Identification of a Fibroblast Growth Factor Receptor 1 Splice Variant That Inhibits Pancreatic Cancer Cell Growth

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Abstract

Fibroblast growth factor receptors (FGFR) play important roles in many biological processes. Nothing is presently known about possible roles of the human FGFR1-IIIb mRNA splice variant. In this study, we characterized for the first time the effects of FGFR1-IIIb expression on the transformed phenotype of human pancreatic cancer cells. The full-length FGFR1-IIIb cDNA was generated and stably expressed in PANC-1 and Mia PaCa-2 pancreatic cancer and TAKA-1 pancreatic ductal cells. FGFR1-IIIb–expressing cells synthesized a glycosylated 110-kDa protein enhancing tyrosine phosphorylation of FGFR substrate-2 on FGF-1 stimulation. The basal anchorage-dependent and anchorage-independent cell growth was significantly inhibited. These effects were associated with a marked reduction of p44/42 mitogen-activated protein kinase (MAPK) phosphorylation in combination with enhanced activity of p38 MAPK and c-Jun NH2-terminal kinase. FGFR1-IIIb expression inhibited single-cell movement and in vitro invasion as determined by time-lapse microscopy and Boyden chamber assay as well as in vivo tumor formation and growth in nude mice. Microscopic analysis of the xenograft tumors revealed a reduced Ki-67 labeling and a lower amount of tumor necrosis in FGFR1-IIIb–expressing tumors. Our results show that FGFR1-IIIb is a functional FGFR that inhibits the transformed phenotype of human pancreatic cancer cells. [Cancer Res 2007;67(6):2712–9]

Introduction

Fibroblast growth factors (FGF) comprise a family of heparin-binding polypeptides that are involved in many biological processes, including pattern formation, cellular differentiation, mitogenesis, and angiogenesis (1-4). Signaling is mediated by a dual receptor system consisting of high-affinity transmembrane FGF receptors (FGFR) that are encoded by four distinct genes and low-affinity heparan sulfate proteoglycans that enhance ligand presentation (4, 5). The extracellular part of FGFRs is usually composed of three immunoglobulin (lg)-like domains (I–III) followed by a hydrophobic transmembrane region and a split tyrosine kinase catalytic domain (4). A special feature of FGFRs is the existence of several receptor isoforms that are generated by alternative mRNA splicing and result in different ligand-binding specificities (5, 6). Analysis of the crystal structure of FGFRs and FGFs showed that splicing of the second half of domain III strongly dictates the interaction of FGF ligands and FGFRs (7). Three possible variants of domain III are known for FGFR1, FGFR2, and FGFR3 (4). The III variant yields a secreted receptor devoid of signaling capacity. Variant IIIb is generally expressed in cell types of epithelial origin, whereas expression of IIIc is restricted to mesenchymal cell types, especially in case of FGFR2 and FGFR3 (8).

Changes in FGFR isoform expression seem to regulate tumorigenesis and malignant transformation (2, 3, 9). Thus, exon switching from the IIIb variant of FGFR2 (KGF) to the IIIc variant of FGFR2 was accompanied with malignant progression in prostate cancer, down-regulation of FGFR2-IIIb with malignant transformation in keratinocytes, whereas reexpression of FGFR2-IIIb resulted in growth inhibition and induction of differentiation in prostate, bladder, and human salivary gland carcinoma cells (9-15). Expression of FGFR1-IIIc in normalgancancer cells resulted in cellular transformation and in vivo tumor formation displaying morphologic features comparable with human pancreatic cancer (16), whereas inhibition of FGFR1-IIIc using a dominant-negative approach resulted in a reversion of the malignant phenotype in pancreatic cancer cells (17).

However, the possible functions of human FGFR1-IIIb are not known because the exact boundaries of the exon encoding the IIIb sequence of the human Fgfr1 gene were unknown. We previously PCR cloned and sequenced this region of the human Fgfr1 gene, revealing that it is composed of 153 nucleotides and encodes a stretch of 50 amino acids only sharing 45% amino acid homology with the human 147-nucleotide IIIc fragment (16). The aim of the present study was to determine possible functions of human FGFR1-IIIb and to characterize its effects on pancreatic cancer cell transformation.

