Blocking Platelet-Derived Growth Factor-D/Platelet-Derived Growth Factor Receptor β Signaling Inhibits Human Renal Cell Carcinoma Progression in an Orthotopic Mouse Model

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Abstract

Renal cell carcinoma is a highly malignant and often fatal disease of the kidney. It is difficult to treat, often because metastases are common at the time of presentation. Platelet-derived growth factor-D (PDGF-D) is a newly discovered member of the PDGF family; its function in tumor progression is largely unknown. Here, we examined the expression level of PDGF-D in human renal cell carcinoma by immunohistochemical staining using tissue arrays. We showed that human renal cell carcinoma expresses high levels of PDGF-D protein. The human renal cell carcinoma cell line SN12-C was stably transfected with *pdgf-d* cDNA. Overexpression of PDGF-D in SN12-C cells promoted tumor growth, angiogenesis, and metastasis of human renal cell carcinoma in an orthotopic severe combined immunodeficient (SCID) mouse model. PDGF-D overproduction in SN12-C cells increased the proliferation and migration of mural cells *in vitro* and improved perivascular cell coverage *in vivo*. Overexpression of PDGF-D led to increased expression of angiopoietin-1 and matrix metalloproteinase-9 in tumor tissues. ShRNAi and Gleevec were used to block PDGF-D expression and PDGF receptor β (PDGFRβ) signaling. Inhibition of PDGF-D expression by short hairpin RNA interference (shRNAi) and blockage of PDGFRβ signaling by Gleevec inhibited the growth and lung metastasis of SN12-C cells grown orthotopically in SCID mice. Thus, PDGF-D is a potential candidate for controlling the progression of metastatic renal cell carcinoma. This opens up an avenue of investigation into novel therapeutic strategies for the treatment of renal cell carcinoma, including the use of recently developed tyrosine kinase inhibitors, such as Gleevec, which inhibit PDGF activity through inhibition of its receptor tyrosine kinase. (Cancer Res 2005; 65(13): 5711-9)

Introduction

Renal cell carcinoma is the most common malignant lesion of the kidney, accounting for 85% of all renal cancers. Most renal cancers are discovered incidentally during radiographic procedures. Almost 20% to 30% of patients with newly diagnosed renal cell carcinoma have evidence of metastases at presentation and their median survival is 6 to 8 months. With metastatic progression, renal cell carcinoma is incurable, and existing systemic therapies are largely ineffective in impacting disease response or patient survival. The lack of effective systemic therapy for metastatic renal cell carcinoma is, in part, due to a fundamental lack of understanding of the molecular events that result in cellular transformation, carcinogenesis, and tumor progression in the human kidney. Four platelet-derived growth factor (PDGF) family members have been identified to date: The classic PDGF-A and PDGF-B are secreted as homodimers or heterodimers and bind to dimeric PDGF receptors composed of α- and/or β-chains. PDGF-C and PDGF-D were only discovered recently (1–4). PDGF-A and PDGF-B, which act on numerous cell types, potentiate tumor growth via multiple processes: (*a*) autocrine stimulation of cancer cells (5–7), (*b*) paracrine stimulation of stromal fibroblasts and perivascular cells (8–10), and (*c*) stimulation of angiogenesis (5, 11). PDGF-A and PDGF-B are also considered to be key molecules mediating vessel maturation (12, 13).

PDGF-D forms homodimers that are secreted in latent form and require extracellular proteolytic cleavage to release the active growth factor domain (3). Although PDGF-A and PDGF-B are well characterized, studies on the expression level and function of PDGF-D in human tumor progression are limited. Many cancer cell lines express PDGF-D mRNA, and PDGF-D is significantly elevated in sera of ovarian cancer and medulloblastoma patients (14). PDGF-D has been shown to stimulate human coronary artery smooth muscle cell proliferation *in vitro* (15) and to transform NIH 3T3 fibroblasts and promote tumor formation *in vivo* (14). Two recent studies showed that overexpression of PDGF-D in human prostate cancer cells and mouse melanoma cells accelerated tumor growth in mouse models (16, 17).

All four PDGF isoforms and both receptor chains are expressed in the kidney (18–20). PDGF-B and PDGF receptor β (PDGFRβ) are overexpressed in many human kidney diseases. PDGF-B signaling through PDGFRβ is considered to be an important mediator in the initiation and progression of renal fibrosis as a result of its biological activity as a mitogen and chemoattractant for fibroblasts (21). PDGFs are up-regulated in glomerulosclerosis, as well as in diabetic and allograft-related nephropathies (22), and PDGF inhibitors can inhibit pathogenic mesenchymal proliferation in the kidney (23).

Because PDGFs are important in both autocrine stimulation of tumor cells (5–7) and in paracrine signaling between the tumor cells and the surrounding stroma (8–10), targeting of the PDGF-D/PDGFRβ pathway may be of significance in treatment of human renal cell carcinoma.

In this study, we investigated the expression and function of the newly discovered PDGF family member, PDGF-D, in human renal cell carcinoma. We found that PDGF-D is strongly expressed in human renal cell carcinoma tumors and overexpression of PDGF-D enhanced tumor progression and metastasis in an orthotopic tumor model in severe combined immunodeficient (SCID) mice. Overexpression of PDGF-D led to increased angiogenesis and higher pericyte coverage of tumor vessels. At the molecular level, PDGF-D overexpression led to overexpression of angiopoietin-1

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and matrix metalloproteinase-9 (MMP-9) in tumor tissue. Lastly, inhibition of PDGF-D expression by shRNA or blocking PDGFRβ signaling inhibited tumor growth and metastasis. These results indicate potential therapeutic use of PDGF-D or PDGFRβ inhibitors to block human renal cell carcinoma tumor progression. This study provides insights into the role and mechanisms of PDGF-D in human renal cell carcinoma progression. By characterizing this novel target, the results of this study will facilitate the improvement of current preclinical and clinical treatment protocols for human renal cell carcinoma.

Materials and Methods

Cell lines. The SN12-C and SN12-L1 renal cell carcinoma cell lines were obtained from Dr. I.J. Fidler (M.D. Anderson Cancer Center, Houston, TX; ref. 24). The UMRC3 and UMRC2 renal cell carcinoma cell lines were obtained from Dr. Peter Ratcliffe (University of Oxford, Oxford, United Kingdom). A Caki-1 renal cell carcinoma cell line was obtained from American Type Culture Collection (Manassas, VA). The tumor cell lines were maintained as adherent monolayers in Eagle's MEM supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, and 2-fold vitamin solution (CMEM, Flow Laboratories, Rockville, MD).

