Aberrant Expression of Neuropilin-1 and -2 in Human Pancreatic Cancer Cells

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ABSTRACT

Purpose: Neuropilin (Np)-1 and -2 are coreceptors for vascular endothelial growth factor (VEGF). This study was designed to assess their role in pancreatic ductal adenocarcinoma (PDAC).

Experimental Design: We assessed Np-1 and Np-2 expression by real-time quantitative PCR in relation to the expression of VEGF ligands and receptors in pancreatic cancer cell lines and tissues.

Results: ASPC-1, CAPAN-1, and PANC-1 pancreatic cancer cells and tumor-derived, laser-captured pancreatic cancer cells exhibited higher Np-1 and Np-2 mRNA levels than VEGF receptor-1, -2, or -3 mRNA levels. Transfection of Np-1 and Np-2 cDNAs in COS-7 cells, and treatment with tunicamycin revealed that both proteins were glycosylated. Both proteins were expressed in pancreatic cancer cell lines, in the PDAC samples, and in acinar cells adjacent to the cancer cells. The normal pancreas was devoid of Np-1 immunoreactivity, whereas Np-2 immunoreactivity was present in the endocrine islets and in some acinar cells, but not in ductal cells.

Conclusions: The aberrant localization of Np-1 and Np-2 in the cancer cells in PDAC suggests that in addition to exerting proangiogenic effects, these coreceptors may contribute to novel autocrine-paracrine interactions in this malignancy.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a deadly malignancy with limited therapeutic options (1). PDACs often exhibit enhanced angiogenesis, and overexpress multiple transmembrane receptors and their ligands, many of which are angiogenic (2). These include epidermal growth factor, transforming growth factor α, transforming growth factor β, hepatocyte growth factor, fibroblast growth factors (FGFs) such as FGF-1, FGF-2, and FGF-5 (2), and vascular endothelial growth factor (VEGF), which is also called VEGF-A (2, 3). VEGF-A consists of five major isoforms generated by differential mRNA splicing and having 121, 145, 165, 189, or 206 amino acid residues in the mature monomer (4). The two high molecular weight species of VEGF-A are sequestered in the extracellular matrix, whereas the three low molecular weight isoforms are freely soluble (5).

Additional members of the VEGF family include VEGF-B, VEGF-C, VEGF-D, placental growth factor, and orf viral VEGF homologues, which are also known as VEGF-E (6).

Because the growth and metastasis of cancer cells is often angiogenesis dependent (7), the roles of VEGF and their receptors in cancer progression has been extensively investigated. Three high-affinity VEGF receptors (VEGFRs), termed VEGFR-1 (flt-1), VEGFR-2 (flk-1/KDR), and VEGFR-3 (flt4), have been identified (5, 6). They possess seven immunoglobulin-like sequences in their extracellular domains and a kinase insert in their intracellular domains (5, 6). In addition, neuropilin (Np)-1, originally identified as a mediator of chemorepulsive guidance for axons in the developing nervous system, acts as a coreceptor for VEGF-A 165, placental growth factor-2, VEGF-B, and VEGF-E (8). Np-1 is a non-tyrosine kinase transmembrane protein of which the overexpression in transgenic mice is associated with various abnormalities, including excess capillary and blood vessel formation (9). The closely related Np-2 also binds VEGF-A 165, as well as VEGF-A 145, VEGF-C (10), and placental growth factor-2 (11), implicating both Np-1 and Np-2 in angiogenesis.

VEGF (VEGF-A) is overexpressed in PDAC (12, 13), and several (12, 14, 15), but not all (13) studies, have reported a positive correlation among blood vessel density, tumor VEGF levels, and disease progression. Although the cancer cells in PDACs sometimes coexpress flk-1/KDR,flt-1, and VEGF in vivo, and act as direct mitogens in some pancreatic cancer cell lines (4, 16, 17), it is not known whether Np-1 and Np-2 expression is altered in PDAC. Therefore, in the present study we analyzed Np-1 and Np-2 expression, and the expression of four VEGF ligands and the three high-affinity VEGF receptors in pancreatic cancer cell lines, and used laser capture microdissection (LCM) and quantitative PCR (Q-PCR) to measure the levels of these mRNA species in cancer cells within the PDAC tumor mass.