Materials and Methods

Materials.

p38 mitogen-activated protein kinase (MAPK) and stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) assay kits were from Cell Signaling Technology (Danvers, MA); human FGF-1 was from R&D Systems (Minneapolis, MN); FGFR antibody, pan-extracellular signal-regulated kinase-2 (ERK-2), phosphorylated (phospho)-specific p44/42 MAPK, and phospho-specific tyrosine P199 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); FGFR substrate-2 (FRS2) antibody was from Upstate Biotechnology (Billerica, MA).

Construction of expression vectors.

Four overlapping cDNA fragments (FRI-A, FRI-B, FRI-C, and FRI-D) encompassing the entire FGFR1 coding region were generated from placental cDNA by PCR amplification, except for FRI-A, which was a XbaI/EcoRI fragment from pCD115 (nucleotides 44-673, X52833) as described (pSVK4/FGFR1-IIIc; ref. 18). FGFR1-IIIb was prepared by substituting FR1B (sequence including the IIIc coding region)
with FR1BIIIb. This resulted in a modified pSVK3 expression vector (18) carrying the full-length EcoRI/XhoI 2.55-kb human FGFR1-IIIb cDNA under the control of the SV40 early promoter (pSVK4/FGFR1-IIIb).

**Cell culture and expression of FGFR1.** PANC-1 and MiaPaCa-2 human pancreatic cancer cells (17) were grown in DMEM, and TAKA-1 spontaneously immortalized Syrian hamster pancreatic ductal cells (16) were grown in DMEM/F12 supplemented with 10% fetal bovine serum. Cell clones carrying FGFR1-IIIb or FGFR1-IIIc were cotransfected (26:1) in a stable manner with pSVK4/FGFR1-IIIb or pSVK4/FGFR1-IIIc and pBSVneo carrying the G418 resistance gene using LipofectAMINE as described (16). Medium of cells carrying a neomycin resistance gene was supplemented with 0.6, 0.8, and 1.2 mg/mL of G418 for TAKA-1, PANC-1, and MiaPaCa-2 cells, respectively.

**Immunoblot and immunoprecipitation analysis.** Immunoblot and immunoprecipitation assays were described previously (16). To analyze N-linked protein glycosylation, cells were incubated in the presence or absence of tunicamycin (5 μg/mL). Whenever indicated, membranes were stripped and reblotted with the indicated antibodies to confirm equal loading (16).

**Detection of active kinases.** To determine basal phosphorylation, immunoblot analysis of exponentially growing cells was carried out using a cocktail of phospho-specific antibodies (Cell Signaling Technology). p44/42 MAPK phosphorylation was determined using a phospho-specific antibody for p44/42 MAPK. Equal loading was confirmed by reblotting with pan-ERK-2 (16).

To determine basal p98 MAPK activity, a p38 MAPK assay kit was used according to the protocol of the manufacturer. In brief, total cell lysates (200 μg in 200 μL of lysis buffer) were incubated for 20 h at 4°C with a resuspended immobilized phospho-p38 MAPK monoclonal antibody (20 μl). After washing twice each with ice-cold lysis buffer and kinase buffer, immunocomplexes were captured by centrifugation and resuspended in 50 μL of kinase buffer supplemented with 200 μmol/L ATP and 2 μg of activating transcription factor-2 (ATF-2) fusion protein as substrate. After incubation for 30 min at 30°C, the reaction mixture was subjected to immunoblot analysis. ATF-2 phosphorylation was detected using a phospho-ATF-2-specific antibody. FGFR1-IIIb–expressing TAKA-1 cells stimulated with FGF-1 (5 nmol/L) for 5 min were used as positive control (data not shown).

To determine basal SAPK/JNK activity, a JNK assay kit was used according to the protocol of the manufacturer. In brief, total cell lysates (250 μg in 250 μL of lysis buffer) were prepared as described above and incubated for 20 h at 4°C with resuspended c-Jun fusion protein beads (20 μL). After washing twice with 500 μL of ice-cold lysis buffer and kinase buffer, respectively, the pellet was resuspended in 50 μL of kinase buffer supplemented with 100 μmol/L ATP. After incubating for 30 min at 30°C, immunocomplexes were captured by centrifugation and subjected to immunoblot analysis using phospho-c-Jun–specific antibodies. FGFR1-IIIb–expressing TAKA-1 cells stimulated with FGF-1 (5 nmol/L) for 5 min were used as positive control (data not shown).

