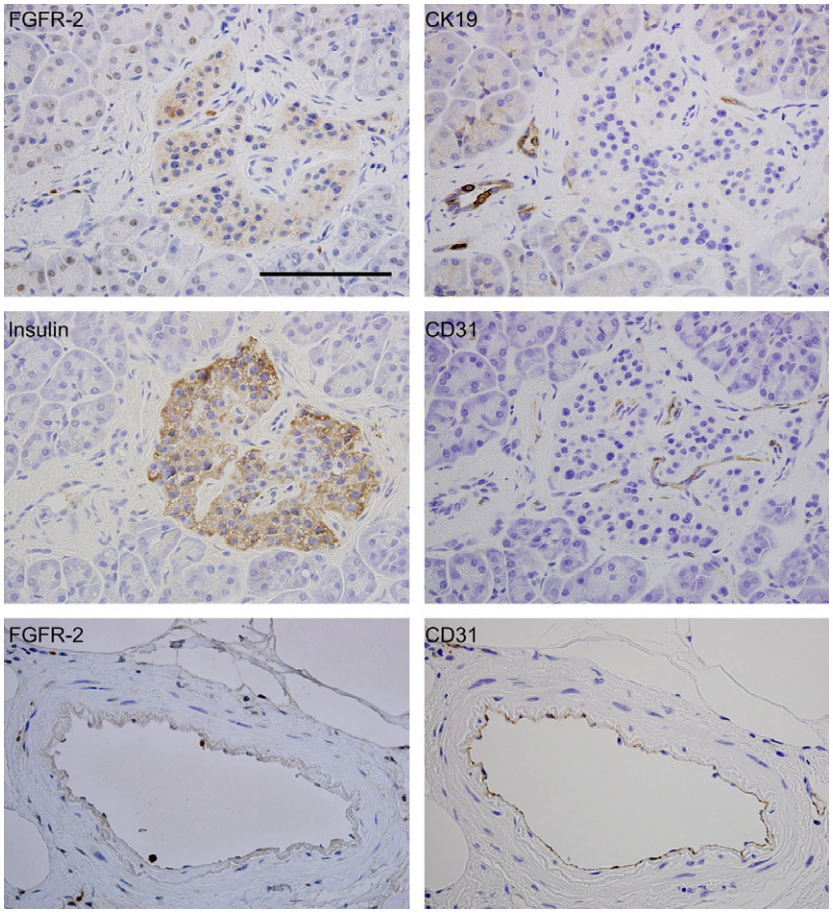

Fig. 2. Fibroblast growth factor receptor (FGFR)-2 is weakly immunoreactive in normal pancreatic tissues. Representative immunohistochemical sections of normal pancreatic tissue are shown, stained with antibodies against FGFR-2, CK19, insulin, and CD31. FGFR-2 immunoreactivity was weakly detected in the islet cells, vascular endothelial cells, and vascular smooth muscle cells in peri-pancreatic tissues. CK19 is expressed in ductal cells, insulin in islet cells, and CD31 in the endothelial cells. Bar = 100 μm.

PDAC cell lines, AsPC-1, PANC-1, and PK-45H (Fig. 3). In contrast, immortalized human pancreatic duct epithelial cell lines, HPDE4 and HPDE6, did not have this gene amplification. PANC-1 cells were used in the subsequent studies because of their common usage, and *FGFR2* amplification.

Inhibition of *FGFR-2* expression on PDAC. To examine the role of FGFR-2 in PDAC cells, we prepared FGFR-2 shRNA, which inhibits both FGFR-2 IIIb and IIIc isoforms, and transfected PDAC cells with the construct (Sh-16 and Sh-21) or with a scramble sequence to create "sham" cells (Sc-4 and Sc-5) as controls. FGFR-2 mRNA levels were lower in FGFR-2-shRNA-transfected clones than in sham cells (Fig. 4a). As analyzed by flow cytometry using the anti-FGFR-2 antibody, the expression levels of FGFR-2 on the cell membrane were lower in the FGFR-2-shRNA-transfected cells than in sham cells (Fig. 4b). Immunofluorescence analysis also showed decreased expression of FGFR-2 protein in FGFR-2-shRNA-transfected cells (Fig. 4c).

