A Novel Mechanism of Resistance to Epidermal Growth Factor Receptor Antagonism *In vivo*

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Abstract

Epidermal growth factor receptor (EGFR) is widely expressed in a number of solid tumors including colorectal cancers. Overexpression of this receptor is one means by which a cell can achieve positive signals for survival and proliferation; another effective means is by constitutive activation of EGFR. We have elucidated the role of constitutive EGFR signaling in malignant progression by stably transfecting colon cancer cells with a human transforming growth factor-α cDNA (a ligand for EGFR) under repressible control by tetracycline. We show that constitutive expression of transforming growth factor-α and its subsequent constitutive activation of EGFR allows for cancer cell survival in response to environmental stress *in vitro* and *in vivo* as well. The reversal of constitutive EGFR activation results in the loss of downstream mitogen-activated protein kinase and Akt activation, and a reduction in xenograft size that is associated with decreased proliferation and increased apoptosis. We used CI-1033, a small molecule antagonist of EGFR, to dissect an activation pathway that shows the ability of ERBb2 to activate Akt, but not Erk in the face of EGFR antagonism. This novel escape mechanism is a possible explanation of why anti-EGFR therapies have shown disappointing results in clinical trials. [Cancer Res 2007;67(2):665–73]

Introduction

Epidermal growth factor receptor (EGFR) signaling has been linked to tumor growth and malignant progression (1–7). EGFR is one of four members of the ErbB family of receptors. Activation of EGFR generates complex signal transduction pathways that modulate many normal cellular processes such as proliferation, survival, adhesion, migration, and differentiation. Aberrant activation of EGFR has been shown to be critical for the maintenance of malignancy in a number of solid tumors, including colon cancer (8–10). EGFR can be activated as a homodimer or as a heterodimer with one of the other three members of the ErbB family. This dimerization results in cross-phosphorylation of SH2 sites of the individual receptors and enables autokinase activity. Phosphorylation of SH2 sites causes signaling complexes to dock to the receptors, which consist of adapter proteins and downstream enzymes. Various ligands including EGF-like molecules, neuregulins, and transforming growth factor-α (TGFα) activate EGFR by binding to the extracellular domain and inducing the formation of receptor homodimers and heterodimers.

TGFα activation of EGFR results in a positive growth stimulus (11, 12). High levels of TGFα have been associated with neoplastic transformation (13, 14) and overexpression of TGFα by stable transfection transforms fibroblasts in culture (15). Transgenic mice overexpressing TGFα develop malignant tumors at a number of sites (16–18). Furthermore, *in vivo* studies have shown that the overexpression of TGFα enhances oncogene-induced carcinogenesis (19–21).

The ability of TGFα to contribute to transformation and oncogenesis occurs through the activation of EGFR. EGFR is widely expressed in a number of solid tumors. In non–small lung cancer, for example, high expression of EGFR has been documented and is associated with a poor prognosis (9, 22, 23). Overexpression of the receptor is only one means by which a cell can achieve positive signals for survival and proliferation. Another means is by constitutively activating the EGF receptor.

We have approached the elucidation of the role of constitutive signaling in malignant progression by stably transfecting early malignant colon cancer cells with a human TGFα cDNA under repressible control by tetracycline. This construct generates constitutive EGFR activation without alteration of EGFR levels (10). The early malignant model used for these studies is a cell line designated FET (24). These cells are well differentiated in tissue culture as they form functional transport domes characteristic of colon epithelial function. Moreover, in athymic mice, FET cells form transient small tumors at a s.c. site which ultimately stop growing and gradually regress over time (10). FET TGFα transfectants show growth factor independence and continue to activate EGFR constitutively in growth-arrested states in contrast to growth factor–dependent FET cells that down-regulate TGFα as well as EGFR activation during growth-arrested states (25). We have shown that inappropriate TGFα expression of transfected cells enables progressive growth of s.c. xenografts in athymic mice (10).

As a tumor grows in size, ultimately, areas of malignant cells outstrip available supplies of oxygen, nutrients, and growth factors in the tumor tissue. The environmental stresses of hypoxia and starvation generate two options for malignant cells. Cells that are able to adapt to environmental stress survive and ultimately undergo expansion as angiogenesis occurs, on the other hand, cells that do not have adequate survival adaptations undergo programmed cell death. FET seems to have a phenotype that cannot adapt to these stresses *in vivo*, whereas TGFα transfection enables cells to survive (10).

It is our hypothesis that constitutive expression of TGFα and its subsequent constitutive activation of EGFR allows for survival in

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the face of environmental stress. Although this hypothesis has been shown to be correct *in vitro* for both native and engineered tissue culture cells, it has not been shown *in vivo* (10, 26). We have used "tetracycline-off" regulation of TGFα to repress the constitutively activated state of EGFR in FET TGFα–transfected cells to one in which EGFR activation is negligible *in vivo*. Moreover, we show that reversal of constitutive EGFR activation results in the expected loss of downstream mitogen-activated protein kinase and Akt activation *in vivo* as well. These effects of tetracycline repression of TGFα in FET TGFα–transfected cells are paralleled by a lack of EGFR and downstream signaling in FET control cells *in vivo* as well. We also show that loss of constitutive EGFR activation in tetracycline-treated FET TGFα–transfected cells results in the reduction of xenograft size that is associated with decreased proliferation and increased apoptosis.

The pathway by which decreased proliferation and increased apoptosis causes regression of these tumors was also explored. CI-1033, a small molecule antagonist of EGFR, which has undergone clinical trials, has been shown to inhibit the growth of cultured EGFR-expressing tumor cell lines and repress the *in vivo* growth of tumors when grown as xenografts in nude mice (27). Characterization of the effects of this drug in the FET model system allowed for the identification of a novel mechanism of escape from the effects of small molecule EGFR inhibitors such as CI-1033. This mechanism involves the formation of an ERBb2 heterodimer with the EGFR in response to ligand (TGFα) binding by the receptor. Complex formation with EGFR leads to conformation-induced activation of ERBb2 kinase despite inhibition of EGFR kinase. The signaling from the ERBb2 kinase can then generate downstream signaling, leading to the activation of the phosphoinositide-3-kinase (PI3K)/Akt pathway.

