

Targeting oncogenic serine/threonine-protein kinase BRAF in cancer cells inhibits angiogenesis and abrogates hypoxia

Alessia Bottos^{a,b,1,2}, Miriam Martini^{b,c,1,3}, Federica Di Nicolantonio^{c,d}, Valentina Comunanza^{a,b}, Federica Maione^{b,e}, Alberto Minassi^f, Giovanni Appendino^f, Federico Bussolino^{a,b,4,5}, and Alberto Bardelli^{b,c,d,4,5}

^aLaboratory of Vascular Oncology, Institute for Cancer Research and Treatment, 10060 Turin, Italy; ^bDepartment of Oncological Sciences, University of Torino, 10060 Turin, Italy; ^cLaboratory of Molecular Genetics, Institute for Cancer Research and Treatment, 10060 Turin, Italy; ^dFondazione Italiana per la Ricerca sul Cancro Institute of Molecular Oncology, 20139 Milan, Italy; ^eLaboratory of Transgenic Mouse Models, Institute for Cancer Research and Treatment, 10060 Turin, Italy; and ^fDipartimento di Scienze Chimiche, Alimentari, Farmaceutiche e Farmacologiche, Università del Piemonte Orientale, 28100, Novara, Italy

Edited* by Napoleone Ferrara, Genentech, South San Francisco, CA, and approved November 18, 2011 (received for review April 1, 2011)

Carcinomas are comprised of transformed epithelial cells that are supported in their growth by a dedicated neovasculature. How the genetic milieu of the epithelial compartment influences tumor angiogenesis is largely unexplored. Drugs targeted to mutant cancer genes may act not only on tumor cells but also, directly or indirectly, on the surrounding stroma. We investigated the role of the BRAF^{V600E} oncogene in tumor/vessel crosstalk and analyzed the effect of the BRAF inhibitor PLX4720 on tumor angiogenesis. Knock-in of the BRAF^{V600E} allele into the genome of human epithelial cells triggered their angiogenic response. In cancer cells harboring oncogenic BRAF, the inhibitor PLX4720 switches off the ERK pathway and inhibits the expression of proangiogenic molecules. In tumor xenografts harboring the BRAF^{V600E}, PLX4720 extensively modifies the vascular network causing abrogation of hypoxia. Overall, our results provide a functional link between oncogenic BRAF and angiogenesis. Furthermore, they indicate how the tumor vasculature can be “indirectly” besieged through targeting of a genetic lesion to which the cancer cells are addicted.

targeted therapy | tumor suppressor gene | personalized medicine

Angiogenesis is a pivotal process for the growth, maintenance, and spread of the majority of solid tumors. In the early steps of carcinogenesis, tumor cells require oxygen and nutrients, which are supplied by newly formed vessels. During later phases of cancer progression, blood vessels provide one of the most important routes for tumor escape and metastasization (1, 2). Notably, the structure of the vascular network dramatically alters tumor features. Tumor vessels are disorganized, with perfusion defects resulting in metabolic changes, hypoxia, and increase in interstitial pressure (3). At the clinical level, these features are associated with a more aggressive phenotype and a reduction of drug delivery to the tumor mass (4, 5).

The angiogenic process is triggered and controlled by the cancer cells, which carry genetic lesions that are causally associated with the neoplastic process. Understanding how individual genetic lesions in oncogenes and tumor suppressor genes affect directly or indirectly the angiogenic process is a fundamental question that has been largely unexplored. It has been suggested that alterations in oncogenes involved in the intracellular MAPK signaling cascade play a central role in regulating tumor angiogenesis (6). For example, KRAS activating mutations are thought to sustain the chaotic tumor vasculature by up-regulating the transcription of angiogenic inducers, including VEGF-A and IL-8 (7–9). BRAF binds to and is the main downstream effector of KRAS. Whether and to what extent genetic alterations affect BRAF-regulated angiogenesis is presently unclear. The most common BRAF mutation is a valine-to-glutamate transition (V600E), which results in constitutive activation of the kinase activity. BRAF^{V600E} is causally involved in the onset and progression of several cancers, including melanoma, papillary thy-

roid, colorectal, and ovarian tumors (10–12), and is often associated with a particularly poor prognosis (13–17).

In addition to activation of aberrant cell proliferation, BRAF^{V600E} modulates tumor–stroma interaction and supports cancer invasiveness by influencing the expression of metalloproteinases and cytokines (IL-6 and IL-10) involved in tumor-immune escape (18, 19). Furthermore, BRAF^{V600E} affects tumor environment by enhancing the expression of HIF1 α (20–22), VEGF-A and IL-8 (23–25) and inhibiting the angiogenic blocker thrombospondin 1 (26).

Specific and selective BRAF kinase inhibitors, such as PLX4032/PLX4720, have recently shown remarkable clinical success in patients with melanoma carrying the BRAF^{V600E} mutation (27–29).

Considering the putative role of mutant BRAF in modulating cancer cell function and microenvironment, we hypothesized that the efficacy of PLX4720 could result from targeting the epithelial and the stromal tumor compartments. In this work, we assessed the contribution of BRAF^{V600E} oncogene in promoting angiogenesis, and investigated whether the BRAF inhibitor PLX4720 affects tumor angiogenesis and hypoxia.

Results

BRAF^{V600E} Drives Angiogenesis in Chicken Chorioallantoic Membrane. To study the specific influence of BRAF^{V600E} on angiogenesis, we used an isogenic model in which BRAF^{V600E} was knocked-in through homologous recombination into the genome of the nontumorigenic human mammary epithelial cell line (hTERT-HME1) (30). As a result, the mutant allele is expressed under its endogenous promoter at levels comparable to wild type (WT) thus overcoming the experimental drawbacks associated with plasmid-driven ectopic overexpression.

