Expression of a functional VEGFR-1 in tumor cells is a major determinant of anti-PlGF antibodies efficacy

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PlGF, one of the ligands for VEGFR-1, has been implicated in tumor angiogenesis. However, more recent studies indicate that genetic or pharmacological inhibition of PlGF signaling does not result in reduction of microvascular density in a variety of tumor models. Here we screened 12 human tumor cell lines and identified 3 that are growth inhibited by anti-PlGF antibodies in vivo. We found that efficacy of anti-PlGF treatment strongly correlates with VEGFR-1 expression in tumor cells, but not with antiangiogenesis. In addition, PlGF induced VEGFR-1 signaling and biological responses in tumor cells sensitive to anti-PlGF, but not in refractory tumor cell lines or in endothelial cells. Also, genetic ablation of VEGFR-1 signaling in the host did not affect the efficacy of PlGF blockade. Collectively, these findings suggest that the role of PlGF in tumorigenesis largely consists of promoting autocrine/paracrine growth of tumor cells expressing a functional VEGFR-1 rather than stimulation of angiogenesis.

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PlGF antibody treatment strictly correlates with VEGFR-1 expression in tumor cells. Efficacy of Anti-PlGF Mabs Is Not Mediated by Antiangiogenesis. To determine whether efficacy of anti-PlGF mAb treatment is mediated by inhibition of angiogenesis, we quantified MVD (CD31 positive vessels) in sections from DU4475, CAKI1, and SKUT1b tumors at the end-point of the studies (SI Materials and Methods). In contrast to anti-VEGF mAb, anti-PlGF treatment did not cause a significant reduction in tumor vasculature (Fig. S3 A–C).

We also wished to evaluate any potential antiangiogenic effects of PlGF Mab in short-term studies. We treated mice bearing exponentially growing tumors of ~400 mm³ with anti-PlGF, anti-VEGF, or control antibodies for 48 h. CD31 IHC analyses of these tumor tissues showed that anti-PlGF did not cause a reduction in MVD. In contrast, anti-VEGF Mab treatment induced a marked reduction in the number of CD31 positive vessels in SKUT1b tumors (Fig. S3D, Upper). Furthermore, qRT-PCR analyses confirmed that the expression of the transcripts for the pan-vascular markers CD31, VE-cadherin, and MCAM were significantly reduced.

Fig. 1. Inhibition of tumor growth by Anti-PlGF mAb treatment is restricted to VEGFR-1 positive xenografts. (A–F, Left) Effects of anti-PlGF mAb C9.V2 on primary growth of human tumor xenografts. (F) Dose-dependent inhibition of Caki-1 tumor growth by anti-PlGF C9.V2 Mab. (A–F, Right) Analysis of VEGFR-1 expression in tumor cells. Tumor cells were incubated with biotinylated anti-VEGFR-1 mAb (blue) and or with Streptavidin-PE only as a control (red) as indicated. VEGFR-1 expression was analyzed by flow cytometry. Positive (pos) indicates the calculated percentage of positive cells. (G and H) Flow cytometry VEGFR-1 positive and negative controls. (G) HEK293-VEGFR-1 cells (blue) are VEGFR-1 positive and HEK293-empty vector (green) are VEGFR-1 negative. (H) Endothelial cells (HUVECs) are VEGFR-1 positive (blue). (I) Anti-PlGF inhibits growth of established DU4475 orthotopic breast carcinoma xenografts. Anti-PlGF or anti-Ragweed mAb was given at 15 mg/kg. Anti-VEGF-A Mab was given at 10 mg/kg. All antibody treatments were administrated biweekly. n = 10–15, *P < 0.05 relative to anti-ragweed treatment. Error bars represent SEM.
duced upon VEGF blockade in SKUT1b. However, anti-PIGF treatment did not decrease the relative mRNA expression levels in any of the vascular markers tested (Fig. S3D, Bottom).

