EGFR Tyrosine Kinase Inhibitors Decrease VEGF Expression by Both Hypoxia-Inducible Factor (HIF)-1–Independent and HIF-1–Dependent Mechanisms

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Abstract

Epidermal growth factor receptor (EGFR) inhibitors can decrease vascular endothelial growth factor (VEGF) expression and tumor angiogenesis. In the current study, we investigate the molecular pathways by which this occurs using two drugs that have been used in the clinic, gefitinib (Iressa) and erlotinib (Tarceva). The decrease in VEGF expression by gefitinib in SQ20B squamous cell carcinoma cells was opposed by adenoviral expression of Akt in these cells. The hypoxia-inducible factor-1 (HIF-1) binding site located at approximately −1 kbp in the VEGF promoter was not required for down-regulation of promoter activity by gefitinib under normoxia. Furthermore, the drug decreased activity of a reporter containing the −88/+54 region. In a gel shift assay, gefitinib led to decreased retardation of a labeled DNA oligonucleotide probe corresponding to the −88/−66 region of the VEGF promoter, which contains Sp1 binding sites. These effects of gefitinib on VEGF promoter activity and DNA binding were both reversed by Akt expression. Phosphorylation of Sp1 was decreased in the presence of gefitinib. Gefitinib also decreases VEGF expression by decreasing HIF-1α expression. This occurs due to decreased protein translation without any change in the level of HIF-1α mRNA. Together, these results suggest that gefitinib decreases VEGF expression both by decreasing Sp1 binding to the proximal core VEGF promoter and by down-regulating HIF-1α expression. Similar results were obtained with erlotinib in SQ20B and gefitinib in HSC3 squamous carcinoma cells. These results indicate that there are at least two separate mechanisms by which EGFR inhibitors decrease VEGF expression. (Cancer Res 2006; 66(6): 3197-204)

Introduction

The epidermal growth factor (EGF) receptor (EGFR), which is highly expressed in many human cancers, including glioblastomas, head and neck squamous cell carcinomas, non–small cell lung cancers, and cancers of the colon and breast, is correlated with disease progression, poor response to cytotoxic agents, and decreased overall survival (1, 2). EGFR is a member of the Erb family of receptor tyrosine kinases and consists of an extracellular ligand-binding domain, a transmembrane hydrophobic domain, and an intracellular domain with tyrosine kinase activity (3–6). EGFR activation triggers multiple signal transduction pathways, including the Ras/Raf/mitogen-activated protein kinase (MAPK) pathway (7) and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (8). EGFR activation leads to many biological processes that are associated with tumor growth, including cell cycle progression, invasion, metastasis, angiogenesis, and decreased apoptosis (6). Therefore, EGFR blockade is a potential approach to inhibit the growth of many tumors and numerous strategies have been explored to target EGFR, including monoclonal antibodies (e.g., cetuximab/C225), tyrosine kinase inhibitors (e.g., gefitinib/Iressa, erlotinib/Tarceva), ligand-linked toxins, and antisense oligonucleotides (2, 9). Numerous preclinical studies have also shown potentiation of the antitumor activity of chemotherapy by treatment with cetuximab/C225 (10, 11) or gefitinib/erlotinib (12, 13).

There has been significant experience with EGFR tyrosine kinase inhibitors and C225 in humans. Although some tumors clearly exhibit dramatic shrinkage in response to EGFR inhibitors, phase III randomized trials have not uniformly shown an advantage to the use of these drugs (9, 14–17). However, there are some data to suggest optimism that these drugs may have a role in cancer therapy. Recent work suggests that gefitinib-responsive non–small cell lung cancers have a specific activating mutation in the EGFR (18–20). A recently reported phase III clinical trial confirmed survival benefit in patients with refractory non–small cell lung cancer receiving erlotinib (21). Cetuximab was found to have clinically significant activity when given alone or in combination with irinotecan in patients with irinotecan-refractory colorectal cancer (22).

Decreased expression of vascular endothelial growth factor (VEGF), a key angiogenic factor, may account for some of the inhibition of tumor growth by EGFR blockade *in vivo*. In several cell lines, EGF induces VEGF expression (23, 24). Conversely, our own data and that of many other groups indicate that EGFR inhibition can decrease VEGF expression and consequently angiogenesis in many tumor types (24–30). Particularly relevant is a study that showed that resistance to anti-EGFR antibody therapy could occur in VEGF-overexpressing human tumor xenografts (31). This acquired lack of responsiveness to the anti-EGFR therapy could be mimicked by VEGF overexpression engineered by gene transfection.

Because of the link between EGFR inhibition and decreased VEGF expression, we chose to investigate the molecular mechanisms underlying this. Hypoxia-inducible factor-1α (HIF-1α) is reportedly induced by EGF stimulation in some cell lines. We had found previously that the PI3K pathway could regulate the proximal core VEGF promoter (32). Therefore, we examined both HIF-1α-dependent and HIF-1α-independent mechanisms by which HIF-1α can be regulated by gefitinib and erlotinib.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Tissue culture and reagents. SQ20B and HSC3, both head and neck squamous cell carcinomas, were cultured in DMEM (4,500 mg/L glucose, Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and grown in an incubator containing 5% carbon dioxide and 21% oxygen. Hypoxic conditions were as described previously (32).

Northern blot analysis. Total RNA was isolated with RNazol (Life Technologies) using the instructions of the manufacturer. Ten to 15 μg RNA were denatured with formaldehyde and formamide and run on a 0.9% agarose gel containing formaldehyde. RNA was transferred by capillary action in 20× SSC [1× SSC is 0.15 mol/L NaCl, 0.15 mol/L sodium citrate (pH 7)] to a Duralon-UV membrane (Stratagene, La Jolla, CA) and UV cross-linked before hybridization. Labeling of radioactive probes was done using [^{32}P]dCTP and a Prime-It kit (Stratagene) using the instructions of the manufacturer. Hybridization was carried out at 65°C, after which the membranes were washed with 0.1× SSC and 0.1% SDS at 65°C. Autoradiography was carried out at −80°C with intensifying screens. A 200 bp VEGF cDNA fragment excised with *Eco*RI from the pGEMh204 plasmid was used to make radioactive probes for hybridization. A HIF-1α cDNA plasmid obtained from G. Semenza (Johns Hopkins University School of Medicine, Baltimore, MD) was used to excise a fragment for HIF-1α Northern blotting. To verify equal loading between lanes, all gels were stained with ethidium bromide and the membranes were probed with a DNA fragment of the 18S rRNA.