Reagents. PDGF-D antibody was obtained from R&D Systems (Minneapolis, MN). Gleevec was a generous gift of Novartis (Basel, Switzerland). PDGFRβ (1:1,000) and phospho-PDGFRβ (1:1,000) antibodies were obtained from Cell Signaling (Beverly, MA).

Plasmid construct and transfection. Full-length *pdgf-d* cDNA was cloned into peak12 vector driven by EF-α promoter (obtained from Dr. Brian Seed, Massachusetts General Hospital, Boston, MA). This expression vector was stably transfected into SN12-C cells using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) as instructed by the manufacturer. The transfected cells were selected with 0.5 μg/mL puromycin.

Cell proliferation assay. One thousand cells were seeded into 38 mm^2 wells of flat-bottomed 96-well plates in triplicate and allowed to adhere overnight. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL, Sigma Chemical, Co., St. Louis, MO) was prepared in PBS. The number of metabolically active cells was determined by MTT assay (25).

Cell migration assay. Cell migration was assessed using Falcon HTS FluoroBlok 24-well inserts (Becton Dickinson, Palo Alto, CA) with 8 mm pores. Cells (6×10^4) were plated in each insert coated with laminin. Conditioned medium from SN12-C and SN12C-PDGFD cells were added to each outer well. At different time points, the cells were incubated with calcein-AM (2 μmol/L; Molecular Probes, Inc., Eugene, OR) for 15 minutes at 37°C to allow epifluorescence visualization of the cells that had migrated through the pores to the bottom of the filter. Four random pictures from the bottom of each insert were acquired, and the area covered by the migrating cells was quantified using NIH Image 1.62 (26).

Tumor cell injections subcutaneous. Viable tumor cells (1×10^6), suspended in 0.2 μmL of HBSS, were injected s.c. into the flanks of SCID mice. Tumor growth was measured every 3 days, and tumor volume was calculated as follows: tumor volume = length × $(\text{width})^2$ / 2 (27).

Orthotopic implantation of tumor cells. The mice were anesthetized with ketamine/xylazine (90/9 mg/kg body weight) in PBS and placed in the left lateral decubitus position. Injection of 1×10^6 viable tumor cells into the renal subcapsule was done as previously described (24). All experiments were approved by the Institutional Animal Care and Use Committee of the Massachusetts General Hospital.

Northern blot analysis. Northern blot was done as previously described (28). PDGF-D and β-actin cDNA probes were synthesized by PCR using primers for PDGF-D5′-ACT CTG ATT GCG GAT GCT CT-3′; and for β-actin: 5′-TGT ATG CCT CTG GTC GTA CC-3′.

Western blot analysis. SN12-C cells were incubated in serum-free medium overnight and then pelleted and resuspended in lysis buffer. Conditioned medium was collected after 3 days of incubation of confluent cell cultures. Thirty micrograms of protein per sample was separated on 10% SDS-polyacrylamide gels (29).

Immunohistochemistry. Proliferating cell nuclear antigen (PCNA, 1:50, DAKO Corp., Carpinteria, CA) staining was carried out on tissue sections of formalin-fixed, paraffin-embedded human renal cell carcinoma xenografts. CD31 (1:800, BD Bioscience, Palo Alto, CA) and αSMA (1:200, clone 1A4, Sigma) double staining was carried out on frozen sections embedded in ornithine carbamyl transferase compound (8 μm thick). The PDGF-D antibody (1:200, R&D Systems) stains specifically and does not cross-react with PDGF-A and PDGF-B. PDGF-D antibody does not cross-react with recombinant human PDGF-C protein (R&D Systems) as determined by dot blot. Tissue microarray slides bearing 50 human renal cell carcinoma tumor sections and nine normal human kidney sections were purchased from Imgenex (San Diego, CA). Antimouse macrophage Scavenger-R antibody was purchased from Serotec (Raleigh, NC). For the quantification of Scavenger-R expression, the number of positive cells was counted in 10 random 0.039 mm^2 fields at ×200 magnification.

Microvessel density were determined as described before (30). Ten areas containing the highest number of vessels were identified by scanning the tumor sections at low power (×40). After the areas of high vascular density were identified, individual vessels were counted in several 0.74 mm^2 fields at ×200 magnification.

Construction of plasmids expressing hairpin RNAs. Silencer plasmid was obtained from Ambion (Austin, TX). The coding sequences for shRNA started with AA and were analyzed by BLAST to ensure that they did not have significant sequence homology with other genes, especially other PDGF family genes. The sequences for the PDGF-D shRNA were taken from Genbank accession no. AF336376 (nucleotides 400-421).

Gleevec treatment. Seven days after implantation of tumor cells into the kidney subcapsule, mice were randomized to receive one of the following treatments by p.o. gavage: daily administration of water (control) or 50 mg/kg Gleevec (31). Treatments continued for 5 weeks. The mice were killed on day 42 after implantation. The weight of the kidney and incidence of lung metastasis were recorded.

Statistical analysis. The significance of differences between two groups was analyzed using the Student's *t* test (two tailed) or Mann-Whitney *U* test (two tailed).

Results

Human renal cell carcinomas overexpress platelet-derived growth factor-D. We first studied the PDGF-D protein expression levels in 50 human renal cell carcinoma sections and nine normal human kidney sections using tissue microarrays to glean a global view of the involvement of PDGF-D in the progression of human renal cell carcinoma. Thirty of the 50 human renal cell carcinoma tumor tissues were clear cell type human renal cell carcinoma; 11 were granular cell and clear cell type; and the rest included collecting duct type, papillary type, and transitional cell carcinoma. The normal tissues were from the same patient. The results showed that PDGF-D was expressed in the collecting tubules of the normal kidney (Fig. 1*A-D*). However, PDGF-D was homogenously strongly expressed in the tumor tissues of all histologic types (Fig. 1*E-H*).

Human renal cell carcinoma cell lines express *pdgf-d* gene. We next examined the expression of PDGF-D and PDGFRβ in five human renal cell carcinoma cell lines, SN12-C, SN12-L1, UMRC3, UMRC2, and Caki-1. All five cell lines expressed both PDGF-D and PDGFRβ as examined by Northern blot and reverse transcription-PCR (RT-PCR, data not shown), indicating that PDGF-D might be an autocrine growth factor for human renal cell carcinoma cells. SN12-C cells were selected and used in the following study because of their moderate level of PDGF-D expression. SN12-C cells express 109.6 ± 60.2 pg/mL of PDGF-A and undetectable levels of PDGF-B using the ELISA kit from R&D Systems.

