Vascular Endothelial Growth Factor-Trap Suppresses Tumorigenicity of Multiple Pancreatic Cancer Cell Lines

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ABSTRACT

Purpose: **Vascular endothelial growth factor A (VEGF-A) is a potent angiogenic agent that binds to two high affinity VEGF receptors (VEGFRs), a process facilitated by the low affinity neuropilin receptors. Although VEGF-A is overexpressed in pancreatic ductal adenocarcinoma, it is not known whether the *in vivo* growth of multiple pancreatic cancer cells can be efficiently blocked by VEGF-A sequestration.**

Experimental Design: **Four human pancreatic cancer cell lines were grown s.c. in athymic nude mice. One cell line also was used to generate an orthotopic model of metastatic pancreatic cancer. The consequences of VEGF-A sequestration on tumor growth and metastasis were examined by injecting the mice with a soluble VEGFR chimer (VEGF-Trap) that binds VEGF-A with high affinity.**

Results: **VEGF-Trap, initiated 2 days after tumor cell inoculation, suppressed the s.c. growth of four pancreatic cancer cell lines and markedly decreased tumor microvessel density. Analysis of RNA from tumors generated with T3M4 cells revealed that VEGF-Trap decreased the expression of VEGFR-1 and neuropilin-1 and -2. VEGF-Trap, initiated 3 weeks after tumor implantation, also attenuated intrapancreatic tumor growth and metastasis in an orthotopic model using PANC-1 cells.**

Conclusions: **VEGF-Trap is a potent suppressor of pancreatic tumor growth and metastasis and also may act to attenuate neuropilin-1 and -2 and VEGFR-1 expression. Therefore, VEGF-Trap may represent an exceedingly useful therapeutic modality for pancreatic ductal adenocarcinoma.**

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is responsible for >20% of deaths caused by gastrointestinal malignancies, making it the fourth most common cause of cancer-related mortality in the United States and other industrialized countries. The prognosis of patients with PDAC is extremely poor, with overall 5-year survival rates <1% (1), 1-year overall survival of 12%, and a median survival of 6 months (2). Survival often is limited to patients who had surgical resection at an early stage of the disease. However, the diagnosis of PDAC often is established at an advanced stage, precluding patients from undergoing tumor resection. Despite recent therapeutic advances (3), these statistics have remained dismal because of the tumor's propensity to metastasize when small and undetectable, the advanced stage at which many patients first develop symptoms, and the intrinsic resistance of pancreatic cancer cells to cytotoxic agents and/or radiotherapy (3–5).

Although PDAC is not a grossly vascular tumor, this malignancy often exhibits enhanced foci of endothelial cell proliferation and frequently overexpresses vascular endothelial growth factor (VEGF), a potent angiogenic factor that is secreted by many tumor cell lines (6). The principal form of VEGF is a homodimeric glycoprotein that has been renamed VEGF-A. It consists of five major isoforms with 121, 145, 165, 189, and 206 amino acid residues, respectively, that originate as a result of alternative splicing from a single gene (7–10). All of the five isoforms are mitogenic toward vascular endothelial cells and act by binding to two related tyrosine kinase receptors, VEGFR-1 and VEGFR-2, on the surface of endothelial cells (11–13). A third high affinity VEGF receptor, VEGFR-3, is expressed in lymphatic vessels (14–15). The three VEGFRs are transmembrane protein tyrosine kinases that possess seven immunoglobulin-like sequences in their extracellular domains and a kinase insert in their intracellular domains.

PDACs overexpress multiple additional angiogenic growth factors, including epidermal growth factor; transforming growth factor α; the three mammalian transforming growth factor β isoforms; hepatocyte growth factor; fibroblast growth factors (FGFs) such as FGF-1, FGF-2, and FGF-5; and platelet-derived growth factor (16). Therefore, it has not been firmly established that VEGF-A is of crucial importance in promoting the angiogenic process in PDAC. To address the potential role of VEGF-A in angiogenesis in PDAC, we used an s.c. nude mouse model of PDAC to assess the consequences of blocking VEGF-A action with VEGF-Trap, a modified soluble VEGFR that consists of the second immunoglobulin-like domain of VEGFR-1 and the third immunoglobulin-like domain of VEGFR-2 (17). We report that VEGF-Trap suppresses the s.c. growth of four distinct pancreatic cancer cell lines in athymic nude mice and that this effect is associated with a marked decrease in microvessel density. In addition, using an orthotopic

model, VEGF-Trap is shown to attenuate intrapancreatic tumor growth and regional and distant metastasis. These findings support the hypothesis that VEGF-A has an important role in pancreatic cancer *in vivo* and raise the possibility that VEGF-Trap may ultimately provide a novel therapeutic option for management of this disease.