Protein extraction and Western blot analysis. For protein isolation, cells were trypsinized and washed once in PBS. The pellets were then solubilized in 0.3 to 0.5 mL of 1× sample lysis buffer [1% Triton X-100, 20 mmol/L Tris (pH 7.6), 150 mmol/L NaCl, 2 mmol/L EDTA, 10% glycerol, 1 mmol/L DTT, 1 mmol/L orthovanadate supplemented with Complete protease inhibitors (Roche, Nutley, NJ)], boiled for 5 minutes, and passed repeatedly through a 26-gauge needle. Samples were centrifuged at 10,000 × *g* and the supernatants were retained. Protein concentrations were determined using a BCA Protein Assay kit (Pierce, Rockford, IL).

For Western blotting, equal amounts of total protein were run in each lane of an SDS-PAGE gel (12% acrylamide). Each protein sample was mixed with an equal volume of 2× Laemmli buffer and boiled at 95°C for 5 minutes before loading onto the gel. After completion of gel electrophoresis, protein was transferred to a Hybond nitrocellulose membrane (Amersham-Pharmacia, Piscataway, NJ) over 1 hour using a blotting apparatus. For detection of the phosphorylated form of Akt protein, we used a monoclonal anti-phospho-Akt antibody (New England Biolabs, Ipswich, MA) followed by a goat anti-mouse antibody (Bio-Rad, Hercules, CA). As a loading control, blots were reprobed with an anti-β-actin antibody (Sigma-Aldrich, St. Louis, MO) at a 1:1,000 dilution followed by a goat anti-mouse antibody (Bio-Rad) at a dilution of 1:500.

Plasmid constructs and transient transfections. The construction of the plasmids pGL3-1.5kbVEGFprom and mut1 have been described previously (32). Transfections were done using Fugene (Roche) according to the instructions of the manufacturer. Briefly, cells were split into 60 mm dishes so that 24 hours later they were ~50% confluent. At this time, each dish was transfected using 6 μL Fugene and 2 μg reporter plasmid and, to control for transfection efficiency, 1 μg pSV-β-galactosidase (Promega, Madison, WI). Cells were harvested by removing the medium, washing twice with PBS, and directly adding 100 μL lysis buffer per dish. Of this lysate, 80 μL was used for luciferase determinations and 10 μL for β-galactosidase assays. These determinations were done using the LucLite kit (Perkin-Elmer, Wellesley, MA) and the β-galactosidase Enzyme Assay System (Promega). Luciferase readings were done on a TopCount Microplate Scintillation and Luminescence Counter (Perkin-Elmer).

Adenovirus. Adenovirus expressing myristoylated Akt capable of replicating in the "packaging" 293 cell line were made using the pAd-Easy protocol as described previously (32). The virus was stored in single-use aliquots at −80°C. Cells were infected at a multiplicity of infection (MOI) of 5 to 10, and cells were harvested 48 hours postinfection.

Gel shift assay. Nuclear proteins were extracted as described previously (33). Oligonucleotides corresponding to −88 to −66 bp of the human VEGF/VPF promoter were synthesized. The complementary sequences 5′-CCGGGGCGGGCCGGGGGCGGGGGT-3′ and 5′-ACCCCGCCCCCGGC-CCGCCCCGG-3′ were labeled with [^{32}P-γ]ATP and T4 polynucleotide kinase. Unincorporated [^{32}P-γ]ATP were removed by centrifugation through G-25 Sephadex column (Boehringer Mannheim) according to the recommendations of the manufacturer. The DNA-binding reaction was done for 30 minutes at room temperature in a volume of 20 μL, containing 5 μg nuclear protein extract, 2.5 mg/mL bovine serum albumin, 10^5 cpm of 0.1 mg/mL poly(deoxyinosinic-deoxycytidylic acid) (Sigma), 5 μL of 4× binding buffer [1× buffer: 10 mmol/L Tris-Cl (pH 7.8), 100 mmol/L KCl, 5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 10% (v/v) glycerol, and 1 mmol/L DTT] with or without excess of unlabeled competitor or Sp1 consensus oligonucleotide (Promega). Samples were subjected to electrophoresis on a native 5% polyacrylamide gel run in 0.5× TGE (50 mmol/L Tris-HCl, 400 mmol/L glycine, and 2 mmol/L EDTA) for 2.5 hours at 120 V.

Orthophosphate labeling and Sp1 immunoprecipitation. Cells were incubated in phosphate-free DMEM (Life Technologies) for 1 hour, labeled in medium containing 1 mCi [^{32}P]P_i (Amersham Pharmacia, Piscataway, NJ) for 8 hours, and harvested with sample lysis buffer as described above for Western blotting.

The protein solution was precleared with agarose A (Invitrogen) and incubated with an anti-Sp1 antibody (Sigma) at 4°C for overnight. Immunoprecipitates were isolated with protein A and the beads were washed four times with buffer. Finally, beads were resuspended in 50 μL of 1× SDS-PAGE loading buffer [0.06 mol/L Tris-HCl (pH 8.0); 1.71% SDS; 6% glycerol; and 0.1 mol/L, 0.002% bromophenol blue] and boiled at 95°C. The released proteins were separated on 12% SDS-PAGE gel. Separated proteins were transferred to a Hybond nitrocellulose membrane (Amersham) and autoradiographed.

[^{35}S]Met-Cys labeling and HIF-1α immunoprecipitation. Medium was replaced with Met-Cys-free DMEM containing 5% serum. After 30 minutes, [^{35}S]Met-Cys was added to a final concentration of 0.2 mCi/mL, and the cells were pulse-labeled for 2 hours in presence of dimethyloxallyl glycine and then harvested. Cells were washed once in ice-cold PBS, trypsinized, and then centrifuged. The pellets were then solubilized in sample lysis buffer described above for Western blotting. The protein solution was passed repeatedly through a 26-gauge needle. Thereafter, samples were centrifuged at 10,000 × *g*, and the supernatants were retained. Fifty microliters of a slurry containing protein A–Sepharose beads was added to the cell lysate in an Eppendorf tube and incubated on ice for 30 to 60 minutes. Then, the mixture was centrifuged at 10,000 × *g* for 10 minutes at 4°C. The supernatant was transferred to a fresh Eppendorf tube and proteins were quantitated using the BCA kit (Pierce). Equal amount of total protein (1 mg) was taken in an Eppendorf tube and 10 μg anti-HIF-1α antibody (H1α67; Novus Biologicals, Littleton, CO) was added and incubated at 4°C for 16 hours. Then, 50 μL washed protein G slurry in prechilled sample lysis buffer was added and incubated for 1 hour at 4°C. Thereafter, the immunoprecipitated proteins were centrifuged thrice at 10,000 × *g* for 30 seconds at 4°C. The supernatant was removed completely and the beads were washed thrice with 500 μL lysis buffer. After the last wash, supernatant was aspirated and 50 μL of 1× Laemmli sample buffer were added to bead pellet. The solution was heated to 90°C to 100°C for 5 minutes. Then, the Eppendorf tube was centrifuged at 10,000 × *g* for 5 minutes. The supernatant was loaded onto a gel.

