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Chronic Activation of Wild-Type Epidermal Growth Factor Receptor and Loss of Cdkn2a Cause Mouse Glioblastoma Formation

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Abstract

Glioblastoma multiforme (GBM) is characterized by overexpression of epidermal growth factor receptor (EGFR) and loss of the tumor suppressors Ink4a/Arf. Efforts at modeling GBM using wild-type EGFR in mice have proven unsuccessful. Here, we present a unique mouse model of wild-type EGFR-driven gliomagenesis. We used a combination of somatic conditional overexpression and ligand-mediated chronic activation of EGFR in cooperation with Ink4a/Arf loss in the central nervous system of adult mice to generate tumors with the histopathologic and molecular characteristics of human GBMs. Sustained, ligand-mediated activation of EGFR was necessary for gliomagenesis, functionally substantiating the clinical observation that EGFR-positive GBMs from patients express EGFR ligands. To gain a better understanding of the clinically disappointing EGFR-targeted therapies for GBM, we investigated the molecular responses to EGFR tyrosine kinase inhibitor (TKI) treatment in this model. Gefitinib treatment of primary GBM cells resulted in a robust apoptotic response, partially conveyed by mitogen-activated protein kinase (MAPK) signaling attenuation and accompanied by BIM_{EL} expression. In human GBMs, loss-of-function mutations in the tumor suppressor PTEN are a common occurrence. Elimination of PTEN expression in GBM cells posttumor formation did not confer resistance to TKI treatment, showing that PTEN status in our model is not predictive. Together, these findings offer important mechanistic insights into the genetic determinants of EGFR gliomagenesis and sensitivity to TKIs and provide a robust discovery platform to better understand the molecular events that are associated with predictive markers of TKI therapy. *Cancer Res; 71(23); 7198–206. ©2011 AACR.*

Introduction

Glioblastoma multiformes (GBM) are classified on the basis of histopathologic features, clinical presentation and molecular characteristics (reviewed in refs. 1, 2). The hallmark features of GBM are uncontrolled cellular proliferation, extensively diffuse infiltration, and a propensity for hypoxia and necrosis that engenders robust angiogenesis and a perennial resistance to therapeutic intervention. The epidermal growth factor receptor (EGFR) plays a crucial role in GBM pathogenesis (3). The importance of this pathway is highlighted by the fact that wild-type EGFR ($EGFR^{WT}$) and its ligands are over expressed and activated in more than 65% of GBM tumors (4–6). While initiation of this tumor subtype requires the overexpression of $EGFR^{WT}$ along with the concomitant loss of the Cdkn2a ($p16^{INK4A}$/$p19^{ARF}$) tumor suppressor locus (7), the role of EGFR signaling in tumor maintenance and sensitivity to tyrosine kinase inhibitors (TKI) is less well studied, especially in animal models. Most of our knowledge of EGFR signaling is based on *in vitro* studies of acute, ligand-mediated activation of the receptor within minutes. This paradigm differs from clinical observations, as EGFR is thought to be chronically active in GBM because of autocrine/paracrine expression of ligands including EGF, TGFα, and HB-EGF (8–11). As such, studies based on clinically relevant mechanisms of response to EGFR inhibition remain largely unexplored, especially in an *in vivo* model system.

The oncogene addiction hypothesis stipulates that a cancer cell is physiologically dependent on the continued activity of an oncogene for maintenance of the malignant phenotype (12). Although the mechanistic details of oncogene addiction are likely to be cancer specific, it appears that oncogenic kinases transduce excessive survival signals through pathways that are

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controlled by canonical growth and survival pathways [e.g., Akt and mitogen-activated protein kinase (MAPK)]. Treatment of oncogenically addicted cancer cells with kinase-inhibitory drugs suppresses these signals, resulting in an oncogenic shock characterized by the cessation of proliferation through a variety of mechanisms including growth arrest, differentiation, senescence, and apoptosis (reviewed in ref. 13). *In vitro* based studies show that inhibition of receptor tyrosine kinases in GBM can lead to the rapid onset of apoptosis, suggesting that GBMs also experience this addiction to oncogenic stimuli (14), although the mechanistic details of oncogene addiction in GBMs remain ill defined.

The use of EGFR TKIs for GBM treatment has proven surprisingly ineffective, resulting in gains of a few months of progression-free survival, with no significant gain in overall survival (reviewed in ref. 15). Retrospective studies show that patients who responded to TKI therapy had tumors harboring $EGFR^{vIII}$ mutant receptors and an intact *PTEN* gene, whereas the nonresponding patients had PTEN-null tumors (16, 17). This finding led to the suggestion that loss of PTEN uncouples PI3K–Akt signaling from the control of EGFR activity. By freeing tumors from their addiction to oncogenic EGFR, PTEN deletion may provide a means of resistance to TKI therapy. However, these observations were not sustained in follow-up phase I/II trial studies (18, 19) showing a vast complexity in the molecular mechanisms of EGFR TKI therapy response.

Overcoming resistance to targeted therapeutics in patients will require an in-depth understanding of the molecular mechanisms of tumor cell resistance. Accurate and realistic model systems can serve as a surrogate paradigm to predict clinical testing, representing a rapid, inexpensive, and powerful approach to this problem. However, there are currently no mouse models of malignant glioma that use $EGFR^{WT}$ as an oncogenic driver of tumorigenesis, making such studies impossible (20). Here we describe and validate a novel genetically engineered mouse (GEM) model of $EGFR^{WT}$-driven GBM. We established that a strict spatiotemporal expression of $EGFR^{WT}$ and chronic autocrine stimulation with a ligand, combined with the loss of clinically relevant tumor suppressor genes efficiently induces gliomagenesis. Using this novel mouse model, we reveal that these GBM tumor cells are oncogenically addicted to EGFR. Treatment with an EGFR TKI results in a rapid BIM_{EL}-mediated apoptotic response. We further show that loss of PTEN posttumor formation does not uncouple PI3K–Akt survival signaling from EGFR control and does not induce TKI resistance. These findings are consistent with the clinical observation that PTEN status is not a predictor of EGFR TKI sensitivity.

