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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 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 CNS of adult mice to generate tumors with the histopathological 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 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 post-tumor formation did not confer resistance to TKI treatment, demonstrating 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.

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Introduction

GBMs are classified on the basis of histopathological features, clinical presentation and molecular characteristics (reviewed in (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 >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 (p16INK4A/p19ARF) tumor suppressor locus (7), the role of EGFR signaling in tumor maintenance and sensitivity to TKIs 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 due to 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 controlled by canonical growth and survival pathways (e.g. Akt and 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 (13)). *In vitro*-based studies demonstrate 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 (15)). Retrospective studies demonstrated that patients who responded to TKI therapy had tumors harboring $EGFR^{vIII}$ mutant receptors and an intact PTEN gene whereas the non-responding 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) demonstrating 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 wild-type EGFR 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 demonstrate that loss of PTEN post-tumor 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 performed in accordance with Tufts University's recommendations for care and use of animals and were maintained and handled under protocols approved by IACUC. Conditional expression of $EGFR^{WT}$ was achieved as previously described (21). Viral vector construction, production, and stereotactic injections are described in Supplementary Materials & 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 IHC and proteins were performed 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 16–24hr and the total number of cells was reported.

Statistical analysis

Statistical analyses were performed using the two-tailed, unpaired Student's 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 histopathological 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 performing a gene set enrichment analysis (GSEA) 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. 1A–C). Our analysis reveals that human tumors that overexpress EGFR preferentially have a relatively high expression of EGF ligand when compared to GBM tumors with low EGFR expression levels ($p=0.000076$). These results, combined with previously reported evidence of ligand-receptor co-expression in GBM, demonstrate 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 demonstrating that overexpression of $EGFR^{WT}$ alone is insufficient to promote gliomagenesis (21). In order to

model $EGFR^{WT}$-driven GBM in mice, we developed a strategy to co-express transforming growth factor alpha (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 TGFα serves as a control vector (eGFP-IRES-iCre) (Fig. 1A). We induced the co-expression of TGFα (or eGFP) and $EGFR^{WT}$ by performing stereotactic intracranial injections of matched titers (Supplementary Fig. 2A,B) 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 co-expressing $EGFR^{WT}$ and TGFα in a $p16^{Ink4a}$/$p19^{Arf}$ null background developed brain tumors with a median survival of 10 weeks post-injection (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 co-expression are required for $EGFR^{WT}$-driven gliomagenesis in mice.

TGFα-$EGFR^{WT}$;InkΔ2/3$^{-/-}$ tumors share many histopathological 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–C). Moreover, these tumors are highly infiltrative with leptomeningial 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, while staining for markers associated with astrocytic (GFAP 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 is similar to those observed in human GBMs with an amplified EGFR locus (Supplementary Fig. 3) and these cells recapitulate growth and histopathological features of the original tumors when orthotopically allografted in mice (Supplementary Fig. 4A,B).

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, demonstrate unrestricted growth in low serum conditions (Supplementary Fig. 5) and produce and secrete TGFα (Supplementary Fig. 6A). This TGFα-driven growth is markedly reduced when the cultures are incubated with an anti-TGFα antibody (Supplementary Fig. 6B) 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, 1045, 1068, 1148 and 1173 decreased 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. 7).

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. 8) and determined that treatment with 10 μM of gefitinib for 24 hr results in maximal growth inhibition. Gefitinib treatment of TGFα-$EGFR^{WT}$;InkΔ2/$3^{-/-}$ tumor cultures (T1-3) resulted in a 50–80% reduction in viability (Fig. 4A). We surmised that the decrease in viability might be due to 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,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. 9). 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 BrdU incorporation in GBM cells are drastically decreased and there is a marked increase in the number of apoptotic cells as measured by 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 pro-apoptotic 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 performed cell growth assays. Fig. 6A,B demonstrates 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 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 shRNA (Supplementary Fig. 10) 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, since gefitinib treatment completely eliminated Akt phosphorylation. PTEN knockdown in TGFα-