Tumor generation and drug treatment in nude mice. Pathogen-free female Ncr-nu/nu mice were obtained from Taconic (Germantown, NY) and were housed aseptically in the animal facilities of University Laboratory Animal Resources and the Institute for Human Gene Therapy of the University of Pennsylvania. All experiments were carried out in accordance with University Institutional Animal Care and Use Committee guidelines. At 5 to 7 weeks of age, mice were inoculated by s.c. injection into the hind flank with 1 × 10^6 SQ20B cells resuspended in 100 μL Matrigel (B-D Collaborative Research, Franklin Lakes, NJ). Visible tumors appeared within 1 week. On day 8, mice were treated with gefitinib (25 mg/kg/d) or DMSO

(control) daily for 4 days i.p. On day 11, mice were sacrificed and tumors were harvested to perform Western blotting for p-Akt and VEGF.

Densitometry. Gels were scanned on an Epson 2450 Perfection Photoscanner using Adobe Photoshop 4.0.1. Bands on the gels were quantified using NIH Image 1.63 software.

Results

Gefitinib down-regulates VEGF expression in SQ20B both *in vitro* and *in vivo*. Figure 1*A* shows that 1 μmol/L gefitinib almost completely abolished both EGFR and Akt phosphorylation in SQ20B cells in tissue culture; therefore, this dose was used for subsequent experiments. The PI3K inhibitor LY294002 was found to inhibit Akt phosphorylation at a dose of 20 μmol/L (data not shown), which was the dose that was subsequently used. Both gefitinib and LY294002 led to a ~50% decrease in VEGF mRNA expression (Fig. 1*B*; *lanes 2* and *3*). In contrast, the MAPK inhibitor U0126 had no effect on VEGF expression (Fig. 1*B*; *lane 4*) although this dose inhibited the MAPK pathway (data not shown). The level of VEGF secreted into the medium was also decreased by gefitinib as measured by ELISA (see Supplementary Fig. S1).

Because inhibition of the Akt pathway decreased VEGF expression, we next investigated whether stimulating this pathway would block the effect of gefitinib on VEGF expression. Transduction of control cells (not treated with gefitinib) with Akt-expressing adenovirus led to a 4-fold increase in VEGF expression (Fig. 1*B*; compare *lanes 5* and *6*). In cells transduced with GFP-expressing (control) adenovirus, gefitinib led to the expected decrease in VEGF mRNA expression (Fig. 1*B*; compare *lanes 7* and *5*). However, in cells transduced with Akt-expressing adenovirus, gefitinib was unable to prevent induction of VEGF expression (Fig. 1*B*; compare *lanes 7* and *8*). Therefore, Akt expression was able to counteract the down-regulation of VEGF caused by gefitinib. This result is consistent with the notion that Akt is downstream of EGFR signaling in the regulation of VEGF mRNA expression.

To verify that gefitinib had the same effect *in vivo*, SQ20B cells were implanted s.c. into nude mice. Mice were either injected i.p. with gefitinib or control carrier. After 4 days of injection, mice were sacrificed and their tumors were removed. The tumors were lysed and Western blotting was done for VEGF and phospho-Akt. Gefitinib treatment led to a decrease in both phospho-Akt and VEGF expression in these tumor cells *in vivo*, consistent with our tissue culture results (see Supplementary Fig. S2).

Gefitinib blocks EGF-induced up-regulation of SQ20B expression. In addition to decreasing the basal expression of VEGF