**Immunofluorescence microscopy.** Cells were grown on glass coverslips to 50% confluence, fixed in 4% paraformaldehyde/PBS for 10 min, and permeabilized with 0.2% Triton X-100/PBS for 10 min. After blocking in 1% bovine serum albumin for 30 min, cells were incubated with the FGFR1 antibody (1:1,000) for 1 h followed by biotin-conjugated anti-rabbit immunoglobulin (1:100) for 1 h and streptavidin–Alexa Fluor 568 (1:200) for 30 min. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Fluorescence and phase-contrast micrographs were taken with an Olympus IX81 microscope.

**Northern blot analysis.** Northern blot analysis of total RNA using the full-length FGFR1-IIIb cDNA probe was carried out as described (16).

**Anchorage-dependent and anchorage-independent growth assays.** Basal cell growth was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (16) and cell counting. To determine basal cell growth, cells (5,000 per well) were propagated for 48 h in complete medium before initiation of the MTT assay. To determine the effect of FGF-1, cells (10,000 per well) were propagated for 24 h in complete medium followed by 48 h in serum-free medium in the presence of heparin (1 μg/mL) and in the presence or absence of FGF-1 (5 nmol/L). For cell counting, PANC-1 cells (50,000 per well) were seeded in six-well plates and propagated for the indicated time in complete medium. Medium was changed daily until cell counting was done.

Basal anchorage-independent growth was assessed by a double-layer soft agar assay as described (16). After colony formation, 300 μg MTT/well was added to stain vital colonies before counting by microscopy.

![Figure 1](https://example.com/f1.png)

**Figure 1.** Characterization of FGFR1 expression and glycosylation in wild-type PANC-1 cells, control-transfected clones PN5 and PN6, and FGFR1-IIIb–expressing clones PF4 and PF40. A, top, FGFR1 immunoblot analysis; bottom, β-actin was used to document equivalent loading. B, FGFR1 glycosylation. PF4 cells were incubated in the absence (–) or presence (+) of 5 μg/mL tunicamycin for 24 h followed by FGFR1 immunoblot analysis. PN6 cells were used as negative control. C, top, Northern blot analysis of total RNA using the full-length FGFR1-IIIb cDNA probe; bottom, a 7S cDNA probe was used as loading control. D, FGFR1 immunofluorescence. Left, strong intensity was found in the perinuclear area in the cytoplasm of FGFR1-IIIb–expressing PF4 cells. Right, arrows, at higher magnification, a clear membranous FGFR1 signal was detectable. Nuclei were counterstained with DAPI. Original magnifications, >300 and >600.
Cell cycle analysis. Fluorescence-activated cell sorting analysis of exponentially growing cells was done using a CycleTest Plus kit according to the instructions of the manufacturer and FACSscan (Becton Dickinson, Franklin Lakes, NJ) analysis system (19).

Cell migration assay. The ability of cells to migrate through filters was measured using a Boyden chamber. Transwell polycarbonate filters (12-μm pore size) were used according to the protocol of the manufacturer. The bottom chamber included medium (1.5 mL) containing 10% FCS, whereas cells (1.0 × 10^5) suspended in 0.5 mL of medium containing 1% FCS were seeded into the upper chamber coated with fibronectin (5 μg/mL in PBS). After incubating the cells for 36 h at 37°C, they were stained for 20 min at room temperature using Cell Stain according to the protocol of the manufacturer. Nonmigrated cells on the upper surface of the filters were removed gently by a cotton-tip swab. Cells migrated to the bottom side of the filter were counted using a light microscope.

Single-cell movement assay. Cells (50,000 per well) were seeded onto fibronectin-coated (5 μg/mL in PBS) six-well plates and grown for 20 h. Cell movement was then monitored for 24 h by an Olympus IX81 motorized inverted microscope taking pictures every 10 min. The total distance of individual cells covered within 24 h was determined using the ImageJ 1.32 program (NIH, Bethesda, MD).

In vivo tumorigenicity assay. To assess the effect of the expression of FGFR1-IIIb on xenograft formation, 10^6 cells per site were injected s.c. into two sites of 4- to 6-week-old female athymic (nude) mice. Animals were monitored for tumor formation every 4 days. Tumor size was measured in three dimensions. Tumor volume was determined by the equation \( vol = l \times w \times d \times 0.5 \), where \( l \) is the length, \( w \) is the width, and \( d \) is the diameter. Animals had to be sacrificed 12 weeks after injection according to our animal protocol (#718) if neither tumor volume (>2 cm³) nor skin ulcerations prompted earlier termination.