Overexpressing platelet-derived growth factor-D in human renal cell carcinoma cell line. To analyze the effect of PDGF-D on

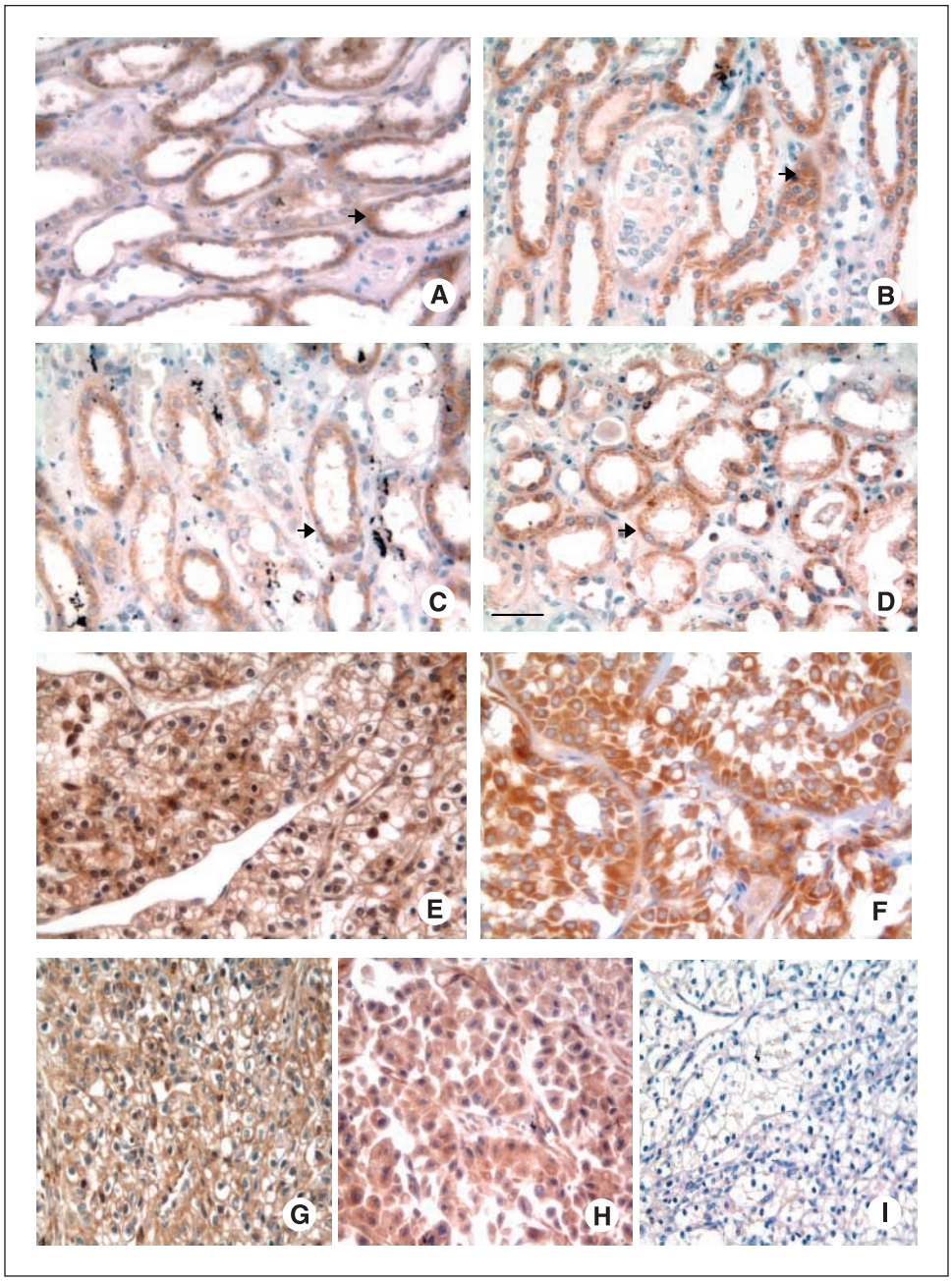

Figure 1. PDGF-D is overexpressed in human renal cell carcinoma samples. Normal kidney tissue in the tissue microarray stains with anti–PDGF-D antibody in collecting tubes (*A-D*). In clear cell type (*E* and *G*), granular cell type (*F*), and granular and clear cell type (*H*) tumor tissues, PDGF-D stains strongly and homogenously. The arrow indicates positive staining. *I*, isotype control staining of the tumor tissues. Antigoat immunoglobulin stains negatively of the tumor tissues. *Bar*, 50 μm.

tumor growth and metastasis, we cloned the full-length human *Pdgf-d* cDNA (Genbank accession no. AF336376) and transfected it into SN12-C cells. The overexpression of PDGF-D was analyzed by Northern and Western blot (Fig. 2*A* and *B*). Western blot of secreted proteins collected from SN12C-PDGFD cells revealed that both the pro-form (M_r 50,000) and the processed forms (M_r 20,000 and 15,000) were detected under reducing condition using anti–PDGF-D antibody (Fig. 2*B*). High expression of PDGF-D was detected in *Pdgf-d*–transfected cells; three highly expressing clones were combined and designated SN12C-PDGFD.

The cellular effects of PDGF-D are exerted through binding to PDGFRβ. Ligand binding induces receptor dimerization and autophosphorylation. The activation of PDGFRβ in PDGF-D–overexpressing cells was studied by Western blotting using a specific antibody against phosphorylated PDGFRβ and total PDGFRβ. Consistent with the RT-PCR analysis of PDGFRβ expression, SN12-C cells express PDGFRβ protein, but parental and mock-transfected SN12-C cells do not have constitutively activated PDGFRβ. Overexpression of PDGF-D activated PDGFRβ *in vitro* (Fig. 2*C*).

Because the PDGF family is important in autocrine stimulation of several cancer cells, and because of the activation of PDGFRβ in PDGF-D overexpressing cells, we next studied whether PDGF-D is an autocrine growth factor for SN12-C cells. The doubling times of the SN12-C, mock, and SN12C-PDGFD cells were 27, 28.2, and 28 hours, respectively.