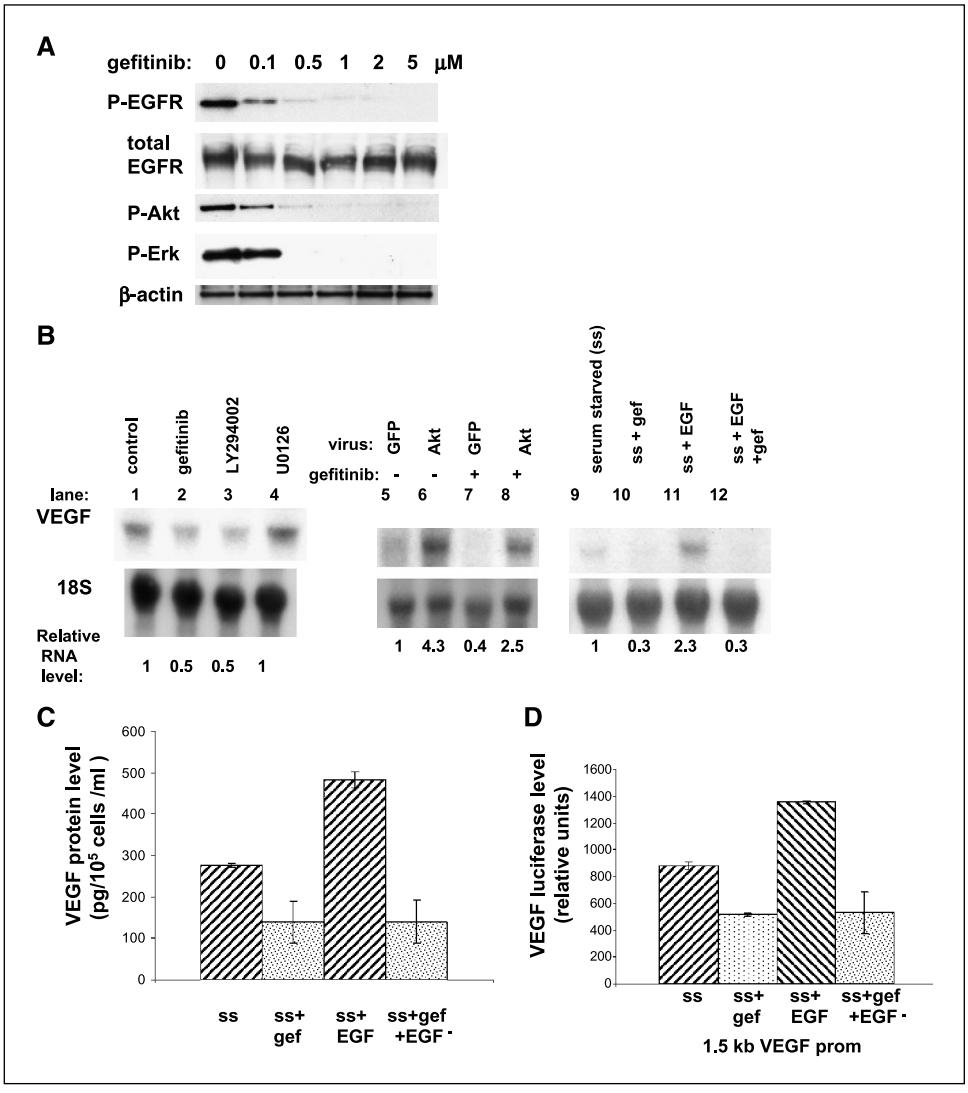

Figure 1. Gefitinib down-regulates basal VEGF expression in SQ20B *in vitro* and *in vivo* and blocks EGF-induced VEGF expression. *A,* SQ20B cells were treated with concentrations of gefitinib as indicated. Sixteen hours later, cells were lysed and Western blotting was done for various proteins as indicated. *B,* Northern blots probed for VEGF and 18S. Relative RNA level was calculated as ratio of intensity of VEGF band to 18S band. *Lanes 1-4,* SQ20B cells were treated with gefitinib (1 μmol/L), U0126 (5 μmol/L), or LY294002 (20 μmol/L). Sixteen hours later, RNA was harvested. *Lanes 5* to *8,* SQ20B cells were transduced with adenovirus expressing either myristoylated Akt or GFP (control). Twenty-four hours later, cells were treated with gefitinib (1 μmol/L) or DMSO (control). After 16 hours, RNA was harvested. *Lanes 9* to *12,* Cells were serum starved (*ss*) for 24 hours. EGF (300 ng/mL) and/or gefitinib (1 μmol/L; *gef*) was then added to selected dishes. Sixteen hours later, cells were harvested for RNA. *C,* cells were serum starved for 24 hours. EGF (300 ng/mL) and/or gefitinib (1 μmol/L) was then added to selected dishes. Twenty-four hours later, aliquots of supernatant were removed from dishes and ELISA for VEGF was done. ELISA values were normalized to the number of cells present. *D,* similar to (*C*), except that cells were transiently transfected under serum-starved conditions with a reporter plasmid containing 1.5 kb of the VEGF promoter. They were also cotransfected with a β-galactosidase-expressing plasmid (pSV2-β-gal). The cells were maintained in serum-starved medium for 24 hours, then gefitinib and/or EGF was added. Sixteen hours later, cells were harvested and assayed for luciferase and β-galactosidase activity. *Y axis,* relative luciferase levels (ratio of luciferase to β-galactosidase readings). *Columns,* mean of three independent transfections; *bars,* SD.

in SQ20B cells, gefitinib also decreased the induction of VEGF in response to EGF stimulation. These experiments were done under serum-starved conditions to eliminate the effect of the low level of EGF in the serum. Figure 1*B* shows that EGF stimulation of SQ20B cells led to a >2-fold increase in VEGF mRNA expression (compare *lanes 9* and *11*), which was completely blocked by pretreatment of the cells with gefitinib (compare *lanes 11* and *12*). Similarly, EGF stimulation of serum-starved cells led to increased VEGF protein secretion, which could be blocked by gefitinib (Fig. 1*C*). Gefitinib also blocked the EGF induction of activity of a reporter plasmid containing 1.5 kb of the VEGF reporter (Fig. 1*D*).

Gefitinib can decrease VEGF promoter activity independently of the HIF-1 binding site. To determine whether gefitinib regulates VEGF at the transcriptional level, we did transient transfection experiments with luciferase reporters. Exposure of cells transfected with the 1.5 kbp wild-type VEGF promoter to gefitinib resulted in a decrease in reporter activity. Our previous results had suggested that the PI3K pathway could activate the VEGF promoter through Sp1 sites located in the proximal promoter (32, 34). Others have suggested that EGFR stimulation can increase VEGF expression by acting through HIF-1 binding sites located in the hypoxia-responsive element (HRE; ref. 35). To separate these two effects, we used reporter constructs containing 1.5 kbp of the wild-type VEGF promoter or with a mutation within the HRE (mut1; see Supplementary Fig. S3). Our previous results confirmed that the mut1 construct displayed a blunted response to hypoxia (32). SQ20B cells were cotransfected with either of these constructs, then treated with gefitinib. Figure 2*A* shows that mutation of the HRE site failed to prevent the drug from down-regulating promoter activity.

To determine whether Akt expression could counteract the effect of gefitinib on VEGF promoter activity, we transfected cells with either the 1.5 kbp wild-type promoter reporter or the mut1 construct and cotransfected with either an activator vector expressing myrAkt or a control (empty) vector. Gefitinib down-regulated promoter activity by ~50% when the empty vector was used as an activator. However, the Akt-expressing activator increased promoter activity even when gefitinib was present (Fig. 2*B*). These results are consistent with the idea that Akt works downstream of gefitinib in the drug regulation of the VEGF promoter. Furthermore, because this effect occurred with both the wild-type promoter construct and mut1, the HRE-mutated construct (Fig. 2*C*), it indicates that Akt can regulate the VEGF promoter independently of HIF-1.

Sp1 is involved in response of VEGF promoter to gefitinib. Previous work from our laboratory indicated that the PI3K/Akt pathway could regulate the −88/+54 bp region of the VEGF promoter (32). Therefore, we tested a luciferase construct containing the −88/+54 region of the promoter and found that its activity could also be down-regulated by gefitinib (Fig. 2*D*). These observations suggest that elements within this region are responsive to the drug. Figure 2*D* also shows that mutation of the Sp1 binding sites within this −88/+54 bp region (Sp1mut construct) decreased the basal level of promoter activity and made it unresponsive to gefitinib, suggesting that these sites were involved in the response to the drug.