Author contributions: A. Bottos, F.D.N., F.B., and A. Bardelli designed research; A. Bottos, M.M., F.D.N., V.C., and F.M. performed research; A.M. and G.A. contributed new reagents/analytic tools; A. Bottos, M.M., F.D.N., V.C., F.M., F.B., and A. Bardelli analyzed data; and A. Bottos, F.D.N., F.B., and A. Bardelli wrote the paper.

Conflict of interest statement: A. Bardelli owns stock in, and has received consultancy fees from, Horizon Discovery.

*This Direct Submission article had a prearranged editor.

¹A. Bottos and M.M. contributed equally to this work.

²Present address: Friedrich Miescher Institute for Biomedical Research, CH-4058 Basel, Switzerland.

³Present address: Department of Genetics, Biology and Biochemistry, Molecular Biotechnology Center, 10100 Turin, Italy.

⁴F.B. and A. Bardelli contributed equally to this work.

⁵To whom correspondence may be addressed. E-mail: federico.bussolino@ircc.it or alberto.bardelli@ircc.it.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1105026109/-DCSupplemental.

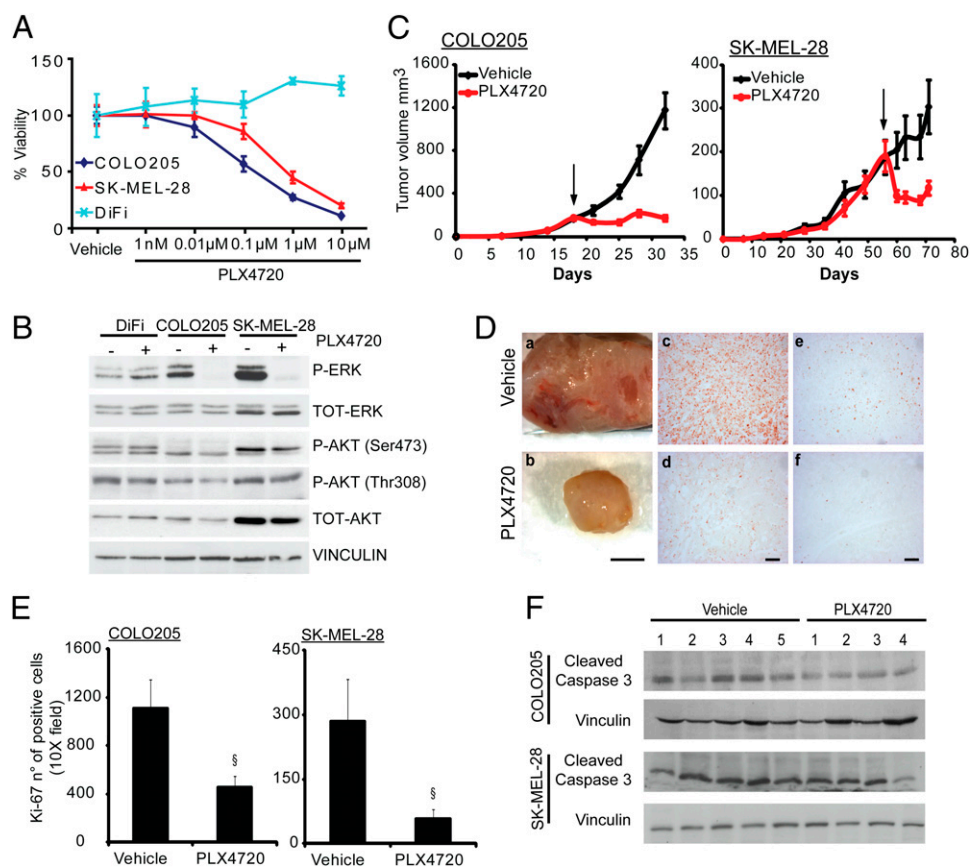


Fig. 2. PLX4720 inhibits proliferation and turns off MAPK signaling in tumor cells carrying BRAF^{V600E}. (A) Proliferation of COLO205 and SK-MEL-28, harboring BRAF^{V600E}, and BRAF WT DiFi cells, was assessed with increased PLX4720 concentration. Data are expressed as percentage of viability compared with vehicle by the ATP assay. Mean \pm SD of two independent experiments performed in quadruplicate is shown. (B) Biochemical analysis of phospho-ERK and phospho-AKT (Ser473 and Thr308) in COLO205, SK-MEL-28, and DiFi cells. Protein loading was normalized by vinculin. Starved cells were stimulated for 30 min with 2.5% FBS plus 1 μ M PLX4720 or vehicle. (C) Tumor growth curve of COLO205 and SK-MEL-28 xenografts. COLO205 and SK-MEL-28 xenografts were treated with PLX4720 60 mg/kg or vehicle for 2 wk after tumor volume reached an average of 150 to 200 mm³. Arrows indicate the time point at which treatment was started. Data are presented as mean tumor volume \pm SEM. (D) Representative images of COLO205 tumors (a and b). (Scale bar: 4 mm.) Representative images of Ki-67 staining in COLO205 (c and d) and SK-MEL-28 (e and f) xenografts. (Scale bar: 100 μ m.) (E) Quantification of proliferating cells by Ki-67 staining in COLO205 and SK-MEL-28 xenografts. Bars show the mean of positive cells per 10 \times field \pm SD ([§] $P < 0.0001$, Student *t* test). For COLO205, vehicle, $n = 4$ mice; PLX4720, $n = 6$ mice; for SK-MEL-28, vehicle, $n = 4$ mice; PLX4720, $n = 5$ mice. (F) Biochemical analysis of cleaved caspase 3 in protein extract of COLO205 and SK-MEL-28 xenografts. Protein loading was normalized by vinculin. At least four independent samples were evaluated.

Indeed, blood vessels were enlarged and chaotic in control tumors and thinner with an organized architecture in PLX4720-treated samples (Fig. 3A, a–c). The average blood vessel diameter decreased from 33 μ m or 31 μ m in start point and vehicle groups, respectively, to 19 μ m in treated samples (Fig. 3C). Surprisingly, despite these evident morphological differences, the blood vessel coverage by pericytes was unaffected by PLX4720 treatment (Fig. S2).