MATERIALS AND METHODS

Materials. The following were purchased: DMEM, RPMI 1640, fetal bovine serum, trypsin-EDTA, and glutamine-penicillin-streptomycin from Irvine Scientific (Santa Ana, CA); BxPC3 and PANC-1 human pancreatic cancer cell lines from American Type Culture Collection (Manassas, VA); and oligonucleotide primers for quantitative PCR from Applied Biosystems (Foster City, CA). The following were gifts: T3M4 and COLO-357 from Dr. R. S. Metzger at Duke University and Chimeric VEGF-Trap protein from Regeneron Pharmaceuticals (Tarrytown, NJ).

Cell Culture. Human pancreatic cancer cells were maintained in monolayer culture at 37°C in a humidified incubator with 5% CO_2/95% air. BxPC3 and T3M4 cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin (complete RPMI), whereas COLO-357 and PANC-1 were grown in DMEM that was similarly supplemented (complete DMEM). These cell lines release variable levels of VEGF-A into conditioned medium, which range from intermediate (PANC-1) to relatively high (BxPC3) levels (18). They also harbor K-*ras*, p53, and/or Smad4 mutations or deletions and therefore are highly representative of cancer cells in PDAC (19–21).

***In Vivo* Tumorigenicity Assay.** The effects of VEGF-Trap on tumor formation and growth were assessed for all of the four cell lines using an s.c. nude mouse model. One million cells per cell line were injected s.c. at one site in the flank region of female, athymic *nu/nu* nude mice (Harlan, Indianapolis, IN) that were 6–8-weeks-old (18, 22). Mice were housed in isolated conditions under pathogen-free conditions (18, 22). Mice were injected s.c. in the nape of the neck (twice weekly) starting 2 days after tumor cell implantation and using either VEGF-Trap (25 mg/kg) or control buffer containing an equal amount of human Fc protein (17). There were five mice/group/cell line and one tumor lesion per mouse to avoid any bystander effects. The tumors were measured externally once weekly, and tumor volume was calculated using the equation: $vol = (l \times h \times w) \times \pi/4$, where *vol* is the volume, *l* is the length, *h* is the height, and *w* is the width (18, 22). Tumors were removed 2–6 weeks after injection, and portions were either immediately frozen in liquid nitrogen and stored at −80°C before RNA extraction or immediately embedded in OCT compound and stored at −80°C before histologic analysis.

The aforementioned s.c. mouse model is nonmetastatic (18, 22). Therefore, to determine whether VEGF-Trap could suppress the metastatic potential of pancreatic cancer cells, we next used an orthotopic mouse model in which tumors derived from the s.c. model are aseptically resected and immediately minced into 2-mm^3 pieces and implanted in the pancreas of nude mice via a surgical flap (23). PANC-1 pancreatic cancer cells were tested in this metastatic model because we have determined previously that these cells express and release high levels of biologically active VEGF-A (18). Mice were randomized to a VEGF-Trap-treated group ($n = 6$) or a control group ($n = 6$) that received buffer containing an equal amount of human Fc protein (17). Injections of VEGF-Trap or control buffer were administered s.c. in the nape of the neck as described previously but were initiated 3 weeks after tumor fragment implantation using a dose of 10 mg/kg twice weekly. Injections were continued for 6 weeks, at which time three of six control mice developed large abdominal masses and appeared cachectic, and all of the mice were killed. At autopsy, primary pancreatic tumors were excised and measured, and the number of metastases was determined. The University of California, Irvine and Dartmouth Medical School Institutional Animal Care and Use committees approved all of the studies with mice. According to their guidelines, whenever the tumors achieved a size >15 mm and/or ulcerated, the mice were killed.