MATERIALS AND METHODS

Materials. The following materials were purchased: fetal bovine serum, DMEM, RPMI 1640, trypsin solution, and penicillin-streptomycin solution from Irvine Scientific (Santa Ana, CA); RiboGreen from Molecular Probes (Eugene, OR); Immo-
Neuropilins in Pancreatic Cancer

First-strand cDNA was used as template for Q-PCR. Reaction was performed at 94°C for 5 min and cooling at 4°C for 5 min. The resulting cDNA was treated with RNase inhibitor. Reverse transcriptase was inactivated by heating in a water bath at 42°C for 5 min and stored at -80°C until RNA extraction. All of the studies were approved by the Human Subjects Committees of the University of California (Irvine, CA), and Yamanashi Medical University (Yamanashi, Japan).

**Tissue Samples.** Normal human pancreatic tissue samples were obtained from 7 previously healthy individuals through an organ donor program. Pancreatic tumor tissues were obtained from 8 patients undergoing pancreatic surgery. Freshly removed pancreatic tissues were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction. All of the studies were approved by the Human Subjects Committees of the University of California (Irvine, CA), and Yamanashi Medical University (Yamanashi, Japan).

**Cell Culture.** ASPC-1 and CAPAN-1 were cultured in RPMI 1640, whereas PANC-1 and COS-7 cells were cultured in DMEM. Cells were maintained at 37°C in 95% air and 5% CO₂, and media were supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

**Laser Capture Microdissection (LCM) and RT-PCR.** Frozen pancreatic cancer tissues sections (5 μm) were mounted on uncoated glass slides and immediately fixed sequentially in 70%, 95%, and 100% ethanol with a final 5 min dehydration step in xylene, followed by air-drying. Morphologically identified pancreatic cancer cells, inflammatory cells, and connective tissues were laser captured separately, using the PixCell I LCM system from Arcturus Engineering (Mountain View, CA), as reported previously (18). Total RNA from each population of laser captured cells, from normal pancreatic tissues, or from pancreatic cancer cell lines was extracted as reported previously (18). DNA was removed by incubating with DNase (10 units) for 2 h at 37°C in the presence of RNase inhibitor (10 units). RNA was then quantified in duplicate with the ultrasensitive fluorescent RiboGreen assay, using a CytoFluor fluorescence probe reader (PerSeptive Biosystems, Framingham, MA). A standard curve representing serial dilutions of 2 μg/ml control RNA was used for RNA measurement, and the amount of RNA in each sample was determined in duplicate. RNA was then incubated with reverse transcriptase for 1 h at 37°C in a total volume of 20 μl containing 1× reverse transcriptase buffer, 0.5 mM of oligo-dT, 1 μM of oligo-dT, and 10 units of RNase inhibitor. Reverse transcriptase was inactivated by heating at 94°C for 5 min and cooling at 4°C for 5 min. The resulting first-strand cDNA was used as template for Q-PCR.

