Supporting Information

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SI Materials and Methods

Flow Cytometry. For flow cytometry, cells were resuspended in complete 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 mmol/L EDTA, and 0.1% (wt/vol) NaN_3 for 12 h on ice or 60 min at RT. After washing, cells were incubated with streptavidin conjugated phycoerythrin (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 CM3050S. 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). 20× image fields (0.51-μm/pixel, x/y resolution) were automatically stitched and loaded into the TissueStudio Analysis package (v1.5, Definens). 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 with10% 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 bFGF(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 PlGF or VEGF, anti-PlGF or anti-VEGF antibodies were preincubated with either hPlGF-2 or hVEGF-A for 1 h at 4 °C. After 6 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 100× 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 (2×10^4 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 hPlGF 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-PlGF antibodies, cells were preincubated with PlGF at 4 °C for 1 h in the presence or 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^2 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-PlGF 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% CO_2. Fluorescence was measured at 530-nm excitation wavelength and 5900nm emission wavelength. IC_{50} 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 hPlGF 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 DharmaFECT-1 reagent (Dharmacon) according to the manufacturers' protocols. Briefly, cells were grown to subconfluency in 6-well plates and transfected with 150 nmnol/L of VEGFR-1 [catalog no. J-003136–12 (siRNA #1) or J-003136–13 (siRNA #2)], PlGF (catalog no. J-016246-07), or negative control siRNAs (catalog no. D-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

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 cells, HUVECs, or mouse-67NR-VEGFR-1 cells (6×10^6/10-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, mPlGF 50 ng/mL plus aPlGF 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 immunoblotting 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 (1:500 dilution) 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 subconfluency (5×10^5/6-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 VEGF (50 ng/mL) for the indicated incubation times and then put on ice. Cells were immediately washed with ice-cold PBS and finally lysed with 2% SDS-containing sample buffer (Bio-Rad). Genomic DNA was separated from the protein lysate by centrifugation at 10,000× *g* for 10 min. For Western blot analysis, after transfer to PVDF membrane (Amersham), membranes were rinsed and blocked for 1 h with 5% no fat milk in TBS. Membranes were incubated with the primary antibody (Anti-p-P44/42 1:1,000 dilution, Cell Signaling Technology Inc. or anti-p-VEGFR-1 1:500 dilution, catalog no. AF4170 R&D) overnight at 4 °C in 5% BSA in Tris buffer saline, 0.1% Tween 20. Membranes were washed and then incubated with an HRP-conjugated secondary antibody (1:2,000 dilution) (Amersham). Blots were developed using the ECL protein detection kit (Amersham). When indicated, cells were preincubated with inhibitors for 20 min at room temperature, followed by treatment with the indicated growth factors.

For VEGFR-2 phosphorylation analysis, HUVECs were starved in serum-free EBM-2 containing 0.25% BSA for 6 h and then were exposed to hVEGF or hPlGF 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 anti-VEGFR-2 antibody (1:500 dilution) (Cell Signaling Technology).

Small Molecule Inhibitors. Axitinib (Santa Cruz Biotechnology), GDC-0973/XL518 (US patent 20110086837), GDC-0879 (1), and SP6000125 (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 H_2O.

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 (1×10^6 cells/mouse). All other tumor cell lines were s.c. inoculated in matrigel in the dorsal flank of immunodeficient mice (5×10^6 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 mm^3, 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.

1. Hoeflich KP, et al. (2009) Antitumor efficacy of the novel RAF inhibitor GDC-0879 is predicted by BRAFV600E mutational status and sustained extracellular signal-regulated kinase/mitogen-activated protein kinase pathway suppression. *Cancer Res* 69:3042–3051.
2. Bais C, et al. (2010) PlGF blockade does not inhibit angiogenesis during primary tumor growth. *Cell* 141:166–177.
3. Liang WC, et al. (2006) Cross-species vascular endothelial growth factor (VEGF)-blocking antibodies completely inhibit the growth of human tumor xenografts and measure the contribution of stromal VEGF. *J Biol Chem* 281:951–961.