Microscopic analysis of xenograft tumors. To assure a standardized analysis, explanted tumors were cut in half through the largest diameters of each tumor. Formalin-fixed and paraffin-embedded 5-μm sections were prepared from the central areas of all xenograft tumors followed by H&E staining or immunohistochemical analysis using a Vectorstain avidin-biotin complex method kit (16). Analysis of Ki-67 staining was carried out in a representative peripheral area of each tumor without necrosis using high-power field microscopy.

Statistical analysis. Results are expressed as mean ± SE or mean ± SD and median and range when indicated. The area of necrosis and Ki-67 labeling of the xenograft tumors are shown as box plots. The box defines the 75th and 25th percentile, and the horizontal line in the box defines the median. The whiskers depict the 5th and 95th percentile, and values in the outer field defined by the whiskers are individually shown as open circles. Student’s t test, Mann-Whitney U rank sum test, or \( \chi^2 \) test was used for statistical analysis (two sided) when indicated. \( P < 0.05 \) was taken as the level of significance.
Results

Expression of FGFR1-IIIb in pancreatic cancer cells. Specific RNase protection assays (16) as well as reverse transcription-PCR (RT-PCR) analysis (data not shown) revealed that cultured human pancreatic cancer cells express both FGFR1-IIIb and FGFR1-IIIc. However, FGFR1-IIIc was the predominantly expressed FGFR1-III variant in all tested human pancreatic cancer lines, including ASPC-1, BXPC-3, CAPAN-1, COLO-357, MIA PaCa-2, PANC-1, and T3M4 (data not shown). Following this initial screening, PANC-1 cells expressing relatively high endogenous FGFR1-IIIc and low FGFR1-IIIb were established overexpressing FGFR1-IIIb in a stable manner. Clones were randomly picked and screened for FGFR1 protein expression. In parental PANC-1 and control-transfected cells (sham), only a faint FGFR1 signal was detectable at 110 and 120 kDa. In contrast, besides the faint signal at 110 kDa, a marked FGFR1 signal was detectable at 120 kDa and between 130 and 150 kDa in several of the transfected clones (Fig. 1A). The diffuse character of the observed FGFR1 bands might have resulted from glycosylation of the FGFR1 protein. To address this possibility, FGFR1-IIIb–expressing PANC-1 cells were incubated in the presence of tunicamycin, an inhibitor of N-linked glycosylation. This treatment resulted in disappearance of the 120-kDa band and the 130- to 150-kDa bands, whereas the 110-kDa band corresponding to the size of the nonglycosylated receptor became more prominent (Fig. 1B). As expected, increased FGFR1 protein levels were associated with increased FGFR1 mRNA transcript levels (Fig. 1C). Immunofluorescence revealed moderate cytoplasmic and strong perinuclear FGFR1 immunoreactivity (Fig. 1D). Moderate membranous FGFR1 immunoreactivity was also detectable in FGFR1-IIIb–expressing cells at higher magnification (Fig. 1D).

FGFR1-IIIb inhibits pancreatic cancer cell growth in vitro. Expression of FGFR1-IIIb inhibited the basal cell growth. Compared with wild-type cells, the growth of FGFR1-IIIb–expressing clones PF4 and PF40 was reduced by 42.7% (±2.3% SE) and 36.0% (±3.1% SE), respectively (P < 0.05). In contrast, the growth of control clones PN5 and PN6 was not altered (−1.7 ± 0.7% SE and 2.0 ± 1.0% SE, respectively). The results of the MTT assay were confirmed by cell counting showing even greater effects after expanding the culture periods (Fig. 2A). Cell cycle analysis revealed that the altered growth abilities were associated with a decrease of cells in G0-G1 and S phase and an accumulation of cells in G2-M (Fig. 2B). The antiproliferative effects of FGFR1-IIIb were more pronounced when analyzing colony formation in soft agar (Fig. 2C).