Materials and Methods

Cell line. The FET human colon cancer carcinoma cell line was originally isolated from a primary human colon cancer (24). Stable transfection with a 930 bp fragment of full-length TGFα cDNA under the control of a tetracycline-repressible promoter, and isolation of resultant transfectants, was done as previously described (10). TGFα transfectants (FETα) and FET control cells were routinely maintained in chemically defined serum-free medium consisting of McCoy's 5A medium (Sigma, St. Louis, MO) supplemented with pyruvate, vitamins, amino acids, antibiotics, insulin (20 μm/mL, Sigma), EGF (10 ng/mL; R&D Systems, Minneapolis, MN), and 650 μg/mL of active geneticin (Life Technologies, Inc., Gaithersburg, MD).

Green fluorescence protein transfection. Packaging cells, 293GP (Clontech, Mountain View, CA), were cotransfected with the plasmid encoding VSVG envelope protein and the retroviral vector encoding green fluorescence protein (GFP) using FuGene (Invitrogen, Carlsbad, CA). Viruses were collected 48 h later and used to infect FETα. After 48 h, infected FETα cells were selected with puromycin for 5 days.

Tetracycline modulation. Tetracycline (Sigma) was dissolved in 50% ethanol and stored at −20°C. Tetracycline was added to the culture medium at a final concentration of 0.1 μg/mL. Fresh tetracycline was added twice weekly when changing the medium. For *in vivo* studies, tetracycline was added to the animals' drinking water at a concentration of 3 mg/mL.

Western blots. Cells were rinsed twice with PBS and lysed in lysis buffer [62.5 mmol/L Tris-HCl (pH 6.8), 6 mol/L urea, 0.5% NP40, and 2% SDS] with freshly added protein inhibitors (50 mmol/L NaF, 1 mmol/L Na3V04, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L benzamidine, 1 mmol/L DTT, 25 μg/mL aprotinin, 25 μg/mL tyrosine inhibitor, 25 μg/mL leupeptin, and 2 mmol/L β-glycerophosphate). Cells were then lysed by a 10-s sonication. Protein concentrations were determined using a bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). Cell lysates were heated for 5 min at 95°C in 2× sample buffer [100 mmol/L Tris (pH 6.8), 2% glycerol, 0.04% bromophenol blue, and 2% β-mercaptoethanol]. Proteins were separated by SDS-PAGE and electroblotted onto Hybond ECL nitrocellulose membranes (Amersham, Arlington Heights, IL). After blocking with 5% nonfat dried milk in TTBS buffer [20 mmol/L Tris (pH 7.5), 500 mmol/L NaCl, and 0.05% Tween 20], the blots were incubated with primary antibody overnight at 4°C. The blots were labeled with peroxidase-conjugated AffinPure goat anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 30 to 60 min and visualized by enhanced chemiluminescence detection (Amersham). Western blots were repeated thrice and protein was quantified using Image Quant software (Piscataway, NJ).

***In vivo* xenografts.** FET control cells and FETα cells were injected s.c. on the dorsal surface of 10 5- to 6-week-old BALB/c nude mice. Mice were maintained in a HEPA-filtered environment. All animal studies were conducted in accordance with the principles and procedures as outlined by federal, state, and institutional guidelines for the care and use of laboratory animals. Xenograft volume was determined by measuring the xenografts with calipers and using the equation volume = (length × width^2) × 0.5.

CI-1033 drug treatment. For *in vitro* studies, drug was added directly to the medium of quiescent cells to make a final concentration of 5 μmol/L. An equivalent volume of DMSO was administered as a control. Cells were harvested 3 h after treatment. For *in vivo* experiments, 10 animals with FETα xenografts were treated with CI-1033. Thirteen days postinjection, animals were treated with CI-1033 at 80 mg/mL in 0.557 mol/L of isothionic acid in lactate buffer (pH 7.0) by gavage. Treatment was continued for up to 15 days. Control animals received gavage treatment with isothionic acid/lactate buffer (vehicle) only. Xenograft volume was again determined by measuring the xenografts with calipers and using the equation volume = (length × width^2) × 0.5. Animals were euthanized and tumors were harvested after 15 days of treatment.

Antibodies. Antibodies were purchased from commercially available sources. EGFR was immunoprecipitated with a mouse monoclonal antibody from Santa Cruz Biotechnology, Santa Cruz, CA (sc-120). Phosphotyrosine analysis was done with RC-20 antibody (E120H; BD Transduction Laboratories). Total protein expression was determined with antibodies to EGFR (E12020; BD Transduction Laboratories, San Diego, CA), Erk (sc-93; Santa Cruz Biotechnology), and Akt (no. 9272; Cell Signaling Technology, Beverly, MA). Phosphospecific antibodies were obtained from Santa Cruz Biotechnology (sc-7383, p-Erk), Cell Signaling Technology (no. 9271, p-Akt, and no. 9204, p-p70S6K), and Calbiochem (no. 324864-p-EGFR, y1173, San Diego, CA). TGFα immunohistochemical staining employed a monoclonal antibody from Oncogene Sciences (Ab2, La Jolla, CA).

Immunohistochemistry. Deparaffinized tissue specimens were subjected to immunohistochemical staining for the detection of TGFα, p-EGFR, p-Erk, and p-Akt using an indirect detection method (28). The catalyzed signal amplification system was used for the phosphospecific antibodies (Dako Corporation, Carpinteria, CA). Each antibody staining was accompanied by a negative control in which slides were incubated with an isotypic IgG (monoclonal) or normal serum (polyclonal) instead of primary antibody. Specimens were processed on the same day to eliminate any variability in conditions. Slides were digitally photographed using the same settings.

***In vivo* proliferation.** Xenografts established from the FETα cells were harvested and placed in 10% neutral buffered formalin fixative for 12 to 24 h and then embedded in paraffin. Slides were cut from these blocks for H&E stains and for immunohistochemical characterizations. Serial sections were cut to complement the H&E sections and were stained with an IgG_1 rabbit polyclonal antibody for Ki-67 (Dako Corporation). Ki-67 Mib-1 is a nonhistone nuclear antigen present in late G_1, G_2, and S phase of the cell cycle but not G_0. The optimal dilution of 1:100 was used. Three- to 4-μm sections were cut, deparaffinized in xylene, and rehydrated in descending grades of ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in water. Immunostaining was done using a variation of the avidin-biotin-peroxidase method. Slides were counterstained with methyl green.

The proliferative rate was determined quantitatively by utilization of the CAS 200 image analyzer. On each harvest day, three to four tumors were

analyzed in each group. Three randomly selected histologically similar fields in each specimen were analyzed in FET TGFα tumors and FET vector control nodules. The mean proliferative activity was determined for each group.