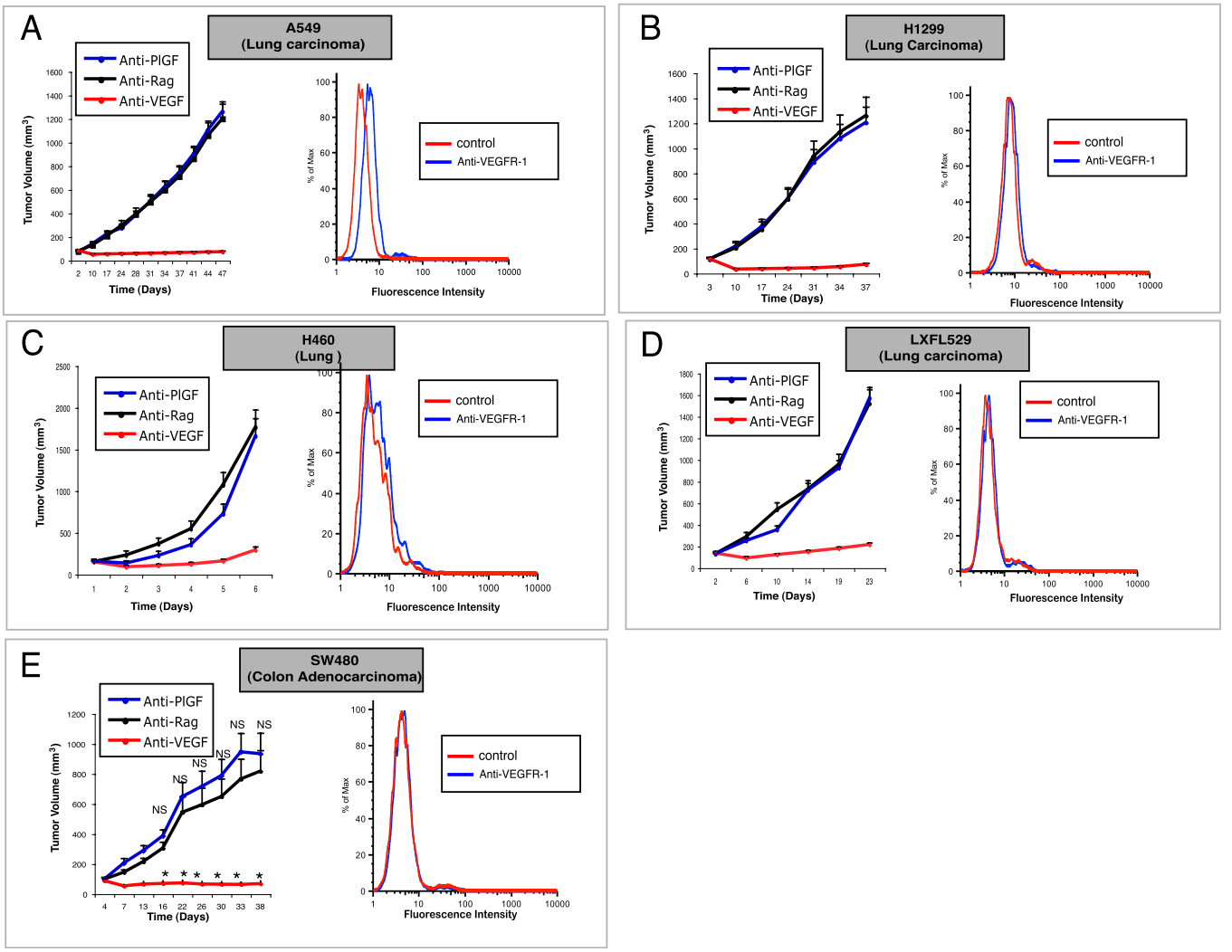

Fig. S1. Inhibition of tumor growth by anti-PlGF mAb treatment is restricted to VEGFR-1 positive xenografts. (*A–E*, *Left*) Effect of anti-PlGF C9.V2 mAb treatment on primary tumor growth of human xenografts. (*A–E*, *Right*) Analyses of VEGFR-1 expression in tumor cells. Tumor cells were incubated 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-PlGF (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.