In SK-MEL-28 xenografts, the vascularized area increased in vehicle-treated tumors whereas PLX4720 reduced the area occupied by vessels (Fig. 3B). Similar to what was observed in COLO205, PLX4720 treatment of SK-MEL-28 tumors increased the vascular surface area compared with start-point tumor. Furthermore, the morphology of the vascular network in SK-MEL-28 was markedly modified and the blood vessel average diameter was reduced from 14 μ m compared with 23 μ m and 22 μ m in start-point and vehicle groups, respectively (Fig. 3A, d–f, and C).

PLX4720 Abrogates Hypoxia in BRAF^{V600E} Tumors. The aberrant morphology of blood vessels was strongly suggestive of altered functionality of the microvasculature. We confirmed this hypothesis by the analysis of tumor perfusion before and after PLX4720 treatment of COLO205 tumors. Tissue perfusion by

blood vessels was strongly increased in PLX4720-treated tumors compared with vehicle or start-point groups, indicating an improved functionality of microvasculature (Fig. 3D). Blood perfusion correlates with improved tissue oxygenation, which causes deep changes in the overall features of tumor parenchyma (3).

We therefore studied the effect of PLX4720 on intratumoral hypoxia in COLO205 and SK-MEL-28 xenografts and found that PLX4720 prominently abolished hypoxia in treated tumors compared with vehicle or start-point tumors (Fig. 4A, a–c and g–i, and B). Moreover, analysis of tumor tissues revealed that the modification of vascular structure by PLX4720 was linked to reduced necrotic area in COLO205 and SK-MEL-28 xenografts (Fig. 4A d–f and j–l, and C). These results indicate that the effect on hypoxia is independent from tumor growth and has implications for the therapeutic activity of PLX4720.

Importantly, targeting of angiogenesis by BRAF inhibition showed significant differences with respect to classical “VEGF directed” antiangiogenic therapy. Treatment of COLO205 tumors with the VEGF-blocking antibody bevacizumab resulted in stabilization of tumor growth and strongly reduced microvascular density (Fig. 5A, B, g and h, and C). Concomitantly, tissue perfusion was impaired, and this led to persistent hypoxia and necrosis (Fig. 5B and D–F). The comparison between the two

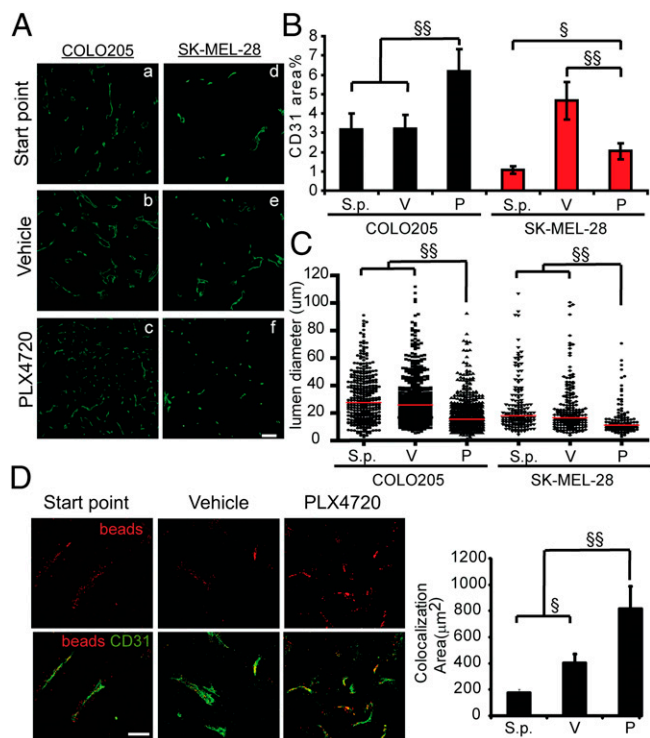


Fig. 3. PLX4720 treatment stabilizes blood vessels in COLO205 and SK-MEL-28 xenograft models. (A) Representative images of immunofluorescence analysis with the CD31 endothelial marker (green) in COLO205 (a–c) and SK-MEL-28 (d–f) tumors. (Scale bar: 100 µm.) (B) Percentage of surface area occupied by vessels quantified by CD31 staining. Bars show mean ± SEM. ($^{SS}P < 0.001$ and $^SP < 0.01$, Student *t* test). For COLO205, start point, *n* = 8 mice; vehicle, *n* = 9 mice; PLX4720, *n* = 14 mice; for SK-MEL-28, start point, *n* = 6 mice; vehicle, *n* = 5 mice; PLX4720, *n* = 5 mice. (C) Quantification of blood vessel lumen diameter. Red bars represent the median for each group ($^{SS}P < 0.0001$, Student *t* test). Dots indicate the individual vessels measured for the indicated cell lines. (D) Representative images of colocalization analysis between perfused beads (red) and CD31 (green) in COLO205. (Scale bar: 50 µm.) Quantification is shown as mean ± SEM of colocalization area (in µm²; $^{SS}P < 0.01$ and $^SP < 0.05$, Student *t* test). COLO205, start point, *n* = 39 blood vessels; vehicle, *n* = 30 blood vessels; PLX4720, *n* = 35 blood vessels. S.p., start point; V, vehicle; P, PLX4720.

approaches suggests that antiangiogenic therapy based on inhibition of oncogenes in the epithelial compartment has a greater chance to positively affect the vascular network compared with blockade of the VEGF pathway.