To further test the role of Sp1 in the gefitinib response, we did a gel shift assay. This showed that treatment of cells with gefitinib dramatically decreased retardation of a labeled DNA oligonucleotide corresponding to the −88/−66 region of the promoter (Fig. 3*A*; compare *lanes 1* and *2*). Binding to this fragment could be

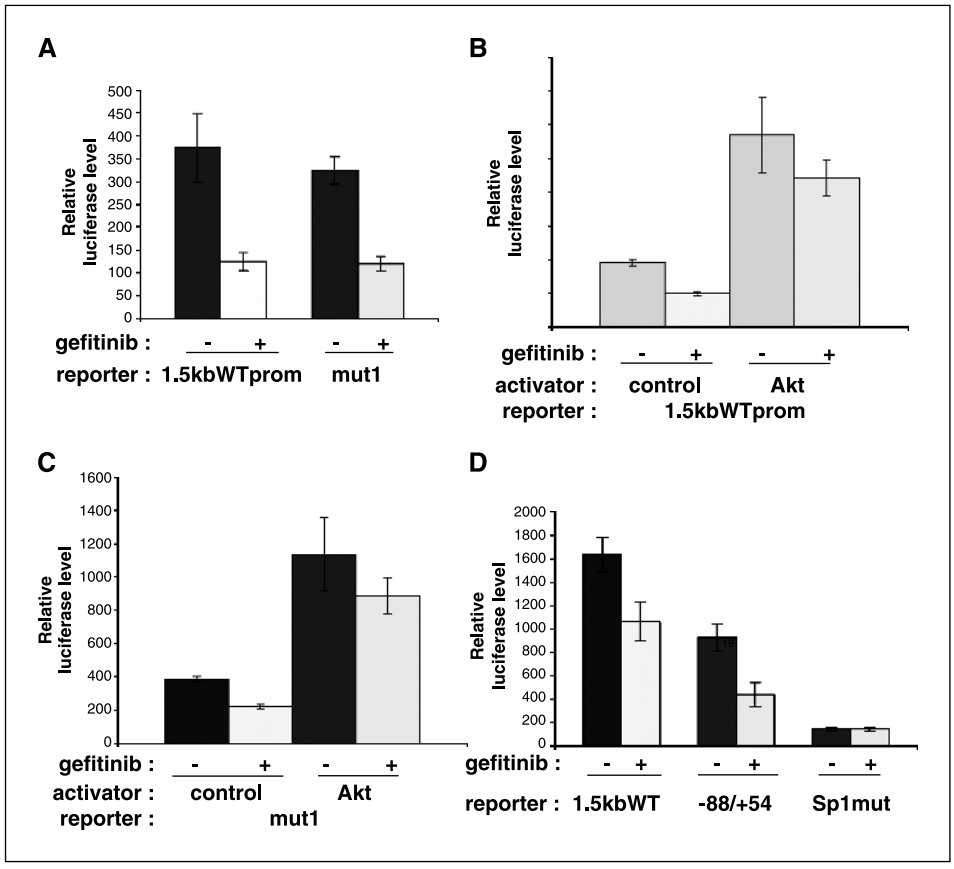

Figure 2. Gefitinib can decrease VEGF promoter activity independently of HIF-1 binding site. Schematics of reporter constructs are shown in Supplementary Fig. S3. Indicated VEGF promoter luciferase construct was cotransfected along with β-galactosidase-expressing plasmid (pSV2-β-gal) and, in some cases, a plasmid expressing myristoylated Akt (*C* and *D*). Twenty-four hours after transfection, cells were treated with gefitinib. After 16 hours, cells were harvested and assayed for luciferase and β-galactosidase activity. *Y axis,* relative luciferase levels (ratio of luciferase to β-galactosidase readings). *Columns,* mean of three independent transfections; *bars,* SD. Representative of results obtained in three separate experiments (*A-D*).