***In vivo* apoptotic rate.** The apoptotic rate in xenografts was determined by the Apotag (Oncor, Gaithersburg, MD) terminal nucleotidyl transferase–mediated nick end labeling (TUNEL) assay kit. The apoptotic rate was determined by counting the number of positively stained apoptotic bodies per 75 μm^2 field at 20× magnification. This was done by two blinded observers (one being a clinical pathologist). The apoptotic rate was determined in each specimen by counting three random fields selected by the pathologist. The mean value for four tumors harvested from the same group on each day was used to determine the representative apoptotic rate.

Results

***In vitro* and *in vivo* characterization of FET and FETα responses to growth factor deprivation stress.** We had previously shown that FETα cells are growth factor–independent for cell cycle re-entry from a growth arrested state (10). It was not known, however, whether the constitutive EGFR activation generated by TGFα also generated constitutive survival signaling. Therefore, we determined whether FETα cells showed enhanced cell survival relative to FET control cells in response to growth factor deprivation stress (GFDS) as reflected by lower levels of PARP cleavage. PARP degradation products were seen in FET control cells, but not FETα cells, at 48 and 72 h following GFDS (Fig. 1*A*). This indicates that the ectopic expression of TGFα generates resistance to stress-induced apoptosis in FETα cells as compared with FET vector control cells.

A Western blot (Fig. 1*B*) shows the differences in downstream effector activation in FETα cells and FETα cells treated with tetracycline. The level of total EGFR for both treated and untreated cells is the same. The level of activated EGFR, however, is decreased in cells treated with tetracycline. Similarly, levels of activated Erk and activated Akt are also decreased in FETα cells treated with tetracycline.

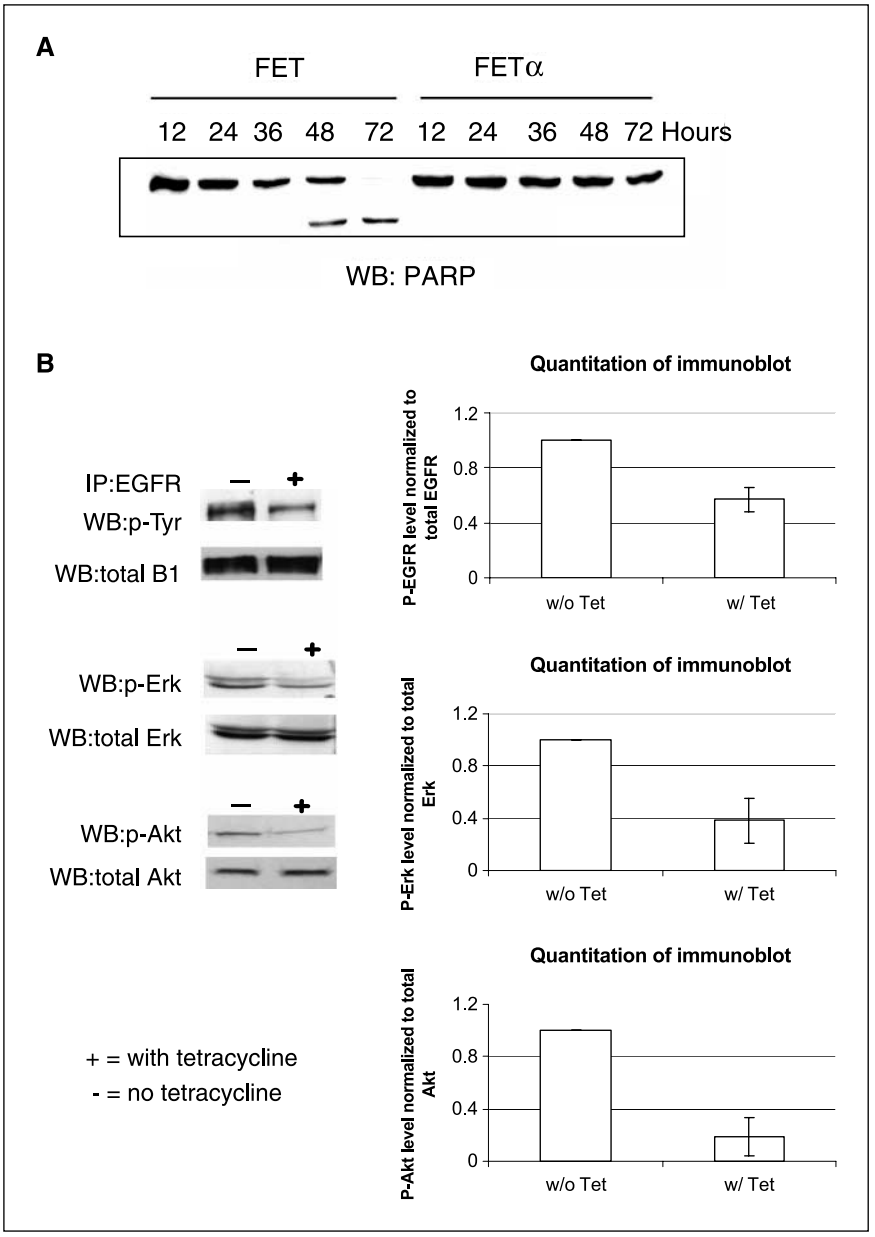

Figure 1. *A,* constitutive activation of EGFR rendered FET cells resistant to GFDS-induced apoptosis. FET vector control and FETα cells were deprived of growth factors after growth to 80% confluence. Cells were harvested at the indicated times and Western blot was done with anti-PARP antibody. *B,* repression of constitutive EGFR activation led to decreased activation of EGFR, Erk, and Akt. FETα cells were treated with 0.1 μg/mL of tetracycline for 3 d to repress constitutive EGFR activation. Cells were harvested and lysates were immunoprecipitated with anti-EGFR antibody. Western blot was done with anti-pTyr, anti-EGFR, anti-pErk, anti-Erk, anti-pAKT, and anti-AKT antibodies. Image Quant software was used to quantify protein levels.