PLX4720 Down-Regulates Expression and Secretion of Proangiogenic Factors in BRAF Mutant Cancer Cells. To further assess whether the antiangiogenic effect of PLX4720 was direct (on the tumor vasculature) or indirect (via epithelial cells), we investigated if PLX4720 affected ECs *in vitro*. We found that proliferation and migration of ECs were unaffected by drug treatment (Fig. S3), thus ruling out a possible direct effect of BRAF inhibition on the endothelial compartment. We next considered if PLX4720 was capable of modulating the production of angiogenic molecules by cancer cells, which in turn might influence the tumor environment. To assess this, we analyzed the effect of BRAF inhibition on the expression of angiogenic factors in colorectal and melanoma cells carrying mutant BRAF. We found that, upon PLX4720 treatment, multiple mediators of angiogenesis (e.g., CXCL1–2–3, IL-1β, and IL-8 and VEGF-A) were down-regulated in COLO205 and SK-MEL-28. On the contrary, the same genes were not modulated in the BRAF WT DiFi colorectal cancer cells (Fig. 6A and B). Additionally, the effect of PLX4720

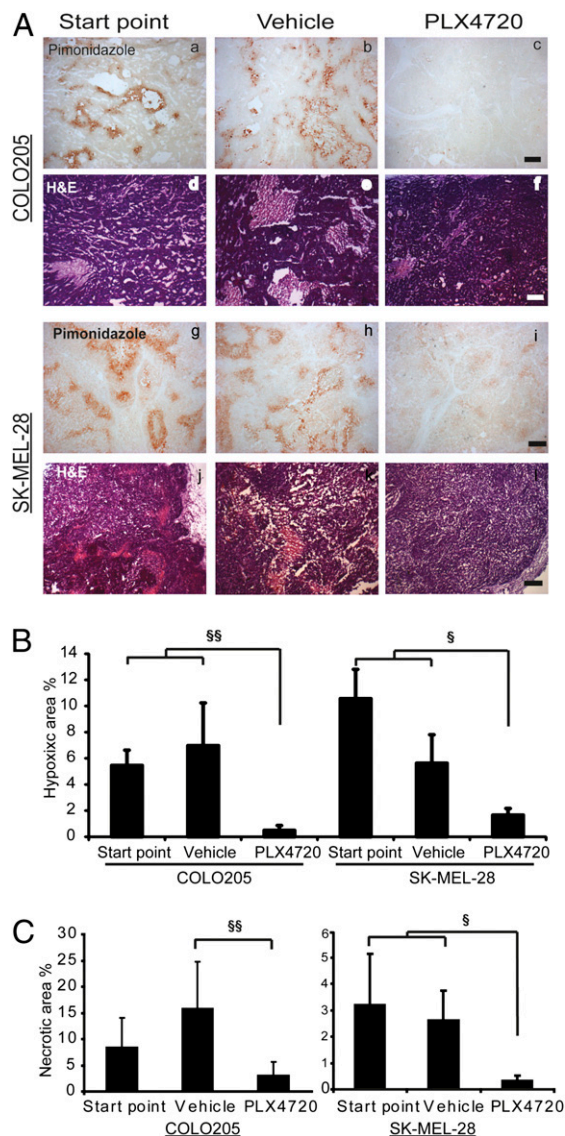


Fig. 4. PLX4720 treatment abolishes tumor hypoxia and decreases necrosis in COLO205 and SK-MEL-28 xenograft models. Histological analysis of COLO205 and SK-MEL-28 xenografts before and after PLX4720 treatment. (A) Representative images of pimonidazole and H&E staining in COLO205 (a–c and d–f) and SK-MEL-28 (g–i and j–l) tumors. (Scale bar: 100 µm.) (B) Quantification of the hypoxic area (percentage) by pimonidazole staining. Bars show mean ± SEM ($^{SS}P < 0.005$ and $^SP < 0.05$, Student *t* test). For COLO205, start point, *n* = 7 mice; vehicle, *n* = 8 mice; PLX4720, *n* = 8 mice; for SK-MEL-28, start point, *n* = 6 mice; vehicle, *n* = 5 mice; PLX4720, *n* = 5 mice. (C) Quantification of necrotic area after H&E staining in COLO205 and SK-MEL-28 xenografts. Bars represent the percentage mean area ± SEM ($^{SS}P < 0.01$ and $^SP < 0.05$, Student *t* test). For COLO205, start point, *n* = 8 mice; vehicle, *n* = 9 mice; PLX4720, *n* = 14 mice; for SK-MEL-28, start point, *n* = 6 mice; vehicle, *n* = 5 mice; PLX4720, *n* = 5 mice.

on VEGF production was not modified by hypoxia, an experimental condition that mimics tumors environment (Fig. 6B). Oncogenic BRAF is a key player in the activation of the MAPK-ERK signaling cascade. We therefore reasoned that the control of VEGF expression by PLX4720 might be caused by the inhibition of MAPK signaling. Treatment with the MEK inhibitor AZD6244 decreased VEGF secretion, thus suggesting that MAPK signaling pathway is essential for VEGF expression (Fig. 6B). ERK has been previously shown to influence protein translation through the control of the phosphorylation status of