$EGFR^{WT}$;Ink$\Delta 2/3^{-/-}$ 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 demonstrated that overexpression of wild-type EGFR leads to cellular transformation only in the presence of ligands (30–36). This requirement for co-expression of receptor and ligand for oncogenic transformation is also exemplified in animal models where simple overexpression of non-mutant EGFR in different tissues, including glia, results in hyperplasia rather than tumor formation (37–39). We have recently demonstrated that somatic overexpression of $EGFR^{WT}$ in the CNS of mice is incapable of forming glioma (21). Moreover, autocrine/paracrine co-expression 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 co-expression of a ligand may be required to initiate tumorigenesis. In this report, we present experimental data that support this hypothesis by demonstrating for the first time the need for an EGFR ligand (TGFα) to initiate GBM tumor formation with $EGFR^{WT}$ in the context of p16Ink4a/p19ARF nullizygosity in the mouse.

Expression of TGFα in gliomas is well established (8, 11, 22–26, 32, 33) and it has been demonstrated that there are no differences between EGF- or 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 pro-peptides 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 post-proteolytic 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 exogeneous TGFα influences the tumor microenvironment in a way that would promote GBM cell growth. Given the emergence of data demonstrating 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 four subgroups termed Proneural, Neural, Classical and Mesenchymal, based on well-defined molecular characteristics (recently reviewed in (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 histopathological features of GBMs including uncontrolled cellular growth, massive invasion and infiltration of tumor cells in surrounding normal parenchyma and pseudopallisading necrosis. Moreover, our model establishes a clinically relevant baseline upon which studies of oncogenic $EGFR^{WT}$ signaling can be performed.

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 phosphotyrosine-dependent sequence-specific recognition and binding, resulting in the transmission of highly precise signals (reviewed in (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 phosphoryation at tyrosine residues 1068, 1148, and 1173 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 1045 creates a binding site for the ubiquitin ligase c-Cbl (44). A decrease in the levels of phosphorylation on tyrosine 1045 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 demonstrate 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.

The canonical Mek1/2-Erk1/2 and PI3K-Akt signaling axes are well-described effector pathways for EGFR. We demonstrate that in our tumor cells, MAPK signaling is utilized 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 longstanding 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 pro-survival 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 hrs) induction of apoptosis, which is associated with the appearance of BIM_{EL} expression. We further demonstrate that the increased BIM_{EL} expression is partly mediated by MAPK activity since inhibition of Mek1/2 leads to a partial apoptotic response and attenuated BIM_{EL} expression as compared to 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 pro-apoptotic protein known to interact with and inhibit the anti-apoptotic activity of Bcl-2, Bcl-X(L) and Mcl-1 (reviewed in (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 (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 demonstrate that eliminating PTEN post-tumor 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 demonstrate that chronic activation of wild-type EGFR is necessary for gliomagenesis and that the resulting tumors are addicted to EGFR activity. Our model is the first wild-type EGFR 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, non mutated EGFR. Loss of PTEN post-tumor formation does not confer resistance to TKI therapy, reaffirming that patient selection for EGFR TKI therapy may not be based on PTEN status alone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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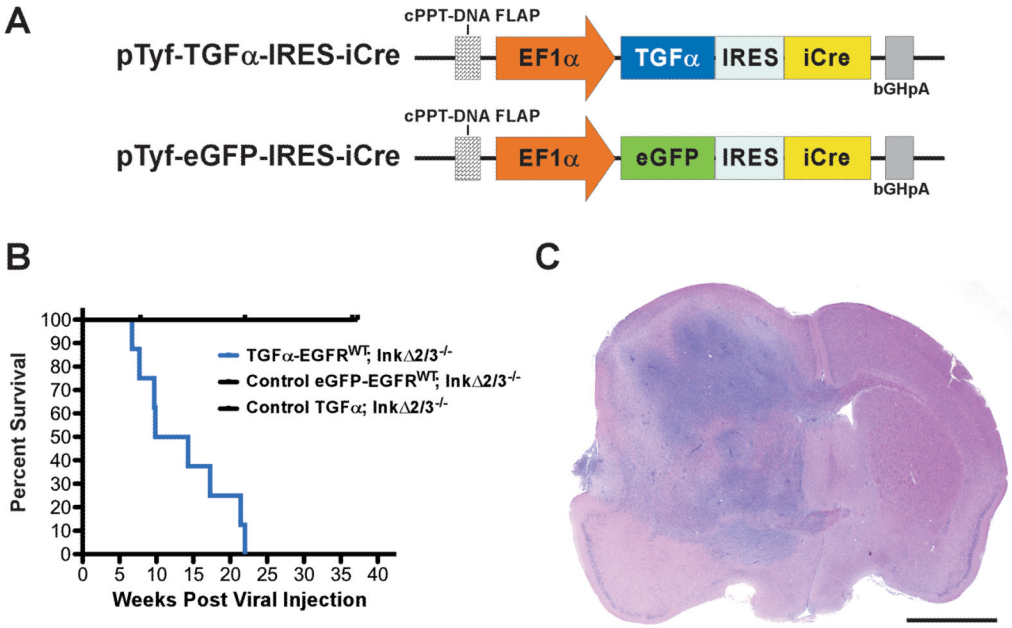