Xenograft analysis. Having established differential FETα and FET control cells *in vitro* responses to GFDS, we sought to determine the effect of constitutive EGFR activation on the growth of FETα xenografts. As previously described, when inoculated s.c. in athymic nude mice, FET cells form small (300 mm^3) nodules that eventually regress (10). In contrast, FETα with constitutive TGFα expression is highly tumorigenic, as shown in the xenograft growth curve in Fig. 2*A*. This figure also shows that with the addition of tetracycline to the drinking water of the mice, and the subsequent down-regulation of TGFα, and therefore, decreased EGFR activation, there is a regression of the xenograft. Even large FETα xenografts (~900 mm^3) regress when animals are provided tetracycline (3 mg/mL) in the drinking water (Fig. 2*A*). Figure 2*B* shows two representative BALB/c nude mice with GFP-labeled FETα s.c. xenografts 30 days postinoculation on the left. On the right of Fig. 2*B*, there are two other BALB/c nude mice that have been treated with tetracycline for 34 days after establishing GFP-labeled FETα xenografts. The regression of green fluorescence is reflective of increased apoptosis and decreased proliferation that occurs as the xenografts regress in the absence of TGFα and subsequent EGFR activation. This indicates that even the growth of large tumors can be reversed when constitutive EGFR signaling for cell survival is blocked. These *in vivo* xenograft studies highlight the importance of constitutive EGFR activation because murine serum was incapable of supporting EGFR activation (as shown below) as well as sustaining xenograft growth in these experiments.

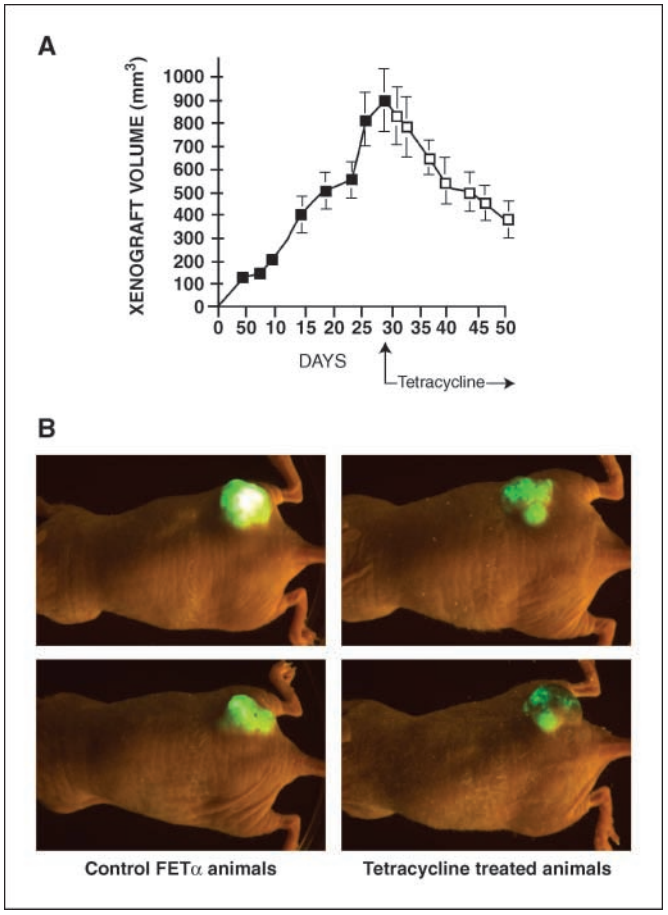

Figure 2. *A,* inhibition of xenograft growth of FETα cells by tetracycline. Exponentially growing cells (5×10^6) were inoculated s.c. in athymic nude mice. Tetracycline (3 mg/mL) was added to the drinking water daily from day 30 onwards. Tumors were measured externally on the indicated days in two dimensions using calipers. Tumor volumes were determined by the equation $V = (L \times W^2) \times 0.5$, where *L,* length; and *W,* width of the tumor. Values are the mean of 10 xenografts. *B,* GFP labeled 5×10^6 FETα cells were inoculated into athymic nude mice. Thirty days after inoculation, tetracycline (3 mg/mL) was added to the drinking water daily for 34 d.

Histology. Xenografts of FET control cells and FETα were excised for histologic analysis (data not shown). The tumor histologies were distinct for the FET control cells, FETα, and FETα + tetracycline xenografts. The FETα + tetracycline and the FET control cells xenografts showed similar histologic architectures. These xenografts were primarily composed of attenuated glandular foci with basolateral polarity of the nuclei. The FETα xenografts, which were not treated with tetracycline, were composed primarily of aglandular/solid tumor cells that had lost basolateral nuclear polarity and showed nuclear pleomorphism as well as cellular atypia.

Proliferation and apoptosis. Evaluation of the Ki-67 Mib1 antigen was used to assess the proliferative fraction of tumor cells, and the TUNEL assay (Apotag) was used to measure apoptosis in FETα versus FETα + tetracycline tumor xenografts. Figure 3*A* illustrates that with the addition of tetracycline, there is a decrease in the proliferative rate seen in the xenografts. Figure 3*B* shows increased Mib1 staining of the FETα xenograft, whereas there is decreased Mib1 staining of the FETα xenograft from that of an animal treated with tetracycline. Figure 3*C* illustrates that the withdrawal of TGFα by the addition of tetracycline results in a 2- to 3-fold increase in the apoptotic fraction. Figure 3*D* shows the difference in TUNEL staining between the FETα and FETα + tetracycline tumor xenografts, respectively. The resultant high rate of apoptosis and tumor regression reflects the poor ability of FET control cells to adapt to cellular stress in the absence of TGFα.

Immunohistochemical validation. FET control cell nodules, FETα, and FETα + tetracycline xenografts were analyzed for TGFα as well as the activation of EGFR, Erk, and Akt as reflected by immunohistochemistry using the appropriate phosphospecific antibodies for staining of tissues (Fig. 4). Results from each of the four experiments are presented in a similar fashion with the negative control in the top left, the FET control cell line in the top right, the FETα with no tetracycline in the bottom left, and the FETα with tetracycline treatment in the bottom right corner.

Figure 4*A* shows that the FETα cell line has a significant level of TGFα. With the addition of tetracycline, the production of TGFα is markedly reduced. Figure 4*B* examines the pEGFR status of the xenografts. With the addition of tetracycline, (TGFα is down-regulated), there is less activation of the EGFR receptor as shown by the decrease in pEGFR staining. The activation of the downstream effector Erk is shown in Fig. 4*C*. This shows that with the addition of tetracycline, the resultant decrease in TGFα and subsequent lack of activation of EGFR also fails to activate the downstream effector Erk. Similarly, Fig. 4*D* shows that with the addition of tetracycline, Akt is also not activated as shown by the lack of Akt phosphorylation.