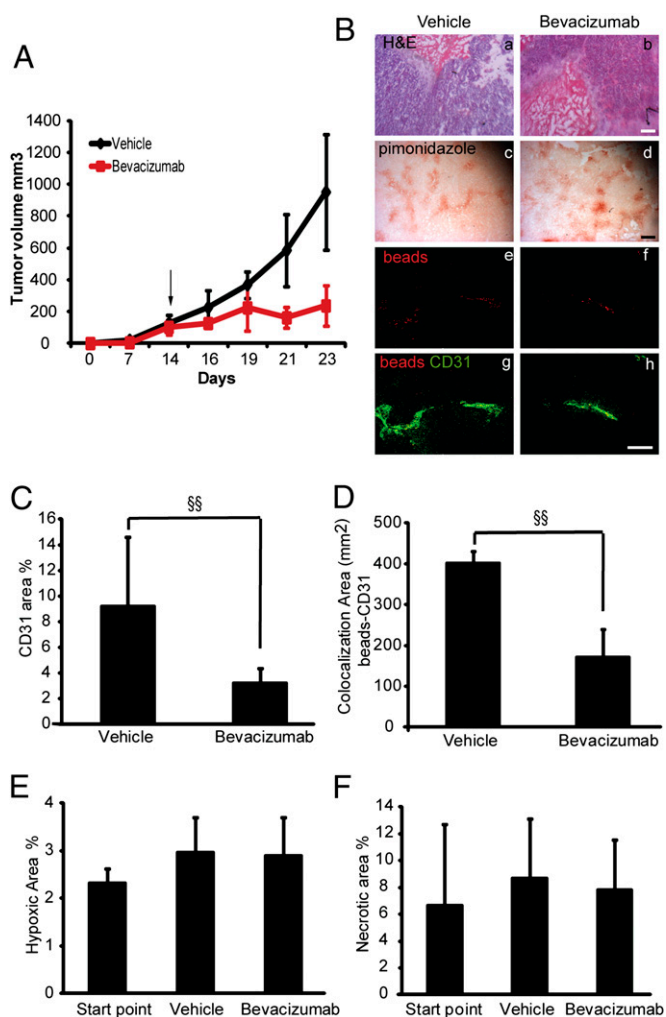


Fig. 5. Effect of bevacizumab on tumor growth and vasculature. COLO205 xenografts were treated with bevacizumab 10 mg/kg or vehicle for 2 wk after tumor volume reached an average of 150 to 200 mm³. (A) Tumor growth curve of COLO205; arrow indicates time point at which treatments were started. Data are presented as mean tumor volume \pm SEM of five mice. (B) Representative images of H&E (a and b) and pimonidazole staining (c and d) in COLO205. (Scale bar: 100 μ m.) Colocalization analysis between perfused beads (red) and CD31 (green) in COLO205 (e–h). (Scale bar: 50 μ m.) (C) Percentage of surface area occupied by vessels quantified by CD31 staining. Bars show mean \pm SEM (^{§§} $P < 0.01$, Student *t* test). Vehicle, $n = 5$ mice; bevacizumab, $n = 6$ mice. (D) Analysis of colocalization area between perfused beads and CD31. Quantification is shown as mean \pm SEM of colocalization area (in μ m²; ^{§§} $P < 0.01$). Vehicle, $n = 35$ blood vessels; bevacizumab, $n = 15$ blood vessels. (E) Quantification of the percentage hypoxic area by pimonidazole staining. Bars show mean \pm SEM. Start point, $n = 4$ mice; vehicle, $n = 5$ mice; bevacizumab, $n = 6$ mice. (F) Quantification of necrotic area by H&E staining. Bars represent the percentage mean area \pm SEM. Start point, $n = 4$ mice; vehicle, $n = 5$ mice; bevacizumab, $n = 6$ mice.

p90RSK1, 4EBP1, and eIF4E translation initiating factor. It is known that activation of ERK induces the phosphorylation of eIF4E by the MNK kinase (31) and of 4EBP1 by inactivation of TSC2 through p90RSK (32). In turn, phosphorylation of 4EBP1 releases eIF4E, thus allowing protein translation. We found that PLX4720 turns off the MAPK pathway in BRAF^{V600E} mutated cells, resulting in decreased phosphorylation of p90RSK1, 4EBP1, and eIF4E. The same effect could not be recapitulated in DiFi cancer cells, demonstrating that it is dependent on the presence of oncogenic BRAF (Fig. 6C). Overall, these results

indicate that BRAF^{V600E} targeted by PLX4720 modifies the proangiogenic program by acting on the MAPK pathway.

Discussion

To support the exponential growth of solid tumors, new blood vessels are continuously formed (1, 2). This active process is mainly sustained by cancer cells that produce and release angiogenic factors into the microenvironment, thus promoting neoangiogenesis. Antiangiogenic therapies have been developed to target this course of action, which is primarily driven by soluble growth factors such as VEGF (33, 34).

Therapies aimed at interfering with the tumor microenvironment and those that directly target cancer cells are often considered to represent two independent approaches. This discrepancy occurs despite the fact that the epithelial and stromal compartments are clearly linked at the structural and functional levels. How activation of a given oncogene in epithelial cells modulates the angiogenic response of the surrounding stromal cells is largely unknown. We and others have shown that the mutational profile of individual tumors affect their response to targeted therapies (35, 36). For example, EGFR kinase inhibitors are mainly active in lung cancer cells carrying mutations in the receptor (37, 38); likewise, the occurrence of KRAS mutations impairs the efficacy of anti-EGFR monoclonal antibodies in colorectal cancer (39–41). These findings are helping clinicians to match patients with appropriate drugs, a process often referred to as personalized medicine. Clinical evidence indicates that antiangiogenic therapies are also mainly effective in distinct patient subpopulations, although the molecular bases for this are still unknown (42–44). It is therefore tempting to speculate that the genetic milieu of the epithelial compartment dictates an individual's response to antiangiogenic therapies by modulating the tumor microenvironment.

In this work, we explored the possibility that the mutational activation of an individual oncogene affects the angiogenic potential of cancer cells. As a model, we chose BRAF^{V600E}, one of the most aggressive oncogenes frequently detected in colorectal tumors and melanomas (10, 12). We took advantage of a KI model, in which BRAF mutation has been introduced into the genome of non-transformed epithelial cells, hence closely recapitulating the situation observed in human neoplasms (30). This strategy overcomes the limitations of previous studies in which expression of cancer alleles was driven by viral promoters, resulting in expression levels many fold greater than in an endogenous setting. We report that BRAF mutant cells display up-regulation of proangiogenic factors and actively recruited the endothelium. In tumor cells, blockage of BRAF^{V600E} with the specific PLX4720 inhibitor not only exerted a cytostatic activity, but influenced the architecture of tumor vasculature. Notably, PLX4720 treatment did not affect ECs directly; rather, it down-regulated the expression of angiogenic factors in tumor cells by switching off the MAPK pathway and p90RSK-dependent translation.