Immunohistochemistry. OCT-fixed sections were stained with an anti-CD31 rat antimouse monoclonal antibody (Clone E13.1; PharMingen, San Diego, CA), counterstained with hematoxylin, and subjected to quantitative analysis of the blood vessel densities (18). Images (10 random areas/slide) were captured by an Olympus DP70 digital camera (Olympus, Tokyo, Japan) at 100× magnification and were imported into an Image-Pro Plus version 4.5.1 (Media Cybernetics, Silver Spring, MD) image analysis program (18). The blood vessel densities then were calculated as the ratio of positively stained areas to the total area of the image or field.

Real-Time Quantitative PCR. RNA extraction, reverse transcription-PCR, and first-strand cDNA synthesis for quantitative real-time PCR analysis (Q-PCR) were carried out as described previously (24). Target gene sequences were from the National Center for Biotechnology Information GenBank databases as we reported previously (25). Q-PCR was performed using an ABI PRISM 7700 sequence detection system (Applied Biosystems; Ref. 24). RNA expression was calculated based on a relative standard curve representing fivefold 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 the fixed threshold above baseline. The target gene copy number in unknown samples was quantified by measuring the threshold cycle and by using a standard curve. Q-PCR data were expressed as a relative quantity based on the ratio of the fluorescent change observed with the target gene to the fluorescent change observed with rRNA.

Statistical analysis. Student's *t* test was used for statistical analysis of the experiments. $P < 0.05$ was taken as the level of significance.

RESULTS

Effects of VEGF-Trap on Tumor Growth. The effects of VEGF-Trap on the tumorigenicity of four well-characterized human pancreatic cancer cell lines (BxPC3, COLO-357, PANC-1, and T3M4) were investigated. Two days following the s.c. injection of cancer cells (1×10^6), the mice were initiated on twice-weekly injections of control buffer or VEGF-Trap (25 mg/kg). At the end of the first week, there were no significant differences in tumor growth between control and VEGF-Trap-injected mice. However, by the second week, VEGF-Trap injections were associated with a marked inhibition of tumor growth in the case of

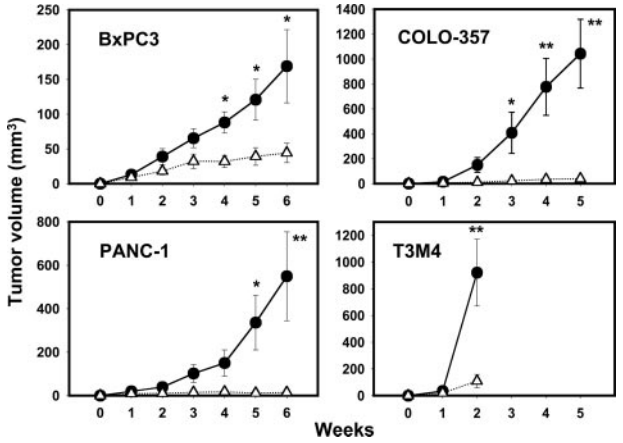

Fig. 1 Effects of vascular endothelial growth factor (VEGF)-Trap on *in vivo* tumorigenicity of pancreatic cancer cells. BxPC3, COLO-357, PANC-1, and T3M4 cells (1×10^6 cells/site) were injected s.c. in athymic nude mice. Two days later, twice-weekly s.c. injections (nape of the neck) of VEGF-Trap protein (or control buffer) were initiated, using a dose of 25 mg/kg, and continued for 2–6 weeks. Tumor volumes (in mm^3) were calculated as described in "Materials and Methods" and expressed as mean ± SE. *, $P < 0.05$; **, $P < 0.01$ when compared with respective controls.