The FET control cells do not show substantial signaling *in vivo*, despite the availability of their EGFR for activation by circulating ligand. These results are in agreement with the modulation of these components by tetracycline in tissue culture cells (10), and thus, also validate this methodology for the *in situ* analysis of the modulation of the activation states of EGFR, Erk, and Akt in response to the inhibition of EGFR activation.

CI-1033 inhibits autocrine TGFα-induced EGFR and Erk activation. FETα cells maintain EGFR activation during growth arrest due to continuous expression of autocrine TGFα (10). We

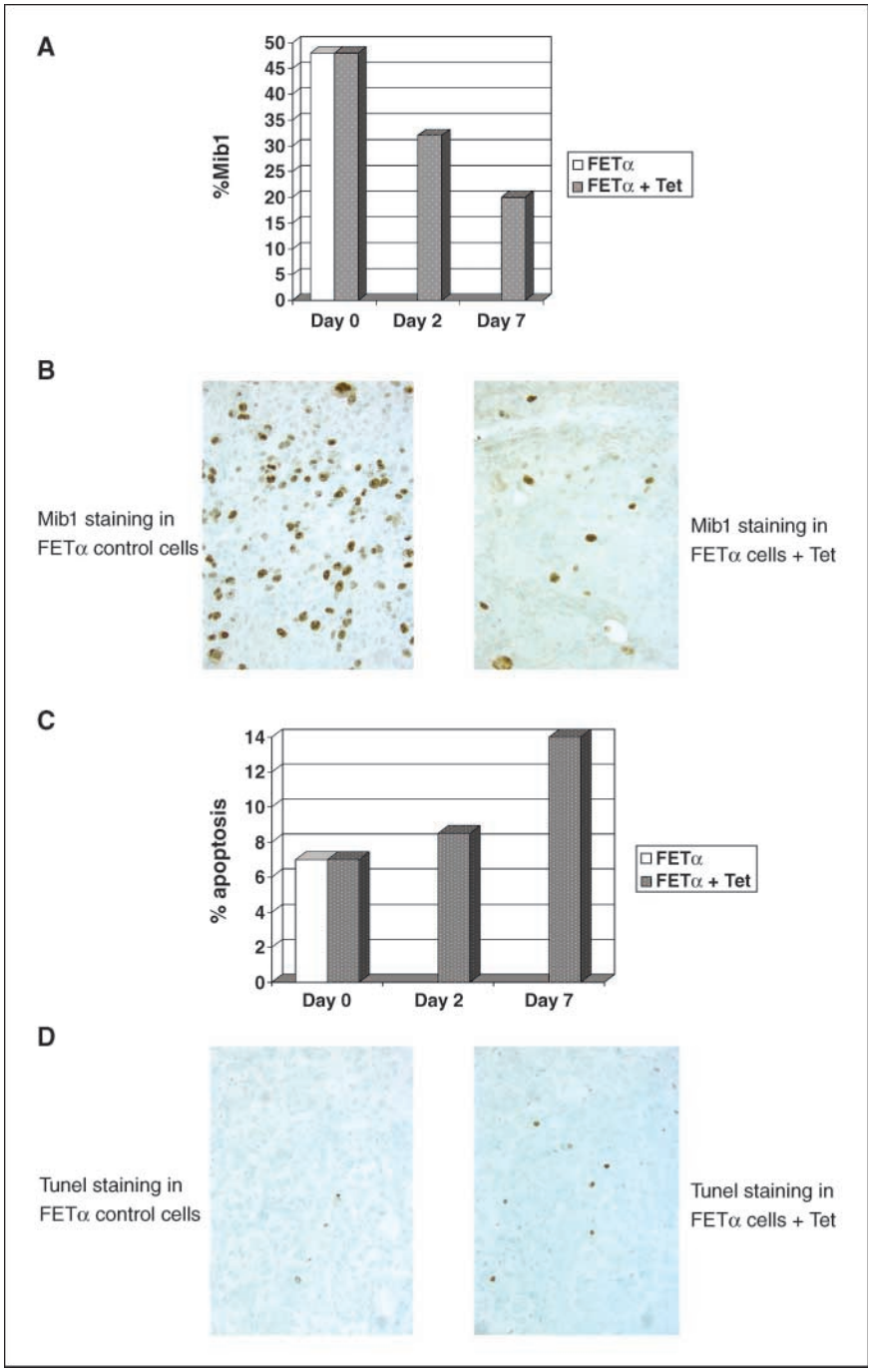

Figure 3. Decreased proliferation and increased apoptosis in FETα cells treated with tetracycline. *A,* four xenografts were harvested on each indicated day. The CAS 200 image analyzer was used to determine the mean proliferative activity for each group. *B,* slides were prepared from the harvested xenografts for standard immunohistochemistry and Ki-67 staining for FETα and FETα cells treated with tetracycline (Dako Corporation). *C,* apoptotic rates were determined by an average of three random 75 μm^2 fields at 20× magnification. *D,* slides were prepared from harvested xenografts for standard Apotag TUNEL assay for both FETα and FETα tetracycline–treated animals (Oncor).

treated FETα cells with CI-1033 to determine if downstream signaling cascades, which could potentially mediate tumorigenicity, were sensitive to the inhibition of EGFR kinase activity. FETα cells growing in a monolayer were treated with CI-1033 (5 μmol/L) or an equivalent volume of DMSO as a control. Receptor activation was determined by EGFR immunoprecipitation followed by a phosphotyrosine immunoblot. Control cells showed readily detectable levels of EGFR phosphotyrosine, whereas cells treated with 1 μmol/L of CI-1033 displayed a dramatic reduction in tyrosine phosphorylation of EGFR immunoprecipitates (Fig. 5*A*). Reblotting of the membrane with EGFR antibody indicated that total receptor protein levels were not affected by drug treatment (Fig. 5*A*).

Next, we examined the activation of EGFR-dependent signal transduction proteins. Immunoblotting with the phospho-Erk antibody revealed that cells challenged with CI-1033 showed a large decrease in detectable phospho-signal compared with control cells (Fig. 5*A*). This was apparent at the same dose that inhibited EGFR phosphotyrosine, thus implying that Erk activation was largely mediated by EGFR signaling.