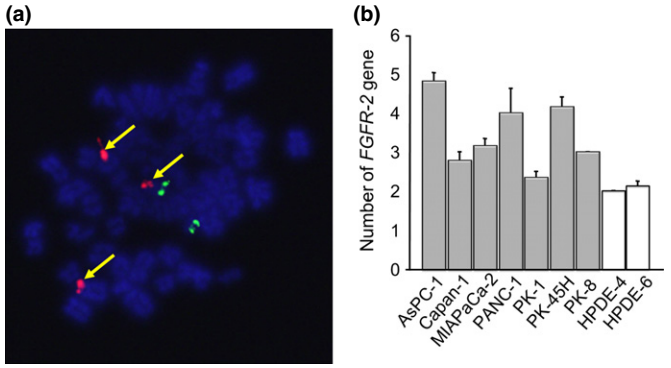

Fig. 3. The *FGFR-2* gene is amplified in pancreatic ductal adenocarcinoma cells. (a) FISH results in PANC-1 cells, showing *FGFR-2* gene amplification. Red, *FGFR-2* gene; green, centromere of chromosome 10. (b) Bar graph showing *FGFR-2* gene copy number in immortalized human pancreatic duct epithelial cell lines HPDE4 and HPDE6.

Effects of FGFR-2 on cell proliferation and cell signaling. Cell proliferation was lower in FGFR-2-shRNA-transfected PANC-1 cells than in sham cells (Fig. 5a). As mitogenic signaling through FGFRs often involves activation of the MAPK pathway,[4,16] the activation of ERK was investigated in FGFR-2-shRNA-transfected cells. FGF-2 caused a rapid increase in p-ERK levels in both wild cells and sham transfected PANC-1 cells. However, the phosphorylation levels of ERK were markedly lower at 20, 30, and 60 min following FGF-2 addition in FGFR-2-shRNA-transfected PANC-1 cells (Fig. 5b).

Effects of FGFR-2 on cell migration and invasion. Next, cell migration was examined using modified Boyden chamber assays. The FGFR-2-shRNA-transfected PANC-1 cells migrated more slowly than the sham cells in the modified Boyden chamber assay, and the number of migrating

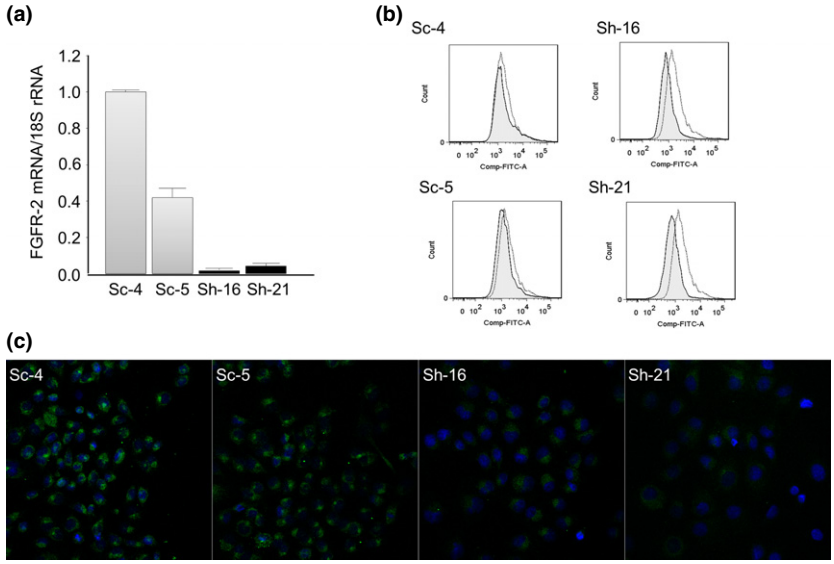

Fig. 4. Fibroblast growth factor receptor (FGFR)-2 expression is reduced in FGFR-2-shRNA stably transfected PANC-1 pancreatic ductal adenocarcinoma cells. (a) Quantitative RT-PCR analysis of FGFR-2 mRNA levels, showing lower expression in FGFR-2 shRNA-transfected clones (Sh-16 and Sh-21) than in sham cells (Sc-4 and Sc-5). (b) Flow cytometry analysis using allophycocyanin-labeled anti-FGFR-2 antibodies, showing decreased protein levels on the cell membrane of shRNA-transfected PANC-1 cells as compared to those on sham cells (dotted line, wild-type PANC-1 cells). (c) By immunofluorescence analysis, FGFR-2 protein (green) levels were lower in FGFR-2-shRNA-transfected cells than in the control cells. Blue, nuclei. Bar = 30 μm.

FGFR-2-shRNA-transfected PANC-1 cells was lower than the number of migrating sham cells (Fig. 6a; $P < 0.01$). Moreover, the number of invading FGFR-2-shRNA-transfected cells was statistically lower by comparison with the sham cells (Fig. 6b; $P < 0.01$).