T3M4 cells (Fig. 1). By contrast, the T3M4-derived tumors from control animals were large and started to ulcerate, necessitating termination of these mice (Fig. 1). By the third week, there was a significant difference in tumor growth with COLO-357 cells. Thus, the growth of tumors in the VEGF-Trap group was markedly blunted, whereas tumors from control animals exhibited relatively rapid growth necessitating termination of the mice at week 5 (Fig. 1). Tumors formed by BxPC3 and PANC-1 cells exhibited a significant difference between control mice and VEGF-Trap mice by week 4, which persisted throughout the 6 weeks of the experiments (Fig. 1). Overall, by comparison of control mice with mice injected with vehicle only, there was 97% inhibition of tumor growth with COLO-357 cells (5 weeks) and PANC-1 cells (6 weeks), 92% inhibition with BxPC-3 cells (6 weeks), and 89% inhibition with T3M4 cells (2 weeks).

Effects of VEGF-Trap on Tumor Angiogenesis. To evaluate the effects of VEGF-Trap on tumor-associated angiogenesis, the tumors from the aforementioned studies were immunostained with anti-CD31 antibodies to delineate the presence of endothelial cells. The tumors from mice treated with control buffer exhibited numerous endothelial cells throughout the tumor mass (Fig. 2*A*). By contrast, tumors from mice treated with VEGF-Trap exhibited a marked decrease in CD31 immunoreactivity, and regions containing scant cellular material (Fig. 2*B*), suggesting that either necrosis or apoptosis of the cancer cells might have occurred in these areas. Moreover, quantitative morphometry with the Image-Pro Plus image analysis system revealed that the mean microvessel density (CD31-positive regions) was markedly decreased in tumors treated with VEGF-Trap compared with control tumors in all of the four cell lines (Fig. 3).

Effects of VEGF-Trap on the Expression of VEGF Receptors and Ligands. In view of the marked decrease in microvessel density in the tumors of VEGF-Trap-treated mice, we next sought to determine whether VEGF-Trap altered the expression of VEGF receptors or ligands in the tumors. Because of the limited amount of material (small tumor size) that was available for analysis in the VEGF-Trap-treated group, ligand and receptor expression was only examined in tumors from T3M4 cells. Q-PCR of tumor RNA revealed that both groups of tumors expressed relatively high VEGF-A levels, moderate VEGF-B and VEGF-C levels, and relatively low VEGF-D levels (Fig. 4*A*). Moreover, VEGF-Trap treatment did not significantly alter the levels of any of these mRNA moieties (Fig. 4*A*).

Detectable levels of VEGFR-1 and VEGFR-3 also were present in tumors from both groups, whereas neuropilin-1 and -2 mRNA levels were relatively high in both groups, and VEGFR-2 mRNA levels were below the level of detection (Fig. 4*B*). In contrast to the lack of an effect with respect to ligand expression, VEGF-Trap injections were associated with small but significant decreases in the expression of VEGFR-1 and neuropilin-1 and -2 mRNA levels, whereas the levels of VEGFR-3 were similar in both groups (Fig. 4*B*).

