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De-repression of PDGFRβ transcription promotes acquired resistance to EGFR tyrosine kinase inhibitors in glioblastoma patients

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Abstract

Acquired resistance to tyrosine kinase inhibitors (TKI) represents a major challenge for personalized cancer therapy. Multiple genetic mechanisms of acquired TKI resistance have been

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Conflict of interest disclosure statement:

Drs. Mischel and Cloughesy served on an advisory board for Celgene's mTOR kinase inhibitor program. Dr. Mischel and Cloughesy also collaborated with Celgene and Sanofi through research contracts on their mTOR kinase, and PI3K/mTOR kinase inhibitor clinical trials. Dr. Kornblum collaborated with Celgene on a research contract for the mTOR kinase inhibitor program. The authors are not aware of any other potential conflicts of interest.

identified in several types of human cancer. However, the possibility that cancer cells may also evade treatment by co-opting physiologically regulated receptors has not been addressed. Here we demonstrate the first example of this alternate mechanism in brain tumors by showing that EGFR-mutant glioblastomas (GBMs) evade EGFR TKIs by transcriptionally de-repressing PDGFRβ. Mechanistic studies demonstrate that EGFRvIII signaling actively suppresses PDGFRβ transcription in an mTORC1 and ERK-dependent manner. Genetic or pharmacologic inhibition of oncogenic EGFR renders GBMs dependent on the consequently de-repressed PDGFRβ signaling for growth and survival. Importantly, combined inhibition of EGFR and PDGFRβ signaling potently suppresses tumor growth in vivo. These data identify a novel, non-genetic TKI resistance mechanism in brain tumors and provide compelling rationale for combination therapy.

Keywords

Receptor Tyrosine Kinase; EGFR; glioma; mTOR and PDGFRβ

Introduction

The epidermal growth factor receptor, EGFR, is commonly amplified and/or mutated in many types of solid cancer including a variety of epithelial cancers and glioblastoma (GBM) (1–3). Despite compelling evidence for EGFR addiction in experimental models, the clinical benefit of most EGFR TKIs has been quite limited. Multiple genetic resistance mechanisms enable cancer cells to maintain signal flux to critical downstream effector pathways, and thus evade EGFR-targeted therapy, including: 1) acquisition and/or selection for secondary EGFR mutations conferring EGFR TKI-resistance (4); 2) additional activating mutations in downstream effectors like PTEN (2), PIK3CA (5) or KRAS (6), and 3) co-occurrence of other amplified or mutated RTKs, including C-MET and PDGFRα (7, 8). In addition to these genetic resistance-promoting mutations, it is suspected that EGFR-dependent cancers may escape targeted therapy by developing dependence on other non-amplified, non-mutated RTKs (9). However, the mechanisms by which cancers, including GBM, evade EGFR TKI treatment by co-opting other physiologically regulated receptors has not been addressed.

Herein, we integrate studies in cell lines, patient-derived tumor cultures, xenotransplants, and tumor tissue from GBM patients in a phase II clinical trial to provide the first demonstration of EGFR TKI resistance mediated by transcriptional de-repression of PDGFRβ. We show that the persistently active EGFR mutation, EGFRvIII, suppresses PDGFRβ expression via mTORC1 and ERK-dependent mechanisms. We demonstrate in cells, mice, and patients that EGFR TKI treatment de-represses PDGFRβ rendering GBMs dependent on PDGFRβ signaling for growth. We further show that combined abrogation of EGFRvIII and PDGFRβ potently prevents GBM growth *in vivo*. These results identify a novel physiological EGFR-TKI resistance mechanism in GBM and suggest a clinically actionable approach to suppress it.