**Real-Time Q-PCR.** Sequences for target genes were obtained from the National Center for Biotechnology Information GenBank databases (Table 1). Q-PCR analysis was performed using an ABI Prism 7700 Sequence Detection System, as reported previously (18). RNA expression was calculated based on a relative standard curve representing 5-fold dilutions of human cDNA. The parameter threshold cycle was defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passes a fixed threshold above baseline. The target gene copy number in unknown samples was quantified by measuring threshold cycle and by using a standard curve. Initial examination of potential endogenous controls revealed that human cyclophilin and human transcription factor IID/TATA binding factor mRNA moieties were either below the level of detection or expressed at very low levels in the LCM samples, whereas glyceraldehyde-3-phosphate dehydrogenase and β-actin mRNA levels were increased in the cancer cells by comparison with the normal cells. Therefore, the amount of RNA in each LCM-derived sample was determined in duplicate, and 1 ng/ml from each sample was used to prepare the corresponding cDNA for Q-PCR analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide primer sequences</th>
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<tr>
<td><strong>VEGF-A</strong></td>
<td>Forward 5'-AATGACGAGGCCTCGAGG-3'</td>
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<tr>
<td></td>
<td>Reverse 5'-TTGATCCGGATCTGCTG-3'</td>
</tr>
<tr>
<td></td>
<td>Probe 5'-TCTGAGCAAGCCACATTGGT-3'</td>
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<td>Reverse 5'-AGCCGATCTAGGATGTCG-3'</td>
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<tr>
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<td></td>
<td>Reverse 5'-CTGAGACGCTCAGTGG-3'</td>
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<td><strong>VEGFR-2</strong></td>
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Transient Transfection, Immunoblotting, and Immuno-histochemistry. COS-7 cells were transiently transfected in serum-free medium with full-length Np-1 or Np-2 cDNAs (19) by using 3 μg of the respective cDNA and a LipofectAMINE PLUS Reagent kit. COS-7 cells were then placed in fresh DMEM and 10% fetal bovine serum, and incubated in the absence or presence of tunicamycin (5 μg/ml) for 16 h. Cell lysates were separated by electrophoresis on 7.5% SDS-PAGE and electrophoretically transferred to Immobilon P membranes (16). Membranes were incubated for 14 h at 4°C with 1:500 dilutions of human anti-Np-1 (A12) or anti-Np-2 (H-300) antibodies. The proteins were detected with horseradish peroxidase-labeled antiserum or antirabbit IgG (1:4000) and chemiluminescence, using the SuperSignal Substrate System and Bio-Max light film. As reported by the manufacturer, the monoclonal anti-Np-1 antibody was raised against the epitope corresponding to amino acids 570–855 of human Np-1, and the polyclonal anti-Np-2 antibody was raised against amino acids 560–858 of human Np-2.

To detect the presence of Np-1 and Np-2 protein in the cancer cell lines, 1 mg/ml of cultured cell lysates were prepared in 0.5 M NaCl, 1 mM CaCl₂, and 1 mM MnCl₂ before incubation for 16 h at 4°C in 50% weight/vol Con-A Sepharose beads (20). The beads were washed three times with a solution consisting of 20 mM Tris (pH 7.5) and 0.5 M NaCl, and once with of 20 mM Tris (pH 7.5) and 50 mM NaCl (20). The Con-A binding proteins were then separated by SDS-PAGE and analyzed by immunoblotting as described above.

Paraffin-embedded tissue sections (4 μm) were subjected to immunostaining using the streptavidin-peroxidase technique, in which endogenous peroxidase activity is blocked by incubation for 30 min with 0.3% hydrogen peroxide in methanol (21). Tissue sections (5 μm thick) were incubated for 15 min (23°C) with 10% normal horse serum and then incubated with the specified antibodies (Np-1, 1:400; Np-2, 1:200) for 24 h at 4°C. Bound antibody was detected with a biotinylated horse antimouse IgG (Np-1) or goat antirabbit IgG (Np-2) secondary antibody and streptavidin-peroxidase complex, using diaminobenzidine tetrahydro-chloride as the substrate (21). Sections were counterstained with Mayer’s hematoxylin. Incubation of some sections with nonimmunized rabbit anti-IgG or without primary antibodies did not yield any immunoreactivity.

RESULTS

Expression of VEGF Ligands and Receptors in Pancreatic Cancer Tissues. Q-PCR of LCM-derived cells revealed the presence of relatively high VEGF-A, VEGF-C, Np-1, and Np-2 mRNA levels in the cancer cells (Fig. 2). By comparison with corresponding levels in the normal pancreas, VEGF-A and VEGF-C mRNA levels in the cancer cells were increased 92-fold and 24-fold, respectively, whereas VEGF-D and -B mRNA levels were increased 15-fold and 4-fold, respectively (Fig. 2A). VEGF-A mRNA levels were also increased in the connective tissue (12-fold) and inflammatory (16-fold) cells, VEGF-C mRNA levels were slightly increased (approximately 5–6-fold) in these cells, whereas VEGF-D was below the level of detection (Fig. 2A).

By comparison with the normal pancreas, Np-1 mRNA levels were 22-fold greater in the cancer cells, and Np-2 and VEGF-B mRNA levels were increased 12- and 3-fold, respectively, in these cells (Fig. 2B). In contrast, VEGF-1 and VEGF-2 mRNA levels were at the lowest limit of detection in the cancer cells. Np-1 mRNA levels were also increased (12-fold) in the inflammatory cells, and both Np-1 and Np-2 were increased ~5-fold in the connective tissue cells. In contrast, Np-2 was not detectable in the inflammatory cells.

