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Cancer Research

HGF/c-Met Acts as an Alternative Angiogenic Pathway in Sunitinib-Resistant Tumors

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Abstract

Molecular and cellular mechanisms underlying resistance/low responsiveness to antiangiogenic compounds are under extensive investigations. Both populations of tumor and stroma (nontumor compartment) seem to contribute in inherent/acquired resistance to antiangiogenic therapy. Here, investigating *in vivo* efficacy of sunitinib in experimental models resulted in the identification of tumors that were resistant/sensitive to the therapy. Analysis of tumor protein lysates indicated a greater concentration of hepatocyte growth factor (HGF) in resistant tumors than in sensitive ones. In addition, using flow cytometry, c-Met expression was found to be significantly higher in endothelial cells than in tumor cells, suggesting that HGF might target the vascular endothelial cells in resistant tumors. Combination of sunitinib and a selective c-Met inhibitor significantly inhibited tumor growth compared with sunitinib or c-Met inhibitor alone in resistant tumors. Histology and *in vitro* analyses suggested that combination treatment mainly targeted the vasculature in the resistant tumors. Conversely, systemic injection of HGF in the sensitive tumor models conferred resistance to sunitinib through maintenance of tumor angiogenesis. In conclusion, our study indicates a role for HGF/c-Met pathway in development of resistance to antiangiogenic therapy and suggests a potential strategy to circumvent resistance to vascular endothelial growth factor receptor tyrosine kinase inhibitor in the clinic. *Cancer Res; 70(24); 10090–100. ©2010 AACR.*

Introduction

Members of vascular endothelial growth factor (VEGF) signaling pathway play a key role in both physiologic and pathologic angiogenesis; hence, recent Food and Drug Administration (FDA) approval of bevacizumab (an anti-VEGF monoclonal antibody) opened a new era in cancer therapy (1). In addition to blocking the ligand, several receptor tyrosine kinase inhibitors (RTKIs) targeting VEGF pathway such as sunitinib and sorafenib are also FDA approved or are being tested in different stages of clinical trials (2). However, despite showing efficacy and survival benefit in multiple treatment settings, the majority of patients will eventually exhibit disease progression while on therapeutic regimen. Molecular and cellular mechanisms responsible for disease progression and resistance are under extensive investigation. From angiogenesis standpoint, several studies have suggested that both tumor cells and stroma (nontumor compartment mainly consisting of fibroblasts, pericytes, myeloid cells, and mesenchymal stem cells) contribute to the development of resistance to antiangiogenic therapy through activation of alternative angiogenic factors such as FGF-2 [fibroblast growth factor (3)], Bv8 [*Bombina variegata* (4)], and PDGF [platelet-derived growth factor (5)].

The HGF/SF (hepatocyte growth factor/scatter factor)/c-Met pathway is known to play a significant role in different stages of development and also in tumorigenesis (6). Although HGF is enriched in cells of mesenchymal origin, c-Met is highly expressed in epithelial cells. In addition, c-Met is broadly expressed in several other cell types including endothelial cells, neural cells, hematopoietic cells, and pericytes. Expression of c-Met and HGF has also been identified in the vast majority of solid tumors (7). Activation of c-Met results in proliferation, survival, and also increased invasiveness of tumor cells through several other signaling pathways such as PIK3/Akt, Src, STAT3, and Ras/Mek (8, 9). In the vasculature, c-Met activation, through paracrine HGF, regulates tumor angiogenesis mainly by induction of proliferation, migration, and survival of endothelial cells resulting in vascular tubulogenesis and branching morphogenesis (10). HGF treatment in human umbilical vein endothelial cells (HUVECs) results in the phosphorylation of tyrosine residues in cytoplasmic domain of c-Met (11, 12). HGF promotes angiogenesis through upregulation of VEGF (a key angiogenic

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inducer) and also via downregulating thrombospondin-1 expression, (a potent angiogenic inhibitor (13). Similar to VEGF, expression of both c-Met and HGF is induced by hypoxia-inducible factor 1α, further providing a role for c-Met/HGF in adverse microenvironment conditions to assist angiogenesis, cell survival, and invasion (14, 15). Consistent with these observations, clinical findings indicate that activation of c-Met pathway is a poor prognostic factor in cancer patients (16–18). However, a full understanding of the role of HGF in tumor angiogenesis and particularly in mediating resistance to VEGF inhibitors remains to be determined.

In the present study, we tested the efficacy of sunitinib in preclinical models and identified tumors that were resistant or sensitive to the therapy. Analysis of protein lysates in the resistant or sensitive tumors found a greater expression for HGF in resistant tumors. Combination treatment using a highly selective c-Met inhibitor, PF-04217903 (19) and sunitinib, provided additive effect in inhibiting tumor growth compared with either a single agent. These results indicated a functional role for HGF/c-Met axis in the sunitinib-resistant tumors.

Materials and Methods

Tumor implantations and treatments

Nude mice were purchased from Jackson Laboratories (Bar Harbor, ME) or Charles Rivers Laboratories (Wilmington, MA) and were maintained under guidelines provided by the Pfizer IACUC (Institutional Animal Care and Use Committee). All the tumor cell lines (B16F1, EL4, LLC, and Tib6) in the current study were obtained from American Tissue Culture Collection (ATCC; Manassas, VA) and were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with glutamine (2 mmol/L) and fetal bovine serum (FBS; 10%). All the cell lines in the current study were authenticated by the supplier (20). For implantation, tumor cells (1×10^6 cells per mouse) were resuspended in 100 μL of media and 100 μL of matrigel growth factor reduced (BD BioSciences, San Jose, CA) and were subcutaneously implanted in one of the flanking areas. Tumor-bearing mice were treated once daily with sunitinib malate at 80 mg/kg or PF-04217903 (45 mg/kg) or the combination of both compounds, using oral route of administration. Tumors volumes were assessed using caliper measurement as described (21). HUVECs and C166 cells were purchased from Lonza Inc. (Lonza, CA) and ATCC, respectively. For *in vitro* assays, HUVECs were grown in EBM2 media supplemented with a cocktail of growth factors provided by the supplier (Lonza Inc.), and C166 were grown in DMEM (Invitrogen) supplemented with FBS (10%).

Histology

Formalin-fixed tumors were embedded in paraffin and were cut (4 μm) using a cryostat machine. Tumor sections were stained with mouse anti-CD31 antibody (BD BioSciences), using DAB immunostaining protocol in the laboratory. To quantify vascular surface area (VSA), images of tumors at 20× magnification were taken from each slide and each treatment. Next, areas of $CD31^+$ cells were highlighted and pixels were quantified using Image-Pro software (MediaCybernetics, Bethesda, MD). Vascular density was calculated by dividing the CD31 pixels to the pixels from total area of the image.