Platelet-derived growth factor-D induces mural cell proliferation and migration. The PDGF family is also important in paracrine stimulation of mural cells. We next studied the effect of PDGF-D on 10T1/2 cell (fibroblast) proliferation and migration

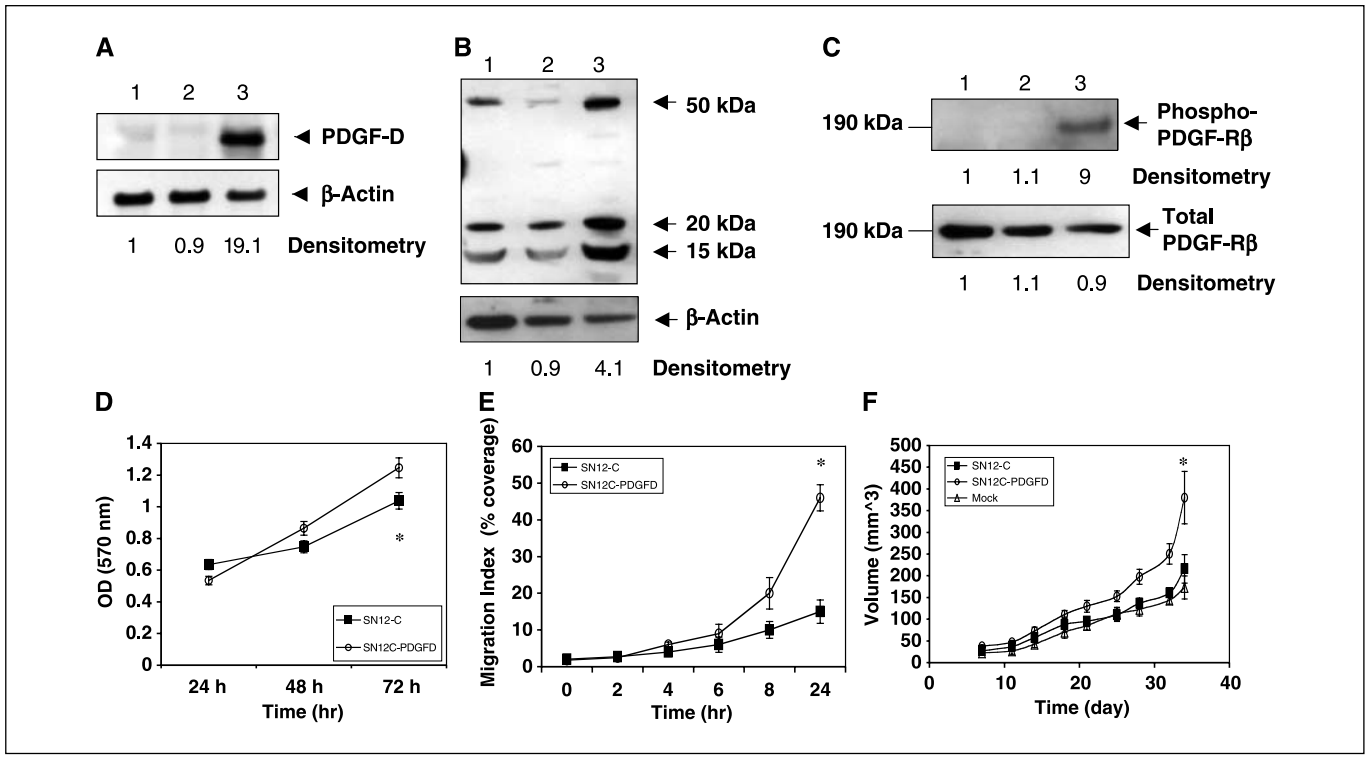

Figure 2. Overexpression of PDGF-D in transfected SN12-C cells. *A,* Northern blot analysis. Parental, mock-transfected, and *pdgf-d*–transfected SN12-C cells were cultured under confluent conditions. mRNA was extracted and a Northern blot analysis was done using 2 μg of mRNA. *B,* Western blot analysis. High levels of PDGF-D were detected in the PDGF-D–transfected cells; PDGF-D migrates as a monomer with a molecular weight of ~50 kDa under reducing conditions. The pro–PDGF-D protein was processed into its active form with molecular weight of 20 and 15 kDa. Equal loading of gels was determined with actin antibody. *C,* activation of PDGFRβ. PDGFRβ activation was determined by Western blotting with primary antibody specific for epitopes corresponding to phosphorylated Tyr^{720} and Tyr^{1021}, respectively. Total PDGFRβ expression was determined with primary antibody against PDGFRβ. *Lane 1,* parental; *lane 2,* mock; *lane 3,* SN12C-PDGFD. Densitometry data was calculated by normalizing the PDGF-D intensity to that of β-actin using NIH Image. *D,* MTT assay. Conditioned medium from SN12-C and SN12C-PDGFD cells were added to 10T1/2 cells. MTT assay was done at different time points. *E,* cell migration assay. *Points,* means; *bars,* SD (*, $P < 0.05$). This is one representative experiment of three. *F,* effect of PDGF-D on human renal cell carcinoma progression in an ectopic model. SCID mice were injected s.c. with 1×10^6 parental, mock, or PDGF-D–overexpressing SN12-C cells. Tumor growth was measured every 3 days (*, $P < 0.05$).

in vitro. Confluent cultures of SN12-C and SN12C-PDGFD cells were incubated for 3 days and supernatants were collected and filtered. The conditioned medium was then added into the culture medium of 10T1/2 cells, and cell proliferation and cell migration were determined at different time points. Conditioned medium from SN12C-PDGFD cells enhanced the proliferation of 10T1/2 cells in a time-dependent manner as determined by MTT assay (Fig. 2*D*). 10T1/2 cells incubated with conditioned medium from SN12C-PDGFD cells migrated faster than cells incubated with medium from parental SN12-C cells in the migration assay (Fig. 2*E*). These findings indicate that PDGF-D may play a predominant role in stimulating stromal cells during tumor growth and angiogenesis.

Platelet-derived growth factor-D enhances human renal cell carcinoma tumor growth and metastasis *in vivo*. To study tumorigenicity and growth rate *in vivo*, we injected parental SN12-C, mock-transfected cells, and SN12C-PDGFD cells s.c. (1×10^6/mouse; $n = 5$) into the flanks of SCID mice. The volumes of the s.c. tumors were measured every 3 days. PDGF-D–overexpressing cells produced faster growing tumors over time than the parental and mock-transfected SN12-C cells (Fig. 2*F*).