Results

EGFR inhibition promotes PDGFRβ upregulation in glioma

To better understand how malignant glioma acquires resistance to EGFR inhibitors *in vivo*, U87 glioma cells expressing the EGFRvIII gain-of-function mutation (designated U87-EGFRvIII herein) were placed in the flank of SCID mice. EGFRvIII, the most common EGFR mutation in GBM, arises from an in-frame genomic deletion of exons 2–7, resulting in a persistently active, highly oncogenic protein (10). Tumor bearing mice were gavaged with the EGFR inhibitor erlotinib (150mg/kg) or vehicle control and tumor growth assessed

over 18 days (Fig. 1a). As expected, erlotinib treatment slowed U87-EGFRvIII tumor growth relative to control, however tumors retained a significant growth rate despite continued erlotinib treatment (Fig. 1b). Immunoblots of tumor lysates confirmed that erlotinib treatment of mice significantly reduced EGFR signaling in xenografts to a level comparable to that of U87 tumor cells expressing kinase-dead EGFR-VIII (Fig. 1c). Thus, U87-EGFRvIII expressing tumors maintain growth despite a significant reduction in EGFR activity when treated with erlotinib; phenocopying the results observed in human trials (2).

We considered the possibility that erlotinib-treated U87-EGFRvIII tumors maintain growth by acquiring neo-receptor tyrosine kinase activity. To directly address this, we performed a phospho-receptor tyrosine kinase array on control and erlotinib-treated tumor lysates. As expected, control U87-EGFRvIII tumors expressed significant levels of phospho-EGFR that were reduced in erlotinib treated mice (Fig. 1d). Erlotinib treated U87-EGFRvIII tumors also had considerable phospho-PDGFRβ activity (Fig. 1d). Heightened activation of the tumor suppressor AXL was also noted, albeit it to a lesser extent than PDGFRβ. Immunoblots of tumor lysates confirmed upregulation and activation of PDGFRβ in response to pharmacologic or genetic inhibition of EGFRvIII (Fig. 1c). Parental U87 tumors expressed significant PDGFRβ (Fig. 1c), which was suppressed by EGFRvIII. Further, erlotinib treatment markedly upregulated PDGFRβ expression in orthotopic U87-EGFRvIII GBM xenografts (Fig. 1e), suggesting that EGFRvIII signaling actively represses PDGFRβ.

To determine if the reciprocal relationship could be extended into other patient-derived glioma models endogenously expressing EGFRvIII, or high levels of wild-type EGFR, we examined phospho-EGFR and PDGFRβ expression in a panel of low passage patient-derived GBM neurospheres (11). Uniformly, erlotinib treatment resulted in upregulation PDGFRβ expression in EGFRvIII expressing GBM neurospheres, as well as GBM neurospheres expressing high levels of EGFR (Fig. 1f). Additionally, GBM39 cells that developed erlotinib resistance in long-term culture maintained suppression of EGFR phosphorylation and concomitant PDGFRβ upregulation (Fig. S1a). Of note and in contrast to PDGFRβ, erlotinib treatment had no effect on PDGFRα expression. To assess whether erlotinib treatment similarly elevated PDGFRβ levels in GBMs endogenously expressing EGFRvIII *in vivo*, we examined PDGFRβ expression up to 10 days of treatment with erlotinib at 150mg/kg. Consistent with our proposed model, erlotinib treatment resulted in elevated phosphorylated and total levels of PDGFRβ (Fig. 1g and S1b). SNP array analysis demonstrated that PDGFRβ was not amplified in these tumor cells, either at baseline or after erlotinib treatment (data not shown). Taken together, these data indicate that EGFRvIII/EGFR signaling negatively regulates PDGFRβ expression in glioma models, and that inhibition of EGFRvIII/EGFR signaling results in upregulation of PDGFRβ.

An RTK switch to *PDGFRβ* occurs in Lapatinib treated patients

Intratumoral heterogeneity of RTK expression is a common feature of malignant gliomas, but it remains unclear if this heterogeneity reflects co-amplification of RTKs within a given tumor cell or differences in RTK expression amongst tumor cells. To distinguish between these possibilities we examined glioma tissue microarrays (TMA) for EGFR and PDGFRβ expression. Similar to our model system studies, we observed a strong inverse correlation between EGFR (total and phosphorylated tyrosine 1086) and PDGFRβ expression in patient glioma tissues (Fig. 2a, p=0.02). To determine if RTK expression was fixed within a given tumor, we utilized patient tissues from a cohort of patients enrolled in a biopsy-treat-biopsy study where patients underwent seven to ten days oral treatment with another EGFR TKI, lapatinib, as part of a phase II clinical trial (12). Post-lapatinib biopsy samples were divided into EGFR-on and EGFR-off groups following immunoblot analysis and demonstrate striking inverse correlation between phospho-EGFR status and PDGFRβ protein expression (Fig. 2b, p=0.04). IHC analysis of one patient was available before and after lapatinib