hPlGF Induces Biological Responses in Anti-PIGF Sensitive (VEGFR-1 Positive) Tumor Cells but Not in Endothelial Cells. We tested the ability of anti-PIGF sensitive tumor cell lines and endothelial cells to respond to VEGFR-1 stimulation in vitro (SI Materials and Methods). We did not observe any responses to PlGF in anti-PIGF refractory (VEGFR-1 negative) tumor cells (Fig. S4). In contrast, anti-PIGF sensitive tumor cell lines proliferated (DU4475, SKUT1b) and migrated (CAKi1 and SKUT1b cells) in response to hPlGF-2 (or hVEGF-A) in a dose-dependent manner (Figs. 2A and 4D). Figure 2A also shows that anti-PIGF Mab blocked PlGF-induced responses in tumor cells. We also evaluated the responses of endothelial cells (HUVECs) to hPlGF-2 and VEGF-A. In agreement with previous reports, HUVECs responded to VEGF-A (or hVEGF-A) in a dose-dependent manner (Figs. 2B and 4D). Figure 2B also shows that anti-PIGF Mab blocked PlGF-induced responses in tumor cells. We also evaluated the responses of endothelial cells (HUVECs) to hPlGF-2 and VEGF-A. In agreement with previous reports, HUVECs responded to VEGF-A but did not show any obvious responses to PlGF in migration (Fig. 2B, Right) and proliferation (Fig. 2B, Left) assays. It has been postulated that endothelial cells do not respond in vitro to exogenous PI GF because they express high levels of endogenous PlGF (13, 23). To test this possibility, we performed PlGF knock-down in HUVECs (SI Materials and Methods). Figure 2C (Left) shows that PlGF knock-down reduces PI GF release by more than 90%. However, HUVECs remained unresponsive to hPlGF-2 but were fully responsive to VEGF-A, hFGF, or FBS (Fig. 2C, Right).

Activation of the Mitogen-Activated Protein Kinase (MAPK) Pathway Is Required for PlGF-Induced Biological Responses in Anti-PIGF Sensitive Tumor Cells. Previous studies have shown that the (MAPK) and PI3K pathways are activated in response to ligand stimulation in some cell lines overexpressing VEGFR-1 (18, 24).

To gain further insights into PlGF/VEGFR-1 signaling in tumor cells, we first performed phospho-kinase antibody array experiments with cell lysates from hPlGF-2 or mock stimulated HEK293-VEGFR-1 cells (SI Materials and Methods). Figure 3A (Left) shows that p42/p44 was activated by PlGF stimulation. No significant differences in phosphorylation of protein kinase B (PKB/AKT) or other proteins included in this array were apparent. Nearly identical results were obtained when lysates from the VEGFR-1 positive uterine sarcoma cell line SKUT1b were analyzed (Fig. 3A, Right). MAPK activation by PlGF was confirmed by Western blot in both SKUT1b (Fig. 3B, Left, and Fig. SS4) and CAKi1 (Fig. 3C, Left, and Fig. SS4, Right). We next used MAPK pathway inhibitors to investigate whether MAPK activation is required for PlGF-induced migration and proliferation. Figure 3B and C (Left) shows that the MEK inhibitor GDC-0973/XL-518 (US patent 20110086837) (25) efficiently blocks PlGF-induced MAPK phosphorylation without affecting cell viability (Fig. 3B and C, Right, and Fig. SS4). In addition, GDC-0973 and the RAF inhibitor GDC-0879 (26) (Fig. 3B and C, right panels), but not Rac, JNK (SP600125), or Rho inhibitors (Fig. SS5), completely suppressed PI GF-responses. However, they only slightly reduced HGF- or FBS-induced CAKi1 and SKUT1b migration and SKUT1b survival/proliferation (Fig. 3B and C, Right, and Fig. 4D). Interestingly, the dose-dependent inhibition of PlGF-induced MAPK phosphorylation by GDC-0973 parallels the inhibition of migration and proliferation induced by this agent (Fig. 3B and C).

Inhibition of PlGF/VEGFR-1 Signaling In Tumor but Not Stromal Cells Is a Major Determinant for Anti-PIGF Efficacy. To confirm the role of VEGFR-1 in PlGF-induced responses in anti-PIGF sensitive tumor cell lines, we knocked-down VEGFR-1 in CAKi1 and SKUT1b cells using siRNA oligonucleotides (SI Materials and Methods).
**Methods.** Figure 4 A and B (Left) shows that VEGFR-1 siRNA but not control siRNA markedly decreases VEGFR-1 expression in both cell lines. VEGFR-1 knock-down also suppressed the ability of these cells to migrate in response to HGF or 10% FBS (Fig. 4 A and B, Right). Consistent with these findings, VEGFR-1 depletion with a different siRNA oligonucleotide sequence (VEGFR-1 siRNA no. 2; Fig. S6d) also specifically inhibited VEGF- and PI GF-induced responses. We found that although PIFG strongly induced tyrosine phosphorylation in HEK293 cells overexpressing hVEGFR-1 (Fig. 4C), it barely affected VEGFR-1 phosphorylation in CAKI1 or SKUT1b (Fig. S5A). This result was not unexpected, because ligand-dependent tyrosine phosphorylation of VEGFR-1 is known to be very low (or undetectable) in cells endogenously expressing this receptor (27–29). To test the potential relevance of tyrosine phosphorylation in the activation of PIFG/VEGFR-1 downstream signaling, we used the VEGFR tyrosine kinase inhibitor axitinib (30). Figure 4C shows that the MEK inhibitor GDC-0973 specifically inhibits MAPK but not VEGFR-1 phosphorylation in HEK293-VEGFR-1 cells. However, axitinib inhibited both PI GF-induced phosphorylation of VEGFR-1 and downstream MAPK activation in a dose-dependent manner. Similar to anti-PIGF mAb (18) (Fig. 4A and Fig. S6C) and MEK inhibitors (Fig. 3 B and C and Fig. S6d), axitinib inhibited PI GF-induced signal transduction (Fig. 4C), SKUT1b cell survival/proliferation (Fig. 4D) and migration of CAKI1 and SKUT1b cells (Fig. S6f). These findings indicate that VEGFR-1 expression and phosphorylation are required for PI GF-induced biological responses in anti-PIGF sensitive tumor cells.