ELISA

Tumors samples were snap frozen in liquid nitrogen and were kept at −70°C. Total protein from each sample was extracted and was quantified using bicinchoninic acid protein assay (Pierce, Rockford, IL). Levels of HGF and total Met in the tumors and serums were measured by ELISA kits (R&D System, San Jose, CA), using protocols provided by the manufacturer. Similar methodology was applied to measure levels of HGF in the conditioned media (CM) or in the tumor lysates.

Flow cytometry

Single-cell suspensions were provided from the tumors by mechanical disruption, followed by RBC lysis using RBC lysis buffer (eBioscience, San Diego, CA). Peripheral blood mononuclear cells (PBMNCs) were also provided by lysing RBCs using RBC lysis buffer (eBioscience). Both tumor cells and PBMNCs were stained with anti-mouse CD11b-APC (BD Biosciences) and anti-mouse Gr1-PE (BD Biosciences) for 30 minutes at 4°C, followed by washing twice with PBS-FBS (3%). The stained samples were analyzed in a FACSCalibur machine (BD Bioscience) and frequency of $CD11b^+Gr1^+$ cells in the tumors and blood were analyzed using Cell Quest (BD Biosciences) or FCS Express (De Novo Inc, Lake Forest, CA) software. Similarly, to measure c-Met expression at the cell surface, *in vitro*–grown cell lines and endothelial cells were stained with PE-conjugated anti-mouse c-Met (eBioscience). To exclude dead cells from the analysis, cells were stained with 7-amino-actinomycin D (BD Biosciences) prior to analysis on a calibur machine.

In vitro assays

Cell lines, including B16F1, Tib6, EL4, and LLC, and endothelial cells, HUVECs and C166, were seeded at 10^4 cells in each well of 24-well tissue culture–treated plates. Cells were grown in the standard media as described earlier. Cells were treated with different concentrations (2, 0.2, and 0.02 μmol/L) of sunitinib, PF-04217903, and combination of both compounds for 4 days. Efficacy of the compounds was measured by counting cells in a Coulter counter machine (BD Biosciences). Similar approach was applied to evaluate the role of HGF or VEGF on cell proliferation, using 3 different concentrations (10, 100, and 200 ng/mL) of each ligand.

HGF purification

For HGF expression and purification, S114 cells [NIH3T3 cells transfected with the human *HGF* and Met (22)] were first cultured in suspension in PFHM II medium (Invitrogen) supplemented with 2.5% FBS to a density of 2×106/mL. The cells were then harvested and washed once with PFHM II medium and resuspended in PFHM II at 0.5×10^6/mL to culture for 96 hours. Next, the supernatant was collected from the growing cultures (1.6×10^6/mL with 95% viability) by centrifugation and was concentrated 26-fold in a Sartocon slice cassette (Sartorius Stedim Biotech, Aubagne, France)

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with a 10-kD cutoff in a cross-flow system. Concentrated supernatant was filtered and applied to a HiPrep 16/10 Heparin FF column (Pharmacia) equilibrated with PBS (pH = 7.4). The unbound materials were depleted by washing the column with PBS twice. HGF was then eluted with a linear gradient of NaCl (0.3–2 mol/L in 1× PBS, 10× column volume) at a flow rate of 1 mL/min. Eluted fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot, using anti-α-HGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA). HGF-containing fractions were pooled and concentrated to about 1 mg/mL with an Amicon centrifugal filter device. Levels of endotoxin in the eluted HGF/SF were reduced to less than 5 EU/mg and the purified HGF was stored at −80°C.

Results

Modes of tumor response to sunitinib in experimental models

Several studies have extensively investigated mechanisms of tumors un/low responsiveness to antiangiogenic therapy in preclinical tumor models. Recent investigation identified populations of murine cell lines that are sensitive/refractory to treatment with anti-VEGF monoclonal antibody (mAb) (23). It was also documented that response to an anti-VEGF mAb is independent of functional B or T cells, as a similar profile of responsiveness to treatment was observed in immunodeficient mice. To focus our efforts on tumor responsiveness to small molecule inhibitors, we sought to investigate sunitinib efficacy in several murine tumor models including B16F1 (melanoma), Tib6 (plasmacytoma), EL4 (lymphoblastic), and LLC (Lewis lung carcinoma) tumors. These studies were initiated in immunodeficient mice (nu/nu) to minimize role of a particular strain of mouse in tumor growth and progression. We administered sunitinib malate at 80 mg/kg, which is projected to result in inhibition of VEGFR and RTK target coverage comparable with clinically utilized dose regimens at 37.5 or 50 mg every day.

As illustrated in Figure 1, although B16F1 (Fig. 1A) and Tib6 (Fig. 1B) tumors were sensitive to sunitinib, EL4 (Fig. 1C) and LLC (Fig. 1D) tumors exhibited resistant to the therapy shortly after the initiation of treatment. These results are in agreement with previous reports investigating efficacy of an anti-VEGF mAb in the same cell lines (23, 24), indicating a similar trend in tumor responsiveness in anti-VEGF versus small molecule inhibitors of VEGF pathway.

In addition to VEGF pathway, several other signaling pathways, such as PDGF-RB receptor, CSF-1R (colony stimulating factor), and c-kit pathways (25), are targeted by sunitinib. To understand whether the inhibition of these additional pathways by sunitinib plays a role in responsiveness to the therapy, we compared efficacy of sunitinib versus axitinib [a selective VEGF inhibitor (26)] in resistant tumors (Fig. 1E and F). Interestingly, sunitinib and axitinib demonstrated a statistically indistinguishable effect on tumor growth in both EL4 (Fig. 1E) and LLC (Fig. 1F), as neither tumor responded to the therapies. Similarly, both sensitive tumors were nearly equally responsive to axitinib and sunitinib (Supplementary Fig. 1A and B). Therefore, with the exception of VEGF, which is targeted by both sunitinib and axitinib, other sunitinib-targeted pathways such as PDGF-RB, CSF-1R, and c-kit do not appear to contribute in tumors response to these RTKIs in the models utilized in the current study. Overall, these efforts identified differential response to sunitinib among several murine lines and provided a reason to investigate alternative angiogenic pathway(s) in the resistant tumors to apply combination treatment.