To produce orthotopic tumors, parental, mock-transfected, or PDGF-D–transfected SN12-C cells were implanted into the renal subcapsule of SCID mice. The mice were killed on day 30 after implantation. All five parental and mock-transfected SN12-C cells produced localized tumor nodules under the kidney capsule. Four of five mice injected with PDGF-D–transfected cells developed larger tumors that covered the entire kidney. The weight of the kidney together with the tumor tissues was measured at the time of autopsy (Table 1). These results clearly show that PDGF-D enhanced tumor growth *in vivo*. To determine the effect of PDGF-D on tumor metastasis, we quantified lung metastasis in mice implanted orthotopically with SN12-C, mock, and SN12C-PDGFD cells. Overexpressing PDGF-D increased the number of lung metastases formed (Table 1).

Platelet-derived growth factor-D increases angiogenesis and perivascular cell coverage in human renal cell carcinoma tumors. Because the hallmark of renal cell carcinoma is its high vascularization, we speculated that PDGF-D expressed by renal cell carcinoma is involved in angiogenesis. To investigate the angiogenic activity of tumors grown in the orthotopic site, tumors grown in the kidney were collected. Immunohistochemical analysis of the kidney tumors revealed that SN12C-PDGFD tumors invaded into the kidney cortex and medulla by day 30, whereas parental SN12-C and mock-transfected cells grew mostly restricted to the kidney subcapsule (Fig. 3*A* and *B*). Normal kidney tissue is highly vascularized. The microvessel density was measured only in tumor tissue (labeled "T" in Fig. 3*C* and *D*) and not in normal kidney tissue (labeled "N" in Fig. 3*C* and *D*). SN12C-PDGFD tumors had a higher microvessel density (32 ± 4) compared with parental and mock-transfected tumors, 15 ± 2 and 12 ± 1.5, respectively, by CD31 staining (Fig. 3*C* and *D*). Immunostaining for PCNA (cell proliferation) and terminal

Table 1. Effect of PDGF-D on renal cell carcinoma grown in the kidney of SCID mice

Cell line	Tumor take	Kidney weight (g)	Kidney weight (mean ± SD)	Body weight (mean ± SD)	Lung metastasis	
					Incidence	Median nodules (range)
SN12C	5/5	0.38, 0.31, 0.24, 0.27, 0.33	0.31 ± 0.05	25.6 ± 0.7	4/5	21 (0-106)
Mock	5/5	0.35, 0.37, 0.28, 0.31, 0.32	0.32 ± 0.04	24.9 ± 1.2	5/5	32 (0-84)
SN12C-PDGFD	5/5	0.90, 0.53, 0.44, 0.73, 0.30	0.58 ± 0.21*	18.7 ± 1.7	5/5	>200†

NOTE: SCID mice (n = 5) were inoculated with SN12C (1×10^6) cells with or without the *pdgf-d* gene. All mice were sacrificed on day 30. Metastasis to the lung was determined on day 30 after injection.
*This is one representative experiment of three.
†$P < 0.05$.

deoxynucleotidyl transferase–mediated nick end labeling (TUNEL, apoptosis) revealed no difference in the percentage of positive staining cells (data not shown).

Most tumor vessels are highly abnormal and lack normal perivascular cell coverage. Pericyte coverage is considered to be a requirement for vessel maturation and normal vascular function (13). PDGF-B has been shown to induce pericyte attachment (32, 33) and, therefore, we speculated that PDGF-D may also enhance perivascular cell coverage. We measured perivascular coverage of ~150 vessels per tumor section. Whereas most of the vessels were not covered by αSMA-positive cells in the SN12-C parental group, overexpression of PDGF-D significantly increased the αSMA-positive fractional coverage from 5.9% to 14.7% (Fig. 3*E-H*). This suggests that PDGF-D may play a role in pericyte recruitment.

A recent study showed PDGF-D induces macrophage recruitment (34). By immunohistochemical staining for Scavenger-R marker for macrophages, we observed SN12C-PDGFD tumors contained a high number of cells staining positive for Scavenger-R (10 ± 5) compared with control tumors (3 ± 2).

PDGF-D expression in the tumor samples was confirmed by Northern blot and Western blot analysis. SN12C-PDGFD tumors expressed significantly higher PDGF-D than parental and mock tumors. SN12C-PDGFD tumors also expressed phosphorylated PDGFRβ, whereas parental and mock-transfected tumors did not (data not shown).

Platelet-derived growth factor-D induces angiopoietin-1 and matrix metalloproteinase-9 expression *in vivo*. To study the molecular mechanisms of PDGF-D–mediated regulation of angiogenesis and metastasis, we analyzed expression profiles in SN12-C and SN12C-PDGFD tumors using pathway-specific cDNA microarrays containing 200 angiogenesis- and metastasis-related genes. Several genes showed a >2-fold change in expression level. Of note, the expression levels of angiopoietin-1 and MMP-9 were significantly increased in PDGF-D overexpressing tumors, but not in SN12C-PDGFD cells *in vitro*. The reliability of the expression changes detected by cDNA microarrays was confirmed using Northern blot analysis (Fig. 3*I*).

Inhibition of platelet-derived growth factor-D expression and blockade of platelet-derived growth factor receptor β signaling inhibits human renal cell carcinoma tumor progression in an orthotopic severe combined immunodeficient mouse model. Several shRNAi sequences spanning different regions in the PDGF-D cDNA were cloned into the pSilencer vector (Ambion), which was driven by the H1 promoter. These sequences were tested in SN12-C cells and the sequence with the most potent inhibition of PDGF-D expression was used in the following experiment. A random sequence that showed no significant homology to any human genes was used as a transfection control. SN12-C cells were stably transfected with PDGF-D RNAi and the expression level of PDGF-D was confirmed by Northern and Western blot (Fig. 4*A* and *B*). Transfection of PDGF-D RNAi inhibited the expression of PDGF-D mRNA and protein. Inhibition of PDGF-D expression in the tumor cells did not affect tumor cell growth and viability as determined by MTT assay (data not shown). The effect of decreased PDGF-D expression on tumor progression was determined in tumor xenografts grown ectopically in the s.c. (Fig. 4*C*) and orthotopically in the kidney subcapsule. Inhibition of PDGF-D expression significantly inhibited tumor formation in the s.c. tumors. The inhibition of PDGF-D by siRNA was confirmed from s.c. tumor tissues by Northern and Western blot analysis (data not shown). When injected into the kidney subcapsule, PDGF-D RNAi cells produced smaller tumors in the kidney compared with parental cells; the incidence of lung metastases did not change (Table 2). To assess the effect of blocking PDGFRβ signaling, we used a pharmacologic tyrosine kinase inhibitor, Gleevec, to treat mice with PDGF-D–overexpressing SN12-C xenografts. Treatment of SN12C-PDGFD tumors grown in the renal subcapsule with Gleevec started 7 days after tumor implantation. Treatment with 50 mg/kg Gleevec for 5 weeks did not affect body weight (data not shown). As shown in Table 2, incidence of tumor formation was 100% in all of the groups. Mice treated with Gleevec had smaller kidney tumors than control mice and lower incidence of lung metastasis (Table 2). Western blot of tumor xenografts showed that Gleevec effectively inhibited PDGF-R phosphorylation (data not shown). CD31 staining revealed that Gleevec-treated tumors had a lower microvessel density (14 ± 2) compared with control tumors (36 ± 2.2). The blood vessels had a significantly decreased αSMA fractional coverage, from 17% in the control-treated mice to 8.5% in the Gleevec-treated mice.