It has been postulated that anti-PIGF efficacy, in the absence of MVD changes, is due to normalization of the vasculature as a consequence of reduced infiltration of VEGFR-1 positive tumor-associated macrophages (TAMs) (8). To probe whether tumor growth inhibition by anti-PIGF indeed requires inhibition of VEGFR-1 signaling in TAMs, hematopoietic stem cells, or other stromal cells, we implanted SKUT1b anti-PIGF sensitive tumor cells in vegfr-1 tk−/−, rag2−/− mice (6). Because these mice express a VEGFR-1 mutant that lacks most of its intracellular domain (including the tyrosine kinase domain), PI GF should be unable to activate VEGFR-1 signaling in host (murine) cells. Figure 4E shows that implantation of SKUT1b cells in vegfr-1 tk−/− does not impair the ability of anti-PIGF to inhibit tumor growth. Similarly, Fig. S6d shows that anti-PIGF treatment has comparable effects on Caki-1 tumor growth in rag2−/− or vegfr-1 tk−/− vs. rag2−/−, vegfr-1 tk−/− mice. These data indicate that anti-PIGF efficacy is mediated by blockade of PI GF/VEGFR-1 signaling in the tumor cells but not by inhibition of VEGFR-1 signaling in host cells.

**Discussion**

Anti-PIGF therapy is currently being evaluated in clinical trials. Nevertheless, the significance of PI GF as a therapeutic target remains incompletely understood. Recent studies suggest that PI GF inhibition reduces tumor growth and angiogenesis by decreasing recruitment of macrophages in tumor tissue (7). However, subsequent reports revealed that inhibition of PI GF-induced signaling does not necessarily inhibit tumor growth, nor does it correlate with pruning of tumor vessels (8). It has been also hypothesized that the efficacy of PI GF inhibition, in the absence of a significant reduction in tumor MVD, is mediated by vascular normalization following reduced TAM infiltration (8, 18). However, this hypothesis does not fully explain the lack of broad antitumor efficacy and the model-dependent efficacy of PI GF inhibition.

Although VEGFR-1 has previously been shown to be expressed in some tumor cells (2–4), the possibility that VEGFR-1 expression may confer sensitivity to PI GF inhibition was not previously investigated. It is interesting to note that of the 12 murine tumor models we recently evaluated (18), inhibition of primary tumor growth by anti-PIGF treatment was restricted to a cell line engineered to overexpress VEGFR-1. Here, we identified three untransfected human tumor cell lines (CAKI1, SKUT1b, and DU4475) sensitive to PI GF neutralization. Remarkably, all anti-PIGF sensitive tumor cell lines identified in the present study were found to be VEGFR-1 positive. Conversely, all anti-PIGF resistant cell lines were VEGFR-1 negative. These data suggest that blockade of PI GF/VEGFR-1 signaling in tumor cells may be required for anti-PIGF mAb efficacy.