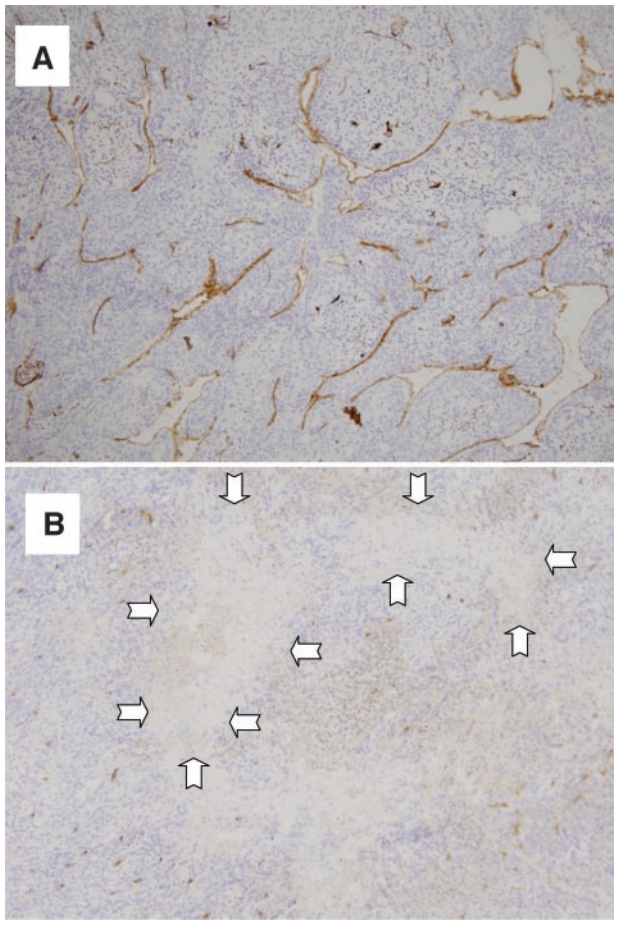

Fig. 2 CD31 immunoreactivity in tumors formed by COLO-357 cells. Immunohistochemical staining for CD31 was performed as described in "Materials and Methods." *A*, tumor from a control mouse. *B*, tumor from a mouse treated with vascular endothelial growth factor-Trap. Open arrows denote areas of scant cellular content in the central portion of the small tumor. Original magnification, ×100

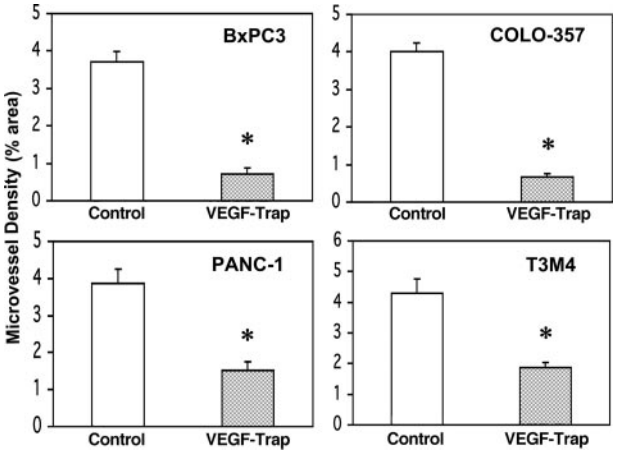

Fig. 3 Effects of vascular endothelial growth factor-Trap (*VEGF-Trap*) on microvessel density. Immunohistochemical staining for CD31 was performed in the tumors from the indicated cell lines, as described in "Materials and Methods," using an Olympus DP70 digital camera and Image-Pro Plus version 4.5.1 image analysis system. Data are the mean ± SE from three tumors/cell line/group. *, $P < 0.01$ when compared with respective controls.

Effect of VEGF-Trap on Tumor Growth and Metastasis in an Orthotopic Model. The s.c. growing PANC-1-derived tumors were implanted into the pancreas of nude mice. Nine weeks following tumor implantation, six of six control mice had large intrapancreatic tumors with extensive local lymph node enlargement and mesenteric lymph node metastasis (Table 1). Five of these mice exhibited peritoneal dissemination, and two mice had ascites. By contrast, the intrapancreatic tumors in the six VEGF-Trap-treated mice were significantly smaller (Table 1). Moreover, one mouse did not exhibit any lymph node enlargement, and five mice exhibited local lymph node enlargement, but these nodes were significantly fewer in number (Table 1) and visibly smaller than in the control group. Only two of the VEGF-Trap-treated mice had mesenteric lymph node metastasis, and only one mouse had peritoneal dissemination (Table 1). None of the VEGF-Trap-treated mice had ascites.

DISCUSSION

Angiogenesis is believed to be essential for growth and metastasis of solid malignancies, and most (26–28), but not all (29), of the studies have reported a positive correlation between tumor VEGF-A levels, blood vessel density, and disease progression in PDAC. Moreover, VEGF-A expression in pancreatic PDAC may be associated with enhanced local spread and liver metastasis and decreased patient survival (26–28). Two additional types of studies suggest that VEGF-A may have an important role in PDAC. *In vitro*, pancreatic cancer cells secrete biologically active VEGF-A, which is the major angiogenic agent produced by these cells (18). *In vivo*, antiangiogenic therapy is effective at suppressing tumor growth in animal models of pancreatic cancer. Thus, the antiangiogenic agent TNP-470 reduces angiogenesis in tumors formed by pancreatic cancer cells, thereby decreasing their growth and metastasis (30); suppression of VEGF-A expression with a VEGF antisense construct attenuates tumorigenicity in nude mice (18); and adenoviral vectors carrying sequences encoding soluble VEGFR-1 and VEGFR-2 (31–32) or the VEGFR tyrosine kinase inhibitor PTK 787 (33) inhibit the growth and/or metastasis of pancreatic cancer cell tumors in mice. Together, these observations suggest that VEGF-A may have an important role in PDAC.