treatment, and demonstrated significant reduction of phospho-EGFR after treatment, with concomitant PDGFRβ expression in the tumor (Fig. 2c). These clinical data support a model where highly active EGFR signaling negatively regulates PDGFRβ expression in primary brain tumors, and indicates that pharmacologic inhibition of EGFR signaling results in an RTK switch to PDGFRβ.

Suppression of PDGFRβ expression is dependent on the AKT/ mTOR signaling pathway

EGFRvIII, and to a lesser extent wild-type EGFR, have been shown to potently activate PI3K signaling in GBM, resulting in phosphorylation of AKT and its downstream effector mTORC1 (12–17). Therefore, we set out to determine whether EGFRvIII suppresses PDGFRβ through AKT and mTORC1 signaling. To examine whether EGFRvIII suppresses PDGFRβ through AKT, U87-EGFRvIII cells were transfected with the constitutively active AKT1 E17K allele (18). Ectopic expression of AKT1 E17K fully abrogated the upregulation of PDGFRβ in response to erlotinib, confirming that EGFRvIII suppresses PDGFRβ through AKT (Fig. 3a). Previous work has identified mTOR as a negative regulator of PDGFRβ expression in mouse embryonic fibroblasts (19), leading us to hypothesize that EGFRvIII signaling to AKT suppresses PDGFRβ expression through mTORC1. To test this, we determined PDGFRβ expression in U87-EGFRvIII cells transiently transfected with siRNA targeting the mTORC proteins, Raptor and Rictor. Immunoblot analysis of U87-EGFRvIII cells transiently transfected with siRNA targeting the mTORC proteins, Raptor and Rictor, indicated that inhibition of mTORC1, and to a lesser extent mTORC2, led to increased levels of PDGFRβ expression (Fig. 3b). Conversely, transfection of a constitutively active mTOR (S2215Y) allele (20) abrogated erlotinib-dependent upregulation of PDGFRβ (Fig. 3c). Further, genetic depletion of the mTORC1 effector p70 S6Kinase by siRNA knockdown similarly upregulated PDGFRβ (Fig. 3d). Confirming mTOR-dependent repression of PDGFRβ, rapamycin robustly upregulated PDGFRβ protein expression in GBM cell lines *in vitro* and *in vivo* (Fig. 3e, f). These results demonstrate that EGFR signals through AKT and mTORC1 to suppress PDGFRβ.

EGFR signaling represses transcription of PDGFRβ gene

Next, we sought to determine if the influence of mTOR signaling on PDGFRβ expression was regulated at the transcriptional level. To that end, U87-EGFRVIII cells were treated with erlotinib or vehicle, and mRNA was collected up to 36 hours after treatment. RT-qPCR demonstrated that PDGFRβ mRNA was upregulated by 8 hours after the addition of erlotinib, and expression progressively increased over a 24 h period (fig. 4a, $p<0.001$). To determine if the increase in *PDGFRβ* expression was a function of increased transcription at the PDGFRβ gene locus, we assessed the expression levels of *PDGFRβ* primary transcripts. RT-qPCR studies revealed that the expression pattern of *PDGFRβ* primary transcript mirrored that of *PDGFRβ* mRNA following EGFR inhibition (fig. 4a, $p<0.001$). Treatment and washout studies revealed that PDGFRβ primary transcript was dynamically regulated by the addition or removal of erlotinib, further suggesting that expression of PDGFRβ is an active transcriptional process (Fig. 4b). Correspondingly, transcriptional reporter studies using the *PDGFRβ* promoter upstream of luciferase indicated that knockdown of EGFR or Raptor significantly increased luciferase activity in U87-EGFRvIII cells (Fig. 4c, $p<0.001$). Lastly, chromatin immunoprecipitation experiments revealed that rapamycin (5nM) treatment results in recruitment of RNA Polymerase II to both the transcriptional start site and exon 1 of *PDGFRβ* (Fig. 4d, $p<0.01$). Taken together, these studies support a model where EGFR signaling dynamically regulates transcription of *PDGFRβ* in an mTOR-dependent manner. However we cannot rule out the possibility that additional factors such as increased stability of the PDGFRβ mRNA pool or heightened translation also contribute to PDGFRβ upregulation.