Treatment with PLX4720 strongly influenced the morphology of the tumor vasculature in the cellular models (COLO205 and SK-MEL-28) that were analyzed. Specifically, the BRAF inhibitor modified the architecture and the functionality of the vessels, which appeared more regularly shaped, and provided a better perfusion of the tumor. Furthermore, in both COLO205 and SK-MEL-28, PLX4720 reduced necrosis and almost completely abrogated tumor hypoxia. We propose that, in addition to the well established growth inhibitory activities on BRAF mutant cancer cells, PLX4720 affects tumor growth by stabilizing the vasculature and ameliorating tissue perfusion. At least in the experimental conditions we tested, BRAF^{V600E} targeting by PLX4720 showed some advantageous features compared with the classical anti-angiogenic therapy with bevacizumab. The latter clearly reduced tumor growth and angiogenesis but did not improve oxygenation or reduce hypoxia. These results are reminiscent of data obtained in mouse models of pancreatic neuroendocrine

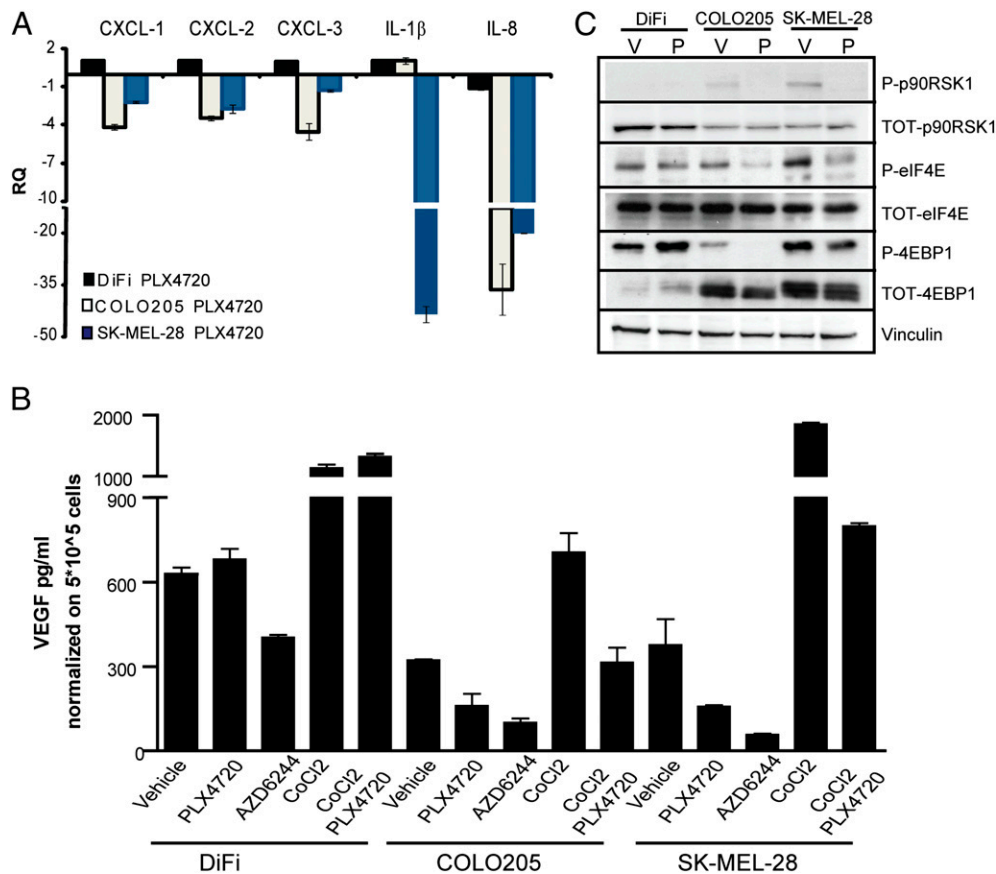


Fig. 6. PLX4720 treatment down-regulates the expression of proangiogenic factors. (A) Expression of proangiogenic factors was evaluated by real-time PCR. Cells carrying BRAF^{V600E}, COLO205, and SK-MEL-28, and BRAF WT DiFi cells were treated for 24 h with PLX4720. Data are expressed as relative quantity (RQ) of PLX4720 compared with vehicle-treated samples. Bars show mean \pm SD of triplicate measurements. (B) Quantification of secreted VEGF-A in cells supernatant by ELISA after 24 h of the indicated treatments. Data are expressed in pg/mL and bars represent mean \pm SEM of duplicate observations. The representative of two experiments is shown. (C) Biochemical analysis of phospho-p90RSK1, -eIF4E, and -4EBP1 in COLO205, SK-MEL-28, and DiFi cells. Protein loading was normalized by vinculin. Starved cells were stimulated for 30 min with 2.5% FBS plus 1 μ M PLX4720 or vehicle.

tumor and glioblastoma, whereby compounds that inhibit VEGF receptor 2 induce a marked hypoxia (45, 46).

Although the overall effect of BRAF inhibition was consistent in the two cell types, treatment of SK-MEL-28 reduced the vascularized area whereas the opposite occurred when COLO205 tumors were treated. A number of explanations could account for this difference. It has been reported that the ratio of the VEGF-A isoforms dictates the final shape of the vasculature and regulates blood flow by influencing vessel size (47, 48). It is possible that the VEGF isoforms and other cytokines are modulated to different extents in the two models. Moreover, VEGF activities may be finely tuned by specific coreceptors of VEGF-receptor 2 (e.g., neuropilins, integrins) that may be differentially expressed on the capillaries of the two xenografts (49). Finally, the effect of PLX4720 on SK-MEL-28 tumor vasculature is in line with the phenotype observed by treating melanoma tumors with VEGF-receptor inhibitor. As a matter of fact, in the SK-MEL-3 melanoma model, treatment with sunitinib caused a significantly decrease in vessel density, but the pressure of oxygen and uptake of contrast agent used in resonance imaging were increased (50).