Soluble forms of VEGFR-1 generally exhibit nonspecific interactions with extracellular matrix and poor pharmacokinetics (17, 34). By contrast, VEGF-Trap, which was created by fusing the second immunoglobulin domain of VEGFR-1 with the third immunoglobulin domain of VEGFR-2, has minimal interactions with the extracellular matrix, an excellent pharmacokinetic profile, and a high affinity for VEGF-A with a k_d that is in the pM range (17). These are important characteristics in PDAC because this cancer often exhibits intense desmoplasia and the fibroblasts within this rich extracellular matrix also express high levels of VEGF-A (25).

In the present study we determined that administration of VEGF-Trap markedly suppressed the s.c. growth of four distinct human pancreatic cancer cell lines in athymic nude mice. Moreover, this growth suppression was associated with a marked decrease in microvessel density. Inasmuch as VEGF-Trap did not alter the expression of either VEGF-A or related moieties, our findings indicate that VEGF-Trap interfered with angiogen-

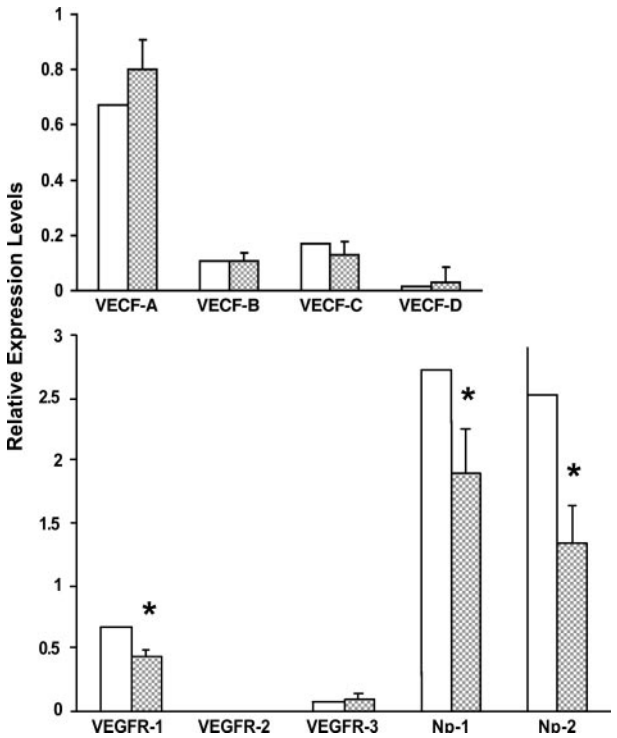

Fig. 4 Effects of vascular endothelial growth factor (*VEGF*)-Trap on the expression of VEGF ligands and receptors in tumors formed by T3M4 cells. Tumor-derived RNA was subjected to quantitative PCR. *A*, expression of VEGF ligands. *B*, expression of VEGF receptors. Relative expression levels were determined in duplicate. Data are the mean ± SE of three tumors/cell line/group. *, $P < 0.01$ when compared with respective controls.

Table 1 Effects of VEGF-Trap in an orthotopic model[a]

Tissue fragments from PANC-1-derived s.c. tumors were implanted into the pancreas of nude mice. VEGF-Trap (10 mg/kg) or control buffer was injected s.c. in the nape of the neck twice weekly. Injections were initiated 3 weeks after tumor implantation and continued for 6 weeks. Data are the mean ± SE from six mice per group.