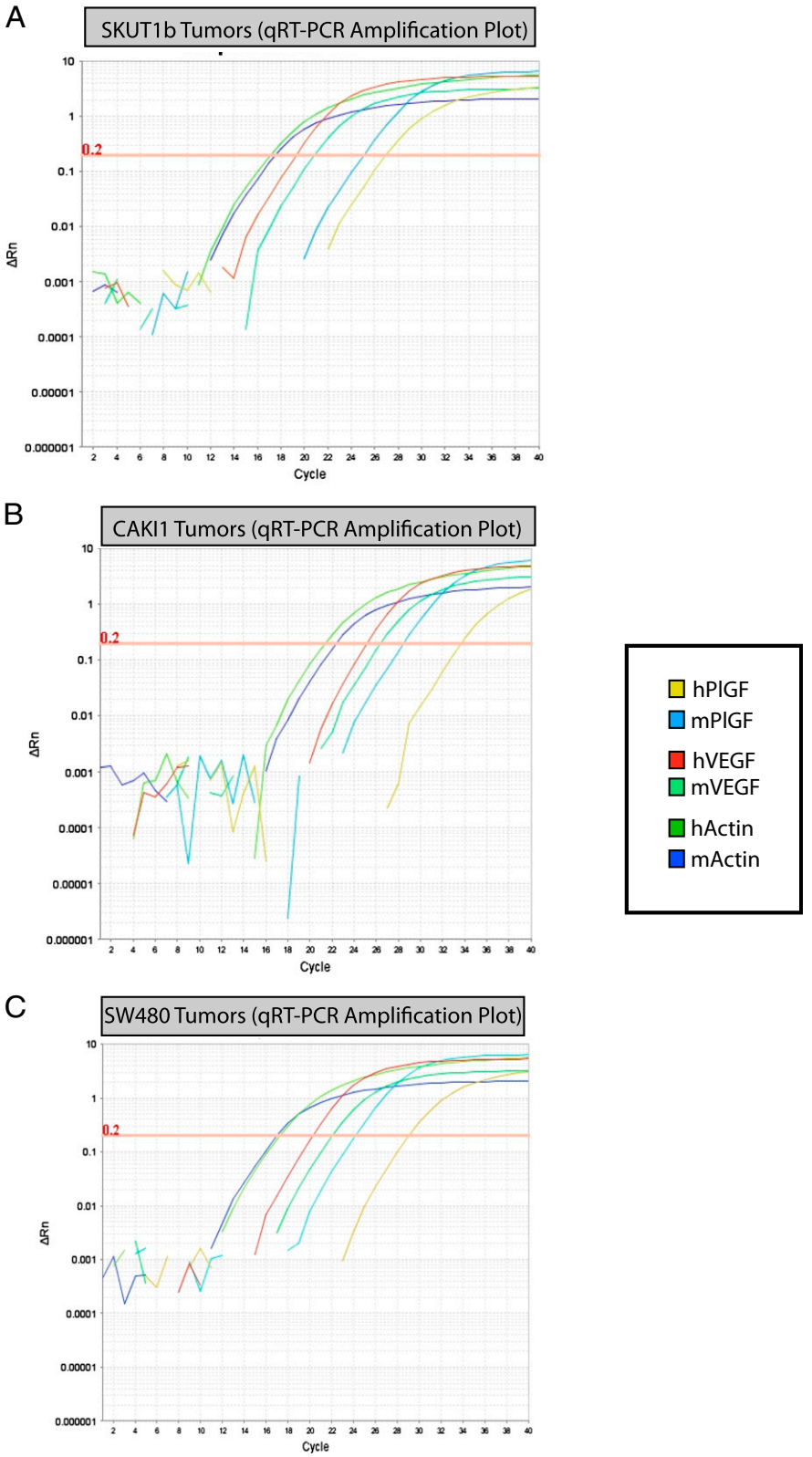

Fig. S2. Expression of PlGF and VEGF mRNA in anti-PlGF refractory and responsive tumors. (*A*–*C*) Real time qRT-PCR amplification plots for the expression of human and mouse PlGF and VEGF mRNA in representative anti-PlGF responsive (*A* and *B*) and refractory tumors (*C*). The respective Ct values 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).