HGF is enriched in the stroma in resistant tumors and mainly targets endothelial cells

We decided to further focus on the mechanisms of resistance to sunitinib as it is in further advanced stages of clinical trials compared to axitinib (27). Given the significance of c-Met in tumor progression (explained in the Introduction section), we fully concentrated our efforts to understand whether HGF/c-Met plays a role in the development of resistance to sunitinib. To provide a rationale to pursue the role of the c-Met pathway in resistant tumors, we first measured levels of HGF in both sera and tumors in all the models. ELISA data showed a gradual reduction in HGF concentration in the serum from non–tumor-bearing mice to all tumor-bearing mice, suggesting that HGF is highly concentrated in the tumors and is not extensively released to the blood circulation (Fig. 2A). This is consistent with another proangiogenic factor, PDGF, which is highly enriched in the tumors but is minimally released into the blood circulation (24). HGF levels in the tumors (Fig. 2B), however, were significantly ($P < 0.05$) higher in resistant tumors than in sensitive ones, particularly in the sunitinib-treated mice. These data indicate that greater concentration of HGF might be associated with resistance to sunitinib. To identify source of HGF in the tumors, we measured levels of HGF protein in all the cell lines as well as endothelial cells, HUVECs and C166, a mouse yolk sac endothelial cells (28), in both normoxic (20% O_2) and hypoxic conditions (1% O_2). Our data demonstrated that irrespective of oxygen concentration, neither tumor nor endothelial cells were enriched in HGF, suggesting that stromal compartment might be a major source of HGF in the tumor mass (Fig. 2C). Interestingly, and consistent with human fibroblast cell lines [Mrc5 (29), NIH3T3 cells found to be highly enriched in HGF in both protein lysates and CM. An increase in HGF levels in NIH3T3-CM compared with NIH3T3-lysate may indicate that extracellular HGF might be a main determinant of HGF concentration and activity in the tumors. HGF expression in the tumor stroma is also consistent with histologic analyses in several studies, indicating localization of HGF to stromal cells in multiple tumor types (30, 31).

As of one of the major components of stroma, hematopoietic cells (originating from the bone marrow) are a major source of proangiogenic factors such as Bv8 (4). To determine whether BMMNCs are enriched for HGF, we isolated these cells from naive as well as from tumor-bearing mice and further fractionated them into subsets of CD11b-enriched and CD11b-depleted cells with high purity (Supplementary

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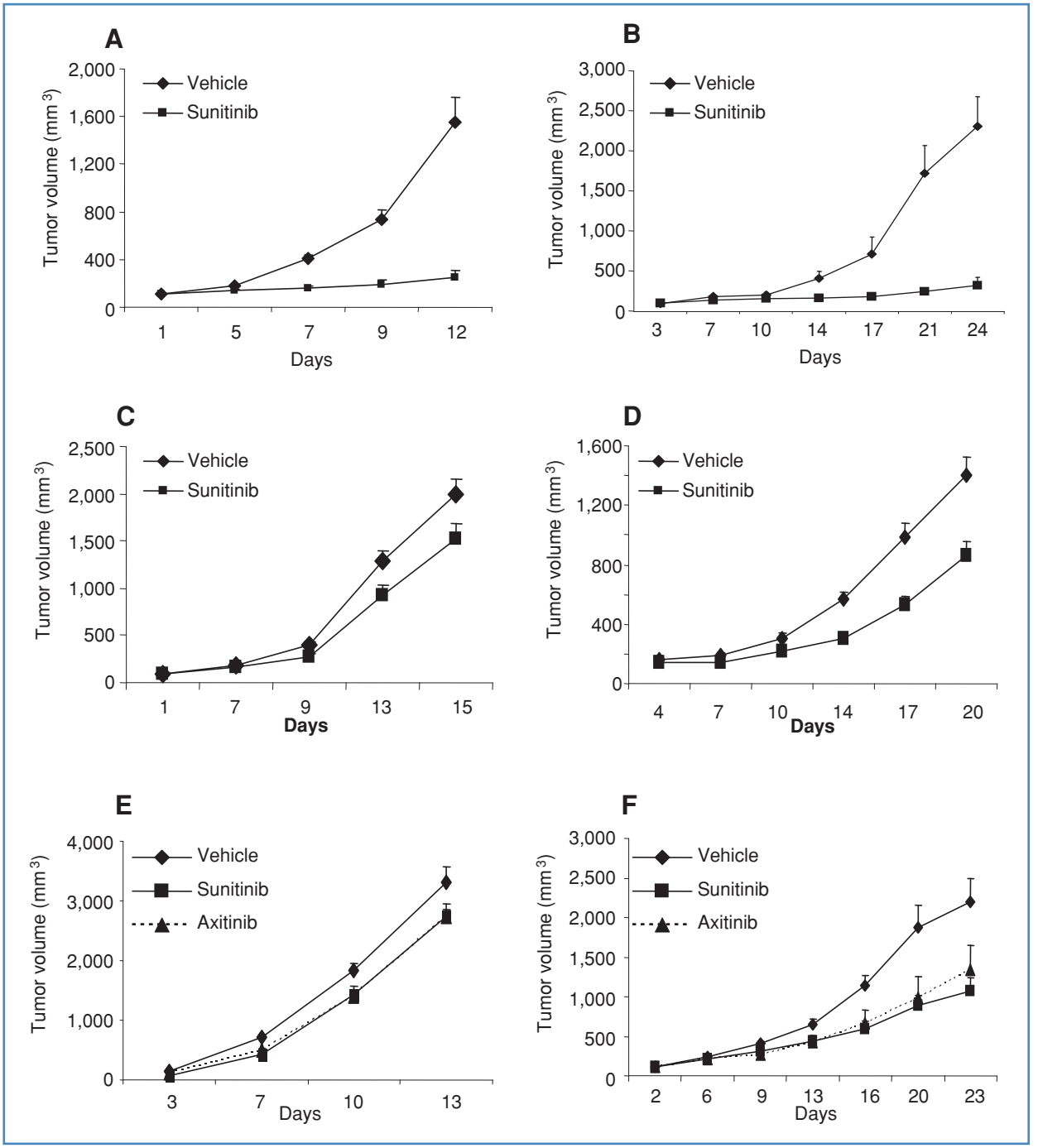

Figure 1. Identification of cell lines resistant or sensitive to sunitinib treatment. A–D, nude mice ($n = 6$) were implanted with murine lines including B16F1 (A), Tib6 (B), EL4 (C), and LLC (D). Cells were implanted in the recipients as described, and treatment with sunitinib (80 mg/kg) or vehicle started the day after implantation. Data are representative of 1 of 2 independent studies. E and F, Inhibition of PDGF-R, CSF-1R, and c-kit pathways does not provide any advantage in tumor growth inhibition compared with inhibition of VEGF alone. Nude mice ($n = 10$) were implanted with EL4 (E) or LLC (F) tumors as described. Treatment with vehicle, sunitinib (80 mg/kg once a day), or axitinib (35 mg/kg twice a day) started 1 day after implantation.