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**Fig. 3.** hPI GF-2-induced responses in anti-PIGF sensitive cell lines require MAPK activation. (A) Phosphoantibody array analyses of hPI GF or mock-stimulated HEK293-VEGFR-1 (Left) and SKUT1b (Right) cells. The figure shows only a relevant section of phopho-array membrane. (B, Left) Effects of MEK inhibitor GDC-0973 on PI GF-induced MAPK phosphorylation in SKUT1b cells. (B, Right) Effects of GDC-0973 or RAF inhibitor GDC-0879 on PI GF-induced SKUT1b cell migration. (C, Left) Effect of MEK inhibitor on PI GF-induced MAPK phosphorylation in Caki-1 cells. (C, Right) Effects of MEK inhibitor and RAF inhibitor on PI GF-induced Caki-1 cell migration. Dotted lines represent basal (control) activity. Experiments were repeated at least three times with comparable results. n = 3–5. Error bars represent SD.
Importantly, no decreases in MVD were observed in the sensitive models, suggesting that efficacy is not mediated by antiangiogenesis. Consistent with these findings, in vitro experiments indicate that anti-PlGF sensitive tumor cells lines, unlike anti-PlGF refractory tumor cells or endothelial cells, respond to PlGF stimulation via VEGFR-1 signaling activation. The divergent ability of VEGFR-1 positive tumor cells and vascular endothelial cells to respond to PlGF ligand stimulation is puzzling. However, it is consistent with previous reports (10, 31) and also with genetic data indicating that, at least during embryonic angiogenesis, endothelial VEGFR-1 acts mainly as a nonsignaling decoy (6, 10). Although it has been proposed that the lack of responsiveness of endothelial cells to PlGF reflects VEGFR-1 occupation due to high levels of endogenous PlGF, our VEGFR-1 knock-down experiment argues against this possibility. Further studies are required to elucidate the mechanisms underlying such cell type-dependent responses.

Implantation of anti-PlGF responsive tumors in vegr-1 f<sup>-/-</sup> background strains may also help reconcile conflicting data in the literature. Indeed, the recently reported ability of an anti-human PlGF Mab to reduce tumor growth of tumors implanted in vegr-1 f<sup>-/-</sup>, rag2<sup>-/-</sup> mice (8) or anti-VEGF-1 mAbs (32) in hepatocellular carcinoma models involves, at least in part, inhibition of release of paracrine growth factors from sinusoidal endothelial cells (e.g., hepatocyte growth factor or IL-6), which has been previously shown to be regulated by endothelial VEGFR-1 (33).

We believe that our findings not only underscore an important and potentially clinically relevant mechanism of action of PlGF Mab, but may also help reconcile conflicting data in the literature. Indeed, the recently reported ability of an anti-human PlGF Mab (8) to reduce MDA-MB-435 tumor growth very likely reflects the presence of a previously described functional VEGFR-1 in these cells (4).

It is presently unclear whether the apparent higher incidence of anti-PlGF efficacy in human xenografts (3 of 15 models tested) compared with the lack of growth inhibition in all 12 murine tumor models truly represents a higher incidence of VEGFR-1 expression/activity in human tumors. In addition, the signaling data we present suggests that the VEGFR-1 pathway contributes to PlGF-induced effects in tumor cells mainly through MAPK activation. Thus, VEGFR-1 expression/activity may provide a selective growth advantage to tumors that are highly dependent on alternative mechanisms may be important in other models. In this context, it is tempting to speculate that the efficacy of anti-PlGF (8) or anti-VEGF-1 mAbs (32) in hepatocellular carcinoma models involves, at least in part, inhibition of release of paracrine growth factors from sinusoidal endothelial cells (e.g., hepatocyte growth factor or IL-6), which has been previously shown to be regulated by endothelial VEGFR-1 (33).
Ras/Raf/MAPK signaling. In this context it is interesting that VEGFR-1 signaling within tumor cells previously has been shown to modulate growth and survival of several Ras/MAPK pathway-driven tumor models and cell lines (3, 34). Growing evidence also supports a possible role for VEGFR-1 signaling in certain human cancers. In vitro studies suggested a role for VEGFR-1 signaling in survival of colorectal and pancreatic cancer cell lines during epithelial to mesenchymal transition (22, 35–37). Also, VEGFR-1 signaling is required for growth of patient-derived malignant melanoma-initiating human cells in mice (38), and anti-hVEGFR-1 mAb treatment increases the survival of mice injected with acute lymphoblastic leukemia cells (39) and also inhibits tumor growth of VEGFR-1-positive breast carcinoma and melanoma xenografts (4). Furthermore, expression of VEGFR-1 in tumor cells has been observed in human biopsies (3, 40, 41). Finally, mutations in VEGFR-1 have been found in human cancers, including ~10% of melanomas (42).

In conclusion, we show that, among the models we tested, efficacy of anti-PlGF mAb treatment is limited to VEGFR-1 expressing tumors, because it requires inhibition of PlGF/VEGFR-1 signaling within tumor cells. These findings may be relevant in the context of ongoing clinical evaluation of anti-PlGF (43), anti-VEGFR-1 (44) Mabs, VEGF-Trap (45), and other VEGFR inhibitor therapies. It is tempting to speculate that VEGFR-1 expression/activity may be a biomarker to select patients and indications likely to benefit from anti-PIGF therapies.