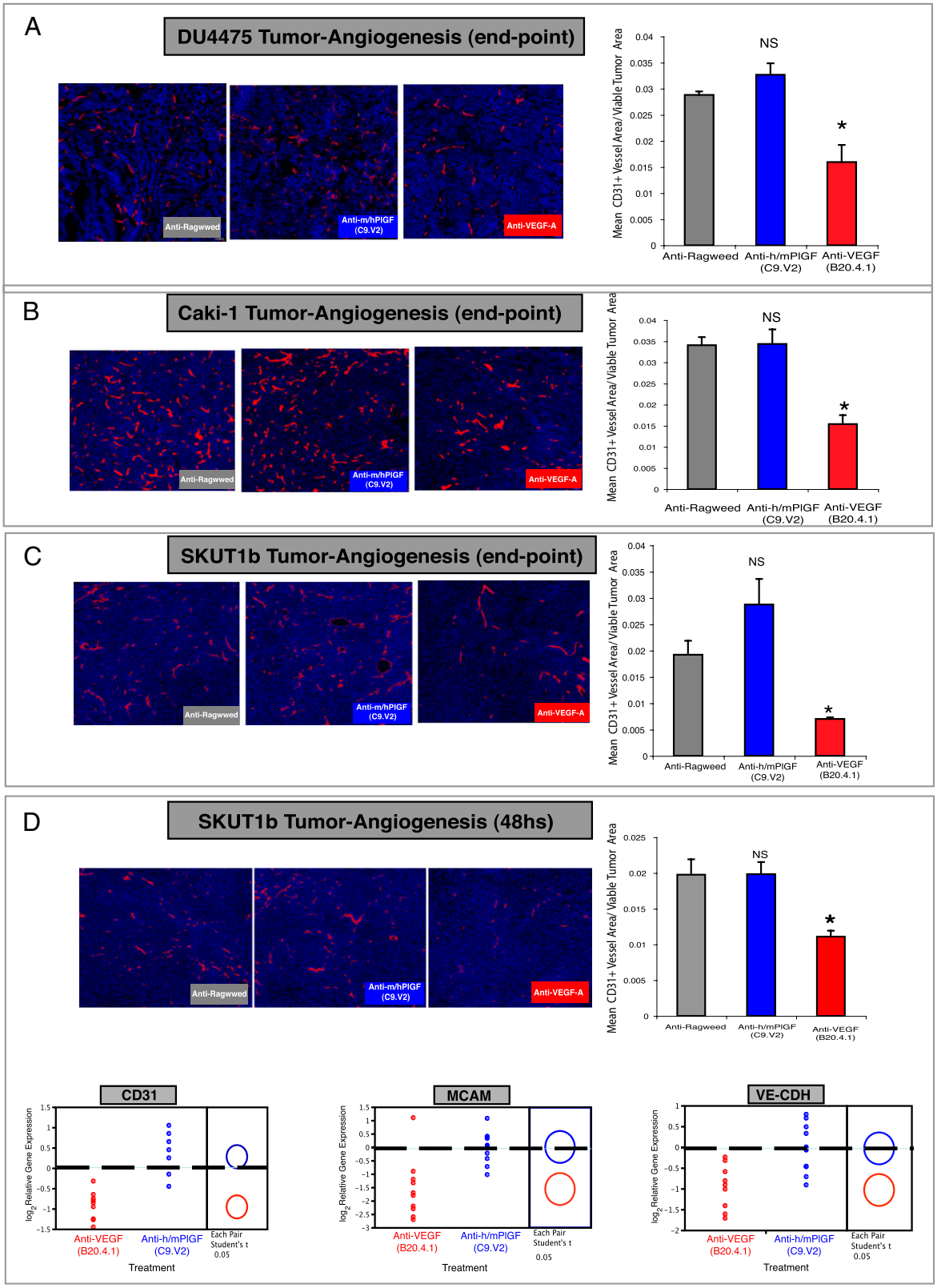

Fig. S3. Anti-PlGF efficacy is not correlated with antiangiogenesis. (*A–D*) Immunohistochemical analysis of MVD in tumor tissue. (*A–D*, *Left*). Representative high magnification (20×, 0.51 μm/pixel) fields from whole-slide tissue sections [vasculature, CD31 (in red) and nuclei, DAPI (in blue)]. (*A–D*, *Right*). Quantification of CD31 vascular area/total tumor (viable) area. Tissues were collected at the terminal time point, as indicated. DU4475 (d 30), SKUT1b (d32), 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-PlGF 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.