Surprisingly, immunoblot with a phosphospecific antibody for Akt showed that CI-1033 did not inhibit the activation of this kinase (Fig. 5*A*). Moreover, an investigation of the effect of CI-1033 on another PI3K pathway intermediate, p70S6K, also indicated that it was not inhibited by EGFR blockade. Similar results were also

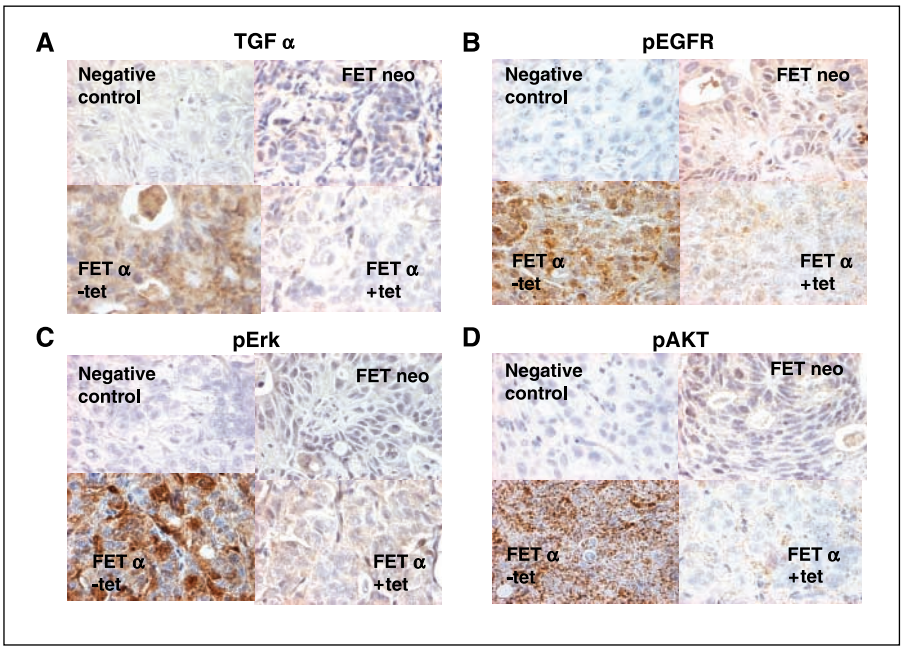

Figure 4. *A* to *D,* immunohistochemical validation of signal transduction. FETα xenografts were harvested and placed in buffered formalin for 24 h and then paraffin embedded. Slides were prepared for standard immunohistochemistry to evaluate the effect of tetracycline on the expression of TGFα, activated EGFR, activated Erk, and activated AKT. Phosphospecific antibodies were used to generate this data. Each experiment has negative control staining (*top left*), FET control cells (*top right*), FETα-untreated cells (*bottom left*), and FETα tetracycline–treated cells (*bottom right*).

obtained with another EGFR inhibitor, AG1478 (data not shown). These results were surprising because previous studies showed that tetracycline treatment in tissue culture blocked both the Erk and PI3K pathways (10), and the immunohistochemistry xenograft results described above also showed that the reversal of ectopic TGFα expression dramatically reduced both Erk and Akt activation, thereby establishing the linkage of EGFR activation to Akt signaling. How can these results be reconciled? The lack of Akt/p70S6K sensitivity to CI-1033 may indicate that the activation of this pathway is initiated by TGFα/EGFR binding, but is not directly dependent on the EGFR kinase activity. One mechanism which would be consistent with both the tetracycline and EGFR small molecule antagonist data would be a model in which ERBb2 or another member of the ErbB family is recruited to heterodimerize with TGFα-bound EGFR resulting in the conformational induction of the activation of the ERBb2 kinase or other ErbB kinase even though CI-1033 binding would inhibit EGFR kinase. We treated FETα cells with the 2C4 antibody against ERBb2, which we have previously shown to inhibit the heterodimerization of ERBb2 and EGFR, to test this hypothesis (29). Cells treated with the 2C4 antibody showed reduced ERBb2 activation as well as diminished activation of p70S6k indicating that PI3K pathway activation was linked to EGFR through the ERBb2 kinase. This result was also confirmed using a small molecule competitive inhibitor of ATP binding, AG879, which is 50-fold more selective for the ERBb2 kinase over that of the EGFR kinase (Fig. 5*C* and *D*).

CI-1033 causes tumor stasis in FETα xenografts. The data from *in vitro* studies described above suggest that CI-1033 is a potent inhibitor of autocrine TGFα-mediated constitutive EGFR tyrosine phosphorylation. Inhibition of EGFR kinase also led to a concomitant reduction in Erk activation. The potential for this drug to block these important signaling mediators, but not the PI3K/Akt pathway, encouraged us to determine if this compound had antitumor effects and whether we could show a similar pattern of downstream signaling as seen with CI-1033 in tissue culture in an *in vivo* setting. FETα xenografts that had formed 200 mm^3 tumors by day 12 postimplantation were treated by gavage with CI-1033 once a day for 13 days (Fig. 5*B*). Control mice that received a drug vehicle had tumors that continued to grow until day 22 postimplantation (Fig. 5*B*). CI-1033-treated mice, however, displayed no further increase in tumor formation (Fig. 5*B*). Growth of the tumor resumed upon termination of CI-1033. These results suggest that CI-1033 is capable of inhibition of tumor growth, but it does not cause tumor regression when faced with the ERB signaling pattern shown by these cells.

Utilization of the aforementioned immunohistochemical techniques for the determination of EGFR, Erk, and Akt activation *in situ* were applied to xenografts from control and CI-1033-treated mice to determine the ability of the drug to inhibit EGFR and the downstream pathways. Analysis of CI-1033 xenografts indicated that TGFα-induced EGFR activation was sensitive to inhibition by small molecule antagonists *in vivo* as shown by the decrease in EGFR phospho-staining (Fig. 6*A*). Additionally, phospho-Erk positivity was considerably less abundant 13 days posttreatment with CI-1033 than in tumors from control mice (Fig. 6*B*). In contrast to the observed modulation in ERK activity, we failed to detect a change in the relative levels of staining with p-AKT antibody after CI-1033 administration (Fig. 6*C*). This was consistent with the *in vitro* data described above and further confirms the lack of sensitivity to CI-1033 by PI3K-dependent pathways in this pattern of staining. Taken together, the findings described here implicate a mechanism that reduces the potential effects of inhibition of the EGFR with respect to downstream signaling in tumors that have EGFR/ERBb2 heterodimers.