The efficacy and safety of antiangiogenic agents has recently been questioned based on the discovery that reducing the blood supply to cancer cells causes hypoxia and could favor metastasization (45, 46, 51). In this regard, our work suggests that the pharmacological inhibition of an oncogenic mutation, which causally contributes to the imbalance between pro- and antiangiogenic mediators, may represent another valuable strategy to target tumor angiogenesis.

Materials and Methods

Isogenic Cell Model. hTERT-HME1 cells were purchased from American Type Culture Collection, and KI cells for BRAF^{V600E} were obtained through adeno-associated viral-mediated homologous recombination as previously described (30).

CAM Assay. Fertilized chicken embryos were incubated for 3 d at 37 $^{\circ}$ C and 70% humidity. A small hole was made over the air sac at the end of the egg, and a second hole was made directly over the embryonic CAM. After 10 d, hTERT-HME1 WT or BRAF^{V600E} KI cells were inoculated onto CAM for 48 h. In detail, 1×10^6 cells were mixed with 50 μ L of serum-free DMEM plus 50 μ L of Matrigel (growth factors reduced; Becton-Dickinson) and dropped onto the CAM to form a plug. CAMs were fixed with PBS solution/3.7% paraformaldehyde for 10 min at room temperature, and images were taken with a QImaging FAST1394 digital color camera (QImaging) connected to the stereomicroscope (model SZX9; Olympus). *SI Materials and Methods* describes the quantification of CAM vasculature.

Mouse Xenografts. All animal procedures were approved by the ethical commission of the University of Turin and the Italian Ministry of Health. COLO205 (5×10^6 cells per mouse) or SK-MEL-28 (8×10^6 cells per mouse) were injected s.c. into the right posterior flanks of 7-wk-old immunodeficient NOD/SCID female mice (6 mice per group; Charles River). Tumor formation was monitored twice per week, and tumor volume based on caliper measurements was calculated by the modified ellipsoid formula (i.e., half the product of length and the square of width). When tumors reached a volume of approximately 150 to 200 mm³, mice were randomized and treated by daily gavage for 2 wk with vehicle (1% methylcellulose, 5% DMSO in sterile water) or PLX4720 60 mg/kg.

Hypoxia Assay. Tumor hypoxia was detected by the formation of pimonidazole adducts with a Hypoxyprobe-1 Plus (FITC-mAb) kit (HP2-200; HPI) following manufacturer instructions. Details about the procedure and quantification are provided in *SI Materials and Methods*.

Tumor Vasculature Quantification. The following primary antibodies were used for histological analysis: rabbit anti-von Willebrand Factor (vWF; A0082; DakoCytomation), mouse anti-HLA-ABC antigen (M0736; DakoCytomation), rabbit anti-Ki67 (RM-9106-F0; Thermo Scientific) rat anti-CD31 (Pecam-1; 550274BD; Pharmingen), anti-NG2 (chondroitin sulfate proteoglycan polyclonal; AB5320; Chemicon). Quantifications on tumor vasculature were performed with Image-Pro Plus 6.2 software (Media Cybernetics). The surface area occupied by vessels was quantified as surface area of anti-CD31 antibody staining compared with total tissue area visualized by DAPI. For each animal, the total vessel area of at least four field/images was quantified.

Quantification of blood vessel lumen diameter was performed on single stack images with Image-Pro Plus 6.2 software (Media Cybernetics). This analysis was performed with the same images used for determination of surface area occupied by vessels.