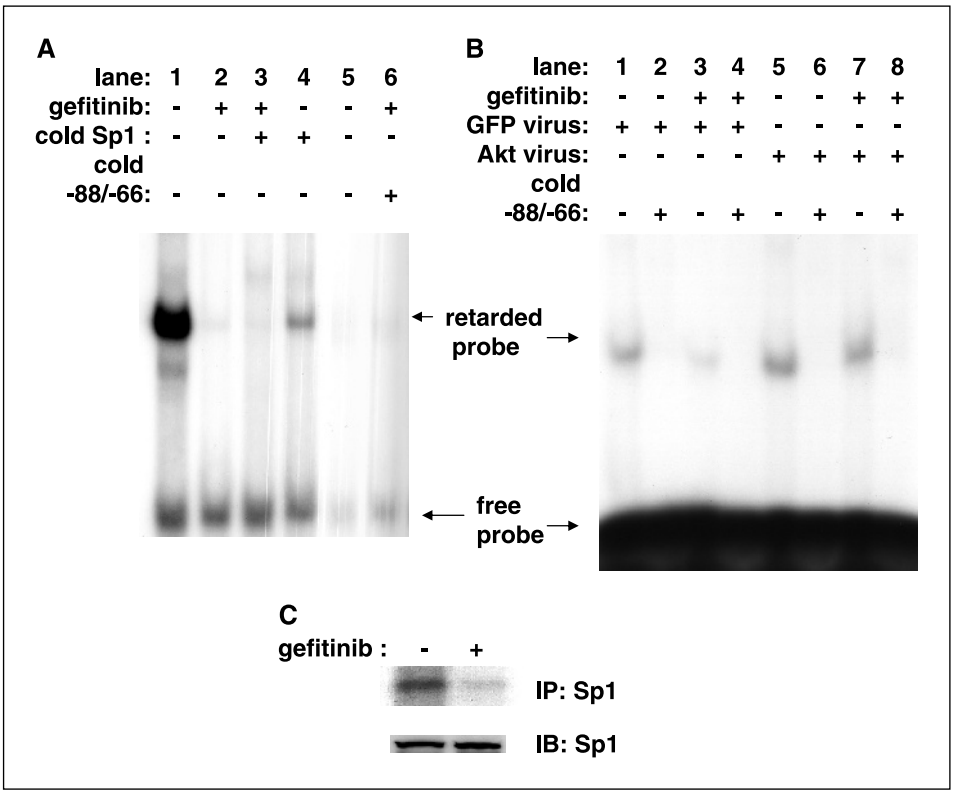

Figure 3. Sp1 phosphorylation and DNA binding are reduced in response to gefitinib. *A* and *B,* oligonucleotides corresponding to −88 to −66 bp in the human VEGF promoter were labeled with [γ-^{32}P]ATP. Gel shift assay was done using nuclear extract from cells treated with gefitinib or control carrier. In (*A*), the DNA-binding reaction was also done with 100-fold molar excess of cold Sp1 consensus oligonucleotides or with cold −88/−66 probe as indicated. In (*B*), cells were transduced with either myrAkt- or GPF-expressing adenovirus (control) at an MOI of 10. Twenty-four hours after infection, cells were treated with gefitinib. Twenty-four hours later, nuclear extracts were harvested for gel shift assay as in (*A*). *C,* SQ20B cells treated with gefitinib or control carrier were *in vivo* labeled with P_i. After 4 hours of labeling, cells were lysed and immunoprecipitated (*IP*) with an anti-Sp1 antibody. Immunoprecipitated complexes were separated on 10% SDS-PAGE gel and transferred to nitrocellulose membrane and autoradiographed. In the lower part of (*C*), these same lysates were separated on 10% SDS-PAGE gel, transferred to nitrocellulose membrane, and probed with an anti-Sp1 antibody to serve as a loading control (*IB,* immunoblot). Representative of results obtained in at least two separate experiments (*A-C*).

effectively competed away by using excess cold Sp1 consensus probe (Fig. 3*A* compare *lanes 1* and *4*). To explore the possible role of Akt, the experiment was modified, this time transducing cells with either control (GFP-expressing) or Akt-expressing adenovirus before treating them with gefitinib. Figure 3*B* shows that gefitinb led to decreased retardation of the DNA probe when control adenovirus was used (compare *lanes 3* and *1*). However, when cells were transduced with Akt-expressing adenovirus, the high level of DNA binding was maintained even when the cells were treated with gefitinib (Fig. 3*B*; compare *lanes 7* and *3*). These results support the idea that Akt acts downstream of gefitinib to regulate Sp1 binding to the proximal core VEGF promoter.

Phosphorylation of Sp1 has been implicated with increased binding of the transcription factor to consensus sites within various promoters (36–38). We did *in vivo* P_i labeling and showed that gefitinib decreases Sp1 phosphorylation, which could explain how it leads to decreased Sp1 binding to the promoter (Fig. 3*C*).

Gefitinib suppresses induction of HIF-1α and VEGF expression under hypoxia by decreasing translation. The previous results indicate that the HRE is not required for regulation of the VEGF promoter by gefitinib under normoxic conditions. Because of reports linking EGF to HIF-1α activation, we determined the effects of the drug on HIF-1α and VEGF expression under hypoxic conditions. EGFR blockade with gefitinib blunted HIF-1α induction under hypoxia although it did not completely eliminate it (Fig. 4*A*; compare *lanes 3* and *4*). Gefitinib treatment also decreased the induction of the HIF-1 target gene *VEGF* (see Supplementary Fig. S4) as well as the VEGF promoter under hypoxia (Supplementary Fig. S5).

Regarding the mechanism by which gefitinib decreases HIF-1α protein expression, it could be by (*a*) decreasing HIF-1α mRNA expression, (*b*) decreasing HIF-1α protein translation independent of a change in the level of the mRNA, or (*c*) by decreasing HIF-1α protein stability. The first possibility was ruled out by showing that the level of HIF-1α mRNA did not change even after 16 hours of gefitinib treatment (Fig. 4*B*). If gefitinib decreased HIF-1α protein expression by decreasing its stability, treatment with proteasomal inhibitors, which stabilize HIF-1α, should prevent this decrease. However, we found that the proteasomal inhibitor MG132 allowed for accumulation of HIF-1α in control cells not treated with gefitinib but failed to do so in gefitinib-treated cells (Fig. 4*C*; compare *lanes 1* and *5*). This strongly suggested that gefitinib does not act by destabilizing HIF-1α but rather by decreasing its synthesis. To directly investigate this possibility, we did metabolic labeling analysis. SQ20B cells were pulse-labeled by means of [^{35}S]methionine-cysteine incorporation, followed by immunoprecipitation of HIF-1α. Dimethyloxallyl glycine, a prolyl hydroxylase inhibitor that stabilizes HIF-1α (39), was added to the medium to prevent the rapid degradation of HIF-1α that normally occurs during normoxia (Fig. 4*D*; compare *lanes 1* and *2*). In cells treated with dimethyloxallyl glycine, the addition of gefitinib decreased the amount of immunoprecipitated HIF-1α (Fig. 4*D*; compare *lanes 2* and *3*). Immunoblots using the lysates were probed for β-actin to show that the total amount of protein was the same in the different lanes. Therefore, Fig. 4*D* confirms that gefitinib leads to decreased HIF-1α translation.

To investigate the pathway downstream of gefitinib that might be responsible for its inhibitory effect on HIF-1α protein translation, we used chemical inhibitors. Inhibition of the PI3K pathway with LY294002 led to decreased HIF-1α induction with hypoxia, whereas inhibition of the MAPK pathway with U0126 did not (data not shown). We then did metabolic labeling analysis with [^{35}S]methionine-cysteine and found that, like gefitinib, LY294002 led to decreased HIF-1α translation (Fig. 4*D*; compare *lanes 4* and *5*).