Figure 1.

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 CNS tissues (48, 49). B, Survival (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 an H&E-stained coronal section of a TGFα-EGFR^{WT};InkΔ2/3$^{-/-}$ brain tumor. Scale bar; 2.0 mm.

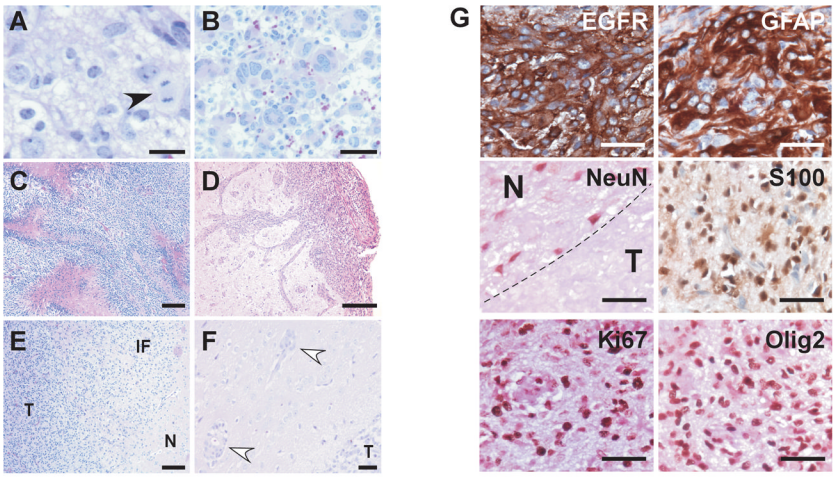

Figure 2.

Representative histological photomicrographs of TGFα-EGFR^{WT};InkΔ2/3$^{-/-}$ tumors. H&E-stained paraffin-embedded tumor sections; A, Tumors are set on a fibrillary background and contain densely packed cells featuring pleimorphic 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 IHC. 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), 125 μm (C, E).