Materials and Methods

Animals and Cell Lines. Female Beige nude and BALB/c nude mice were obtained from Charles River. Rag2−/−/ mice were from Jackson Laboratories. flt1−/− mice were generated as described (6). flt-1−/−, rag-2 double ko mice were generated by crossing flt1−/− with with rag2−/− mice. Tumor cell lines were obtained from the ATCC. Tumor cells were maintained in RPMI-1640 containing 10% FBS (Sigma, Sigma-Aldrich), penicillin (100 units/mL), streptomycin (100 μg/mL), and l-glutamine (2 mM/L). Hek293 cells were cultured in DMEM supplemented with 10% FBS (Sigma, Sigma-Aldrich), l-glutamine (2 mM/L), and puromycin (1 μg/mL). Primary HUVECs were purchased from Lonza and maintained in EGM-2 medium (Lonza). Only low-passage HUVECs were used in our experiments. All cells were cultured at 37 °C in a humidified incubator containing 5% CO2, Hek293+hVEGFR-1 and HEK293-control cell lines were generated by transfection followed by puromycin selection.

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Supporting Information

Yao et al. 10.1073/pnas.1109029108

SI Materials and Methods

Flow Cytometry. For flow cytometry, cells were resuspended in DMEM and incubated for 5 h at 4 °C. Then 10^6 cells were blocked with Fc-blocking reagent (BD Biosciences) for 20 min on ice and stained with biotin-conjugated monoclonal hamster-anti-human VEGFR-1 antibodies (Clone 1D11.3.15, Genentech, Inc.) in PBS containing 2% FCS, 2 nmol/L EDTA, and 0.1% (wt/vol) NaN3 for 12 h on ice or 60 min at RT. After washing, cells were incubated with streptavidin conjugated phycocerythrin (PE) (BD Biosciences) for 60 min on ice. Dead cells were excluded using DAPI (Molecular Probes, Invitrogen). Samples were measured on a LSR Fortessa (BD Biosciences) and analyzed with FlowJo software (TreeStar).

Imaging and Analysis. Five tumors per treatment group were dissected and embedded in OCT blocks. Tissues were cryo-sectioned to 10-μm thickness on Leica CM3508S. Images were processed to identify and exclude staining artifacts such as skin tissues and folds. The remaining tumor area was subjected to angiogenesis assays to quantify CD31 positive staining. Two batches of tumor sections at different position were quantified and showed consistent results. Whole-slide digital images were acquired with a CCD camera on a Zeiss AxioImager Z1 fluorescence microscope, controlled by TissueFAXS software (v1.2.4, TissueGnostics). 20x image fields (0.51-μm/pixel, x/y resolution) were automatically stitched and loaded into the TissueStudio Analysis package (v1.5, Definiens). Viable tumor tissue was isolated from necrotic tissue and host skin, and CD31 positive vessel area was calculated using an empirically determined fixed threshold. Vessel density was then calculated as the ratio of CD31 positive pixels to the total viable tumor area.

Cell Migration Assays. Migration assays for Caki-1, Skut-1B, and HUVE cells were performed in transwell membranes (8-μm pore size) inserted in 24-well plates (Corning Costar). Membranes were precoated with 1% gelatin (Caki-1 and Skut-1B cells) or 1 μg/mL of human fibronectin (Sigma-Aldrich) for HUVEC assays. Cells were grown in RPMI-1640 supplemented with 10% FBS (Caki-1 and Skut-1B) or EGM-2 (HUVECs) until they were 80% confluent. Cells were then trypsinized and plated in the corresponding migration inserts (20000 Caki-1 cells/insert, 1000 SKUT1b cells/insert in RPMI-1640, and 10000 HUVEC/insert in EBM-2 plus 0.25% BSA). VEGF, hPlGF-2 (R&D), HGF (Genentech), or hFGF(BD) were added to the corresponding lower wells filled with RPMI-1640 or EBM-2 containing 0.25% BSA as indicated. For the inhibitor assays, cells on the migration inserts were pretreated for 1 h with the indicated inhibitors and then the migration assay was performed as indicated above. For the purpose of assessing neutralization of PI GF or VEGF, anti-PI GF or anti-VEGF antibodies were preincubated with either hPI GF-2 or hVEGF-A for h 1 at 4 °C. After h (Caki-1 and HUVEC) or 18 h (Skut-1B cells) of incubation at 37 °C, cells on the upper (internal) surface of the membrane were carefully scraped off. Cells present on the external side of the membrane were fixed with 100% ethanol for 5 min and subsequently stained with hematoxylin for 10 min, then rinsed off gently with running water. The number of migrated cells in each well was quantified by counting five randomly chosen fields at 100x magnification. Each determination represents the mean of three individual wells ± SD. Experiments were repeated at least three times.