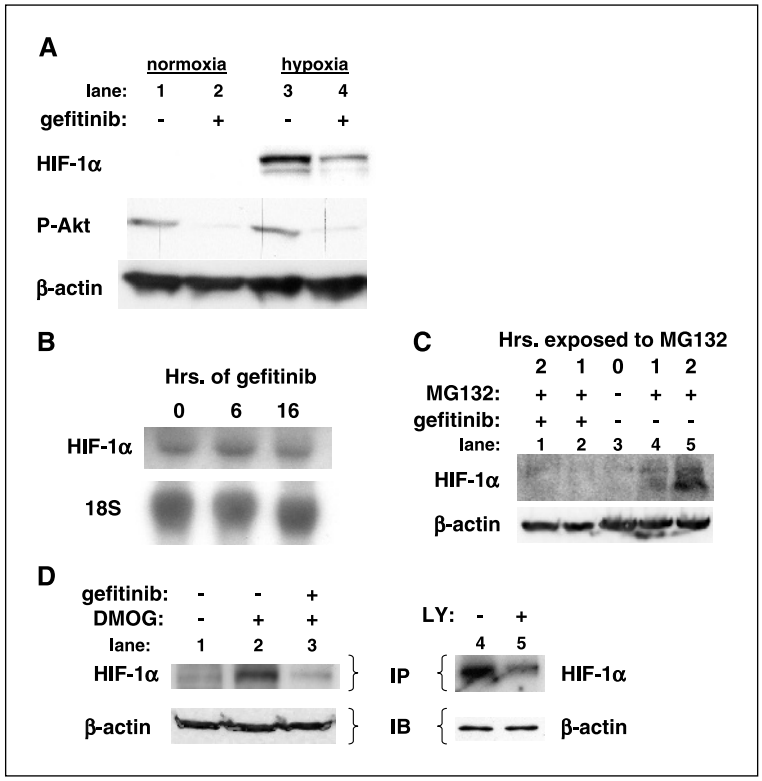

Figure 4. Gefitinib suppresses induction of HIF-1α and VEGF expression under hypoxia by decreasing translation. *A,* SQ20B cells were treated with gefitinib or control carrier for 16 hours, then exposed to hypoxia (0.2% O_2) for 3 hours. Then, cells were harvested and Western blotting was done for proteins as indicated. *B,* cells were treated with gefitinib for indicated periods of time. Cells were then harvested and Northern blotting was done. *C,* cells were pretreated with gefitinib (1 μmol/L), or not pretreated, as indicated. Sixteen hours later, cells were treated with proteasomal inhibitor MG132. Cells were harvested at different intervals following the addition of MG132 (10 μmol/L) as indicated, and Western blotting was done. *D,* HIF-1α immunoprecipitation following pulse labeling. *Lanes 1* to *3,* cells were treated with gefitinib for 24 hours. Then, regular medium was replaced with DMEM (met-cys free) containing [^{35}S]Met-Cys, gefitinib, and dimethyloxallyl glycine (100 μmol/L). Three hours later, cells were lysed and equal amounts of proteins were immunoprecipitated with HIF-1α antibody (*top*). Equal amounts of protein samples were run on a gel and Western blotting was done for β-actin to serve as loading control (*bottom*). *Lanes 4* and *5,* same as above except that cells were treated with LY294002 (*LY*, 20 μmol/L) for 3 hours before pulse labeling.

Erlotinib has similar effect as gefitinib in SQ20B cells. To determine whether the results described above were specific to the drug gefitinib, we repeated the studies using another small-molecule EGFR inhibitor, erlotinib. Ten micromoles per liter of the drug led to near-complete disappearance of both phospho-EGFR and phospho-Akt (Supplementary Fig. S6). Treatment with this concentration of drug under normoxia led to an ~50% decrease in VEGF mRNA expression (Fig. 5*A*). Erlotinib decreased activity of both the 1.5 kb VEGF promoter construct as well as the −88/+54 construct (Fig. 5*B*). Consistent with an effect on Sp1-mediated transactivation of the promoter, gel shift assay showed that gefitinib decreased retardation of a labeled DNA probe corresponding to nucleotides −88 to −66 in the VEGF promoter (Fig. 5*C*; compare *lanes 1* and *3*). Similar to gefitinib, erlotinib also decreased HIF-1α induction by hypoxia (Fig. 5*D*; compare *lanes 3* and *4*).

Gefitinib decreases VEGF expression in HSC3 cells. To generalize our results, we also did studies with a second cell line, HSC3. Gefitinib at 5 μmol/L effectively decreased phosphorylation of both EGFR and Akt (Supplementary Fig. S7*A*). This dose of drug decreased VEGF expression under normoxia (Supplementary

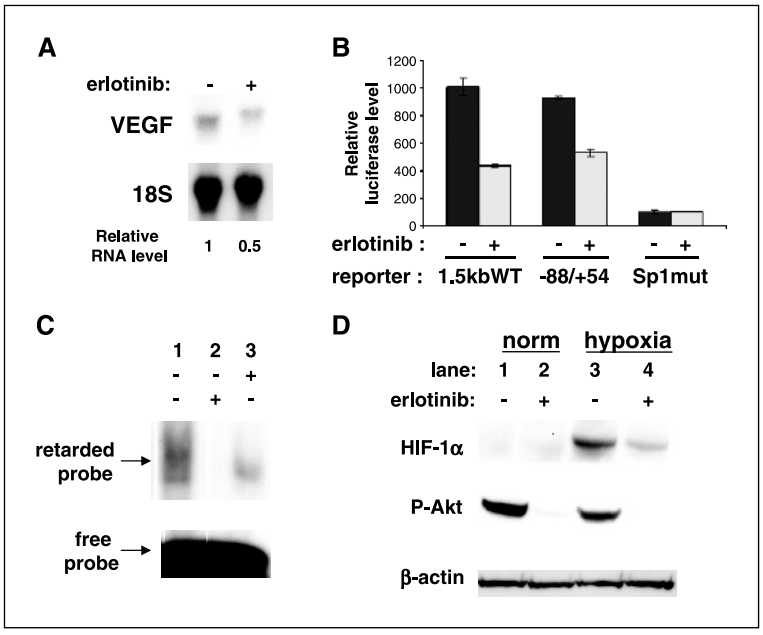

Figure 5. Effects of erlotinib in SQ20B cells. *A,* SQ20B cells were treated with erlotinib (10 μmol/L). Sixteen hours later, RNA was harvested and Northern blotting was done. *B,* plasmids containing 1.5 kb, −88/+54, and Sp1 mut of VEGF promoter upstream to a luciferase reporter gene were cotransfected with β-galactosidase expressing plasmid (pSV2-β-gal). Twenty-four hours later, cells were treated with erlotinib. Sixteen hours later, samples were collected and assayed for luciferase and β-galactosidase activity. *Y axis,* relative luciferase levels (ratio of luciferase to β-galactosidase readings). *Columns,* mean of three independent transfections; *bars,* SD. *C,* oligonucleotides corresponding to −88 to −66 bp in the human VEGF promoter were labeled with [γ-^{32}P]ATP. Gel shift assay was done using nuclear extract from cells treated with erlotinib. The DNA-binding reaction was also done with 100-fold molar excess of unlabeled Sp1 consensus oligonucleotides or with cold probe as indicated. *D,* SQ20B cells were treated with erlotinib for 16 hours and thereafter exposed for hypoxia (0.2% oxygen) for 3 hours. Cells were harvested and Western blotting was done for proteins as indicated.