Fibroblast growth factor receptor-2 expression in 3-D culture of PDAC cells. A 3-D culture system was used to assess the expression of FGFR-2 in the invasive front. Recent studies have shown that cells in 3-D culture have more similar characteristics to the *in vivo* environment, as compared with cells in 2-D culture.[30] PANC-1 cells formed irregular and island-shaped spheroids, and FGFR-2 localized to the surface of these spheroids, with its highest expression at their peripheral regions (Fig. 6c, arrows). At the central portion of the spheroids, FGFR-2 weakly localized, and actin (red) or nuclei (blue) were observed on their cell surface. These results suggest that the receptor contributes to the migration and invasion of these cells.

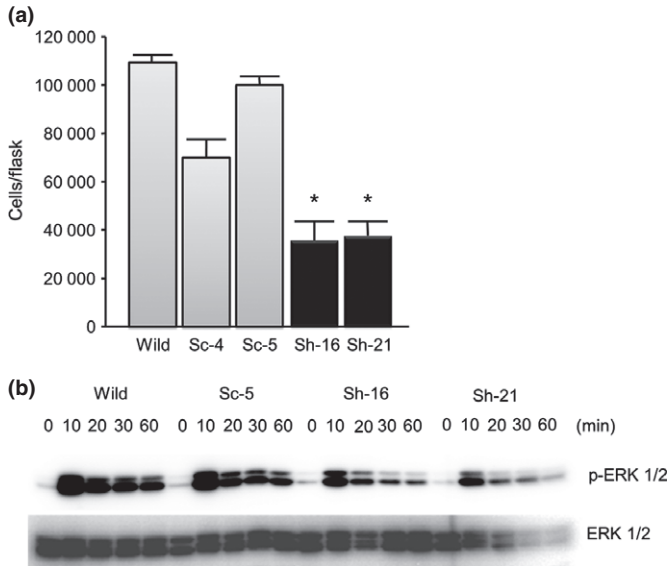

Fig. 5. Reduced fibroblast growth factor receptor (FGFR)-2 expression decreases pancreatic ductal adenocarcinoma cell proliferation *in vitro* and blocks ERK signaling. (a) Bar graph showing results from a cell proliferation assay. Cell proliferation was lower in FGFR-2-shRNA-transfected PANC-1 cells than in control cells (*$P < 0.01$ *vs* wild, Sc-4, and Sc-5 cells). (b) Immunoblot analysis of p-ERK 1/2 in PANC-1 cells. Phosphorylation of ERK was lower in FGFR-2-shRNA-transfected PANC-1 cells (Sh-16 and Sh-21) than in control cells (Wild and Sc-5) after FGF-2 treatment.

Expression levels of VEGF-A in FGFR-2-shRNA-transfected PANC-1 cells. We previously showed that expression of VEGF-A was regulated by FGFR-2 IIIb expression.[3] Therefore, to examine the effect of FGFR-2 shRNA on this function of FGFR-2 IIIb in PDAC cells, VEGF-A expression levels were examined in FGFR-2-shRNA-transfected cells. Immunofluorescence analysis showed that VEGF-A expression levels were lower in FGFR-2-shRNA stably transfected PANC-1 cells than in the sham cells (Fig. S1; $P < 0.01$).

Effects of FGFR-2 on s.c. tumor formation in nude mice. To determine whether FGFR-2 modulated the *in vivo* proliferation of PDAC cells, FGFR-2-shRNA-transfected PANC-1 cells or sham cells were s.c. injected into nude mice. The FGFR-2-shRNA-transfected cells formed significantly smaller tumors than two different sham cell lines in nude mice ($P < 0.05$; Fig. 7a), but there were no characteristic histological changes in the cells (Fig. 7b).

Discussion

Our previous studies in PDAC have shown that the expression level of FGFR-2 IIIb correlates with venous invasion and VEGF-A expression,[3] and FGFR-2 IIIc correlates with faster development of liver metastasis after surgery.[4] Here, we found that the expression level of FGFR-2 in PDAC was