Materials and Methods

EGFR conditional mice and procedures

Procedures were carried out in accordance with Tufts University's recommendations for care and use of animals and were maintained and handled under protocols approved by Institutional Animal Care and Use Committee (IACUC). Conditional expression of $EGFR^{WT}$ was achieved as previously described (21). Viral vector construction, production, and stereotactic injections are described in Supplementary Materials and Methods section.

Histology and immunodetection

Brains were either used to isolate primary cultures or processed for histology (Supplementary Materials and Methods). Immunodetection of cytologic markers by immunohistochemistry and proteins were carried out using antibodies and standard protocols (see Supplementary Materials and Methods).

Survival assays and inhibitor treatments

Cell viability was measured by trypan blue exclusion and XTT assays. Cells were treated with gefitinib (LC Labs) or PD325901 (LC Labs) for 16 to 24 hours and the total number of cells was reported.

Statistical analysis

Statistical analyses were carried out using the 2-tailed, unpaired Student t test in Prism 5.0 (GraphPad Software).

Results

Ligand-mediated activation of wild-type EGFR in the context of tumor suppressor loss in mice induces tumors with histopathologic characteristics of human GBM

Many studies have reported the presence of autocrine and/or paracrine expression of EGFR and its ligands in GBM tumors (8–11). We validated these observations using The Cancer Genome Atlas (TCGA) public database by conducting a gene set enrichment analysis in GBMs with an amplified EGFR gene locus that overexpress wild-type and point-mutant EGFR versus non–EGFR-expressing tumors to determine whether EGFR ligands are indeed preferentially expressed in EGFR-positive tumors (Supplementary Fig. S1A–S1C). Our analysis reveals that human tumors that overexpress EGFR preferentially have a relatively high expression of EGF ligand than GBM tumors with low EGFR expression levels ($P = 0.000076$). These results, combined with previously reported evidence of ligand–receptor coexpression in GBM, show that physiologically relevant overexpression of EGFR is associated with ligand expression. This strong correlation between ligand and receptor overexpression suggests that $EGFR^{WT}$ signaling can be chronically active in GBMs.

We recently supported these observations experimentally *in vivo* by showing that overexpression of $EGFR^{WT}$ alone is insufficient to promote gliomagenesis (21). To model $EGFR^{WT}$-driven GBM in mice, we developed a strategy to coexpress TGFα, an EGFR ligand expressed in human GBMs (8, 11, 22–26), and $EGFR^{WT}$ in the adult mouse brain. We used a Cre/Lox conditional $EGFR^{WT}$ transgenic strain in which overexpression of human $EGFR^{WT}$ is Cre-dependent (21). Robust $EGFR^{WT}$ expression is triggered by the removal of a floxed translational and transcriptional stop cassette (LSL), which attenuates the activity of an artificial ubiquitous promoter (*CAG*). To simultaneously express Cre recombinase and TGFα, we created a bicistronic lentiviral vector that expresses TGFα and Cre (TGFα–IRES–iCre). A construct expressing eGFP in lieu of