To confirm that the LCM-derived cells were enriched for the target population, we measured cytokeratin-20 and CD45 in the three populations of cells. Cytokeratin-20 is expressed at high levels in pancreatic cancer cells (22), whereas CD45 is expressed at high levels in inflammatory cells. Q-PCR revealed that the connective tissue cells expressed low levels of both markers, the cancer cells expressed high levels of cytokeratin-20 mRNA, and the inflammatory cells expressed relatively high levels of CD45 (Fig. 2C).

Expression of Np-1 and Np-2 Proteins in Pancreatic Cancer Cell Lines. To confirm antibody specificity, transient transfection of Np-1 and Np-2 cDNAs was performed in COS-7 cells, followed by immunoblotting analysis (Fig. 3A). A single protein band with an approximate molecular weight of M₅₇₀,000 was observed with the anti-Np-1 antibody in cells transfected with the Np-1 cDNA, but not in cells transfected with the Np-2 cDNA. By contrast, two major protein bands (M₅₁₁3,000 and 107,000) and a minor protein band (~121,000) were observed with the anti-Np-2 antibody in cells transfected with the Np-2 cDNA, but not with the Np-1 cDNA (Fig. 3A). Thus, each tested antibody was specific for the respective neuropolin. To determine whether the presence of multiple protein bands was due to Np-2 glycosylation, COS-7 cells were next incubated in the absence or presence of the glycosylation inhibitor tunicamycin (5 μg/ml for 16 h), followed by immunoblotting with the same antibodies (Fig. 3B). In the presence of tunicamycin, a single band corresponding to unglycosylated Np-1 (M₇₁₁4,000) and Np-2 (M₅₁₀7,000) was readily evident, indicating that both Np-1 and Np-2 were glycosylated, and that the presence of several Np-2 protein bands was due to protein glycosylation.
The same antibodies were next used to confirm that pancreatic cancer cell lines express Np-1 and Np-2 at the protein level. Immunoblotting of total cell lysates failed to reveal the presence of either Np-1 or Np-2 (data not shown). By contrast, immunoblotting of cell lysate-derived Con-A binding proteins (20) revealed that ASPC-1 and CAPAN-1 expressed both Np-1 and Np-2 proteins, whereas PANC-1 cells expressed only the Np-1 protein (Fig. 4).

**Immunohistochemical Analysis of Np-1 and Np-2 Expression in PDAC.** To determine whether Np-1 and Np-2 proteins are expressed in either the normal pancreas or in the cancer cells in PDAC, the above highly specific antibodies were used next for detecting Np-1 and Np-2 immunoreactivity in 5 human pancreatic tissue samples. Np-1 immunoreactivity was not detectable in any of these normal samples (Fig. 5A), whereas faint to moderate Np-2 immunoreactivity was present in many of the endocrine islet cells and in some of the acinar cells in all 5 of these samples (Fig. 6A). In contrast, weak Np-1 immunoreactivity was present in the cancer cells in 3 samples (Fig. 5B), and moderate Np-1 immunoreactivity was present in the cancer cells in 2 of 5 PDAC samples (Fig. 5C). All 5 of the PDAC samples exhibited moderate to strong Np-2 immunoreactivity in the cancer cells (Fig. 6, B and C). Furthermore, there was moderate to strong Np-1 (Fig. 5B) and Np-2 (Fig. 6B) immunoreactivity in many of the acinar cells adjacent to the cancer cells, and mild to moderate Np-1 and Np-2 immunoreactivity in the tumor microvasculature (Fig. 5C; Fig. 6C).
Fig. 2  Expression of vascular endothelial growth factor (VEGF) ligands and receptors in pancreatic cancer tissues. RNA from the indicated laser-captured cell types was subjected to quantitative PCR. A, expression of VEGF ligands. B, expression of VEGF receptors. C, expression of cancer (CK-20) and inflammatory cell (CD-45) markers. Relative expression levels were determined in duplicate, using RNA isolated from three normal pancreatic tissues and three pancreatic ductal adenocarcinoma samples. Data (means) are shown as the fold increase in the indicated cell populations by comparison with the corresponding levels in the normal pancreas; bars, ±SE.
DISCUSSION