Discussion

EGFR activation has been shown to play a role in tumor progression in a number of solid tumor malignancies including colon cancer. A greater understanding of the EGFR signal transduction cascade involved in tumor progression may result in the development of novel, selective combination anticancer approaches. The ability to modulate the TGFα construct in this study shows that the constitutive activation of EGFR by

constitutive expression of TGFα allows for tumor cell survival and the ability to overcome environmental stress. This was extended into an animal model along with *in situ* analysis of signaling. The reversibility of TGFα expression by tetracycline treatment coincided with a decline in EGFR, Erk, and Akt activation by immunoblot. This was validated *in vivo* when xenografts were stained with corresponding phospho-antibodies to detect the activation of these signaling markers. The mechanisms by which *in vivo* growth is enabled include both increased proliferation and decreased apoptosis, as reflected by Ki-67 and TUNEL analyses. Significantly, even large xenografts were dependent on endogenous EGFR activation by ectopic TGFα expression rather than acquiring nutrients from the host sera because tetracycline treatment led to a reduction of the tumor volume.

Xenograft studies are limited in that they may not reflect the complicated biological interplay between the host and an *in situ* tumor that arises *de novo*. However, they do provide an *in vivo* biological model to study tumor biology. The *in vivo* results with xenografts suggest that the importance of constitutive ErbB family activation by autocrine mechanisms *in vivo* may be underestimated.

To help dissect the signal transduction pathway through which EGFR activation results in tumor progression, we studied the effects of the EGFR kinase inhibitor CI-1033. Initially, we defined the signaling events *in vitro* which were mediated by TGFα autoactivated EGFR and then determined their susceptibility to inhibition by CI-1033. In contrast to the results obtained with tetracycline reversal of TGFα, Akt activation was not susceptible to inhibition by EGFR tyrosine kinase blockade with CI-1033. This

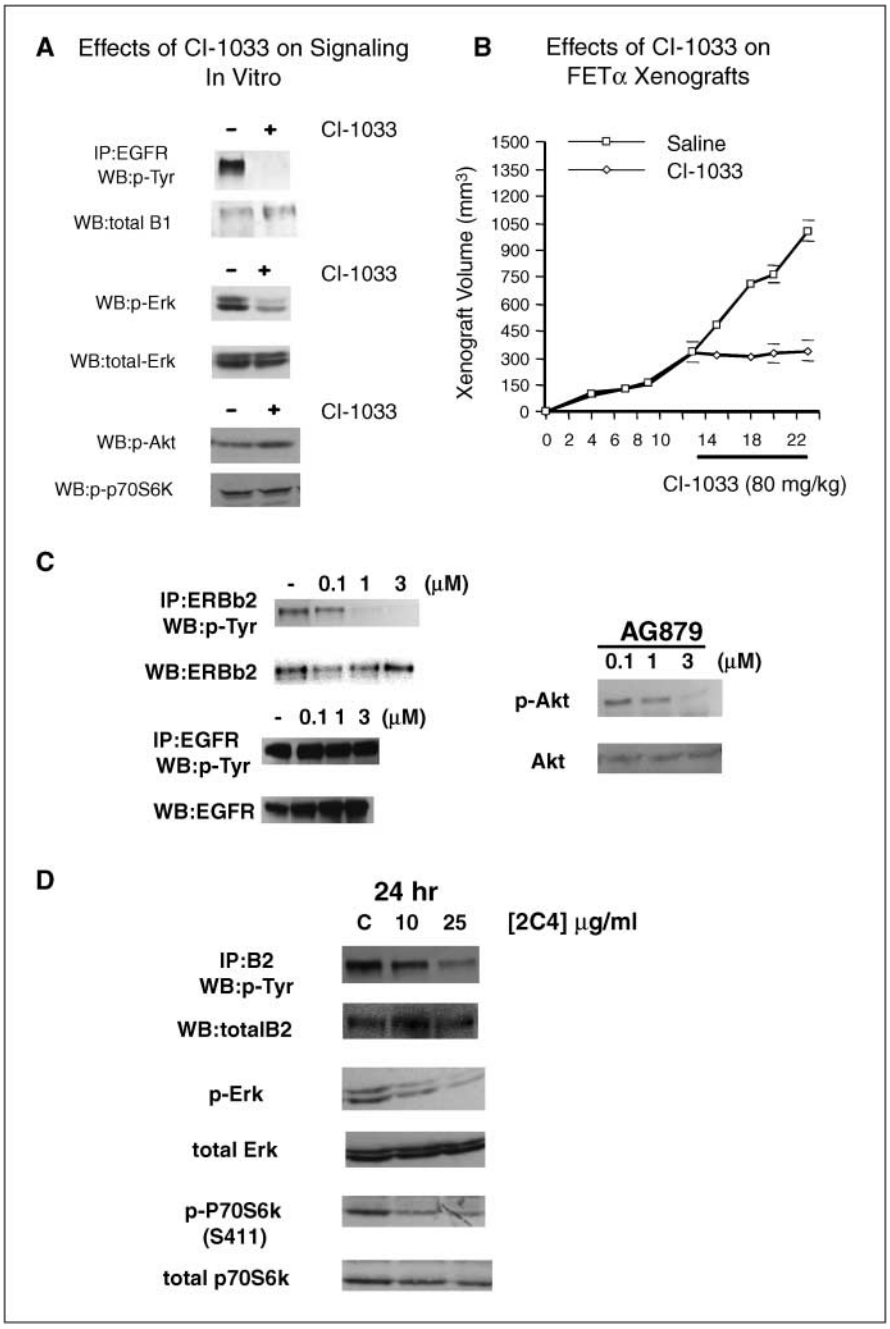

Figure 5. Effects of clinical EGFR antagonist on FETα cells *in vivo* and *in vitro*. *A,* FETα cells were treated with 5 μmol/L of CI-1033 for 3 h. Cells were harvested and lysates were immunoprecipitated with anti-EGFR antibody. Western blot was done with anti-pTyr, anti-EGFR, anti-pErk, anti-Erk, anti-pAkt, and anti-Akt antibodies. *B,* ten animals with FETα xenografts were treated with CI-1033. Thirteen days postinjection, animals were treated with CI-1033 at 80 mg/mL in 0.557 mol/L of isothionic acid in lactate buffer (pH 7.0) by gavage. Treatment was continued for up to 15 d. Control animals received gavage treatment with isothionic acid/lactate buffer (vehicle) only. Animals were euthanized and tumors were harvested after 15 d of treatment. Tumor volumes were determined by the equation $V = (L \times W^2) \times 0.5$, where *L,* length; *W,* width of the tumor. *C,* FETα cells were treated with AG879, an ERBb2 antagonist, and Western blots evaluating phosphorylated ERBb2 and p-Akt. *D,* FETα cells were treated with the 2C4 antibody to ERBb2 and Western blots to p-ERBb2 and p-Erk, and p70S6k are shown.