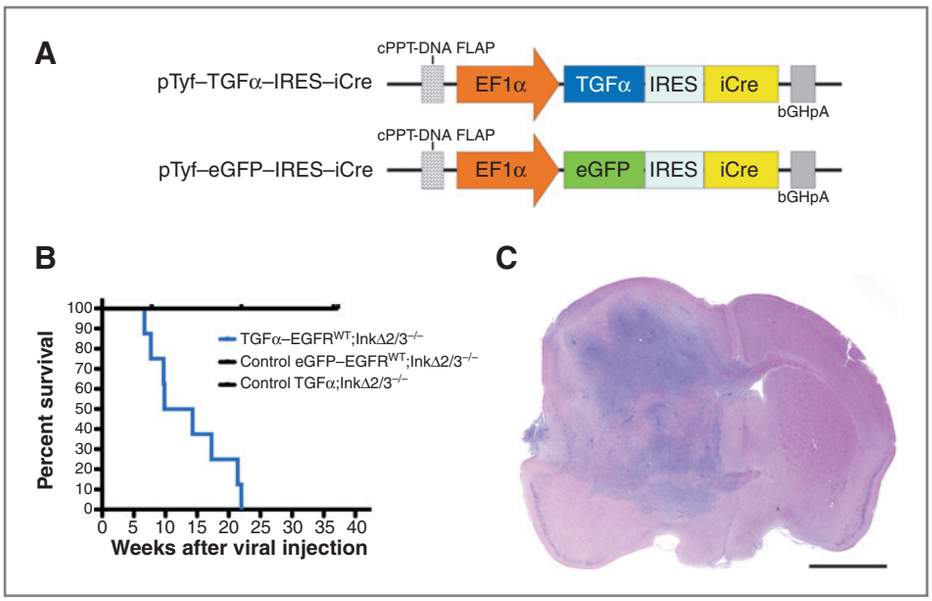

Figure 1. Activated EGFR^{WT} cooperates with loss of tumor suppressor genes to form brain tumors that have characteristics of GBM. A, schematic representation of the pTyf lentiviral transducing vectors. This bicistronic vector is derived from a previously described self-inactivating virus (46) modified to contain the human TGFα cDNA followed by a poliovirus1 IRES and improved Cre (iCre) cDNA (50) driven by the human elongation factor-1α (EF1α) promoter. For control experiments, TGFα is replaced with the *eGFP* gene. The presence of a central polypurine tract (cPPT)-DNA FLAP element upstream of the multiple cloning site significantly improves the transduction efficiency in central nervous system tissues (48, 49). B, survival rate (Kaplan–Meier) analysis of conditional EGFR mice. Cohorts of mice of the indicated genotypes were stereotactically injected in the striatum with titer-matched pTyf–TGFα–IRES–iCre or pTyf–eGFP–IRES–iCre and monitored for survival over time. C, photomicrograph of a hematoxylin and eosin–stained coronal section of a TGFα–EGFR^{WT};InkΔ2/3$^{-/-}$ brain tumor. Scale bar, 2.0 mm.

TGFα serves as a control vector (eGFP–IRES–iCre; Fig. 1A). We induced the coexpression of TGFα (or eGFP) and EGFR^{WT} by conducting stereotactic intracranial injections of matched titers (Supplementary Fig. S2A and S2B) of TGFα–IRES–iCre and eGFP–IRES–iCre viruses in cohorts of conditional CAG–LSL–EGFR^{WT};InkΔ2/3$^{-/-}$ compound mice and monitored tumor formation and survival over time. Mice coexpressing EGFR^{WT} and TGFα in a p16^{Ink4a}/p19^{Arf} null background developed brain tumors with a median survival of 10 weeks postinjection (Fig. 1B). Neither expression of EGFR^{WT} in the absence of ligand, nor expression of TGFα in the absence of transgenic EGFR^{WT} resulted in tumor formation in p16^{Ink4a}/p19^{Arf} null mice, supporting the hypothesis that receptor and ligand coexpression are required for EGFR^{WT}-driven gliomagenesis in mice.

TGFα–EGFR^{WT};InkΔ2/3$^{-/-}$ tumors share many histopathologic features with human GBMs (Fig. 1C). They are highly cellular, very proliferative (numerous mitoses), and are composed of cells displaying pleomorphic nuclei present on a fibrillary background (Fig. 2A). In addition, the tumors include giant multinucleated cells and areas of pseudopallisading necrosis, both prominent features of human GBM (Fig. 2B and C). Moreover, these tumors are highly infiltrative with leptomeningeal spread (Fig. 2D) and diffuse infiltration into normal parenchyma (Fig. 2E). Tumor cells are also found in the perivascular space and can be observed at significant distance from the bulk mass (Fig. 2F). Immunohistochemical staining of TGFα–EGFR^{WT};InkΔ2/3$^{-/-}$ tumors for EGFR revealed robust membrane expression, whereas staining for markers associated with astrocytic (glial fibrillary acidic protein and S100) and neuronal (NeuN) differentiation revealed that the neoplastic cells express markers of astrocytic lineage (Fig. 2G). In addition, the levels of EGFR expression seen in these tumors are similar to those observed in human GBMs with an amplified EGFR locus (Supplementary Fig. S3) and these cells recapitulate growth and histopathologic features of the original tumors when orthotopically allografted in mice (Supplementary Fig. S4A and S4B).

Signaling through constitutively activated EGFR^{WT} in GBM cells

Signaling events resulting from a chronic activation of EGFR^{WT} in GBM have yet to be studied in detail. To understand EGFR^{WT} signaling events in this context, we established a series of primary cultures from TGFα–EGFR^{WT};InkΔ2/3$^{-/-}$ tumors. These primary cultures are stable, show unrestricted growth in low serum conditions (Supplementary Fig. S5), and produce and secrete TGFa (Supplementary Fig. S6A). This TGFα−driven growth is markedly reduced when the cultures are incubated with an anti−TGFα antibody (Supplementary Fig. S6B) thus indicating that TGFα–EGFR^{WT}-driven signaling is sufficient to support tumor cell growth and maintenance. We next used these primary GBM cells to ascertain the effects of EGFR inhibition on cell growth and signaling.

To decipher EGFR signaling in our GBM tumor cultures, we surveyed EGFR's phosphotyrosine levels by immunoblot analysis. The phosphorylation levels of the canonical tyrosine residues 845, 1,045, 1,068, 1,148, and 1,173 decreased