Fig. 2). Interestingly, HGF level was significantly higher in the BMMNCs (Fig. 2C) versus tumor or endothelial cells (Fig. 2A and B); however, ELISA data did not reveal a clear trend in sensitive or resistant tumors in either CD11b-enriched or -depleted population, suggesting that both subsets may act as source of HGF in the tumors.

To identify whether tumor or endothelial cells are the main targets for HGF, we assessed receptor expression in

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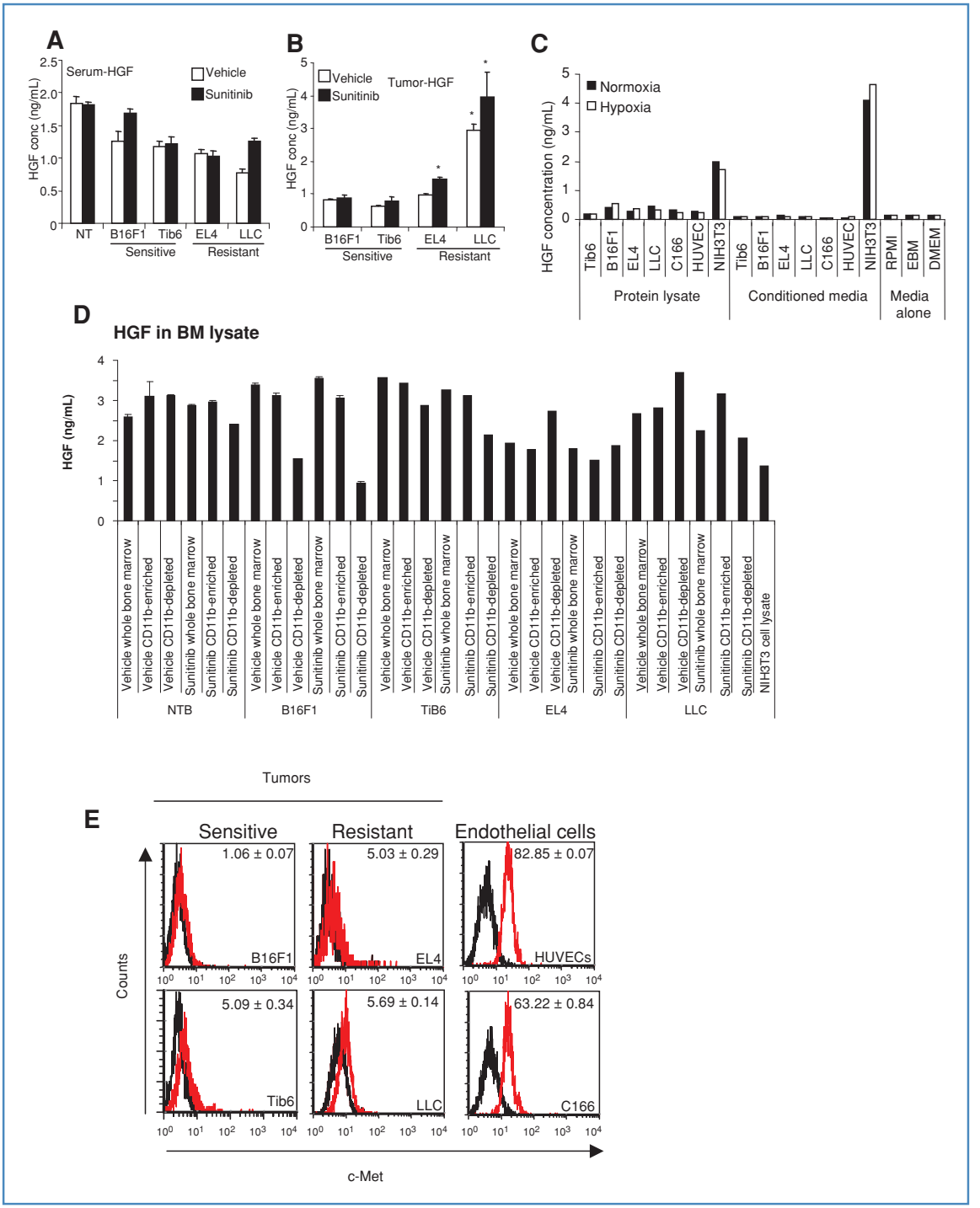

Figure 2. Endothelial cells, but not tumor cells, are mainly targeted by HGF/c-Met axis. A and B, HGF measurement in the sera (A) and tumors (B) in both vehicle- and sunitinib-treated tumors. Using ELISA, levels of HGF in serum and tumor samples were quantified as described in the Materials and Methods section. Bars represent mean ± SEM concentration of HGF in sunitinib- or vehicle-treated tumors. C, HGF is mainly expressed in stromal compartment in the tumor mass. Both resistant and sensitive cell lines as well as endothelial cells (HUVECs and C166) and NIH3T3 (positive control for HGF expression) were incubated in either normoxic (20% O_2) or hypoxic (1% O_2) conditions and HGF was measured in the lysate and condition media as explained in the Materials and Methods section. D, bone marrow (BM) cells are enriched in HGF. Nude mice were implanted with sensitive or resistant tumors and were treated with vehicle or sunitinib as described. Levels of HGF in CD11b-enriched and CD11b-depleted subpopulation were measured using ELISA. E, c-Met is mainly expressed in endothelial cells. Using flow cytometry, expression of c-Met at the cell surface in both tumor cells and endothelial cells (HUVECs and C166) was investigated. Images are the representative histograms from each line comparing c-Met expression (red line) versus isotope control (black line).