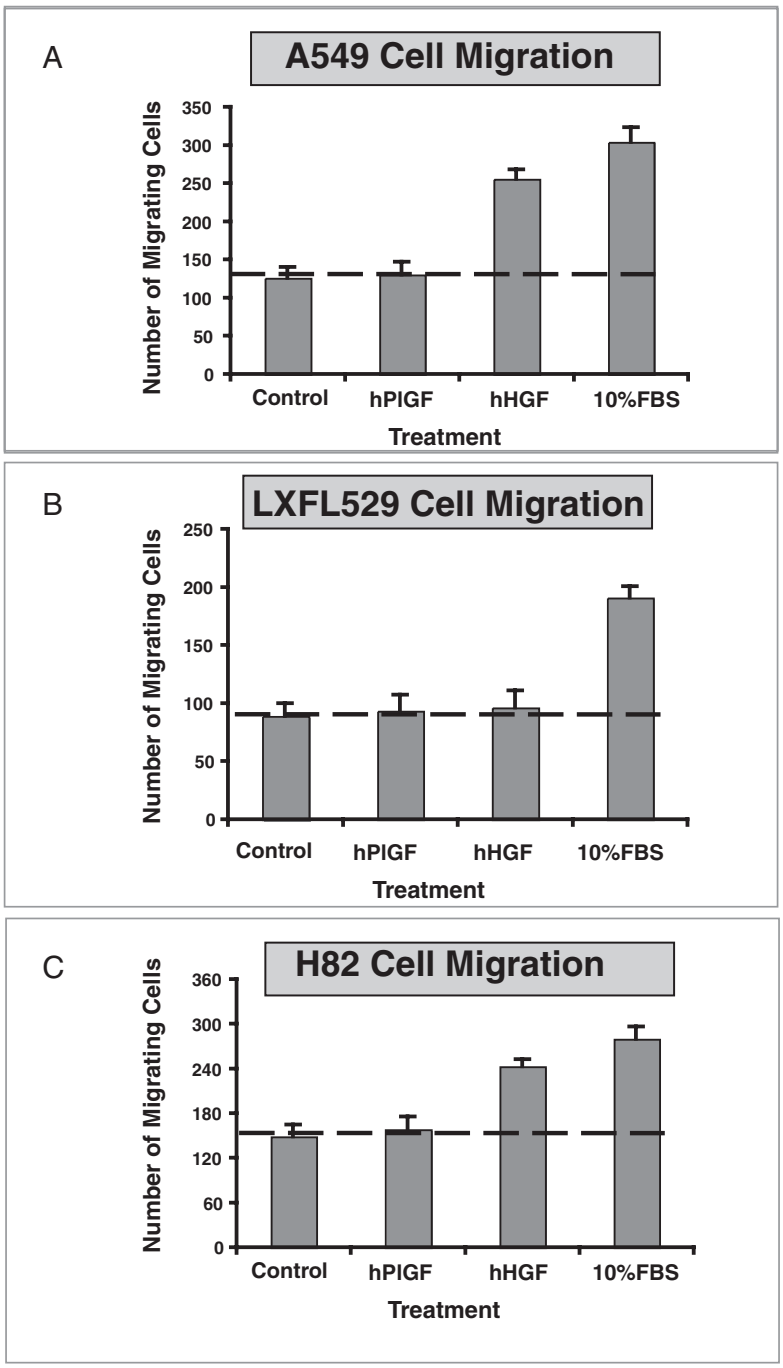

Fig. S4. PlGF does not induce migration in anti-PlGF (VEGFR-1 negative) refractory tumor cells. hPlGF-2 (50 ng/mL) does not induce migration of A549 (*A*), LFXFL529 (*B*), or H82 (*C*) VEGFR-1 negative tumor cells. In contrast, HGF (10 ng/mL) and/or FBS (10%) induce migration in these cell lines. Dotted lines represent basal (control) activity. Experiments were repeated at least three times with comparable results. n = 3–5, Error bars represent SD.