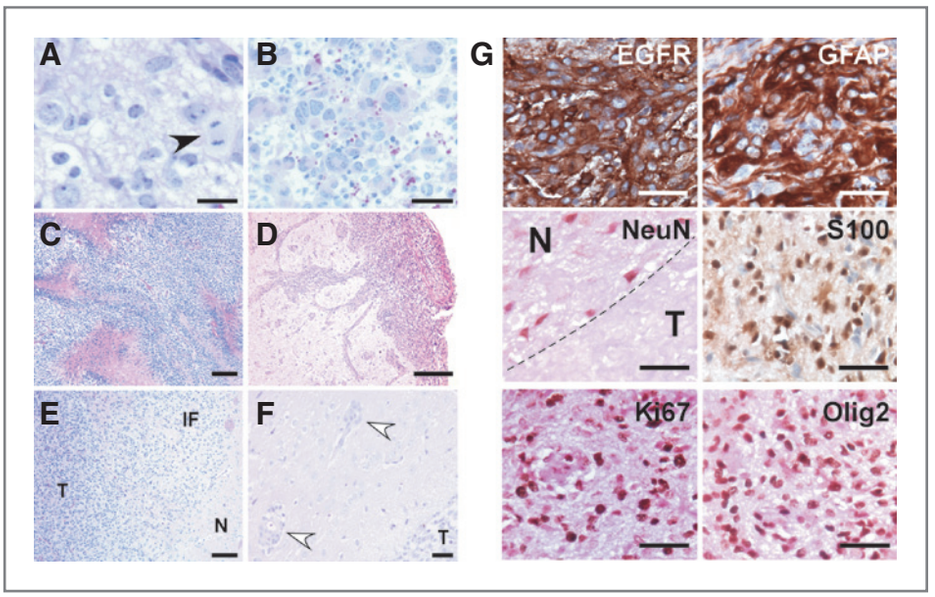

Figure 2. Representative histologic photomicrographs of TGFα–EGFR^{WT};InkΔ2/3$^{-/-}$ tumors. Hematoxylin and eosin–stained paraffin-embedded tumor sections. A, tumors are set on a fibrillary background and contain densely packed cells featuring pleomorphic nuclei with prominent nucleoli and mitoses (black arrowhead). B, giant multinucleated cells are present within tumors. C, tumors exhibit marked pseudopallisading necrosis. D–F, the highly infiltrative nature of TGFα–EGFR^{WT} tumor cells is depicted, (D) tumor cells migrate within meninges in the subarachnoid space and invade the Virchow–Robin space and (E) are infiltrating normal parenchyma (N) by forming a loose infiltrating front (IF) away from the bulk tumor (T) and (F) tumor cells migrate along blood vessels and invade the perivascular space (white arrow head) distant from the bulk tumor (T). G, EGFR^{WT} GBM tumors express markers of astrocytic differentiation. Representative photomicrographs of tumors stained with cell lineage markers using immunohistochemistry. Tumors stain positive for markers of astrocytic lineage [glial fibrillary acidic protein (GFAP) and S100] and negative for markers of neuronal (NeuN) lineage. GBM tumors also stain positive for human EGFR, the proliferation marker Ki67, and for Olig2. EGFR, GFAP, and S100 sections were counterstained with hematoxylin and sections for the nuclear NeuN, Olig2, and Ki67 markers were counterstained with eosin. N, normal brain; T, tumor. Scale bars, 25 μm (A), 50 μm (B, F, G), 62.5 μm (D), and 125 μm (C, E).

dramatically upon EGFR kinase inhibition (Fig. 3A). In contrast, the levels of pTyr^{992} increased upon gefitinib treatment. Next, we determined the levels of activation and the effect of EGFR inhibition on the canonical EGFR signaling pathways driven by MAPK and PI3K–Akt. We found that the MAPK pathway (Mek1/2–Erk1/2) is highly active in TGFα–EGFR^{WT};InkΔ2/3$^{-/-}$ tumor cells and inhibition of EGFR with gefitinib dramatically reduces Mek1/2–Erk1/2 signaling (Fig. 3B). Surprisingly, the PI3K–Akt pathway is not activated in TGFα–EGFR^{WT};InkΔ2/3$^{-/-}$ tumor cells (Supplementary Fig. S7).

We then ascertained the effect of EGFR kinase inhibition on cell growth. We first calculated the IC_{50} values for gefitinib using an *in vitro* cell growth assay (Supplementary Fig. S8) and determined that treatment with 10 μmol/L of gefitinib for 24 hours results in maximal growth inhibition. Gefitinib treatment of TGFα–EGFR^{WT};InkΔ2/3$^{-/-}$ tumor cultures (T1–T3) resulted in a 50% to 80% reduction in viability (Fig. 4A). We surmised that the decrease in viability might be because of an increased rate of apoptosis. Using flow cytometry, the levels of apoptosis in TGFα–EGFR^{WT};InkΔ2/3$^{-/-}$ tumor cultures, as measured by the percentage of cells expressing cleaved caspase-3, increased dramatically after 24 hours of gefitinib treatment (Fig. 4B and C). This increase in apoptosis is confirmed by detecting the presence of cleaved PARP (Fig. 4D) and can be observed as early as 4 hours after gefitinib treatment (Supplementary Fig. S9). A similar apoptotic response was brought about by identical concentrations of erlotinib (data not shown).

To validate these results, we treated animals with actively growing orthotopically allografted TGFα–EGFR^{WT};InkΔ2/3$^{-/-}$ GBM cells with erlotinib and assessed tumor response (Fig. 5A–C). Within 48 hours of treatment, the levels of phospho-EGFR are no longer detectable, the levels of bromodeoxyuridine (BrdUrd) incorporation in GBM cells are drastically decreased and there is a marked increase in the number of apoptotic cells as measured by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay (Fig. 5A–C).

Elevation of BIM_{EL} expression upon attenuation of EGFR signaling

Acute inhibition of constitutively activated EGFR using TKIs has been shown to result in an apoptotic response that is mediated by an increase in the expression of the proapoptotic protein BIM (27) as a result of the attenuation of MAPK signaling (28, 29). To gain insight into the mechanisms responsible for the EGFR TKI–mediated apoptosis observed in our EGFR^{WT}-driven tumor cell cultures, we measured the expression of BIM in cells treated with gefitinib or the Mek1/2 inhibitor PD325901 and carried out cell growth assays. Figure 6A and B show that inhibition of EGFR causes an increase in the expression of the long form of BIM (BIM_{EL}) and that BIM_{EL} expression is partly mediated by the