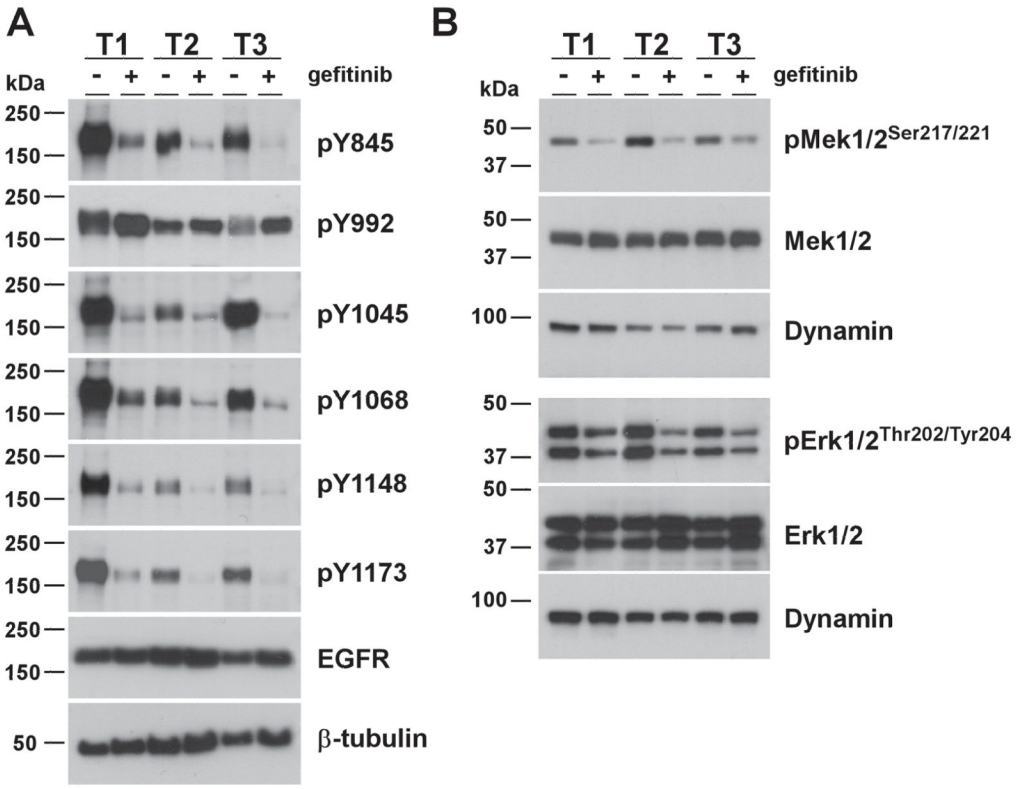

Figure 3.

EGFR^{WT} kinase inhibition attenuates signaling pathways. A–B, Immunoblot of total cell extracts from vehicle- and gefitinib-treated (10 μM) 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.

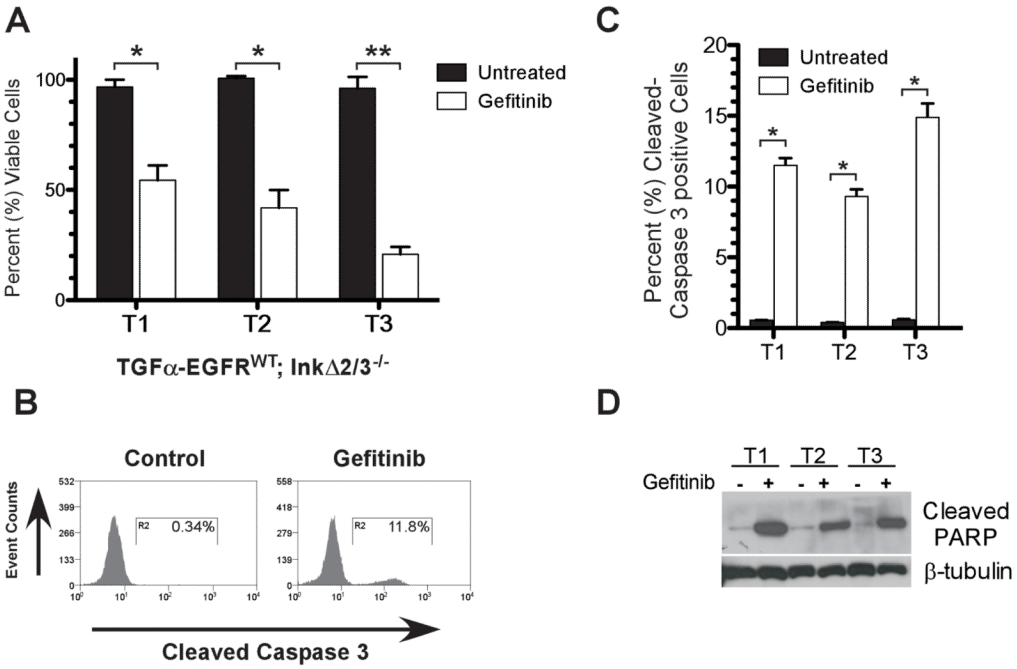

Figure 4.