VEGF is critical for tumor angiogenesis, as evidenced by the observations that VEGF mRNA is up-regulated in vivo in many human malignancies (4), and that blockade of VEGF actions with anti-VEGF or anti-VEGFR-2 antibodies, VEGF anti-sense or soluble VEGFR-1 expression, and VEGFR tyrosine kinase inhibitors, leads to inhibition of tumor growth (23–26). Furthermore, gene knockout studies revealed that loss of a single VEGF allele results in embryonic lethality between day 11 and 12, with impaired angiogenesis and blood-island formation (27), and that homozygous knockout mice for VEGFR-1 or VEGFR-2, or double knockouts for Np-1 and Np-2 die in utero between day 8.5 and 9.5 (28). In the VEGFR-1/H11002/H11002 mice, the defect occurs in the later stages of angiogenesis. In VEGFR-2/H11002/H11002 mice, there is a reduction in hematopoietic precursors and complete failure to organize blood vessels. By contrast, the Np-1/H11002/H11002/Np-2/H11002/H11002 exhibit avascular yolk sacs, and mice that are deficient for Np-1 but heterozygous for Np-2, or deficient for Np-2 but heterozygous for Np-1, die at day 10.0–10.5 and exhibit diffuse vascular abnormalities that are more marked than either Np-1 or Np-2 single knockouts (28). Together, these observations suggest that Np-1 and Np-2 are as important as the other components of the VEGF pathway in embryonic angiogenesis.

In the present study, we determined that cultured human pancreatic cancer cell lines and the cancer cells in PDAC exhibited high Np-1 and Np-2 mRNA levels, intermediate levels of VEGFR-3, and very low levels of VEGFR-1 and VEGFR-2. The presence of Np-1 and Np-2 in the cultured cancer cells was confirmed by immunoblotting with antibodies of which the specificity was documented in transient transfection experiments, which also revealed, for the first time, that both Np-1 and Np-2 are glycosylated. In agreement with the Q-PCR data, all three of the tested pancreatic cancer cell lines expressed Np-1 protein, and the only cell line in which Np-2 protein was below the level of detection (PANC-1) expressed very low levels of Np-2 mRNA. The presence of Np-1 and Np-2 in the cancer cells in PDAC was confirmed by immunohistochemistry. Moreover, Np-1 and Np-2 immunoreactivity was also evident in the tumor microvasculature and the acinar cells adjacent to the cancer cells. These observations suggest that Np-1 and Np-2, from which there can arise natural soluble forms by alternative splicing (20), may have multiple roles in PDAC. In addition to a potential role in angiogenesis, these may include suppression of cancer-directed immune mechanisms by interference with dendritic cell and T-cell activation (29), and enhanced cancer cell survival through Np-mediated activation of survival pathways (30). Because acinar cells are known to be susceptible to degeneration in PDAC, it is also possible that the Np-1 and Np-2 modulate acinar cell survival in PDAC.

Although VEGF-B was the most abundant VEGF mRNA moiety in the cancer cell lines, VEGF-A mRNA was the most abundant VEGF mRNA moiety in the laser captured cancer cells. Therefore, it is possible that conditions within the tumor microenvironment contribute to the preferential expression of VEGF-B.
VEGF-A. For example, VEGF-A expression may be induced by hypoxia and by growth factors that are abundant in PDAC (31), such as FGF-2, transforming growth factor β, and transforming growth factor α (3). In addition to promoting angiogenesis, these high levels of VEGF-A may suppress cancer directed immune responses (32). Furthermore, in conjunction with the increased expression of Np-1 and Np-2, VEGF-A may participate in aberrant autocrine/paracrine loops that directly promote pancreatic cancer cell growth in vivo. Several lines of evidence support this hypothesis. First, VEGF-A is mitogenic in some pancreatic cancer cells in vitro (16, 17). Second, when VEGF-A-165 associates with Np-1, there is a 10-fold increase in the binding affinity of the complex to VEGFR-2 (33). Thus, the relative abundance of Np-1 and Np-2 in PDAC, and their ability to form complexes with VEGFR-2 (34) and VEGFR-1 (35), respectively, may facilitate signaling even when the levels of VEGFR-1 and VEGFR-2 in the cancer cells are relatively low. Third, inducible expression of Np-1 in Dunning rat prostate carcinoma AT2.1 cells results in larger and more vascular tumors in rats, indicating that the availability of Np-1 in the cancer cells promotes cancer growth and enhances angiogenesis (36). Fourth, Np-1 expression in human prostate cancer cells is often associated with concomitant increases in VEGF-A expression and correlates with more aggressive disease (37). Fifth, concomitant overexpression of VEGF-A and Np-1 also occurs in estrogen-induced rat pituitary tumors, raising the possibility that both receptors may contribute to tumor angiogenesis in this model (38).