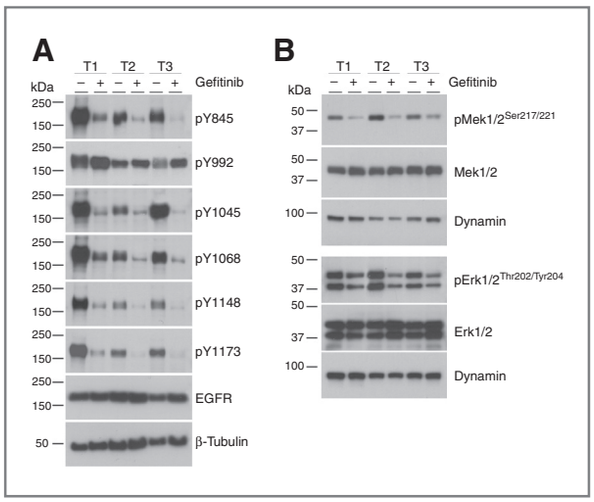

Figure 3. EGFR^{WT} kinase inhibition attenuates signaling pathways. A and B, immunoblot of total cell extracts from vehicle- and gefitinib-treated (10 μmol/L) TGFα–EGFR^{WT};InkΔ2/3$^{-/-}$ tumor cultures analyzed for the presence of (A) the indicated phosphotyrosine residues (B) the activation status of the canonical MAPK members Mek1/2 and Erk1/2. β-Tubulin and dynamin are used as internal loading controls.

Mek1/2–Erk1/2 signaling axis. Inhibition of Mek1/2 led to a more modest apoptotic response as measured both molecularly and physiologically.

Modulation of PTEN expression in established tumor cultures has no therapeutic consequence

Loss of function of the tumor suppressor gene *PTEN* is a common occurrence in GBM tumors. Originally, studies in patients indicated that GBM tumors with an intact PTEN were more sensitive to EGFR kinase inhibitors than those with PTEN deficiencies (16). However, these observations were not sustained in follow-up studies suggesting that PTEN status has little predictive value for EGFR TKI treatment response. To determine whether PTEN contributes to the sensitivity of our GBM tumor cell cultures to EGFR kinase inhibition, we eliminated PTEN expression in our TGFα–EGFR^{WT};InkΔ2/3$^{-/-}$ tumor cells using a potent short hairpin RNA (Supplementary Fig. S10) and analyzed these cultures for their sensitivity to gefitinib treatment.

Elimination of PTEN expression in these cells resulted in the activation of the PI3K–Akt signaling axis as measured by the appearance of activated Akt (Fig. 7A). However, this newly acquired PI3K–Akt signaling remains dependent on the activity of EGFR, as gefitinib treatment completely eliminated Akt phosphorylation. PTEN knockdown in TGFα–EGFR^{WT};InkΔ2/3$^{-/-}$ tumor cells did not result in an increased resistance to gefitinib treatment, as measured by similar levels of apoptosis in control and PTEN knockdown cells when ascertained by immunoblot (Fig. 7A) or cell viability assay (Fig. 7B).

Discussion

Numerous *in vitro* studies showed that overexpression of EGFR^{WT} leads to cellular transformation only in the presence of ligands (30–36). This requirement for coexpression of receptor and ligand for oncogenic transformation is also exemplified in animal models where simple overexpression of nonmutant EGFR in different tissues, including glia, results in hyperplasia rather than tumor formation (37–39). We have recently shown that somatic overexpression of EGFR^{WT} in the central nervous system of mice is incapable of forming glioma (21). Moreover, autocrine/paracrine coexpression of EGFR and its ligands EGF, TGFα, or HB-EGF has been shown in various human tumors, including gliomas (8–10, 22–26, 40), an observation that we validated using the TCGA public database. Together, these studies propose the notion that physiologically relevant overexpression of EGFR^{WT} is not an oncogenic event in and of itself and that coexpression of a ligand may be required to initiate tumorigenesis. In this report, we present experimental data that support this hypothesis by showing for the first time the need for an EGFR ligand (TGFα) to initiate GBM tumor formation with EGFR^{WT} in the context of p16^{Ink4a}/p19^{Arf} nullizygosity in the mouse.

Expression of TGFα in gliomas is well established (8, 11, 22–26, 32, 33) and it has been shown that there are no differences between EGF- and TGFα-stimulated EGFR signaling events (41), thus offering a compelling rationale for its use in our studies. Moreover, soluble EGFR ligands are produced as membrane-bound propeptides that are proteolytically cleaved to release an active ligand from their membrane tethers. We found that mature, active TGFα but not EGF (data not shown) can be expressed from an artificial cDNA corresponding to the postproteolytic product.

Our GEM model offers a unique system to study the potential effects that TGFα may exert on the parenchyma. It is conceivable that the expression of exogenous TGFα influences the tumor microenvironment in a way that would promote GBM cell growth. Given the emergence of data showing the importance of the microenvironment on tumor behavior (42), it is likely that TGFα impacts tumor growth beyond its autocrine role and our model represents a relevant stage to research this phenomenon.