Discussion

The current study characterized the expression and function of PDGF-D in the progression and metastasis of human renal cell carcinoma, and unveiled inhibition of PDGF-D or PDGFRβ as potential new therapies against renal cell carcinoma.

We chose to study human renal cell carcinoma because it has a high mortality rate and has proven challenging for conventional

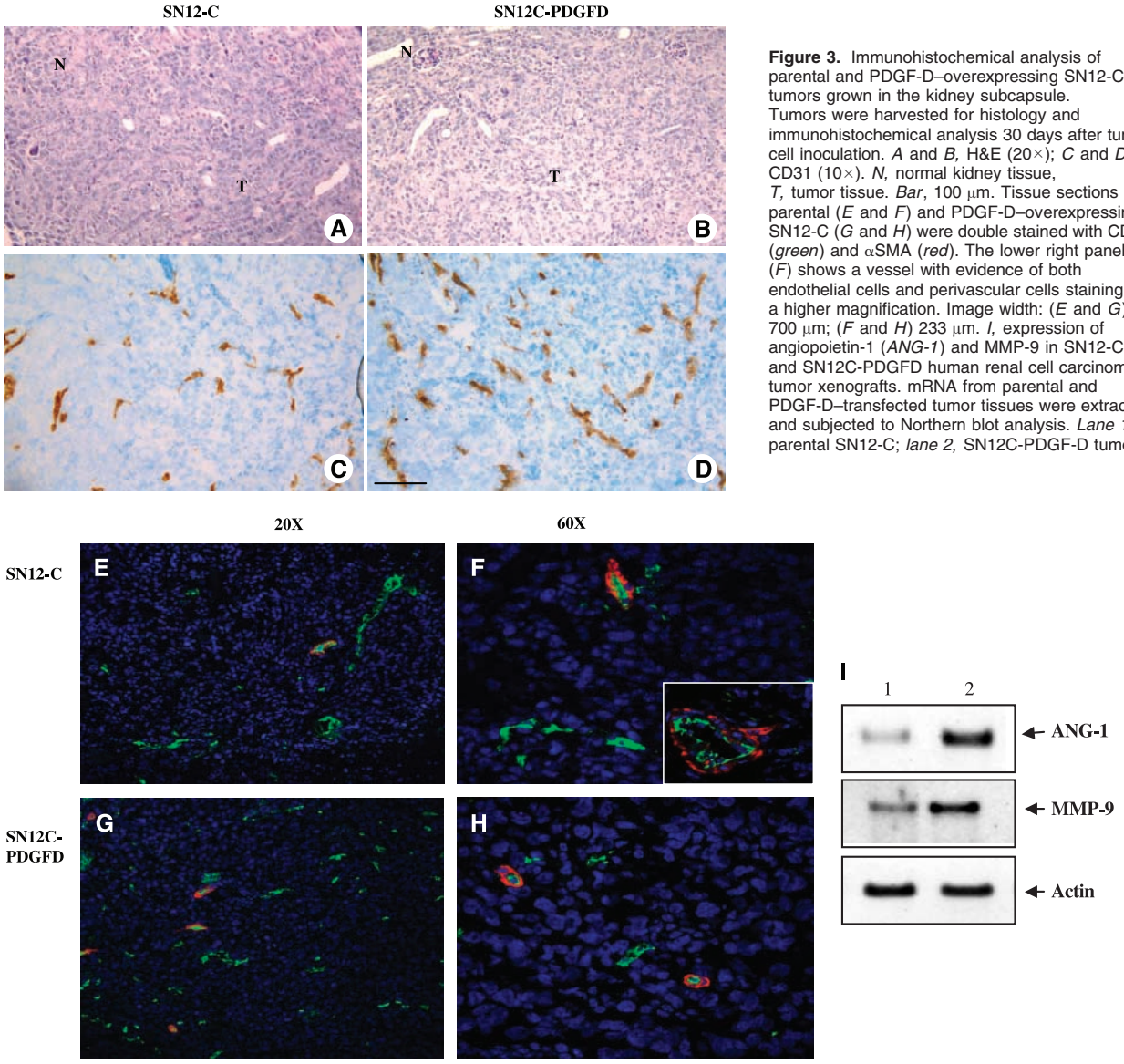

Figure 3. Immunohistochemical analysis of parental and PDGF-D–overexpressing SN12-C tumors grown in the kidney subcapsule. Tumors were harvested for histology and immunohistochemical analysis 30 days after tumor cell inoculation. *A* and *B,* H&E (20×); *C* and *D,* CD31 (10×). *N,* normal kidney tissue, *T,* tumor tissue. *Bar*, 100 μm. Tissue sections of parental (*E* and *F*) and PDGF-D–overexpressing SN12-C (*G* and *H*) were double stained with CD31 (*green*) and αSMA (*red*). The lower right panel in (*F*) shows a vessel with evidence of both endothelial cells and perivascular cells staining at a higher magnification. Image width: (*E* and *G*) 700 μm; (*F* and *H*) 233 μm. *I,* expression of angiopoietin-1 (*ANG-1*) and MMP-9 in SN12-C and SN12C-PDGFD human renal cell carcinoma tumor xenografts. mRNA from parental and PDGF-D–transfected tumor tissues were extracted and subjected to Northern blot analysis. *Lane 1*, parental SN12-C; *lane 2,* SN12C-PDGF-D tumors.

treatments, thus providing a fertile ground for the development of novel therapeutic strategies. The PDGF family, especially PDGF-B and its receptor PDGFRβ, has been shown to be important in both the embryonic development of the kidney and the progression of several kidney diseases. PDGFB and PDGFRβ are overexpressed in many human kidney diseases and the expression of PDGF-A was correlated with clinicopathologic parameters of patients with clear cell renal cell carcinoma (35). A recent study showed that PDGF-D is expressed in visceral glomerular epithelial cells and some vascular smooth muscle cells (19). It is overexpressed in mesangioproliferative glomerulonephritis and mediates mesangial cell proliferation (36). However, whether PDGF-D is expressed in human renal cell carcinoma is not known. Our study showed for the first time that PDGF-D is highly expressed in all histologic types of human renal cell carcinoma tumors and in five different human renal cell carcinoma cell lines, indicating that PDGF-D may be important in human renal cell carcinoma progression.