Site	Control group	VEGF-Trap group	*P*
Primary tumor			
Volume (mm^3)	4408 ± 980	646 ± 209	0.0038
Lymph nodes			
Local			
Number of affected mice	6	5	
Number of lymph nodes (per mouse)	14.50 ± 2.81	3.33 ± 1.41	0.0052
Mesenteric region			
Number of affected mice	6	2	
Number of lymph nodes (per mouse)	13.67 ± 3.13	1.17 ± 0.98	0.0034
Peritoneum			
Number of affected mice	6	1	
Number of affected sites (per mouse)	7.17 ± 2.15	0.67 ± 0.67	0.0016

[a] VEGF, vascular endothelial growth factor.

esis because it efficiently sequestered VEGF-A within the tumor microenvironment following its release from the cancer cells. Because injections of the decoy receptor were started 2 days after tumor inoculation in the s.c. model, it also is possible that VEGF-Trap interfered with co-option of host vasculature, which occurs early in tumor development, and this effect might represent an additional advantage of VEGF-Trap compared with other forms of antiangiogenic therapies (35).

In the present study we also determined that VEGF-Trap administration was associated with decreased expression of VEGFR-1 and neuropilin-1 and -2. By contrast, VEGFR-2 mRNA levels were below the level of detection, whereas VEGFR-3 levels were not altered by VEGF-Trap. Decreased VEGFR-2 expression following VEGF-Trap administration was reported previously with SK-NEP-1 cultured human Wilms' tumor cell xenografts and was proposed to reflect a decrease in neovasculature (36). The decreased expression of VEGFR-1 and neuropilin-1 and -2 in the present study may reflect a similar phenomenon.

Neuropilin-1 is a coreceptor for VEGF-A165 and VEGF-B (37), whereas neuropilin-2 binds VEGF-A165, VEGF-A145, and VEGF-C (38). Both coreceptors are nontyrosine kinase transmembrane proteins that are expressed in endothelial cells and that have been implicated in promoting angiogenesis. Moreover, cultured human pancreatic cancer cell lines and PDAC-derived, laser-captured pancreatic cancer cells exhibit relatively high neuropilin-1 and -2 levels (34). Their ability to form complexes with VEGFR-1 and VEGFR-2 suggests that in addition to promoting angiogenesis, neuropilins may allow for aberrant signaling in the cancer cells in PDAC (39–41). In this context, the observation that VEGF-Trap attenuated neuropilin expression in T3M4-derived tumor xenografts raises the possibility that, in addition to suppressing angiogenesis, VEGF-Trap also may act to abrogate VEGF-A-dependent aberrant autocrine/paracrine loops that promote pancreatic cancer cell growth survival *in vivo*. Three lines of evidence support this hypothesis. First, expression of neuropilin-1 in Dunning rat prostate carcinoma AT2.1 cells results in larger and more vascular tumors in rats (42). Second, neuropilin-1 expression in breast cancer cells has been associated with a VEGF-A-induced survival signal that may enhance breast cancer metastasis (43). Third, VEGF is mitogenic in some pancreatic cancer cells *in vitro* (44–45).

VEGF-Trap induces the regression of SK-NEP-1 cell-derived tumors in nude mice and decreases the size of lung micrometastases that were already established before VEGF-Trap therapy (36). In this model, in addition to endothelial cell apoptosis, there is apoptosis of the recruited perivascular cells within the tumors (36). VEGF-Trap also suppresses ascites formation in nude mice by ovarian cancer cells engineered to overexpress VEGF and inhibits the growth of metastatic lesions in this model (46). Moreover, in the present study we determined that a relatively low dose of VEGF-Trap (10 mg/kg) initiated 3 weeks following tumor implantation induced a significant reduction of the mean intrapancreatic tumor volume compared with the mean tumor volume in control mice, and a marked decrease in metastatic frequency. Together, these observations suggest that VEGF-Trap may be useful to manage established tumors and their metastases. The present findings also are noteworthy in the context of the clinical course of PDAC, which is characterized by an early propensity to metastasize and a high risk for disease recurrence following resection. The marked overexpression of VEGF-A in PDAC (25), its ability to act as a survival factor for endothelial cells and perivascular cells and to render endothelial cells more radioresistant (47), and its capacity to promote cancer cell survival (43, 48–50) and suppress cancer-directed immune mechanisms (51) suggest that a VEGF-Trap-based strategy designed to sequester VEGF-A and block its actions in PDAC may have a unique therapeutic benefit for PDAC patients who have unresectable tumors and for patients who have undergone resection and who are at high risk for disease recurrence.

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