GBM tumors are now categorized into 4 subgroups termed proneural, neural, classical, and mesenchymal, based on well-defined molecular characteristics (recently reviewed in ref. 1). The combination of EGFR overexpression and Cdkn2a loss is found in nearly 65% of all GBMs and is a key molecular component that defines the classical GBM subgroup (6). Our knowledge of EGFR signaling is mostly derived from *in vitro* studies of acute, short-term stimulation of the receptor with exogenous ligands. Although informative in many respects, including the establishment of EGFR signaling networks (3), this paradigm falls short in clinical relevancy in that it does not address signaling events that emanate from chronically activated receptors. More importantly, these short-term *in vitro* studies are inadequate to determine the cellular effects of inhibition of a chronically active receptor. Our model, which is based on relevant genetic aberrations, recapitulates hallmark histopathologic features of GBMs including uncontrolled cellular growth, massive invasion, and infiltration of tumor cells in

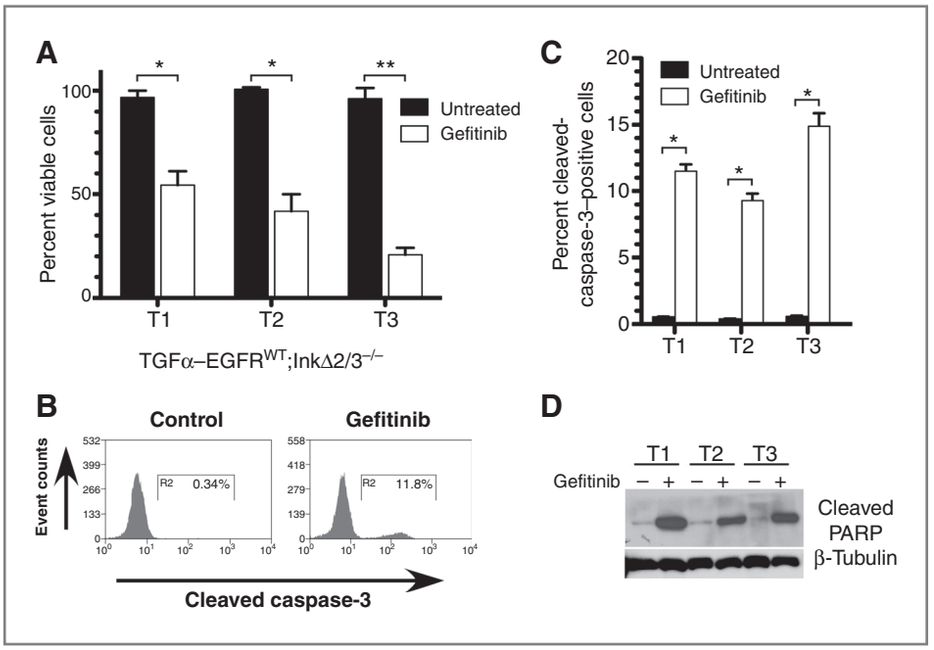

Figure 4. EGFR kinase inhibition in TGFα–EGFR^{WT};InkΔ2/3$^{-/-}$ GBM tumor cells is cytotoxic. A, tumor cells are sensitive to gefitinib treatment. Viability assay of 3 independent tumor cell cultures (T1–T3) after vehicle or gefitinib treatment (10 μmol/L) for 24 hours. Data are plotted as percentage of viable cells of treated over mock treatment (mean ± SD; $n = 3$ in each group; *, $P < 0.005$; **, $P < 0.0005$; 2-tailed t test). B, representative flow cytometric analysis and C, graphical representation of TGFα–EGFR^{WT};InkΔ2/3$^{-/-}$ GBM primary cell cultures mock- and gefitinib-treated indicating an increase in cleaved caspase-3–positive cells upon EGFR kinase inhibitor treatment (mean ± SD; $n = 3$ in each group; *, $P < 0.0001$; 2-tailed t test). D, immunoblot of total cell extracts from vehicle- and gefitinib-treated (10 μmol/L) cultures of the TGFα–EGFR^{WT};InkΔ2/3$^{-/-}$ GBM tumor cells analyzed for the presence of the apoptotic marker cleaved PARP. β-Tubulin is used as an internal loading control.

surrounding normal parenchyma and pseudopallisading necrosis. Moreover, our model establishes a clinically relevant baseline upon which studies of oncogenic EGFR^{WT} signaling can be carried out.

Activation of EGFR leads to the creation of phosphotyrosine (pTyr) residues on the receptor itself and on substrate proteins. These pTyr sites are beacons for a host of SH2 and PTB domain-containing signaling proteins capable of