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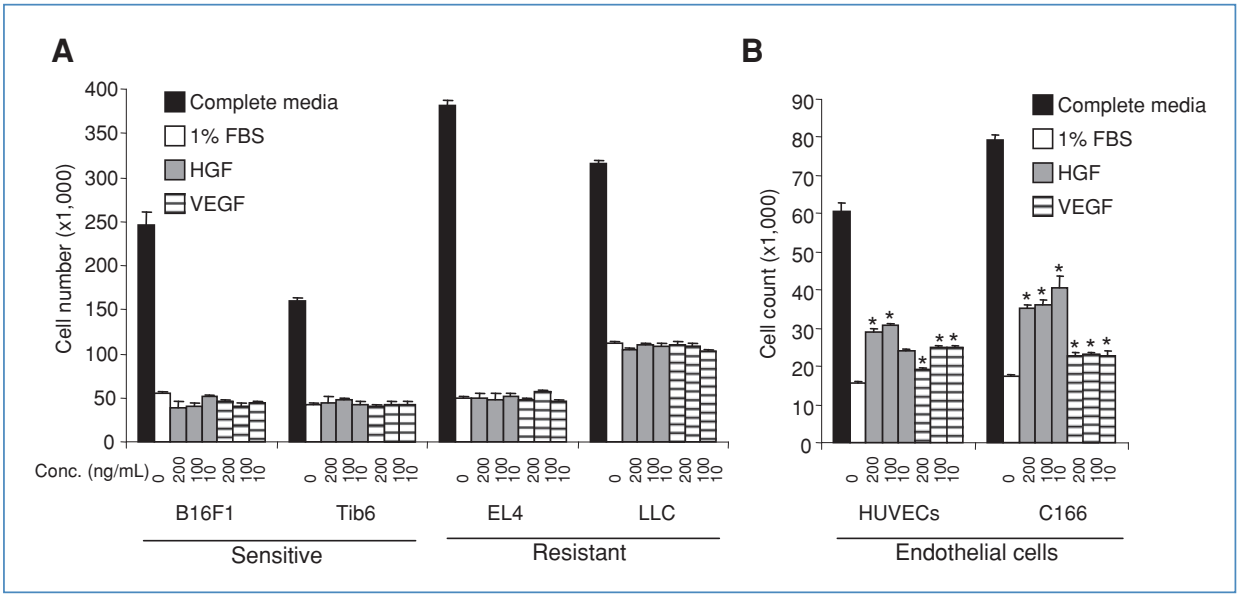

Figure 3. Proliferation of endothelial cells, but not tumor cells, is induced by HGF. Tumor cells, B16F1, Tib6, EL4, and LLC (A), and endothelial cells, HUVECs and C166 (B), were seeded at 10^4 in each well of 24-well tissue culture–treated plates and were grown in plain media supplemented with 1% FBS. Cells were treated with 3 different concentrations (10, 100, and 200 ng/mL) of HGF and VEGF. After 4 days of incubation, cells were counted using a Coulter counter machine. Asterisks are indicative of a significant difference in HGF- or VEGF-treated cells versus PBS.

both cell types. Flow cytometry analysis indicated that although tumor cells express minimal levels of c-Met at the cell surface, endothelial cells showed greater expression of c-Met ($P < 0.05$), indicating that vasculature might be the major target for HGF in the tumor mass (Fig. 2D). To provide functional relevance for greater expression of c-Met, we investigated tumor/endothelial cell proliferation. *In vitro* treatment of endothelial and tumor cells with different concentrations of HGF and VEGF showed that neither molecule affected cellular proliferation in the tumor cells (Fig. 3A). Conversely, both growth factors promoted proliferation of endothelial cells, providing additional rationale that the vasculature might be main a target of HGF in the tumors (Fig. 3B).

Combination of sunitinib and PF-04217903 has additive effect in growth inhibition in the resistant tumors

The *in vitro* analysis of tumor and endothelial cells provided a strong rationale to test *in vivo* efficacy of combination treatment, using sunitinib and PF-04217903 [a highly selective c-Met inhibitor (19)], in the resistant tumors. As illustrated in Figure 4A and B, PF-04217903 monotherapy did not inhibit tumor growth in any of the sensitive tumors, indicating a lack of activation of c-Met pathway in either vasculature or tumor cell compartments in these models. Furthermore, there was no significant difference in tumor growth in the combination treatment versus sunitinib monotherapy in B16F1- (Fig. 4A) and Tib6 (Fig. 4B)-sensitive tumors, suggesting a lack of contribution of c-Met pathway in these tumors. However, growth of both resistant tumors (EL4, Fig 4C; LLC, Fig. 4D) was significantly ($P < 0.05$) inhibited by the combination regimen compared with either single-agent regimen, indicating a role for c-Met activation, possibly through paracrine HGF, in these tumors. In addition, combination therapy was efficacious in resistant tumors when treatment started in established tumors (data not shown).

To understand the mechanism of tumor growth inhibition for the combination regimen, vasculature was analyzed in both sensitive and resistant tumors (Fig. 5A). In the sensitive tumors, sunitinib significantly ($P < 0.05$) reduced the vascular density, as measured by CD31-positive areas, compared with the vehicle-treated tumors. However, combination treatment did not change vascular density in sunitinib as single agent versus the combination regimen. PF-04217903 monotherapy did not significantly alter the vasculature in either sensitive or resistant tumors, further signifying the role of VEGF in promoting tumor growth and angiogenesis. In the resistant tumors, sunitinib inhibited the vascular expansion compared with the vehicle-treated tumors. In addition, combination therapy significantly reduced VSAs compared with sunitinib alone in the resistant tumors. To further investigate histologic findings, we treated all the cell lines as well as endothelial cells with different concentrations of sunitinib, PF-04217903, or the combination of both compounds *in vitro*. As illustrated in Figure 5B, although sunitinib and the combination significantly inhibited endothelial cells proliferation at pharmacologically relevant concentrations (0.2 and 0.02 μmol/L), tumor cells were not affected by either the agent or the combination (Fig. 5C). Together, histologic and *in vitro* data suggest that targeting the vasculature is one of the mechanisms by which combination treatment inhibits growth of resistant tumors.

We also studied kinetics of myeloid cells, identified by the expression of CD11b and Gr1 (32), in both resistant and sensitive tumors, as recent studies have suggested a role