To show that the observed alterations in pancreatic cancer cells were due to the expression of a functional transmembrane receptor, TAKA-1 cells were used as model. They do express several FGFs (FGF-1, FGF-2, and FGF-5) and FGFR2, FGFR3, and FGFR4 (20). In contrast to pancreatic cancer cells, they do, however, not express endogenous FGFR1. Accordingly, TAKA-1 clones carrying FGFR1-IIIb were established after transfection with FGFR1-IIIb cDNA. A FGFR1 signal was detectable at 110 and 120 kDa and between 130 and 150 kDa in FGFR1-IIIb–expressing TAKA-1 cells and was paralleled by increased FGFR1 mRNA transcript levels (data not shown). Control-transfected and FGFR1-IIIb–expressing TAKA-1 cells were incubated in the presence of FGF-1, known to be able to ubiquitously bind to FGFs followed by immunoprecipitation of FRS2. Whereas no alteration of FRS2 tyrosine phosphorylation was seen in control-transfected cells, FGF-1 enhanced tyrosine phosphorylation of FRS2, showing that FGFR1-IIIb expression is capable of activating intracellular signaling cascades following incubation with exogenous FGF ligands (Fig. 2D). FGF-1 (5 nmol/mL) in the presence of heparin (1 µg/mL) for 48 h also enhanced the growth of FGFR1-IIIb–expressing TF9 TAKA-1 cells in serum-free medium by 40.3% (±4.4% SE) compared with untreated controls in the MTT assay. The growth of the control-transfected TAKA-1 clone TN6 was not significantly altered (−8.3 ± 2.4% SE). Under similar conditions, FGF-1 enhanced the growth of control-transfected PANC-1 PN6 cells by 43.3% (±2.2% SE) and the growth of FGFR1-IIIb–expressing PANC-1 clones PF4 and PF40 by 54.3 (±12% SE) and 53.3 (±6.4% SE), respectively.

Although FGF-1 enhanced proliferation in serum-free medium, a subsequent analysis of the basal anchorage-dependent and
anchorage-independent growth of TAKA-1 clones confirmed the inhibitory functions of FGFR1-IIIb in these noncancerous pancreatic ductal cells. Compared with wild-type cells, the growth of FGFR1-IIIb–expressing TAKA-1 clones TF1 and TF9 was reduced by 32.2% (±0.9% SE) and 38.7% (±0.9% SE), respectively (P < 0.05). The growth of control clones TN5 and TN6 was not altered (−1.7 ± 0.9% SE and 1.0 ± 0.6% SE, respectively). Colony formation (25,000 cells per dish) of TAKA-1 cells was 106 (±3 SE), 108 (±5 SE), and 110 (±6 SE) colonies for wild-type, TN5, and TN6 cells, respectively. No colony formation was observed for clone TF9 and only 26 (±3 SE) colonies per dish were observed for TF1 cells (P < 0.05).

**FGFR1-IIIb expression alters MAPK activities.** To determine possible alterations in signaling cascades induced by FGFR1-IIIb expression, a phospho-protein screening assay for p90 RSK, AKT, p44/42 MAPK, and S6 kinase was done. FGFR1-IIIb expression inhibited p44/42 MAPK phosphorylation, whereas no effects were seen on AKT or S6 phosphorylation (Fig. 3A). No p90 RSK phosphorylation was detectable. The results were confirmed in PANC-1 clones and also in TAKA-1 clones using a phospho-specific p44/42 phosphorylation was detectable. The results were confirmed in PANC-1 clones (Fig. 3B). Activity assays of the related MAPKs p38 and JNK revealed that FGFR1-IIIb–expressing clones displayed a mild enhancement of p38 activity determined by the in vitro ability to phosphorylate ATF-2 (Fig. 3C) and a marked up-regulation of the activity of JNK determined by the in vitro ability to phosphorylate c-Jun (Fig. 3D).

**Effects of FGFR1-IIIb expression on cell motility and migration.** Single-cell movement and cell migration were characterized next to determine whether FGFR1-IIIb is also involved in other functions important for cellular transformation. Single-cell movement was monitored by time-lapse microscopy (Fig. 4A). The distances covered within 24 h were 860, 880, and 820 μm for wild-type PANC-1, TN5, and TN6 cells, respectively. In contrast, expression of FGFR1-IIIb resulted in a marked reduction of motility in PF4 and PF40 cells with covered distances of 410 and 510 μm, respectively (Fig. 4B).

A Boyden chamber assay was used to determine migration. FGFR1-IIIb expression resulted in an inhibition of the ability of the cells to move to the other side of the chamber within 36 h compared with wild-type and control-transfected PANC-1 cells (Fig. 4C and D).