- Hanahan D, Folkman J (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 86:353–364.
- Bergers G, Benjamin LE (2003) Tumorigenesis and the angiogenic switch. *Nat Rev Cancer* 3:401–410.
- Jain RK (2005) Normalization of tumor vasculature: An emerging concept in antiangiogenic therapy. *Science* 307:58–62.
- Bottaro DP, Liotta LA (2003) Cancer: Out of air is not out of action. *Nature* 423:593–595.
- Vaupel P, Kelleher DK, Höckel M (2001) Oxygen status of malignant tumors: pathogenesis of hypoxia and significance for tumor therapy. *Semin Oncol* 28(2 suppl 8)29–35.
- Rak J, Yu JL, Kerbel RS, Coomber BL (2002) What do oncogenic mutations have to do with angiogenesis/vascular dependence of tumors? *Cancer Res* 62:1931–1934.
- Rak J, et al. (1995) Mutant ras oncogenes upregulate VEGF/VPF expression: Implications for induction and inhibition of tumor angiogenesis. *Cancer Res* 55:4575–4580.
- Okada F, et al. (1998) Impact of oncogenes in tumor angiogenesis: Mutant K-ras up-regulation of vascular endothelial growth factor/vascular permeability factor is necessary, but not sufficient for tumorigenicity of human colorectal carcinoma cells. *Proc Natl Acad Sci USA* 95:3609–3614.
- Sparmann A, Bar-Sagi D (2004) Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. *Cancer Cell* 6:447–458.
- Davies H, et al. (2002) Mutations of the BRAF gene in human cancer. *Nature* 417:949–954.
- Wan PT, et al.; Cancer Genome Project (2004) Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell* 116:855–867.
- Garnett MJ, Marais R (2004) Guilty as charged: B-RAF is a human oncogene. *Cancer Cell* 6:313–319.
- Sartore-Bianchi A, et al. (2009) Multi-determinants analysis of molecular alterations for predicting clinical benefit to EGFR-targeted monoclonal antibodies in colorectal cancer. *PLoS ONE* 4:e7287.
- Souglakos J, et al. (2009) Prognostic and predictive value of common mutations for treatment response and survival in patients with metastatic colorectal cancer. *Br J Cancer* 101:465–472.
- Xing M, et al. (2005) BRAF mutation predicts a poorer clinical prognosis for papillary thyroid cancer. *J Clin Endocrinol Metab* 90:6373–6379.
- Xing M (2010) Prognostic utility of BRAF mutation in papillary thyroid cancer. *Mol Cell Endocrinol* 321:86–93.
- Tol J, Nagtegaal ID, Punt CJ (2009) BRAF mutation in metastatic colorectal cancer. *N Engl J Med* 361:98–99.
- Sumimoto H, Imabayashi F, Iwata T, Kawakami Y (2006) The BRAF-MAPK signaling pathway is essential for cancer-immune evasion in human melanoma cells. *J Exp Med* 203:1651–1656.
- Mesa C, Jr., et al. (2006) Conditional activation of RET/PTC3 and BRAFV600E in thyroid cells is associated with gene expression profiles that predict a preferential role of BRAF in extracellular matrix remodeling. *Cancer Res* 66:6521–6529.
- Kumar SM, et al. (2007) Mutant V600E BRAF increases hypoxia inducible factor-1alpha expression in melanoma. *Cancer Res* 67:3177–3184.
- Zerilli M, et al. (2010) BRAF(V600E) mutation influences hypoxia-inducible factor-1alpha expression levels in papillary thyroid cancer. *Mod Pathol* 23:1052–1060.
- Kikuchi H, Pino MS, Zeng M, Shirasawa S, Chung DC (2009) Oncogenic KRAS and BRAF differentially regulate hypoxia-inducible factor-1alpha and -2alpha in colon cancer. *Cancer Res* 69:8499–8506.
- Sharma A, et al. (2005) Mutant V599EB-Raf regulates growth and vascular development of malignant melanoma tumors. *Cancer Res* 65:2412–2421.
- Liang S, Sharma A, Peng HH, Robertson G, Dong C (2007) Targeting mutant (V600E) B-Raf in melanoma interrupts immunoevasion of leukocyte functions and melanoma extravasation. *Cancer Res* 67:5814–5820.
- Niault TS, Baccarini M (2010) Targets of Raf in tumorigenesis. *Carcinogenesis* 31:1165–1174.
- Nucera C, et al. (2010) B-Raf(V600E) and thrombospondin-1 promote thyroid cancer progression. *Proc Natl Acad Sci USA* 107:10649–10654.
- Bollag G, et al. (2010) Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. *Nature* 467:596–599.
- Flaherty KT, et al. (2010) Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med* 363:809–819.
- Chapman PB, et al.; BRIM-3 Study Group (2011) Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* 364:2507–2516.
- Di Nicolantonio F, et al. (2008) Replacement of normal with mutant alleles in the genome of normal human cells unveils mutation-specific drug responses. *Proc Natl Acad Sci USA* 105:20864–20869.
- Ueda T, Watanabe-Fukunaga R, Fukuyama H, Nagata S, Fukunaga R (2004) Mnk2 and Mnk1 are essential for constitutive and inducible phosphorylation of eukaryotic initiation factor 4E but not for cell growth or development. *Mol Cell Biol* 24:6539–6549.
- Roux PP, Ballif BA, Anjum R, Gygi SP, Blenis J (2004) Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase. *Proc Natl Acad Sci USA* 101:13489–13494.
- Ferrara N, Mass RD, Campa C, Kim R (2007) Targeting VEGF-A to treat cancer and age-related macular degeneration. *Annu Rev Med* 58:491–504.
- Ferrara N, Kerbel RS (2005) Angiogenesis as a therapeutic target. *Nature* 438:967–974.
- Bardelli A, Siena S (2010) Molecular mechanisms of resistance to cetuximab and panitumumab in colorectal cancer. *J Clin Oncol* 28:1254–1261.
- Normanno N, et al. (2009) Implications for KRAS status and EGFR-targeted therapies in metastatic CRC. *Nat Rev Clin Oncol* 6:519–527.
- Lynch TJ, et al. (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 350:2129–2139.
- Paez JG, et al. (2004) EGFR mutations in lung cancer: Correlation with clinical response to gefitinib therapy. *Science* 304:1497–1500.
- Lièvre A, et al. (2006) KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. *Cancer Res* 66:3992–3995.
- Benvenuti S, et al. (2007) Oncogenic activation of the RAS/RAF signaling pathway impairs the response of metastatic colorectal cancers to anti-epidermal growth factor receptor antibody therapies. *Cancer Res* 67:2643–2648.
- Karapetis CS, et al. (2008) K-ras mutations and benefit from cetuximab in advanced colorectal cancer. *N Engl J Med* 359:1757–1765.
- Kabbinavar FF, et al. (2005) Addition of bevacizumab to bolus fluorouracil and leucovorin in first-line metastatic colorectal cancer: Results of a randomized phase II trial. *J Clin Oncol* 23:3697–3705.
- Johnson DH, et al. (2004) Randomized phase II trial comparing bevacizumab plus carboplatin and paclitaxel with carboplatin and paclitaxel alone in previously untreated locally advanced or metastatic non-small-cell lung cancer. *J Clin Oncol* 22:2184–2191.
- Burger RA, Sill MW, Monk BJ, Greer BE, Sorosky JI (2007) Phase II trial of bevacizumab in persistent or recurrent epithelial ovarian cancer or primary peritoneal cancer: A Gynecologic Oncology Group Study. *J Clin Oncol* 25:5165–5171.
- Pàez-Ribes M, et al. (2009) Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell* 15:220–231.
- Ebos JM, et al. (2009) Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. *Cancer Cell* 15:232–239.
- Lee S, Jilani SM, Nikolova GV, Carpizo D, Iruela-Arispe ML (2005) Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. *J Cell Biol* 169:681–691.
- Gerhardt H, et al. (2003) VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol* 161:1163–1177.
- Ferrara N, Gerber HP, LeCouter J (2003) The biology of VEGF and its receptors. *Nat Med* 9:669–676.
- Eichhorn ME, et al. (2008) Contrast enhanced MRI and intravital fluorescence microscopy indicate improved tumor microcirculation in highly vascularized melanomas upon short-term anti-VEGFR treatment. *Cancer Biol Ther* 7:1006–1013.
- Chan DA, Giaccia AJ (2007) Hypoxia, gene expression, and metastasis. *Cancer Metastasis Rev* 26:333–339.