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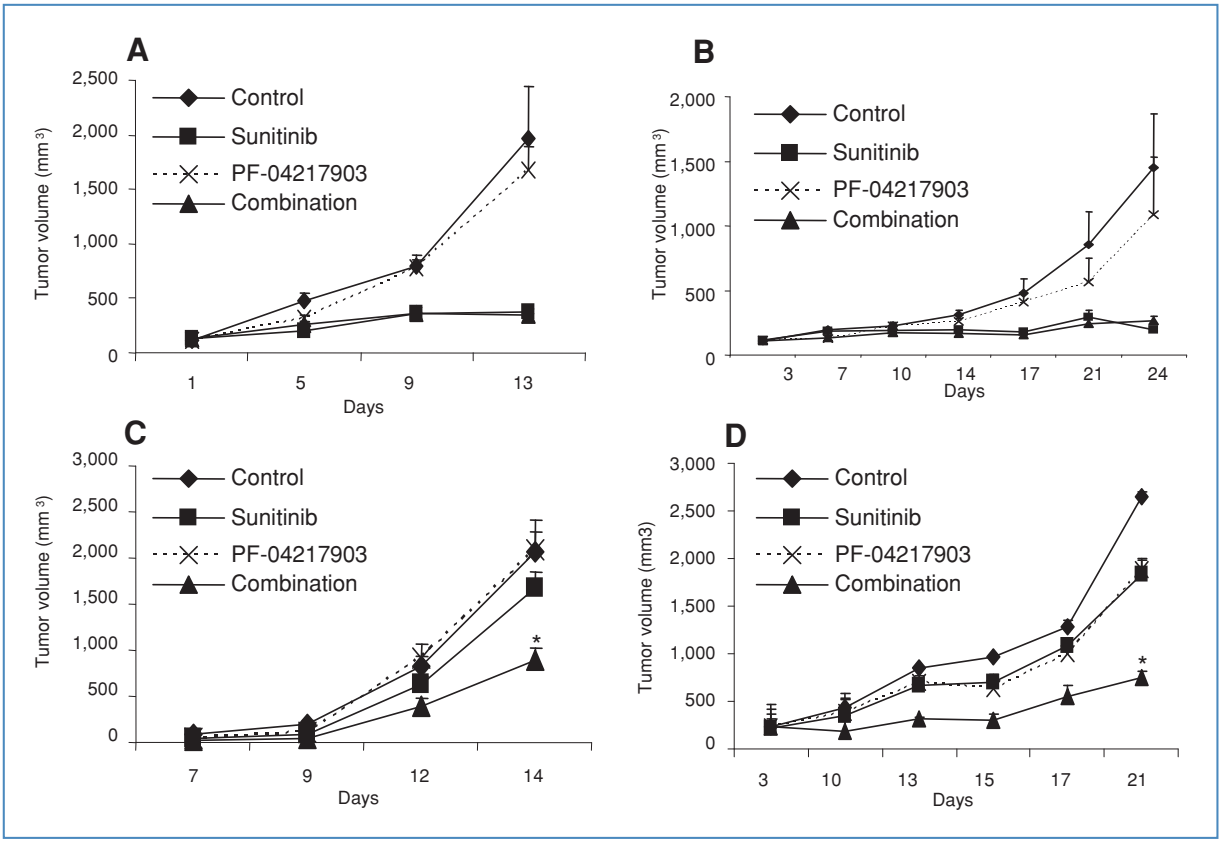

Figure 4. Combination of sunitinib and PF-04217903 has additive effect compared with sunitinib monotherapy. Efficacy of combination treatment (sunitinib plus PF-04217903) in sensitive or resistant tumors. Nude mice ($n = 6$) were implanted with B16F1 (A), Tib6 (B), EL4 (C), and LLC (D) cells (1×10^6 cells per mouse). Treatments started a day after implantation, and tumors volumes were measured twice a week. Data are representative of 1 of 2 independent studies. Graphs represent mean tumor volumes, and bars represent SEM. Asterisks indicate significant difference ($P < 0.05$) when comparing sunitinib versus combination treatment.

for these cells in mediating refractoriness to VEGF inhibitors (33). Myeloid cells have shown to be enriched in proangiogenic factors such as Bv8, which promote tumor growth through induction of both local angiogenesis and mobilization of myeloid cells to the tumors. Therefore, it is important to know whether treatment with sunitinib or combination affects kinetics of myeloid cells in the tumors utilized in the current study. Analysis of tumor-associated myeloid cells indicated a greater ($P < 0.05$) frequency of these cells in the resistant tumors than in the sensitive ones (Fig. 5D). This is in agreement with previous reports, suggesting that resistant tumors instruct the bone marrow to induce proliferation, mobilization, and homing of myeloid cells to the tumors (23). Interestingly, sunitinib seems to increase the frequency of tumor-associated myeloid cells in both resistant tumors. Therefore, greater frequency of myeloid cells may result in an increase in HGF concentration in the tumor microenvironment. However, PF-04217903 alone or in combination with sunitinib did not alter myeloid cell infiltration to the tumors, indicating that HGF may not play a significant role in the kinetics of these cells in the resistant tumors. Combined, our data suggest that HGF/c-Met pathway may directly target the vasculature in the resistant tumors.

HGF upregulation confers resistance to sunitinib in the sensitive tumors

To further recapitulate the role of c-Met pathway in development of resistance to sunitinib, and using a gain-of-function approach, we hypothesized that HGF upregulation in sensitive tumors confers resistance to sunitinib. Therefore, we treated mice bearing sensitive tumors with systematic (intraperitoneal route) injection of purified HGF (Fig. 6A and B). Treatment with sunitinib started 4 days after implantation to allow sufficient exposure of tumor/endothelial cells to HGF, leading to the activation of c-Met pathway. Similar to previous data (Figs. 1A and B and 4A and B), both B16F1 or Tib6 tumors in mice that did not receive HGF were sensitive to sunitinib treatment (Fig. 6A and B). However, the B16F1 or Tib6 tumors treated with HGF did not respond to sunitinib and showed a significant increase ($P < 0.05$) in tumor volume compared with the corresponding sunitinib-treated population. To understand the mechanism of tumor growth promotion in the sunitinib-treated tumors, we examined the vasculature in all the treatments. Analysis of sections of tumors stained with CD31 indicated an increased vascular density ($P < 0.05$) in the sunitinib-treated tumors cotreated with HGF versus tumors that did not receive HGF