**In vivo tumorigenicity.** In a next step, tumorigenicity of control clones PN5 and PN6 and of FGFR1-IIIb–expressing clones PF4 and PF40 was compared in a xenograft model. Thirteen of 16 and 16 of 24 sites injected with FGFR1-IIIb–expressing PF4 and PF40 cells, respectively, developed measurable tumors after 6 weeks. In contrast, almost all sites injected with control clones developed tumor nodules after 6 weeks, 8 of 8 injected with PN5 cells and 22 of 24 injected with PN6 cells. There was a decreased frequency of tumor formation in FGFR1-IIIb–expressing clones (P < 0.05). In addition, animals carrying tumors arising from control-transfected clones had to be sacrificed after 8 to 10 weeks due to tumor size and the occurrence of skin ulceration (Fig. 5A). Animals carrying FGFR1-IIIb–expressing tumors survived for at least 12 weeks (Fig. 5A) because the in vivo growth was less rapid compared with tumors of control-transfected cells (Fig. 5B).

**Morphology and immunohistochemical analysis of xenograft tumors.** All tumors were paraffin embedded. Section of the largest area of each tumor was used for the microscopic analysis. Xenograft tumors of control animals displayed a morphology comparable with liver metastases of solid tumors. They showed a central area of necrosis surrounded by areas of highly proliferating cells (Fig. 6A). In contrast, FGFR1-IIIb–expressing tumors showed a

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**Figure 4.** Effect of FGFR1-IIIb expression on single-cell movement and in vitro invasion of PANC-1 cells. A, single-cell movement was monitored by time-lapse microscopy taking pictures every 10 min for 24 h. Red, yellow, and blue dots, the positions of control-transfected PANC-1 cells and FGFR1-IIIb–expressing PF4 cells, respectively, at the beginning (0 h). Left, red, yellow, and blue lines, tracks of the cells covered within 24 h. B, the total distance (μm in 24 h) of individual cells (n = 50) covered within 24 h was evaluated using the ImageJ 1.32 and Simple Track program. Columns, mean distances (in μm) covered within 24 h for wild-type PANC-1, control-transfected PN5 and PN6, and FGFR1-IIIb–expressing PF4 and PF40 cells; bars, SD. *, P < 0.05, compared with wild-type PANC-1, PN5, and PN6. C, Boyden chamber assay. The number of cells that moved to the lower side of the chamber within 36 h was determined. Migrated control-transfected PN6 cells and FGFR1-IIIb–expressing PF4 cells at low and high magnification. D, columns, mean number of migrated cells within 36 h of three separate experiments; bars, SD. *, P < 0.05, compared with wild-type PANC-1, PN5, and PN6.
more homogenous pattern and exhibited only small necrotic areas interspersed within the cell mass (Fig. 6A). These observations were independent of the tumor size. The median relative area of necrosis was 33% and 20% for control and FGFR1-IIIb–expressing tumors in comparison with the corresponding total area, respectively (P < 0.003; Fig. 6B).

Analysis of the proliferative index of serial sections revealed that Ki-67 immunoreactivity was high in the peripheral regions of the control tumors. It was present in 30% to 40% of all cells in the total areas of these tumors (Fig. 6C). In contrast, perinuclear Ki-67 immunoreactivity was observed in <5% of the cells in FGFR1-IIIb–expressing tumors. The median number of Ki-67–positive cells per high-power field area in representative peripheral regions without necrosis was 460 and 101 in control and FGFR1-IIIb–expressing tumors, respectively (P < 0.03; Fig. 6D).

Expression of FGFR1-IIIb inhibits, whereas FGFR1-IIIc enhances, cell growth. Additional clones overexpressing either FGFR1-IIIb or FGFR1-IIIc were established to confirm the growth-inhibitory effects of FGFR1-IIIb. Although FGFR1-IIIc is the predominant FGFR1 expressed in PAN-1 cells, overexpression of FGFR1-IIIc (clones PFc8 and PFc13, respectively) resulted in enhanced basal cell growth of 53.7% (±2.3% SE) and 33.3% (±4.9% SE), respectively, compared with wild-type and control-transfected cells (P < 0.05). Overexpression of either FGFR1-IIIb or FGFR1-IIIc in PAN-1 cells did not alter the endogenous expression of the corresponding Ig domain III variant (data not shown). Basal cell growth of the already characterized TAKA-1 cells expressing FGFR1-IIIc (Tfc15 and Tfc16; ref. 16) was enhanced under the present conditions by 32.3% (±4.7% SE) and 28.7% (±7.7% SE), respectively (P < 0.05). The establishment of MIA PaCa-2 clones, predominantly expressing endogenous FGFR1-IIIc but also relatively high levels of FGFR1-IIIb (data not shown), revealed similar results. Basal cell growth of clones overexpressing IIIb was inhibited by 31.3 ± 5.2% SE (MF12), 21.7 ± 2.2% SE (MF22), and 27.3 ± 2.8% SE (MF39), respectively. In contrast, basal cell growth of clones overexpressing FGFR1-IIIc was enhanced by 30.7 ± 8.1% SE (MFc1), 31.3 ± 3.7% SE (MFc23), and 34.3 ± 7.3% SE (MFc24), respectively (P < 0.05).