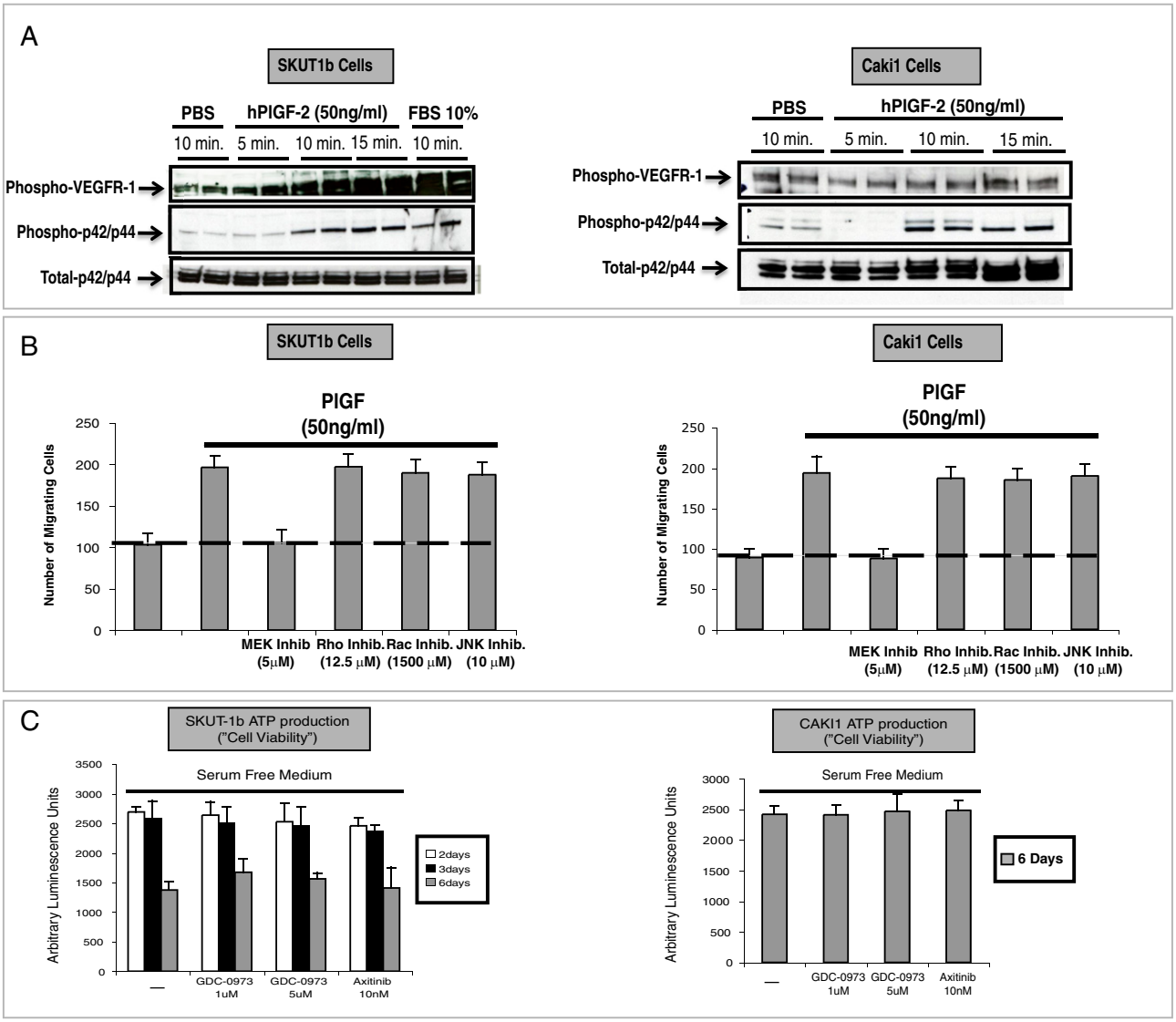

Fig. S5. hPlGF-2-induced responses in anti-PlGF sensitive cell lines require MAPK activation. (*A*) Time-course activation of phospho-VEGFR-1 and p42/p44 by PlGF stimulation in SKUT1b (*Left*) and Caki-1 cells (*Right*). (*B*) Effects of MEK inhibitor GDC-0973, Rho Inhibitor (EMD, catalog no. 555550), 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.