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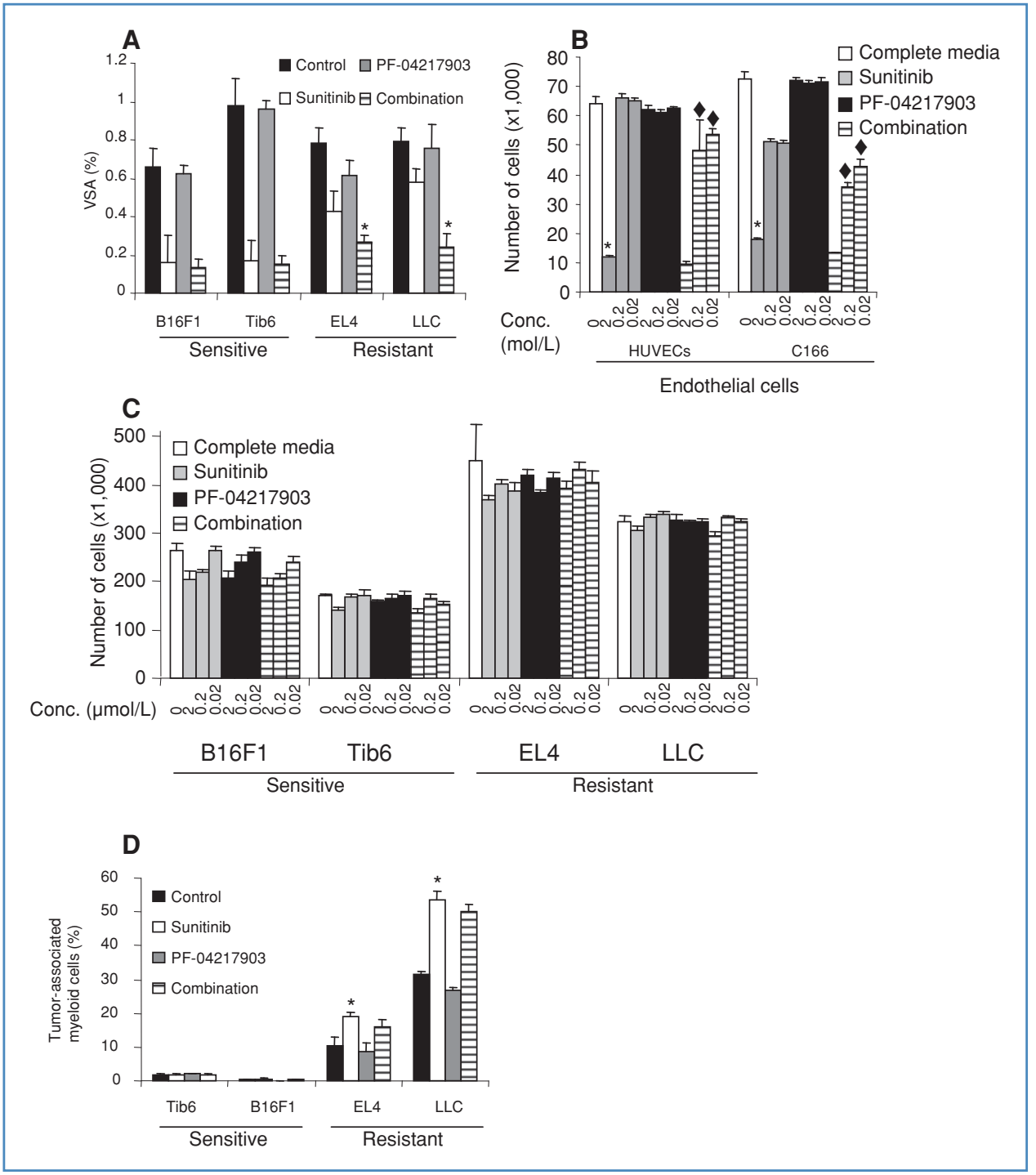

Figure 5. Inhibition of angiogenesis is one of the mechanisms by which combination treatment affects tumor growth. A, vascular quantification in the resistant or sensitive tumors. Images of CD31 staining were selected from each section (4 images from each section and up to 24 images in total), and vascular density was calculated as described. VSA, vascular surface area. B and C, endothelial cells, but not tumor cells, are sensitive to combination treatment using sunitinib and PF-04217903. Tumor or endothelial cells treated with sunitinib, PF-04217903, or the combination of both compounds and were incubated in an incubator for 4 days. Cell proliferation was assessed by counting content of each well in a Coulter counter machine as described. D, kinetics of myeloid cells in the tumors. Nude mice were implanted with sensitive or resistant tumors and were treated with vehicle, sunitinib, PF-04217903, or combination as described. At terminal analysis, tumors were isolated and were stained with anti-mouse CD11b-APC and anti-Gr1-PE antibodies and frequency of myeloid cells was measured in a FACS calibur. Asterisks mean significant difference ($P < 0.05$) in the frequency of myeloid cells in the vehicle versus sunitinib-treated tumors.

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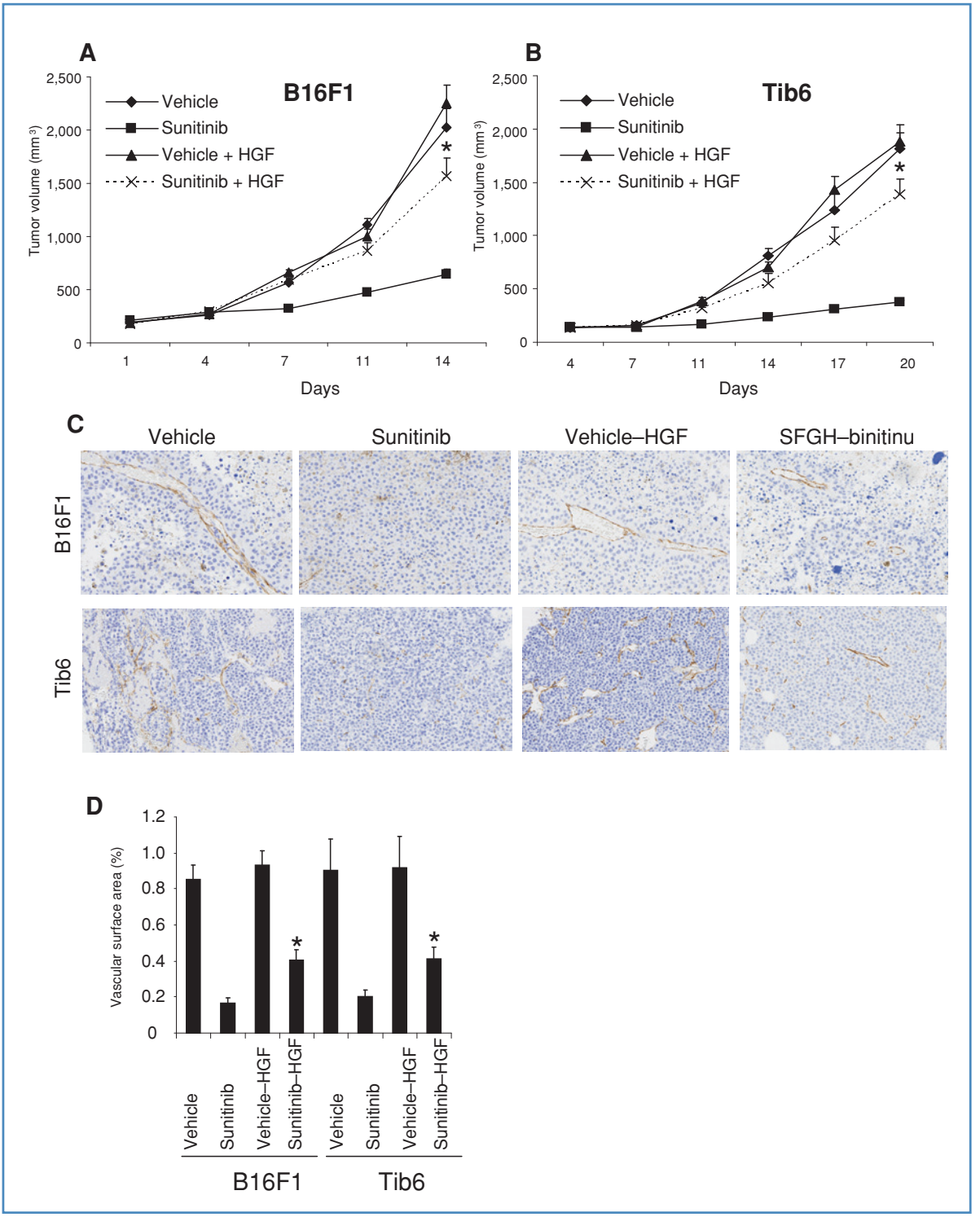