Discussion

Alterations in receptor tyrosine kinase pathways are involved in the genesis of most cancers (21). Numerous studies showed that the tissue-specific expression of FGFs and FGFRs is a critical factor regulating FGFR signaling pathways and malignant transformation (2, 11, 16, 22-24). Investigations on the roles and actions of Ig domain III mRNA splice variants of human FGFR1 were thus far limited to IIIc (16). Ornitz et al. (25) used a synthesized murine FGFR1-IIIb cDNA to show the effects of FGFs on cell proliferation. Beer et al. (26) were able to clone a full-length cDNA encoding murine FGFR1-IIIb from a skin wound cDNA library. FGFR1-IIIb knockout experiments showed that FGFR1-IIIb is dispensable for skin development and wound repair in mice (27). Therefore, the current knowledge about FGFR1-IIIb actions is scarce and possible functions of human FGFR1-IIIb remain unclear.

In this study, we described effects of human FGFR1-IIIb after overexpression in pancreatic cancer cells. Our results revealed that expression of FGFR1-IIIb inhibited the transformed phenotype of cultured human pancreatic cancer cells. These inhibitory effects were associated with a reduced basal p44/42 MAPK phosphorylation in parallel with enhanced activity of JNK and p38. The antiproliferative effects of FGFR1-IIIb were confirmed in a xenograft model. A significant inhibition of tumor formation and growth in combination with reduced cell proliferation determined by Ki-67 labeling was shown. To compare the effects of FGFR1-IIIb with those of FGFR1-IIIc and to rule out interfering actions due to plasmid expression, cell clones overexpressing either FGFR1-IIIb or FGFR1-IIIc were established in PAN-1 and MIA PaCa-2 cells. Additionally, FGFR1-IIIb–expressing clones were established in TAKA-1 spontaneously immortalized pancreatic ductal cells and compared with FGFR1-IIIc–expressing clones established earlier (16). The two cDNA expression systems only differ in the coding sequence of the second half of Ig domain III, consisting of 153 nucleotides (IIIb exon) and 147 nucleotides (IIIc exon, ref. 16). The region encodes a stretch of 50 and 48 amino acids, respectively, with the two respective variants only sharing 45% amino acid homology (16). Analysis of the cell growth of clones overexpressing either IIIb or IIIc revealed that IIIc enhanced and IIIb inhibited basal cell proliferation in pancreatic cancer cells as well as in noncancerous TAKA-1 pancreatic ductal cells.

To confirm that FGFR1-IIIb is a functional transmembrane receptor, we took advantage of TAKA-1 cells, which do not express endogenous FGFR1 (20). Incubation of FGFR1-IIIb–expressing
TAKA-1 cells with the ubiquitously binding FGF-1 resulted in strong tyrosine phosphorylation of FRS2 known to link FGFR to the MAPK pathway but was without effect in control-transfected TAKA-1 cells. Our studies with tunicamycin indicated that FGFR1-IIIb is glycosylated. Although membranous FGFR1-IIIb expression was present, immunofluorescence revealed that FGFR1-IIIb was predominantly localized in the perinuclear region of the cells, an observation also made for FGFR1-IIIc (28).