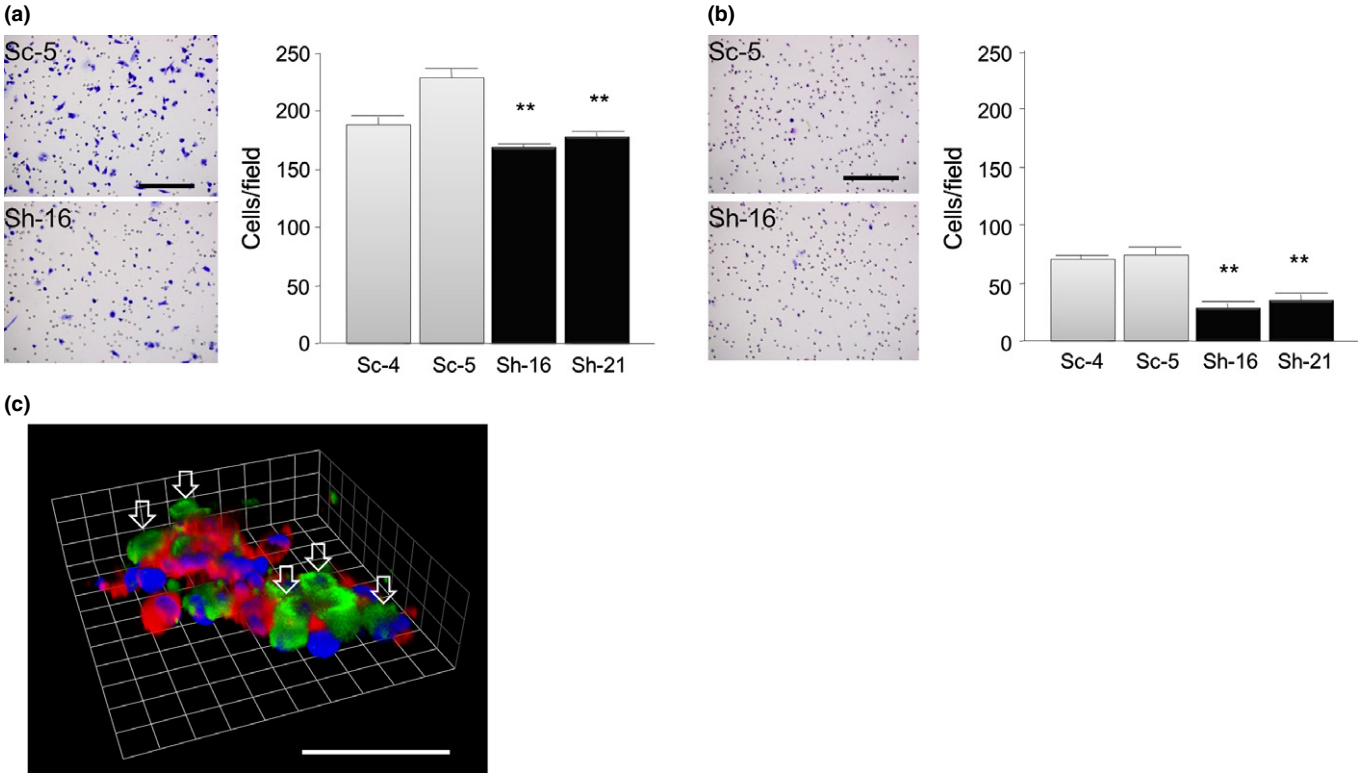

Fig. 6. Fibroblast growth factor receptor (FGFR)-2 shRNA reduces cell migration and invasion of PANC-1 pancreatic ductal adenocarcinoma cells, and FGFR-2 is expressed on the invasive front in 3-D culture. Cell migration (a) and invasion (b) were significantly inhibited in FGFR-2-shRNA stably transfected cells as compared with sham cells (**$P < 0.01$ *vs* Sc-4 and Sc-5). Stained, migrated cells in the bottom chamber are shown on the left, with the quantified cells/field represented in bar graph form on the right. Bar = 200 μm. (c) Immunofluorescence analysis shows FGFR-2 expression at the cell surface of spheroids, with greatest intensity at the peripheral lesions of the colonies in 3-D culture (arrows). Bar = 100 μm.