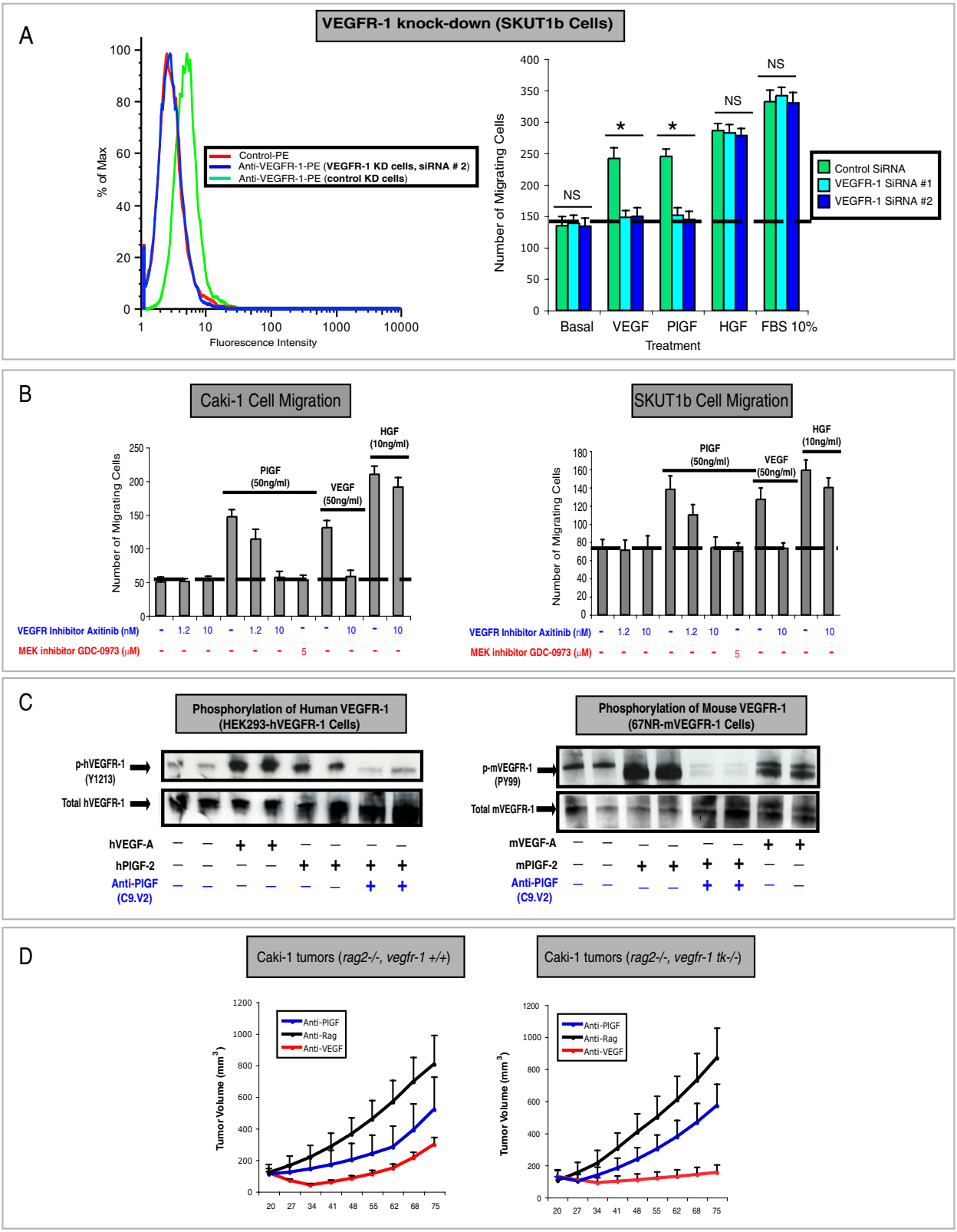

Fig. S6. Inhibition of PlGF/VEGFR-1 signaling in tumor but not stromal cells is a major determinant of anti-PlGF efficacy. (*A*, *Left*) Effects of VEGFR-1 siRNA #2 on VEGFR-1 membrane expression as measured by flow cytometry. (*A*, *Right*) Effect of VEGFR-1 knock-down (with VEGFR-1 siRNA #1 or 2) on 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.