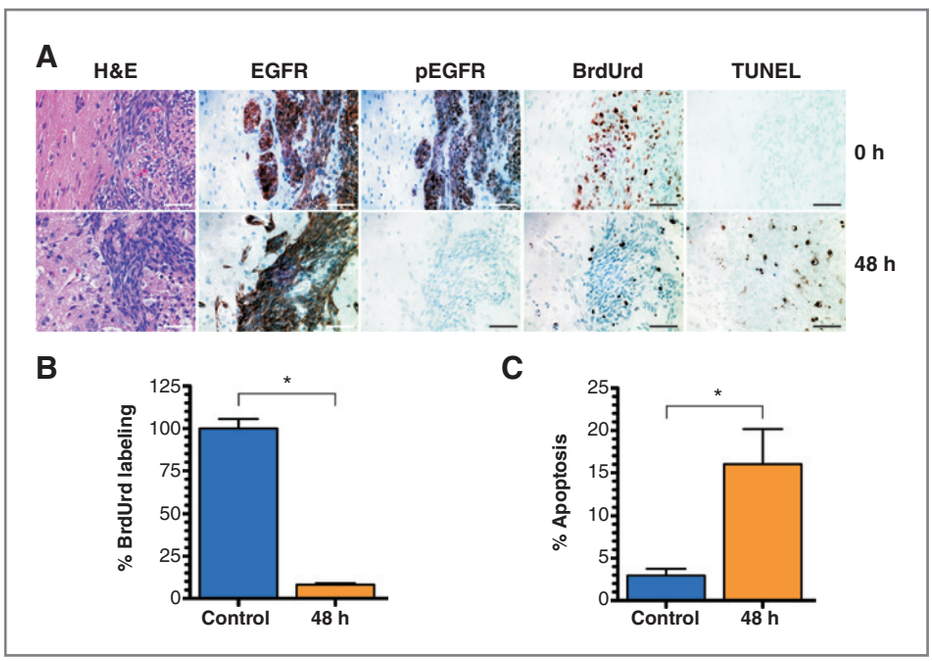

Figure 5. Orthotopic allograft tumors of TGFα–EGFR^{WT};InkΔ2/3$^{-/-}$ GBM cells are sensitive to EGFR inhibition. A, representative photomicrographs of paraffin-embedded tumor tissue sections stained for the indicated markers from control (0 hour) and treated tumor-bearing animals 48 hours posttreatment. B, graphical representation of the quantification of proliferation assayed by BrdUrd incorporation. The BrdUrd staining data are presented as the percentage of BrdUrd-positive cells in treated tumors over control tumors. C, graphical representation of the quantification of the percentage of apoptotic cells as measured by the number of TUNEL-positive cells. Quantification of apoptosis is presented as percentage of TUNEL-positive cells per field of view. (mean ± SD, $n = 6$ in each group; *, $P = 0.0001$; 2-tailed t test). Scale bar, 250 μm. H&E, hematoxylin and eosin.

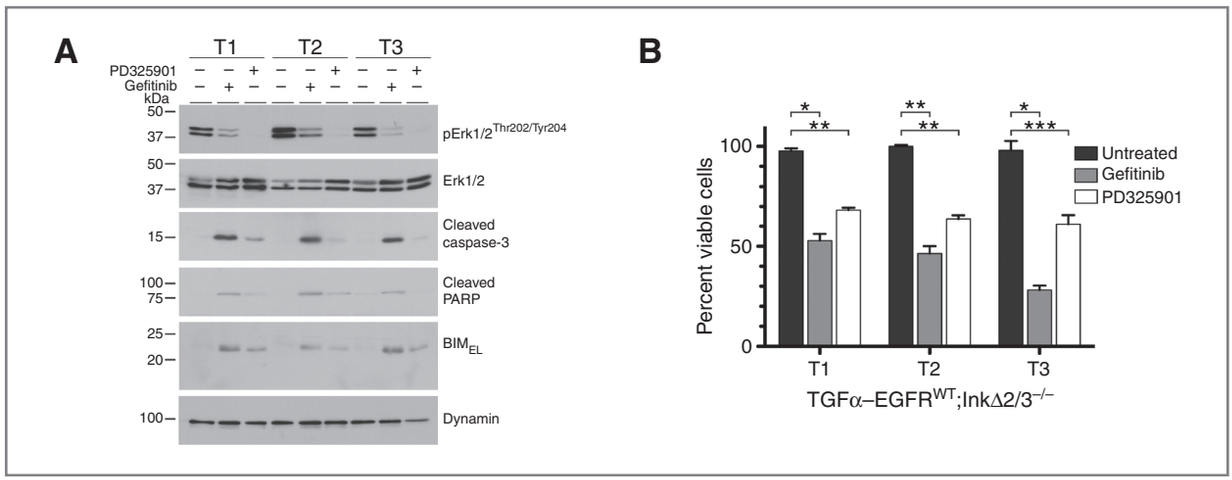

Figure 6. EGFR^{WT} inhibition-induced apoptosis is partly mediated by MAPK signaling attenuation. TGFα–EGFR^{WT};InkΔ2/3$^{-/-}$ GBM tumor cell cultures (T1–T3) were treated with gefitinib (10 μmol/L) or Mek1/2 inhibitor PD325901 (100 nmol/L) for 24 hours and A, analyzed by immunoblot analysis for the apoptotic markers cleaved caspase-3 and cleaved PARP and for the proapoptotic protein BIM$_{EL}$ and B, analyzed for viability in a growth assay. Data are plotted as percentage of viable cells of treated over mock treatment (mean ± SD; $n = 3$ in each group; *, $P = 0.0002$; **, $P < 0.0001$; ***, $P < 0.005$; 2-tailed t test).

phosphotyrosine-dependent sequence-specific recognition and binding, resulting in the transmission of highly precise signals (reviewed in ref. 43). Knowledge of these sites is an invaluable tool in determining the signaling events that emanate from a receptor. In our studies, the inhibition of EGFR kinase activity with gefitinib resulted in a drastic decrease in the levels of the canonical pTyr residues we surveyed, with the exception of pTyr^{992}, which was increased. Decreases in levels of phosphorylation at tyrosine residues 1,068, 1,148, and 1,173 are expected to result in an attenuation of MAPK signaling (44). Tyrosine 845 is a target of Src family kinases (44) and a reduction in the levels of phosphorylation at tyrosine residue 845 indicate a reduction in Src activity. Phosphorylation on tyrosine 1,045 creates a binding site for the ubiquitin ligase c-Cbl (44). A decrease in the levels of phosphorylation on tyrosine 1,045 would possibly lead to a lower rate of receptor degradation.

EGFR pTyr^{992} is a substrate for the tyrosine phosphatase SHP-2 (45). The observed increase of pTyr^{992} levels upon gefitinib treatment may result from a shift in the balance between the activities of EGFR and SHP-2. On the other hand, binding of a high affinity SH2 or PTB domain–containing protein to pTyr^{992} may be increased upon gefitinib treatment, which would then result in protection of this residue from the activity of phosphatases. Regardless of the mechanism involved, phosphorylation on Tyr^{992} creates a binding site for the SH2 domains of phospholipase C-γ, RAS-GAP, and Vav2 (45–47). Our results suggest that a sustained increase in signaling from these effector proteins in our cells may result from gefitinib treatment. Alternatively, other as of yet unidentified signaling molecules may be recruited and activated by this increase in pTyr^{992}. We show that inhibition of a chronically activated receptor has different consequences than that of an acutely stimulated receptor. Under these clinically relevant parameters, there is a renewed interest in studying downstream signaling upon inhibition of EGFR kinase activity.