VEGF-C mRNA levels were 24-fold higher in the laser-captured cancer cells in comparison with the normal pancreas, whereas VEGF-D and VEGF-B mRNA levels were 10-fold and 4-fold higher in the cancer cells, respectively. Both VEGF-C and VEGF-D bind and activate VEGFR-2, and are the only known ligands for VEGFR-3 (5–6). VEGF-C also binds Np-2, and Np-2-deficient mice exhibit marked to complete absence of the small lymphatic vessels and capillaries, underscoring the importance of Np-2 in lymphangiogenesis (39). VEGF-C and VEGFR-3 immunoreactivity occurs in both the cancer cells and lymphatic cells in PDAC, and expression of VEGF-C in the cancer cells correlates with increased lymphatic invasion and lymph node metastasis (40). Furthermore, VEGFR-3-dependent pathways enhance leukemic cell survival and chemoresistance (41). Taken together, these observations suggest that VEGF-C, in conjunction with VEGFR-3 and Np-2, may contribute to a prolymphangiogenic profile in PDAC, and imply a potential for autocrine and paracrine growth-promoting effects by VEGF-C and VEGF-D acting via the relatively high levels of VEGFR-3 in PDAC.

Np-1 and Np-2 are up-regulated in the endothelial cells of human neuroblastomas (42), and their concomitant overexpres-
sion is associated with increased vascularity and poor prognosis in non-small cell lung cancer (43). Furthermore, Np-1 promotes the angiogenic process during wound healing (44), whereas Np-2 is expressed in pancreatic endocrine islet cells and in endocrine tumors in the gastrointestinal tract (45). In the present study, Np-1 mRNA levels were increased 11-fold in the inflammatory cells, and both Np-1 and Np-2 were increased ~8-fold in the connective tissue cells, whereas Np-2 was not detectable in the inflammatory cells. To our knowledge, this is the first report documenting the expression of Np-1 and Np-2 in such cell types. Moreover, VEGF-A and VEGF-C mRNA levels were increased in the inflammatory cells, as well as in the connective tissue cells adjacent to the cancer cells. It has been recognized recently that the expression of VEGF by cultured fibroblasts and activated inflammatory cells may also contribute to angiogenesis in pathological situations (46, 47). Thus, our findings raise the possibility that VEGF-A and VEGF-C expression in the inflammatory infiltrates and desmoplastic regions in PDAC may also contribute to the angiogenic process in this malignancy.

It is notoriously difficult to study gene expression in PDAC, because the tumor mass consists of cancer, acinar and ductal cells, endocrine islet cells, endothelial and nerve cells, inflammatory cells, proliferating fibroblasts, and desmoplasia. Using LCM and Q-PCR to assay multiple mRNA species in specific cell types in PDAC samples, we documented that there are concomitant increases in Np-1, Np-2, and VEGFR-3 mRNA levels in the cancer cells in PDAC, and that VEGF-A and VEGF-C mRNA levels are markedly elevated these cells. Np-1 and Np-2 may, thus, contribute to the pathobiology of PDAC by promoting angiogenesis and by enhancing direct actions by VEGF on pancreatic cancer cells in vivo. Therefore, our findings suggest that Np-1 and Np-2, as well as VEGFR-3, may represent novel therapeutic targets in PDAC.

REFERENCES
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