Figure 6. HGF treatment in sensitive tumors confers resistance to sunitinib. Nude mice ($n = 10$) were implanted with B16F1 (A) or Tib6 cells (B). Each mouse was implanted with 1×10^6 cells in a mixture of 100 μL of media and 100 μL of growth factor–reduced matrigel. Treatment with sunitinib (80 mg/kg; once a day) started at day 5 postimplantation. Treatment with HGF (80 mg/kg; intraperitoneal) started at day 1 postimplantation and continued on alternative days in the assigned groups. Graphs represent mean ± SEM tumor volumes. C, sections of tumors obtained from all the treatments were cut and were stained with rat anti-mouse CD31 antibody as described. Representative images show tumor vasculature in each treatment. D, vascular density was measured in each treatment to assess whether resistance to sunitinib is associated with the development of new vasculature. Bars represent mean ± SEM of the vascular surface area. Asterisks indicate significant difference ($P < 0.05$) when comparing sunitinib alone versus sunitinib-treated tumors in mice that received HGF.

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(Fig. 6C and D). Combined, these data further confirm a role for HGF/c-Met pathway in development of resistance to sunitinib via targeting the vasculature.

Discussion

Agents targeting tumor angiogenesis, particularly VEGF inhibitors, have provided new cancer treatment options. Similar to other anticancer agents, tumor recurrence has been one of the major challenges in patients treated with angiogenesis inhibitors. Recent reports in preclinical models suggest that activation of alternative angiogenic pathways in anti-VEGF–treated tumors is one of the mechanisms of unresponsiveness to the therapy (34). In addition, 2 recent independent studies suggested that antiangiogenic therapy may induce tumor progression and metastasis due to increased invasiveness of tumor cells (35, 36). Overall, these observations have emphasized further investigations to optimize application of this class of agents in the clinical setting.

Here, we investigated modes of resistance to sunitinib, which is clinically approved for metastatic renal cell carcinoma and imatinib-resistant or intolerant gastrointestinal stromal tumor and is in several trials for other tumor types including lung cancers (37, 38). Sunitinib efficacy in murine lines in the current study, and in comparison with recent reports (23, 24), indicates that blocking the receptor-mediated signaling is as efficacious as blocking the ligand in inhibiting tumor growth. Future studies may determine whether there are distinct indications in preclinical models in blocking the VEGF pathway versus inhibiting the ligand.

We focused our investigations on the c-Met pathway, as several studies have pointed a significant role for this pathway in development, tumorigenesis, and angiogenesis. In general, c-Met pathway can be activated through: i) paracrine or autocrine HGF, ii) amplification of c-Met gene locus, and iii) activating mutations in the kinase domain of the c-Met receptor (37, 38). Of note, our data in the present study suggest that c-Met is activated through binding of HGF, as none of the cell lines exhibited a notable response to different concentrations of c-Met inhibitor *in vitro* or *in vivo*. Therefore, in our models, greater concentration of HGF and/or proximal interaction of tumor and stromal cells in a relatively hypoxic microenvironment are critical for the activation of c-Met pathway in intact tumors.

Lack of HGF expression in tumor or endothelial cells signifies a role of stroma in the resistant tumors as a source of HGF. Of the tumor stroma compartments, BMMNCs was found to be an enriched source of HGF, though there was no significant difference in levels of HGF in non–tumor-bearing mice versus tumor-bearing ones. There has been extensive focus on the role of stromal cells in mediating resistance to angiogenic inhibitors in preclinical models. For example, proangiogenic factors, such as Bv8 (4, 39), PDGF-C (40), FGF-2 (3), and PlGF (5), have been identified in different stromal compartment including myeloid cells and tumor-associated fibroblasts. Therefore, in addition to Bv8, HGF is also enriched in the BMMNCs. Furthermore, flow cytometry data indicated a significant increase in the frequency of $CD11b^{+}Gr1^{+}$ cells in the resistant tumors, which is consistent with previous reports that indicate an increase not only in the frequency but also in the total number of these cells in the resistant tumors (24). Therefore, we hypothesize that even though there is marginal difference in HGF level in the BMMNCs isolated from resistant or sensitive tumors, greater infiltration of BMMNCs to the resistant tumors provides more HGF to the vasculature than that in sensitive ones (23). In addition, we observed a significant difference in the levels of secreted versus intracellular HGF in NIH3T3 cells and therefore intracellular HGF may not be a true representative of its bioavailability in the tumor microenvironment. Future studies will test levels of secreted HGF in BMMNCs isolated from resistant versus sensitive tumors. Interestingly, the *in vitro* data indicate that although both tumor and endothelial cells are exposed to HGF, only the latter is capable of proliferation, providing a hypothesis that angiogenesis is the main target of HGF in these tumors. Assessment of myeloid population further confirms a direct angiogenic role for HGF, as neither c-Met inhibition nor HGF upregulation in the sensitive tumors affected kinetics of myeloid cells in the peripheral blood or in the tumors.

Overall, the current investigation unveils yet another molecular mechanism of resistance to VEGF inhibitors. Using a loss of function (inhibition of c-Met pathway in the resistant tumors) and a gain of function (upregulation of HGF in the sensitive tumors) approach indicate a role for HGF in tumors resistance to sunitinib. Future studies will determine whether there is clinical application using combination treatment to circumvent lack of response to VEGF RTKIs in cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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