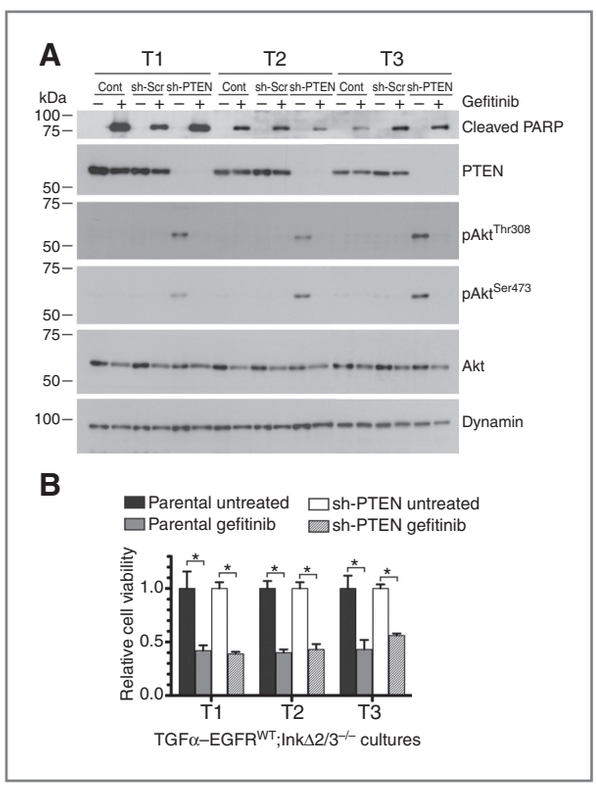

Figure 7. PTEN loss does not confer resistance to EGFR kinase inhibition. A, cells expressing a scrambled control (cont) short hairpin RNA (shRNA; sh-Scr) or a PTEN shRNA (sh-PTEN) were analyzed by immunoblot for cleaved PARP, phospho-Akt^{Thr308}, Akt^{Ser473}, and PTEN expression. Total Akt and β-tubulin are used as an internal loading control. B, parental TGFα–EGFR^{WT};InkΔ2/3$^{-/-}$ cultured tumor cells (T1–T3) and their PTEN knockdown counterpart were treated with gefitinib (10 μmol/L) and assayed for cell viability. The results are presented as values relative to untreated conditions (mean ± SD; $n = 3$ in each group; *, $P < 0.005$; 2-tailed t test).

The canonical Mek1/2–Erk1/2 and PI3K–Akt signaling axes are well-described effector pathways for EGFR. We show that in our tumor cells, MAPK signaling is used by EGFR (Fig. 3). However, to our surprise, we did not detect PI3K–Akt activation (as measured by the levels of phospho-Akt). This result is surprising given the long-standing notion that EGFR strongly signals through PI3K. Perhaps in the chronic setting of our *in vivo* GBM model, tumor cells select for non–PI3K-dependent prosurvival signals. The cells from this tumor model are addicted to EGFR activity for maintenance, as inhibition of EGFR with gefitinib results in a rapid (4–8 hours) induction of apoptosis, which is associated with the appearance of BIM_{EL} expression. We further show that the increased BIM_{EL} expression is partly mediated by MAPK activity as inhibition of Mek1/2 leads to a partial apoptotic response and attenuated BIM_{EL} expression as compared with gefitinib treatment. These results indicate that EGFR signals through additional, as of yet unidentified pathways that when inhibited, feed into the mechanism of BIM_{EL} expression. BIM is a proapoptotic protein known to interact with and inhibit the antiapoptotic activity of Bcl-2, Bcl-X(L) and Mcl-1 (reviewed in ref. 29). Our observations are reminiscent of examples in non–small cell lung cancers (NSCLC) that are addicted to oncogenic EGFR, where TKI treatment results in apoptosis (reviewed in ref. 48). The mechanistic details connecting loss of EGFR kinase activity and initiation of apoptosis still remain unclear but reported data in NSCLC suggest that the apoptosis is mediated by a Mcl-1/Bim axis (49).

Loss of PTEN is commonly associated with GBMs. Molecularly, loss of PTEN is thought to uncouple PI3K activity from the control of EGFR, thus rendering tumor cells insensitive to EGFR TKI therapy. However, this simplistic molecular view of PTEN function does not harmonize with clinical data and reveals the complexities associated with PTEN-modulated signaling events. Here, we show that eliminating PTEN posttumor formation does not uncouple PI3K from EGFR and does not confer resistance to EGFR TKI treatment. Our results are in line with the clinical observations that PTEN status does not predict response to EGFR TKI treatment.

The results presented here show that chronic activation of $EGFR^{WT}$ is necessary for gliomagenesis and that the resulting tumors are addicted to EGFR activity. Our model is the first $EGFR^{WT}$ glioma model, which provides a paradigm for studies of signaling events in the clinically relevant context of human GBMs with amplification and overexpression of wild-type, nonmutated EGFR. Loss of PTEN posttumor formation does not confer resistance to TKI therapy, reaffirming that patient selection for EGFR TKI therapy may not be based on PTEN status alone.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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