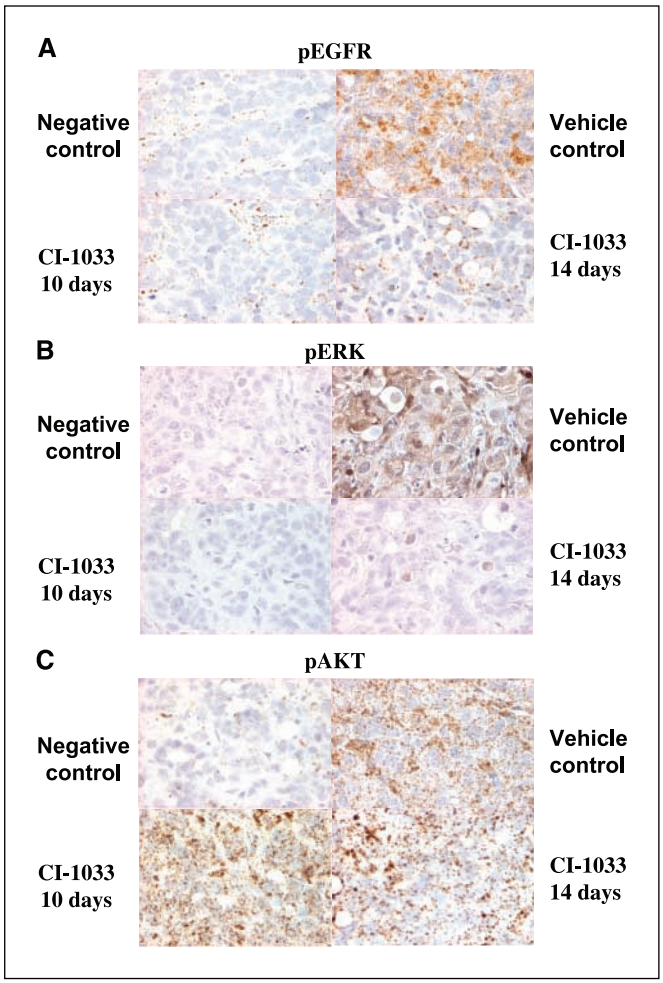

Figure 6. *A* to *C,* CI-1033 inhibits activation of EGFR and Erk, but not AKT. Thirteen days postinoculation; xenografts were treated with CI-1033 and harvested after 10 or 14 d of treatment. Tissues were placed in buffered formalin for 24 h and paraffin embedded. Immunohistochemistry was done for p-EGFR, p-Erk, and p-AKT on negative control, vehicle control, 10 day–treated and 14 day–treated xenografts.

effect was apparent from both cell culture experiments and in drug-treated xenografts. The mechanism which would be consistent with both the tetracycline-off and the EGFR small molecule antagonist data would be a model in which ErbB2 or another member of the ErbB family is recruited to heterodimerize with TGFα-bound EGFR (activated EGFR), thereby leading to the conformational induction of the activation of ErbB2 kinase or other ErbB family member even though CI-1033 binding would inhibit EGFR kinase activity. Thus, the data suggest that blockade of EGFR tyrosine kinase activity alone does not necessarily lead to the inhibition of all its apparent ligand-associated pathways. Given that other members of the ErbB family such as ErbB2 can participate in EGFR signaling, it seems that all aspects of EGFR signaling are not necessarily silenced by EGFR kinase inhibition alone. It has been shown that ErbB2 remains capable of signaling to activate Akt in response to EGF in the presence of a catalytically impaired EGFR kinase (30). Previously, we showed that ErbB2 is recruited to EGFR in the FET model system (10). Here, we have shown that this results in the conformational induction of the activation of ErbB2 kinase and its downstream effectors, even though CI-1033 binding inhibits EGFR kinase.

In vivo data showed that CI-1033 prevented further tumor growth in staged tumors when administered to mice bearing FETα xenografts. From the signaling results obtained, we conclude that downstream activation of Erk by EGFR results in tumor growth. This is reflected by the associated reduction in Erk activity by inhibition of EGFR with CI-1033 and a stable xenograft volume which did not increase as long as treatment was continued. Of note, however, is the difference in response to CI-1033 by FETα versus the tetracycline-off experiment in which there is not just tumor stasis, but tumor regression. This suggests that EGFR inhibition with the kinase inhibitor CI-1033 does not result in increased apoptosis. Data from our group using a combination of selective EGFR and ErbB2 inhibitors or a dual EGFR/ERBb2 selective inhibitor with FETα and other cell lines, shows that when both members of the ErbB family are targeted in combination, the effects on activation status, apoptosis, and inhibition of growth are synergistic *in vitro* (31, 32).

This mechanism of dimer partner activation shown by EGFR and ErbB2 is a possible explanation for why molecular targeted therapies have shown disappointing responses in clinical trials as single agents (33–35). In addition to this escape from inhibition of signal transduction through a mechanism involving incomplete inhibition of a heterodimerized receptor, we have shown that inhibition of one receptor can be compensated for by the increased expression and activation of another receptor (36). ErbB2 inactivation by stable transfection of a single chain antibody against ErbB2 resulted in the inactivation of ErbB2 tyrosine phosphorylation and reduced heterodimerization of ErbB2 and ErbB3. This, in turn, led to the reorganization in ErbB family signaling involving increased expression and activation of the EGFR as well as increased heterodimerization between EGFR and ErbB3.

Thus, blockade of only one ErbB family member can result in the generation of signaling escape mechanisms such as dimer partner activation or recruitment and activation of remaining family members that can sustain signal pathways that ultimately may allow for tumor progression. The role of the ErbB family in tumor growth and progression is well documented but, by the same token, is very complex. Efforts directed at clinical targeting of this family continue to reveal new complexities involving the flexibility of this signaling system, especially with respect to the inhibition of individual kinases and the resultant effects on the system as a whole. The effects of targeting heterologous signaling receptors such as the insulin-like growth factor receptor and G proteins are as yet unexplored, but these interactions clearly have the potential to contribute to this complexity. Added to these considerations is the well-known variability of cell context and signaling. The ErbB family remains an attractive and promising target for cancer treatment; however, it is unlikely that its potential for broad clinical application will be realized until more is known about the complex interplay among the ErbB family members, the effects of modulation of ErbB signaling on other receptor signaling modules, and the cellular contexts that are most frequently encountered in cancer target tissues.

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