Cell Proliferation Assays. Low-passage HUVE cells were routinely cultured in EGM-2 medium. HUVEC (2 × 10^5 cells/well) were plated in 6-well plates and allowed to attach overnight. After starvation in EBM-2 for 3 h, cells were exposed to the indicated concentrations of hP lGF diluted in EBM-2 containing 2% FBS medium. hVEGF was used as positive control. Cell numbers were counted after 72-h exposures using Z2 coulter particle count and size analyzer (Beckman Coulter).

DU4475 cells were routinely cultured in RPMI1640 plus 10% FBS. For proliferation assays, DU4475 cells were seeded at a density of 2 × 10^5 cells/well into 24-well plates in serum-free conditions (0.1% BSA) for 24 h. Subsequently, cells were incubated with VEGF or PlGF in RPMI1640 medium containing 2% FBS for an additional 36 h. To assess the inhibitory effects of anti-PI GF antibodies, cells were preincubated with PlGF at 4 °C for 1 h in the presence of absence of antibodies. Cell numbers were counted in triplicate using Z2 coulter particle count and size analyzer (Beckman Coulter).

Cell Viability Assays. ATP production was measured from viable cells (Cell Titer Glo assay, Promega). Caki-1 and Skut-1B cells (700 cells/well) were seeded onto 96-well plates overnight and starved in serum-free medium for 24 h, then exposed to various concentrations of inhibitors for 6 d. CellTiter-Glo Reagent (100 μL) was then added to each well (30 min, room temperature) before detection of luminescence (Wallac 1420 VICTOR plate reader, Perkin-Elmer).

Skut-1B cell proliferation/survival was also quantified by Alamar Blue (Invitrogen) following manufacturer instructions. Briefly, cells (700/well) were plated in 96-well plates in serum-containing medium and allowed to attach overnight. Subsequently, adherent cells were washed with PBS and then growth arrested in serum-free RPMI-1640 medium for 24 h. Finally, growth factors were added. Medium was preincubated with anti-PI GF or anti-VEGF antibody for 1 h at 4 °C, and cells were preincubated with axitinib or GDC-0973 for 20 min at room temperature. Three days after stimulation, media were removed from the wells and replaced with 100 μL of 10 μmol/L prewarmed resazurin (Alamar blue) in RPMI-1640 and the plates were incubated for another 4 h at 37 °C, 5% CO2. Fluorescence was measured at 530-nm excitation wavelength and 5900nm emission wavelength. IC50 values were calculated using KaleidaGraph.

siRNA (Knock-Down) Studies. Four different On-target plus siRNA oligonucleotides/gene target were purchased from Dharmacon. Individual siRNA sequences were first evaluated by their ability to decrease target gene expression. VEGFR-1 knock-down expression was measured by FACS and hP lGF was measured by ELISA (R&D Systems). To minimize possible off-target effects, siRNA oligos that cause nonspecific inhibition of migration in response to 10%FFBS or HGF were not used in these experiments. Transfection procedures were performed with DharmoFECT-1 reagent (Dharmacon) according to the manufacturers’ protocols. Briefly, cells were grown to subconfluency in 6-well plates and transfected with 150 nmol/L of VEGFR-1 [catalog no. J-003136–12 (siRNA #1) or J-003136–13 (siRNA #2)], PI GF (catalog no. J-016246-07), or negative control siRNAs (catalog no. p-001206-13-05) diluted in antibiotic-free medium. After incubation for 6 h at 37 °C, RPMI-1640 supplemented with serum was added to Caki-1 and Skut-1B cells and EGM-2 medium was added to HUVEC. Cells were cultured for an additional 30 h at 37 °C and were starved by serum-free medium for

Yao et al. www.pnas.org/cgi/content/short/1109029108
24 h (Caki-1 and Skut-1B) or 3 h (HUVEC) and analyzed for cell migration as described above.

**Human Phospho-Kinase Antibody Array.** Hek-293-VEGFR-1 and skut-1B cells were starved for 24 h and then stimulated with or without hPlGF (R&D) for 10 min. Then cells were washed with ice-cold PBS and lysed in lysis buffer provided by kit. Total protein concentrations were determined by Bio-Rad protein assay. Signal transduction downstream of hPlGF-2 was assessed using the Human Phospho-Kinase Antibody Array (R&D systems) following the manufacturer’s instructions.