Fig. S7*B*). Activity of the 1.5 kb VEGF promoter reporter was decreased by gefitinib as well as the activity of the −88/+54 VEGF promoter construct (Supplementary Fig. S7*B*). However, mutation of the Sp1 binding sites in the −88/+54 promoter led to lowered activity and unresponsiveness to gefitinib. These results are almost identical to those seen in SQ20B cells (Fig. 2*D*) and implicate the Sp1 sites in the response of the promoter to gefitinib. Gefitinib also decreased the induction of HIF-1α by hypoxia in this cell line (Supplementary Fig. S7*D*).

Discussion

The literature supports a link between EGFR activation and VEGF expression that is likely to be important in tumor progression (24–30). Interruption of this pathway with EGFR inhibitors leads to decreased VEGF expression, which may contribute to the antitumor activity of these agents. Therefore, we studied the signaling pathway linking EGFR inhibition with VEGF down-regulation. Our data indicate that the PI3K/Akt pathway operates downstream of gefitinib to regulate VEGF expression. PI3K inhibition leads to decreased VEGF expression, and Akt counteracts the down-regulation of VEGF expression and VEGF promoter activity by gefitinib. Because previous reports had suggested that EGF might induce the transcription factor HIF-1α (35, 40), we analyzed the effects of gefitinib on HIF-1α expression. Treatment of cells with the drug blocked HIF-1α induction and, consequently, VEGF induction in response to hypoxia. Luwor et al. (41) recently reported that the cetuximab (C225) monoclonal antibody could decrease HIF-1α expression and consequently VEGF expression. However, in contrast to their report, our results indicate that the story is more complicated. We describe a pathway that is distinct from the HIF-1 pathway by which VEGF expression is decreased in response to EGFR inhibition. The proximal core VEGF promoter contains Sp1 binding sites, and binding of Sp1 to these sites is modulated by EGFR activation. In a gel shift assay, gefitinib-treated cells showed decreased binding of factors to a DNA probe corresponding to the −88/−66 region in the VEGF promoter. This decreased binding could be reversed by inducing cells with Akt-expressing virus, indicating that Akt plays a role downstream of EGFR in this effect. Furthermore, gefitinib-treated cells showed decreased Sp1 phosphorylation. Because phosphorylation of Sp1 generally leads to its increased binding to DNA containing Sp1 sites (36–38), we hypothesize that gefitinib prevents Sp1 phosphorylation, thereby decreasing binding to the promoter and leading to decreased transactivation of the VEGF promoter (Fig. 6).

The down-regulation of HIF-1α expression caused by gefitinib results from a decrease in HIF-1α protein synthesis despite the fact that the HIF-1α mRNA levels are not altered. Treatment of cells with the PI3K inhibitor LY294002 had the same effect on decreasing HIF-1α translation, suggesting that the effect of gefitinib is mediated through the PI3K/Akt pathway. Consistent with this, Akt has been implicated in increasing protein translation through multiple mechanisms (42).

Therefore, we have found that there are two distinct pathways by which EGFR tyrosine kinase inhibitors can decrease VEGF transcription. Which of these predominates is likely to be dependent on the cell line and the particular conditions to which the cells are exposed. In SQ20B cells, it seems that the HIF-1 pathway does not play a major role in regulating the VEGF promoter under normoxia as mutation of the HRE did not lead to a tremendous decrease in promoter activity. Furthermore, gefitinib and erlotinib could substantially down-regulate VEGF promoter activity even when the HRE was mutated. It is likely that both pathways are important in regulating VEGF expression in human tumors. Human tumors often contain regions of hypoxia, but these regions are often heterogeneous and interspersed between regions that are better oxygenated (43–45).

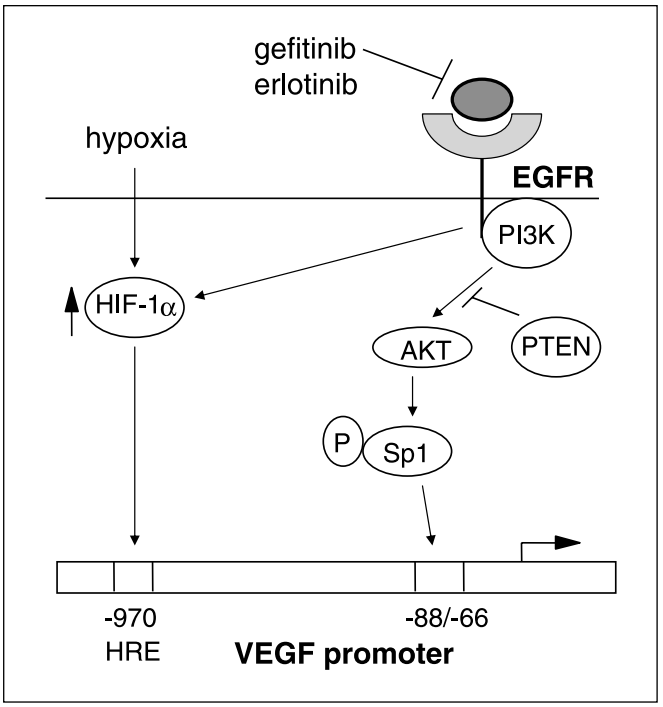

Figure 6. Schematic of pathway of VEGF promoter regulation by EGFR stimulation. Inhibition of EGFR with tyrosine kinase inhibitors leads to down-regulation of PI3K/Akt pathway that decreases VEGF promoter activity by two different pathways, one involving Sp1 and another involving HIF-1α.

Our findings have some implications regarding targeted therapy for patients. EGFR inhibition, both with tyrosine kinase inhibitors and C225, is currently being used in clinical trials; however, many patients who have EGFR expression do not show a favorable response (16, 17, 22, 46). There are many potential reasons why this might occur, but one might be activation of the PI3K/Akt pathway by EGFR-independent mechanism. We found that overexpression of Akt using adenovirus interfered with the down-regulation of VEGF by gefitinib. Human tumors often have loss of PTEN, overexpression of PI3K subunits, or overexpression or mutation of Akt, all of which can increase Akt expression independently of EGFR (8). It has previously been reported that loss of PTEN opposes the antitumor effect of gefitinib (47, 48). Our results offer a concrete example by which Akt activation might oppose the effect of EGFR inhibition through loss of sensitivity to VEGF suppression.

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