Because PDGF-D, like PDGF-B, signals through the PDGFRβ, overlapping biological activity is to be expected. On the other hand, PDGF-D binds only to PDGFRβ and thus differs from PDGF-B, which binds both to α- and β-receptors. Several different downstream signaling events have been shown for PDGFRα and PDGFRβ (37). In the developing mouse kidney, PDGF-D is expressed in the metanephric mesenchyme (38), whereas PDGF-B is mainly expressed in the endothelial cells. The differences in receptor preferences and biochemical properties and in the spatial difference in expression of PDGF-B versus PDGF-D in the kidney imply potential functional differences in their roles in kidney tumor progression. We showed that in several human renal cell carcinoma cell lines, PDGF-D was abundant as evidenced by Northern blotting and Western blotting; however, the expression of PDGF-B was not detectable, indicating that PDGF-D may have a more significant role in human renal cell carcinoma progression.

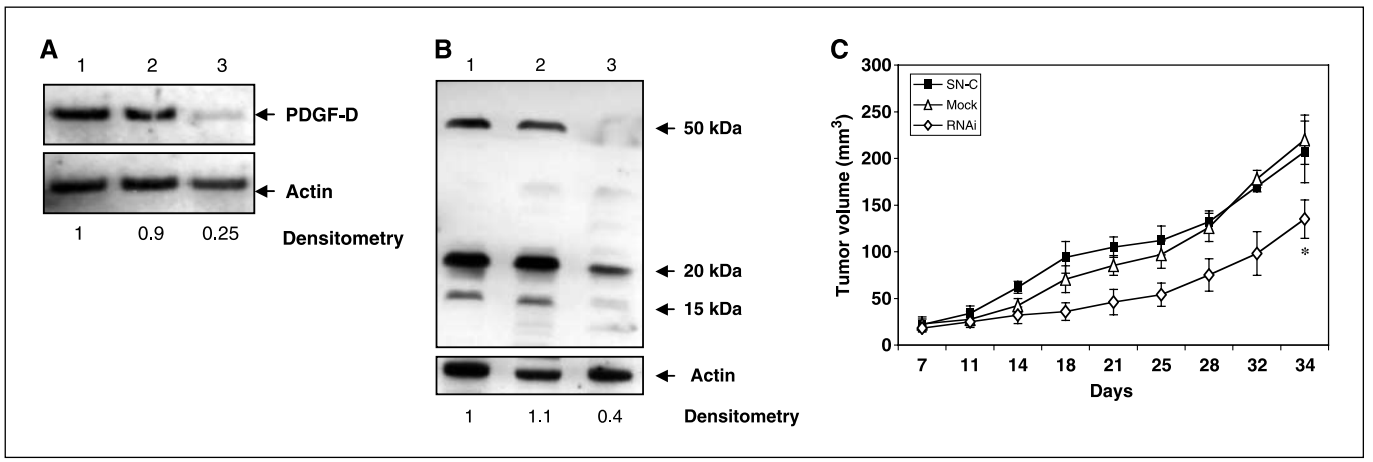

Figure 4. PDGF-D RNAi inhibits PDGF-D expression. Parental, mock-transfected, and PDGF-D RNAi–transfected SN12-C cells were cultured under confluent conditions. *A,* mRNA was extracted and a Northern blot analysis was done using 2 μg of mRNA; *B,* protein extracts were analyzed by Western blot; PDGF-D expression was decreased in the PDGF-D RNAi–transfected cells. PDGF-D migrates as a monomer with a molecular weight of ~50 kDa under reducing conditions. The pro–PDGF-D protein was processed into its active form with molecular weight of 20 and 15 kDa. *Lane 1,* parental; *lane 2,* mock; *lane 3,* PDGF-D RNAi. Densitometry data was calculated by normalizing the PDGF-D intensity to that of β-actin using NIH Image. *C,* development of parental, mock, and PDGF-D RNAi tumors after s.c. inoculation. SCID mice were injected into the s.c. with 1×10^6 parental, mock, or PDGF-D RNAi–transfected SN12-C cells. Tumor growth was measured every 3 days. *, $P < 0.05$. This is one representative experiment of three.

PDGF-A and PDGF-B, and PDGFRα and PDGFRβ are coexpressed in many human carcinomas, including stomach, pancreas, lung, and prostate (39), which indicates that PDGF may be an autocrine growth factor for these tumor types. However, in our study, overexpression of PDGF-D did not increase proliferation of SN12-C human renal cell carcinoma cells *in vitro*. This might be because PDGF-D requires extracellular proteolytic cleavage to release the active growth factor domain, and under cell culture conditions, the protease activity is low. Thus, the level of the active form of PDGF-D is not high enough to affect tumor cells. However, when we did PCNA and TUNEL staining of the kidney tumor tissues to study tumor cell proliferation and apoptosis, we observed that overexpression of PDGF-D did not change tumor proliferation and apoptosis rate *in vivo* (data not shown), indicating that PDGF-D is not an autocrine growth factor for SN12-C cells. The effect of PDGF-D on tumor progression was mostly due to its paracrine stimulation of host cells during tumor growth and angiogenesis.