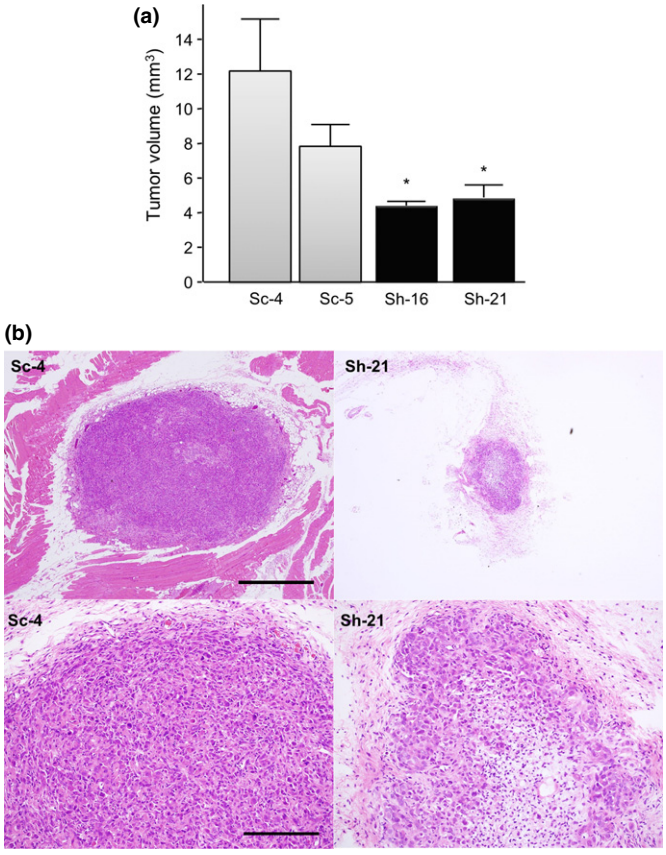

Fig. 7. Fibroblast growth factor receptor (FGFR)-2-shRNA stably transfected PANC-1 pancreatic ductal adenocarcinoma cells formed smaller s.c. tumors than those in sham cells in nude mice. (a) Graphical representation of tumor volume in nude mice derived from PANC-1 cells. (*$P < 0.05$ *vs* Sc4 and Sc-5). (b) Characteristic s.c. tumors in nude mice. Stained with H&E. Bar, upper panel = 1 mm; lower panel = 100 μm.

correlated with advanced stage cancer. These findings suggest that FGFR-2 and its isoforms clinically contribute to the aggressiveness in PDAC.

Gene amplification of *FGFR2* has been reported in gastric and breast cancers,[22,31,32] and 43% of PDAC cell lines in this study showed *FGFR2* amplification, whereas immortalized human pancreatic duct epithelial cells do not have this amplification. Mechanisms of FGFR-2 expression have not been well clarified, however, gene amplification may partly play a role in PDAC. To further understand the role of FGFR-2 in PDACs, the effects of other regulatory mechanisms of FGFR-2, including transcription factors, epigenetic regulation, or single nucleotide polymorphisms in intron 2, will require investigation.

A shRNA targeting FGFR-2 effectively inhibited FGFR-2 mRNA and surface protein expression on PDAC cells, which possess gene amplification of *FGFR2*. Decreased *in vitro* cell proliferation, migration, and invasion in FGFR-2-shRNA cells were similar to the results of FGFR-2 IIIc inhibition, whereas inhibition of VEGF-A was correlated with the function of FGFR-2 IIIb in PDAC. Furthermore, the marked decrease of tumor formation by the FGFR-2 shRNA cells suggests that FGFR-2 is a novel therapeutic target for PDAC. To clarify the roles of FGFR-2 in pancreatic cancer cases, clinical studies with a greater number of pancreatic cancer patients are needed because FGFR-2 expression levels and survival rates were not correlated in this experiment.

Several FGFR inhibitors, as well as selective inhibition with mAbs against FGFRs, have also been reported as new therapeutic approaches, and have been evaluated in clinical trials. AZD4547, a selective inhibitor of FGFR-1, -2, and -3 was developed, and is under clinical investigation for the treatment of FGFR-dependent tumors.[33] Monoclonal antibodies against FGFR-2 IIIb or IIIc isoforms successfully inhibited the proliferation of gastric tumor xenografts.[34] However, there has been no report on FGFR-2 targeting therapy for PDAC. This study is the first to show the possible effectiveness of FGFR-2 targeting therapy in PDAC; therefore, further translational research will be needed.

In summary, *FGFR2* gene amplification was observed in some of the PDAC cell lines investigated in this study, and the inhibition of FGFR-2 effectively reduced *in vitro* proliferation, migration, and invasion, as well as proliferation *in vivo*. These findings suggest that FGFR-2 targeting therapy is effective for at least some PDAC cases.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Immunohistological section obtained from a patient with invasive pancreatic ductal adenocarcinoma, stained for fibroblast growth factor receptor-2 with specific blocking peptide.

Fig. S2. Vascular endothelial growth factor-A (VEGF-A) expression in PANC-1 pancreatic ductal adenocarcinoma cells.

Table S1. Engraftment ratio of fibroblast growth factor receptor (FGFR)-2-shRNA transfected PANC-1 pancreatic ductal adenocarcinoma cells and sham cells.