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 three independent tumor cell cultures (T1-3) after vehicle or gefitinib treatment (10 μM) for 24 hours. Data is plotted as percentage of viable cells of treated over mock treatment (mean ± SD; n=3 in each group, *p<0.005, **p<0.0005, two-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, two-tailed t-test). D, immunoblot of total cell extracts from vehicle- and gefitinib-treated (10 μM) cultures of the TGFα-EGFR^{WT};InkΔ2/3$^{-/-}$ GBM tumor cells analyzed for presence of the apoptotic marker cleaved PARP. β-tubulin is used as an internal loading control.

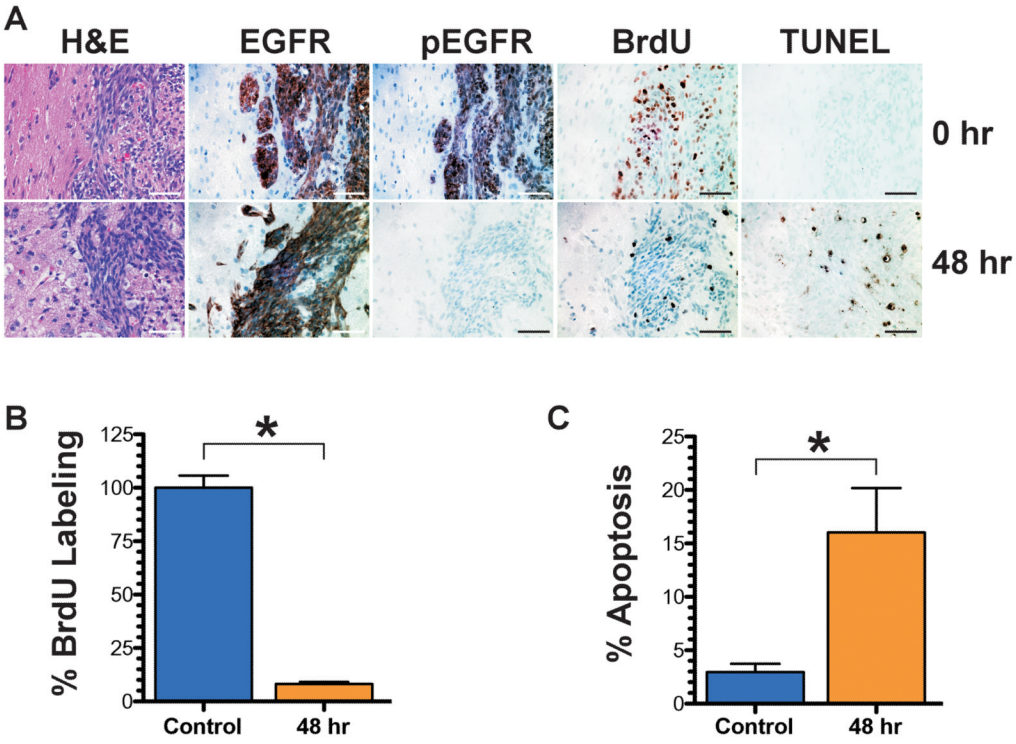

Figure 5.

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 hr) and treated tumor-bearing animals 48 hr post treatment. B, graphical representation of the quantification of proliferation assayed by BrdU incorporation. The BrdU staining data is presented as the percentage of BrdU 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 ± S.D., n=6 in each group *p=0.0001, two-tailed t-test). Scale bar: 250 μm.

