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

Yoko Matsuda,1,2,4 Hisashi Yoshimura,1,4 Taeko Suzuki,1 Eiji Uchida,2 Zenya Naito1 and Toshiyuki Ishiwata1

1Departments of Pathology and Integrative Oncological Pathology, Nippon Medical School, Tokyo; 2Department of Pathology, Tokyo Metropolitan Geriatric Hospital, Tokyo; 3Surgery for Organ and Biological Regulation, Graduate School of Medicine, Nippon Medical School, Tokyo, Japan

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

Correspondence
Toshiyuki Ishiwata, Departments of Pathology and Integrative Oncological Pathology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan. Tel.: +81-3-3822-2131 ext. 5232; Fax: +81-3-3685-3067; E-mail: ishiwata@mns.ac.jp
4These authors contributed equally to this study.

Funding information
Japan Society for the Promotion of Science (25462127), Pancreas Research Foundation of Japan, Japan Society for the Promotion of Science (25-11084).

Received February 3, 2014; Revised June 9, 2014; Accepted June 19, 2014

Cancer Sci 105 (2014) 1212–1219
doi: 10.1111/cas.12470

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive human malignancy with an overall 5-year survival rate of 6%. 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 FGFR receptors (FGFR).1,2,4

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 breast11,12 and endometrial cancers.13 Amplification and overexpression of FGFR2 are 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, FGF3, 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.20 Furthermore, FGFR-2 IIIc promotes the proliferation and migration of PDAC cells, and confers cancer stem cell-like features onto these cells.21 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 Peroxi-dase conjugated streptavidin were from Nichirei (Tokyo, Japan).
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); the goat polyclonal anti-human FGFR-2 (N-20), rabbit polyclonal anti-insulin). For the evaluation of positivity, the following antibodies (1:400 anti-FGFR-2; 1:50 anti-CD31; 1:100 anti-ERK1, K-23) antibodies were used: 0, no staining; 1, mild staining; 2, moderate staining; and 3, intense staining. Thus, the positively stained cells were classified into the following categories: low (1–10%), mild staining; 1:100 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).

Fluorescence in situ hybridization analysis. The FISH analysis was carried out as previously described. The probes were generated from appropriate clones from a library of human genomic clones (GSP Laboratories, Kawasaki, Japan). Ten microliters of probe was heated for 5 min at 73–75°C. The slides were then sealed with rubber cement, 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.)

Construction of expression vector for FGFR-2-shRNA. Expression vectors for human FGFR-2-shRNA were constructed as previously described. The sense target sequence for FGFR-2 was synthesized and inserted into the pBAsi-hU6 Neo DNA vector.

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

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

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

Expression vectors for human FGFR-2-shRNA were constructed as previously described. The sense target sequence for FGFR-2 was synthesized and inserted into the pBasi-hU6 Neo DNA vector. 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.
(5 × 10^5 per 25 µL) were incubated with 1 µg aliphococyanin-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 FACS Aria 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 Insiteck, Kanagawa, Japan), and analyzed using control software EZ-C1 (Nikon Insiteck). 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, non-radioactive 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. 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. Nanoculture plates, which have a specific microsquare pattern on the bottom, allow the formation of spheroids for PDAC cells, 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 × b^2 × 0.5, where a is the longest diameter and b is the shortest. 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
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).

**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...
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.

**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, 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 isoforms inhibition, whereas inhibition of VEGF-A was correlated with the function of FGFR-2 IIib in PDAC. Furthermore, the marked decrease of FGFR-2 IIIb or IIIc isoforms successfully inhibited the proliferation of gastric tumor xenografts. 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.

Acknowledgments

We express our appreciation to Dr. Murray Korec (Departments of Medicine and Biochemistry and Molecular Biology, Indiana University School of Medicine, and the Melvin and Bren Simon Cancer Center, Indianapolis, IN, USA) for helpful discussions, and Ms. Yoko Kawamoto and Ms. Kiyoko Kawahara (Department of Pathology, Nippon Medical School) for their excellent technical assistance. We also thank Dr. Ryuji Ohashi (Division of Surgical Pathology, Nippon Medical School Hospital) for preparing tissue blocks.

Disclosure Statement

The authors have no conflict of interest.