We showed that overexpression of PDGF-D leads to increased microvessel density. PDGF-BB and PDGF-AB have been shown to increase angiogenesis via the up-regulation of vascular endothelial growth factor in porcine aorta endothelial cells (40, 41). However, using cDNA microarray, we did not observe detectable differences in vascular endothelial growth factor (VEGF) RNA level between parental and PDGF-D–overexpressing kidney tumors (data not shown). This indicates that VEGF may not play a role in conjunction with PDGF-D. Macrophages and mural cells that surround the tumor vessels are known to produce VEGF (42), which serves as a survival factor for endothelial cells. PDGF-B and PDGFRβ play a central role in pericyte recruitment and blood vessel stabilization (12, 43). *Pdgfb* and *Pdgfrβ* knockout mice die perinatally with extensive hemorrhaging, and numerous vessels lack or are incompletely covered by mural cells (44). Our study showed that overexpression of PDGF-D improved perivascular cell coverage in SN12-C tumors grown orthotopically. This confirmed a recent study that showed PDGF-D production by mouse melanoma cells leads to an increase in the number of tumor vessel pericytes (17). We also showed that PDGF-D overexpression increases perivascular cell proliferation, migration, and macrophage infiltration. This indicates

Table 2. Effect of inhibiting PDGF-D expression and Gleevec on renal cell carcinoma progression in the kidney of SCID mice

	Tumor take	Kidney weight (g)	Kidney weight (mean ± SD)	Lung metastases	
				Incidence	Nodules
SN12C	5/5	0.25, 0.35, 0.27, 0.31, 0.36	0.30 ± 0.05	5/5	32 (5-86)
Mock	5/5	0.38, 0.27, 0.30, 0.25, 0.36	0.31 ± 0.06	5/5	36 (10-96)
PDGF-D RNAi	3/5	0.21, 0.24, 0.19, 0.18, 0.22	0.20 ± 0.02*	3/5	31 (0-42)
Control†	9/9	1.038, 0.942, 0.925, 0.536, 0.525, 0.372, 0.314, 0.298, 0.282	0.58 ± 0.3	8/9	142 (24-184)
Gleevec	10/10	0.357, 0.323, 0.32, 0.287, 0.273, 0.23, 0.228, 0.199, 0.197, 0.189	0.26 ± 0.06*	7/10	54 (0-172)‡

NOTE: SCID mice ($n = 5$) were inoculated with SN12C (1×10^6) cells with or without the PDGF-D RNAi. All mice were sacrificed on day 30.
*This is one representative experiment of three.
†SCID mice ($n = 10$) were inoculated with SN12C-PDGFD cells (1×10^6). Daily p.o. gavage of either H_2O or 50 mg/kg Gleevec started day 7, and continued for 5 weeks. All mice were sacrificed on day 42.
‡$P < 0.05$.

that the PDGF-D–enhanced angiogenesis is, at least in part, due to the recruitment of host macrophage and pericytes to neovessels.

At the molecular level, PDGF-D–overexpressing tumors express higher levels of angiopoietin-1. Angiopoietin-1 maintains and stabilizes mature vessels by tightening the junctions between endothelial cells, smooth muscle cells, and pericytes (45–47). Whereas others have reported the expression of angiopoietin-1 in glioblastomas, our present study showed that SN12-C cells do not express angiopoietin-1 *in vitro* and the expression of angiopoietin-1 is not induced in SN12-C cells by overexpression of PDGF-D. Thus, the increased expression of angiopoietin-1 in the tumor tissues is likely due to the paracrine stimulation of PDGF-D in host cells. Indeed, a recent study showed PDGF-B stimulation up-regulated angiopoietin-1 expression in smooth muscle cells (48). The effect and mechanisms of PDGF-D on angiopoietin-1 expression in host cells needs further investigation. Human renal cell carcinoma cells infiltrate surrounding kidney tissues by secreting proteases, including MMPs. Using a cDNA microarray, we showed that PDGF-D–overexpressing tumors express higher levels of MMP-9, thus providing a molecular mechanism for the higher incidence of lung metastasis and pericyte coverage observed in PDGF-D–overexpressing tumors.

Even with major advances in primary tumor diagnosis and therapy, treatment of metastatic renal cancer has made little progress in the past 30 years. Radical nephrectomy is still the primary therapy for human renal cell carcinoma. Standard chemotherapy agents have exhibited dismal results in the treatment of renal cell carcinoma (49). Strategies to combine tumor cell inhibition with modulation of the host microenvironment could provide a more effective approach to the treatment of human renal cell carcinoma. Indeed, a recently reported randomized phase II trial of anti-VEGF (Avastin) for metastatic renal cancer showed promising results with significantly prolonged time to progression of disease (50). Our study showed that the growth and metastasis of SN12-C renal cell carcinoma xenografts depended on PDGF-D. When PDGF-D expression was knocked down by shRNA, the growth of kidney tumors was inhibited, but the incidence of lung metastasis from the primary kidney tumors did not change, indicating that other factors and signaling pathways may compensate for the inhibition of PDGFR-β. This finding suggests that a blockade of PDGF-D–dependent pathways may be an effective strategy to inhibit the growth of human renal cell carcinoma.

Recent studies have shown that Gleevec inhibits tumor growth and metastasis by blocking PDGF-R activation in animal models of dermatofibrosarcoma protuberans (51), human ovarian carcinoma (52), human pancreatic carcinoma, and prostate cancer bone metastases (31, 53). Because SN12-C cells do not express phosphorylated PDGFRβ *in vitro*, and the level of phosphorylated PDGFRβ is not detectable in kidney tumor xenografts by Western blot, we studied the effect of Gleevec on PDGF-D–overexpressing SN12-C tumors. Our study confirmed that Gleevec effectively blocked PDGF-R phosphorylation, mice that received this treatment had significantly smaller kidney tumors and lower incidence of lung metastases than control mice implanted with SN12C-PDGFD cells, and Gleevec treatment led to decreased microvessel density in PDGF-D–overexpressing tumors. Furthermore, we showed that Gleevec decreased pericyte coverage of tumor vessels. This finding has important clinical implications; in the absence of mural cells, the endothelium is more vulnerable to antiangiogenic therapies targeting endothelial cells, such as VEGF blockade (13, 54).

In conclusion, our results indicate that the PDGF-D ligand and its receptor are present in human renal cell carcinoma cell lines. Overexpression of PDGF-D enhanced tumor growth and metastasis. The inhibition of PDGF-D expression by shRNA and the blockade of PDGFRβ signaling by Gleevec in an orthotopic mouse model of renal cell carcinoma decreased the growth of primary renal cell carcinoma, and in the case of PDGFRβ blockade, decreased the incidence of lung metastases. Therefore, further investigation of the PDGF-D signaling pathway as a possible target for the development of novel therapeutic strategies for the treatment of human renal cell carcinoma is clearly necessary.

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Blocking Platelet-Derived Growth Factor-D/Platelet-Derived Growth Factor Receptor β Signaling Inhibits Human Renal Cell Carcinoma Progression in an Orthotopic Mouse Model

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