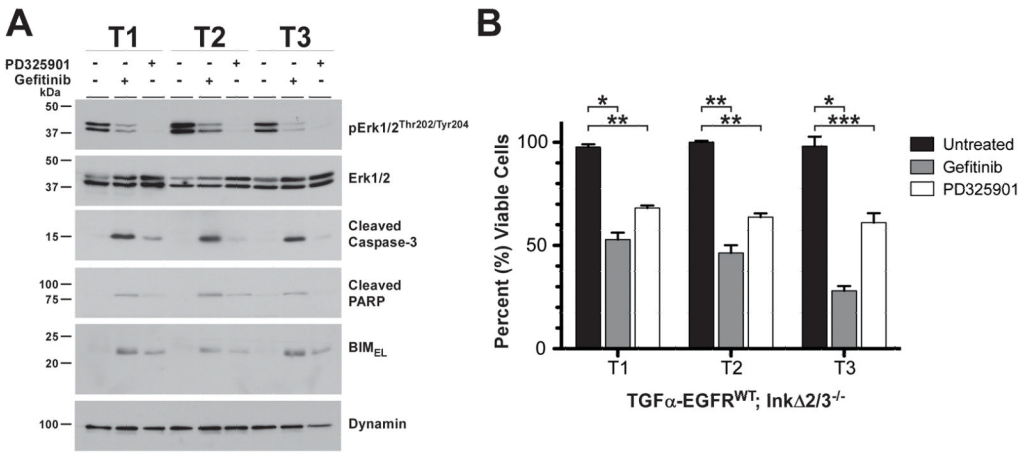

Figure 6.

$EGFR^{WT}$ 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 μM) or Mek1/2 inhibitor PD325901 (100 nM) for 24 hr and A, analyzed by immunoblot analysis for the apoptotic markers cleaved caspase-3 and cleaved PARP and for the pro-apoptotic protein BIM_{EL} and B, analyzed for viability in a growth assay. Data is 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, two-tailed t-test)..

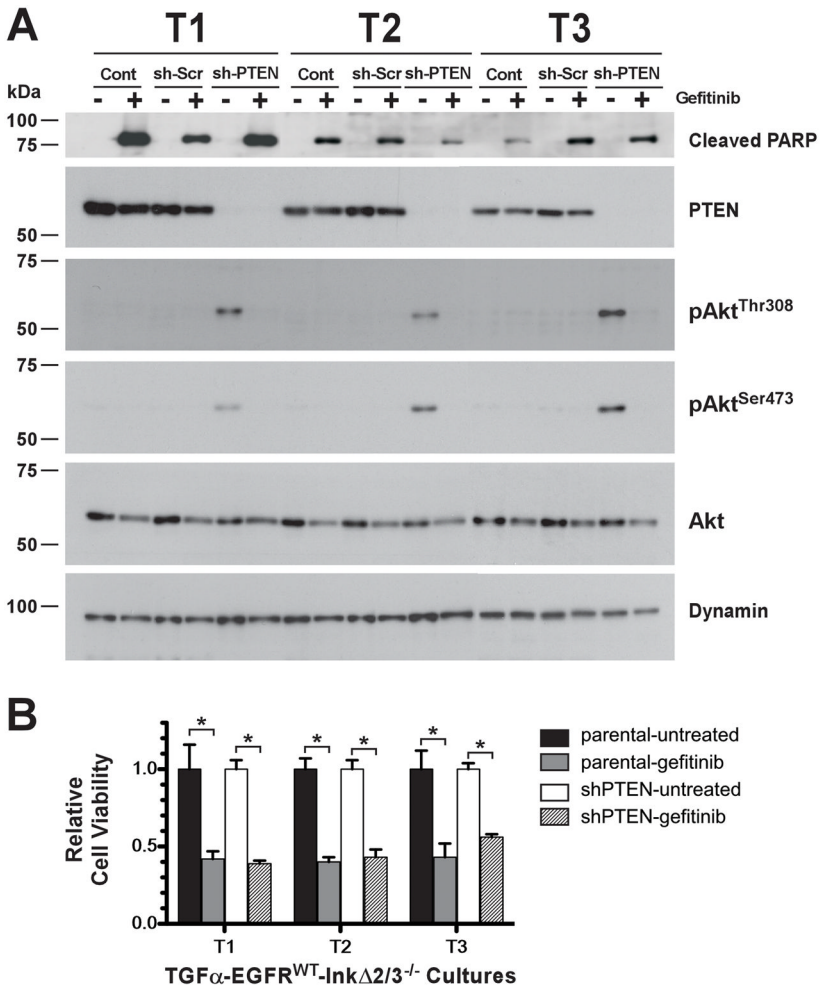

Figure 7.

PTEN loss does not confer resistance to EGFR kinase inhibition. A, cells expressing a scrambled control sh-RNA (sh-Scr) or a PTEN sh-RNA (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 μM) 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, two-tailed t-test).