**Phosphorylation Assays.** Hek 293-VEGFR-1, HUVECs, or mouse-67NR-VEGFR-1 cells (6 × 10⁶/10-cm dish) were grown in 10-cm dish until 80–90% confluent. After 24 h (293), 6 h (HUVECs), or 5 h (67NR-VEGFR-1) serum starvation, cells were stimulated without or with hPlGF 50 ng/mL, hPlGF 50 ng/mL plus anti-PlGF C9 50 μg/mL, hVEGF 50 ng/mL, or mPlGF 50 ng/mL plus mPlGF C9, or 7A10 (10 μg/mL), mVEGF 50 ng/mL. After 10-min incubation, the cells were lysed in cell lysis buffer (Cell Signaling Technology) containing Phosphatase Inhibitor Mixture 1 1:100 dilution (Sigma) and PMSF 1 mmol/L of PMSF (Sigma). The cell lysates were centrifuged at 13,000 × g for 10 min at 4 °C, and the resulting supernatant was incubated with anti-VEGFR-1 antibody (2 μg/mL) (Santa Cruz Biotechnology) overnight at 4 °C. Immunocomplexes were captured by incubating with EZview Red Protein A Affinity Gel (Sigma) and immunoprecipitated proteins were subjected to immun blotting analysis using the p-Tyr (PY99) (1:2,000 dilution) (Santa Cruz Biotechnology, Inc.) Proteins were visualized by using an ECL kit. Blots were reprobed with an anti-hVEGFR-1 antibody (2 μg/mL) (Santa Cruz Biotechnology, Inc.) or anti-mVEGFR-1 antibody (1:500 dilution) (R&D Systems).

For p42/p44 MAPK and VEGFR-1 phosphorylation assays, Hek293-VEGFR-1, Caki-1, and Skut-1B cells were grown to subconfluence (5 × 10⁶/6-plate well). Cells were then starved for 24 h (293) or 72 h (Caki-1 and Skut-1B) in serum-free medium and Caki-1 cells were starved in low glucose DMEM. Cells were stimulated by hPlGF or hVEGF for 5 or 10 min. Cells were then lysed with 2% SDS sample buffer. Anti phospho-VEGFR-2 (Cell Signaling Technology) was diluted 1:1,000 in 5% BSA in PBS 0.1% Tween 20. Blots were reprobed with an antibody-VEGFR-2 antibody (1:500 dilution) (Cell Signaling Technology).

**Small Molecule Inhibitors.** Axitinib (Santa Cruz Biotechnology), GDC-0973/XL518 (US patent 20110088637), GDC-0879 (1), and SP600125 (EMD Bioscience) were dissolved in DMSO and the final concentration of DMSO was kept below 0.1%. Rho Kinase inhibitor and Rac1 inhibitor (EMD Chemicals) were dissolved in H₂O.

**Human PlGF ELISA.** Human PlGF concentrations were measured using Quantikine human PlGF Immunoassay kit (R&D systems, McKinley Place, NE) following the manufacturer’s instructions.

**Tumor Models.** DU4475 cells were orthotopically implanted in the mammary fat pad (1 × 10⁶ cells/mouse). All other tumor cell lines were s.c. inoculated in matrigel in the dorsal flank of immunodeficient mice (5 × 10⁶ cells/mouse). Antibodies were i.p. administered at the doses indicated in the corresponding figure legends. Treatment with anti-PlGF Mab C9.V2 (2) or with anti-VEGF Mab B-20.4.1 (3) was initiated 24 h after tumor cell inoculation or after tumors had reached 400 mm³, as indicated in the appropriate figure legends. All tumor growth experiments were performed at least two times and conducted in accordance with the Guide for the Care and Use of Laboratory Animals. An Institutional Animal Care and Use Committee approved all animal protocols.