In contrast to our previous findings about FGFR1-IIIc (16), the present study revealed that human FGFR1-IIIb is a tyrosine kinase receptor exerting inhibitory functions on the transformed phenotype of cancer cells. To our knowledge, this is the first report of a naturally occurring FGFR1 variant that inhibits the growth of epithelial cell types. Thus far, it has been shown that interactions of FGFR1 with SPARC can inhibit endothelial cell proliferation (29) and that FGFR1-mediated FGF-2 stimulation can induce growth inhibition of nasopharyngeal cancer cells (30). Our hypothesis that FGFR1-IIIc promotes, whereas FGFR1-IIIb inhibits, the transformed phenotype of epithelial cells is further supported by the following findings. Inhibition of FGFR1-IIIc signaling using dominant-negative strategies or soluble forms of the receptor resulted in significant growth inhibition of several cancer cell lines, including pancreas and prostate (17, 31–33). Moreover, similar possible counteracting functions have been described for the Ig domain III variants of FGFR2 (9–15). Thus, switching from FGFR2-IIIb to FGFR2-IIIc expression has been implicated in the progression from a nonmalignant, stromal-dependent, epithelial tumor to an invasive, stromal-independent and undifferentiated tumor in

Figure 6. Morphology and Ki-67 immunoreactivity of xenograft tumors. Analysis was done from sections of each tumor, including the largest diameters. A, double-headed arrow, even small control tumors displayed a large area of central necrosis; arrowheads, tumors of FGFR1-IIIb–expressing clones displayed a more homogenous pattern with only a few or no areas of necrosis; arrows, area of magnification of the respective bottom panels. B, area of necrosis. The total area of each tumor and the area of necrosis were determined from central tumor sections (largest diameters) after H&E staining using square patterns under low magnification (∼16). Results are the relative area of necrosis in % in relation to the total tumor area and are box plots of control (n = 30) and FGFR1-IIIb–expressing xenografts (n = 29). The median values differ significantly. *, P = 0.00236, Mann-Whitney U rank sum test. C, immunohistochemistry of serial sections using a Ki-67 antibody revealed that 30% to 40% of the cells in the vital peripheral areas of the control tumors displayed nuclear Ki-67 immunoreactivity. In contrast, only a few cells (∼3%) in FGFR1-IIIb–expressing tumors showed the presence of Ki-67 immunoreactivity. Arrows, areas of magnification. Original magnifications, ×16, ×63, and ×250 (insets). D, Ki-67 labeling. The total number of Ki-67–positive cells per representative high-power square field (∼63 magnification) was determined in representative peripheral fields for each tumor. Results are number of positive cells per high-power field and are box plots of control (n = 30) and FGFR1-IIIb–expressing xenografts (n = 29). The median values differ significantly. *, P = 0.0229, Mann-Whitney U rank sum test. B and D, box, the 75th and 25th percentile; horizontal line in the box, median. Whiskers, 5th and 95th percentile; ○, values below and above.
prostate cancer cells (10). Down-regulation of FGFR2-IIIb (KGFR) was accompanied with malignant transformation in keratinocytes, whereas reexpression of FGFR2-IIIb resulted in growth inhibition and induction of differentiation in prostate, bladder, and human salivary gland adenocarcinoma cells (12–15).

Generally, IIIb mRNA splice variants of FGFRs are expressed in cell types of epithelial origin, whereas expression of IIIc is restricted to mesenchymal cell types (8). By using specific mRNA protection assays (16) and RT-PCR analysis, we showed that cultured human pancreatic cancer cell lines predominantly express the FGFR1-IIIc variant, whereas FGFR1-IIIb was only coexpressed at very low levels in most of the cell lines. Both receptors were also expressed at various levels in normal and cancerous human pancreatic tissues (16). FGFR1-IIIc expression in spontaneously immortalized TAKA-1 pancreatic ductal cells, which do not express endogenous FGFR1, resulted in an enhanced activation of the p44/42 MAPK pathway and malignant transformation (16). These observations underscore the potential importance of Ig domain III variants of FGFR1 and exon switching from IIIb to IIIc for the malignant phenotype of human pancreatic cancer. Establishment of FGFR1-IIIb may inhibit proliferation, motility, and invasion (24), thereby exhibiting important impeding roles for cancer spread and metastasis in human pancreatic cancer. Although it is not readily evident how FGFR1-IIIb inhibits MAPK, it is unlikely to be through a dominant-negative action inasmuch as the same receptor was able to cause FRS2 phosphorylation and to induce FGF-1-dependent proliferation. Future studies will address whether FGFR1-IIIb activates specific MAPK phosphatases (29) and delineate the regulation and consequences of exon switching and reexpression of endogenous FGFR1-IIIc in pancreatic cancer cells.

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