References

1 Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin 2012; 62(1): 10–29.
2 Kommann M, Becker HG, Korec M. Role of fibroblast growth factors and their receptors in pancreatic cancer and chronic pancreatitis. Pancreas 1998; 17(2): 169–75.
3 Cho K, Ishiwata T, Uchida E et al. Enhanced expression of keratinocyte growth factor and its receptor correlates with venous invasion in pancreatic cancer. Am J Pathol 2007; 170: 1964–74.
4 Ishiwata T, Matsuda Y, Yamamoto T, Uchida E, Korc M, Naito Z. Enhanced expression of fibroblast growth factor receptor 2 IIic promotes human pancreatic cancer cell proliferation. Am J Pathol 2012; 180: 1928–41.
5 Itoh N, Ornitz DM. Evolution of the Fgfr and Fgf gene families. Trends Genet 2004; 20: 563–9.
6 Potthoff MJ, Kliwer SA, Mangelsdorf DJ. Endocrine fibroblast growth factors 15/19 and 21: from feast to famine. Genes Dev 2012; 26: 312–24.
7 Hynes NE, Dey JH. Potential for targeting the fibroblast growth factor receptor in breast cancer. Cancer Res 2010; 70: 5199–202.
8 Katoh M. FGFR2 abnormalities underlie a spectrum of bone, skin, and cancer pathologies. J Invest Dermatol 2009; 129: 1861–7.
9 Katoh Y, Katoh M. FGFR2-related pathogenesis and FGFR2-targeted therapeutics (Review). Int J Mol Med 2009; 23: 307–11.
10 Katoh M. Cancer genomics and genetics of FGFR2 (Review). Int J Oncol 2009; 33(2): 333–4.
11 Hunter DJ, Kraft P, Jacobs KB et al. A genome-wide association study identifies alleles in FGFR2 associated with risk of sporadic postmenopausal breast cancer. Nat Genet 2007; 39: 870–4.
12 Meyer KB, Mata AT, O’Reilly M et al. Allele-specific up-regulation of FGFR2 increases susceptibility to breast cancer. PLoS Biol 2008; 6: e108.
13 Pollock PM, Gartsdie MG, Dejeza LC et al. Frequent activating FGFR2 mutations in endometrial carcinomas parallel germline mutations associated with craniostenositis and skeletal dysplasia syndromes. Oncogene 2007; 26: 7158–62.
14 Hatton Y, Itoh H, Uchino S et al. Immunohistochemical detection of K-sam protein in stomach cancer. Clin Cancer Res 1996; 2: 1373–81.
15 Esvarakumar VP, Lax I, Schlessinger J. Cellular signaling by fibroblast growth factor receptors. Cytokine Growth Factor Rev 2005; 16: 139–49.
16 Mohammadi M, Olsen SK, Ibrahimi OA. Structural basis for fibroblast growth factor receptor activation. Cytokine Growth Factor Rev 2005; 16: 107–37.
17 Yamazaki Y, Friess H, Buchler M et al. Overexpression of acidic and basic fibroblast growth factors in human pancreatic cancer correlates with advanced tumor stage. Cancer Res 1993; 53: 5289–96.
18 Siddiqui I, Funatomi H, Kobrin MS, Friess H, Buchler MW, Korec M. Increased expression of keratinocyte growth factor in human pancreatic cancer. Biochem Biophys Res Commun 1995; 215(1): 309–15.
19 Ishiwata T, Friess H, Buchler MW, Lopez ME, Korec M. Characterization of keratinocyte growth factor and receptor expression in human pancreatic cancer. Am J Pathol 1998; 153(1): 213–22.
20 Furukawa T, Duguid WP, Rosenberg L, Viallet J, Galloway DA, Tsao MS. Long-term culture and immortalization of epithelial cells from normal adult human pancreatic ducts transfected by the E6E7 gene of human papilloma virus 16. Am J Pathol 1996; 148: 1763–70.
21 Matsuda Y, Ishiwata T, Yamahatsu K et al. Overexpressed fibroblast growth factor receptor 2 in the invasive front of colorectal cancer: a potential therapeutic target in colorectal cancer. Cancer Let 2011; 309(2): 209–19.
22 Matsumoto K, Arai T, Hamaguchi T et al. FGFR2 gene amplification and clinicopathological features in gastric cancer. Br J Cancer 2012; 106: 727–32.
23 Matsuda Y, Naito Z, Kawahara K, Nakazawa N, Korc M, Ishiwata T. Nestin is a novel target for suppressing pancreatic cancer cell migration, invasion and metastasis. Cancer Biol Ther 2011; 11: 512–23.
24 Matsuda Y, Ishiwata T, Kawamoto Y et al. Morphological and cytoskeletal changes of pancreatic cancer cells in three-dimensional spheroidal culture. Med Mol Morphol 2010; 43: 211–7.
25 Sakai T, Larsen M, Yamada KM. Fractonectin requirement in branching morphogenesis. Nature 2003; 423: 876–81.
26 Padron JM, van der Wilt CL, Smid K et al. The multilayered postconfluent cell culture as a model for drug screening. Crit Rev Oncol Hematol 2000; 36 (2–3): 141–57.
27 Yamada M, Moritoh C, Kawaguchi M, Okigaki T. Growth, morphology, function, and morphogenetic properties of rat renal glomerular epithelial cells in vitro: effects of retinyl acetate. Eur J Cell Biol 1989; 49(2): 252–8.
28 Smalley KS, Lioni M, Herlyn M. Life isn’t flat: taking cancer biology to the next dimension. In Vitro Cell Dev Biol Anim 2006; 42: 242–7.
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.