Fig. S1. Inhibition of tumor growth by anti-PIGF mAb treatment is restricted to VEGFR-1 positive xenografts. (A–E, Left) Effect of anti-PIGF C9.V2 mAb treatment on primary tumor growth of human xenografts. (A–E, Right) Analyses of VEGFR-1 expression in tumor cells. Tumor cells were incubated with biotinylated anti-VEGFR-1 mAb (blue) or with streptavidin-PE only as a control (red) as indicated in the figure. VEGFR-1 expression was analyzed by flow cytometry. Tumor cells were implanted s.c. in the flank of immunodeficient mice. Anti-PIGF (C9.V2) or anti-ragweed antibodies were given at 15 mg/kg. Anti-VEGF-A mAb (B20.4.1) was given at 10 mg/kg. All antibody treatments were administered biweekly. n = 10–15, *P < 0.05 relative to anti-ragweed treatment. Error bars represent SEM.
Fig. S2. Expression of PlGF and VEGF mRNA in anti-PlGF refractory and responsive tumors. (A–C) Real time qRT-PCR amplification plots for the expression of human and mouse PlGF and VEGF mRNA in representative anti-PlGF responsive (A and B) and refractory tumors (C). The respective Ct values for hVEGF, hPlGF, hActin, mVEGF, mPlGF, and mActin are: SKUT1b (A): 19.3, 26.9, 17.1, 20.8, 25.1, 17.4; CAKI1 (B): 25.1, 33.7, 21.4, 26, 28.5, 22.3; SW480 (C): 20.3, 29, 17.2, 22, 24.2, 16.9. Tumors were grown in immunodeficient mice and samples were collected at the end of the tumor studies. (A–C) Expression of PlGF and VEGF in pooled RNA samples (from five tumors).
Fig. S3. Anti-PIGF efficacy is not correlated with antiangiogenesis. (A–D) Immunohistochemical analysis of MVD in tumor tissue. (A–D, Left). Representative high magnification (20×, 0.51 μm/pixel) fields from whole-slide tissue sections [vasculature, CD31 (in red) and nuclei, DAPI (in blue)]. (A–D, Right). Quantification of CD31 vascular area/total tumor (viable) area. Tissues were collected at the terminal time point, as indicated. DU4475 (d 30), SKUT1b (d 32), Caki-1 (d 37), or 48 h after treatment (SKUT1b; D, Upper). D, Lower, shows relative gene expression (compared with anti-ragweed) of pan-vascular markers after 48 h of anti-PIGF or anti-VEGF-treatment. Dots represent fold differences in gene expression (compared with anti-ragweed) for individual tumors. For all tumor studies, the last dose of the Abs was given 24 h before tissue collection. n = 5–10, *P < 0.05 relative to anti-ragweed treatment. Error bars represent SEM.

Yao et al. www.pnas.org/cgi/content/short/1109029108

5 of 8
Fig. S4. PlGF does not induce migration in anti-PlGF (VEGFR-1 negative) refractory tumor cells. hPlGF-2 (50 ng/mL) does not induce migration of A549 (A), LXFL529 (B), or H82 (C) VEGFR-1 negative tumor cells. In contrast, HGF (10 ng/mL) and/or FBS (10%) induce migration in these cell lines. Dotted lines represent basal (control) activity. Experiments were repeated at least three times with comparable results. $n = 3–5$, Error bars represent SD.
Fig. S5. hPlGF-2-induced responses in anti-PlGF sensitive cell lines require MAPK activation. (A) Time-course activation of phospho-VEGFR-1 and p42/p44 by PlGF stimulation in SKUT1b (Left) and Caki-1 cells (Right). (B) Effects of MEK inhibitor GDC-0973, Rho Inhibitor (EMD, catalog no. 555550), Rac inhibitor (EMD, catalog no. 553502), and JNK inhibitor (SP600125) on PlGF-induced cell migration in SKUT1b cells (Right) and Caki-1 cells (Left). (C) Effects of GDC-0973 and VEGFR inhibitor (axitinib) on cell viability (ATP production) measured after starvation for 2–6 d (SKUT1b, Left) or after 6 d (Caki-1, Right). Dotted lines represent basal (control) activity. Experiments were repeated at least three times with comparable results. n = 3–5, Error bars represent SD.
**Fig. S6.** Inhibition of PlGF/VEGFR-1 signaling in tumor but not stromal cells is a major determinant of anti-PlGF efficacy. (A, Left) Effects of VEGFR-1 siRNA #2 on VEGFR-1 membrane expression as measured by flow cytometry. (A, Right) Effect of VEGFR-1 knock-down (with VEGFR-1 siRNA #1 or 2) on hPlGF-2, and VEGF-A induced SKUT1b cell migration. (B) Effects of axinitinib and GDC-0973 on hPlGF-2-induced migration of Caki-1 (Left) and SKUT1b (Right) cells. Dotted lines represent basal (control) activity. Experiments were repeated at least three times with comparable results. \( n = 3–5 \), Error bars represent SD. (C) Effect of anti-PlGF C9.V2 in hPlGF (Left) and mPlGF (Right) induced VEGFR-1 phosphorylation. (D) Effects of anti-PlGF (C9.V2), anti-VEGF-A (B20.4.1), or anti-ragweed on the growth of tumors implanted in vegfr-1-/-, rag2-/- (Left) or vegfr-1 tk-/-, rag2-/- (Right) littermate mice. Antibodies were administered as indicated in Fig. 1 and in Materials and Methods. \( n = 10 \), relative to anti-ragweed treatment. Error bars represent SEM.