ERK signaling contributes to the regulation of PDGFRβ

The MAPK pathway is also activated by EGFRvIII signaling (Fig. 5a), thus we investigated whether the MAPK signaling pathway also contributes to the regulation of PDGFRβ expression. The MEK inhibitor U0126 upregulated PDGFRβ expression, although to a lesser extent than erlotinib (Fig. 5a), which was not abrogated by over expression of wild type S6K1 or constitutively active S6K1 or S6K1 and S6K2 alleles (Fig. 5b). Taken together, these results demonstrate the presence of a parallel pathway by which EGFRvIII/EGFR signaling regulates PDGFRβ through a MAPK (Fig. 5c). Other RTKs such as MET have been shown to engage PI3K signaling to confer resistance to erlotinib in GBM (7). Therefore, we asked whether MET signaling, which can activate both AKT/mTORC1 and MAPK pathways, could similarly promote PDGFRβ upregulation. In U87-EGFRvIII or GBM-39 neurospheres, the MET inhibitor PHA-665752 (PHA, 0.05–4μM) was not sufficient to promote PDGFRβ upregulation like erlotinib (Fig. 5d, e). However, addition of exogenous HGF ligand promoted AKT and ERK phosphorylation and suppressed erlotinib-mediated upregulation of PDGFRβ in a dose-dependent fashion (Fig. 5f). These results suggest that HGF-mediated activation of MET can also repress PDGFRβ by engaging AKT/mTOR and MAPK signaling.

PDGFRβ is dispensable for EGFRvIII-driven GBM growth, but becomes required for the growth of EGFRvIII-inhibited tumors

Next, we asked if PDGFRβ signaling influences proliferative capacity in EGFR-inhibited glioma. To that end, U87-EGFRvIII cells were cultured with erlotinib or vehicle and PDGFbb (0–20ng/mL/day) for 4 days. The addition of PDGFR ligand to untreated U87-EGFRvIII cells had little effect on proliferative capacity (Fig. 6a). As expected, treating U87-EGFRvIII cells with erlotinib alone significantly reduced both EGFR signaling (Fig. S2a) and proliferation (Fig. 6a). The addition of PDGFbb to cultures restored proliferative capacity of erlotinib treated cells (Fig. 2a) in a receptor-specific (Fig. S2b) and dose-dependent manner (Fig S2, c). Similarly, addition of PDGFRβ ligand to cultures signifcantly restored proliferative capacity of U87-EGFRvIII cells transfected with siRNA targeteing EGFRvIII (Fig. 6b and S2d). Next, we asked whether PDGFRβ signaling was required for tumor growth *in vivo* in GBM cells expressing EGFRvIII, and whether abrogation of EGFRvIII rendered these tumor cells PDGFRβ-dependent. To that end, U87-EGFRvIII and U87-EGFRvIII-kinase dead cells were stably transduced with shRNAs targeting PDGFRβ or control shRNA and implanted in flanks of SCID mice. Consistent with our *in vitro* studies, silencing of PDGFRβ had little effect on U87-EGFRvIII tumor growth (Fig. 6c). In contrast, silencing PDGFRβ significantly attenuated the growth of tumors expressing kinase dead-EGFRvIII (Fig. 6d). Immunoblots of xenograft lysates confirmed a relationship between PDGFRβ and EGFR activation in tumors (Fig. 6e).

To determine whether PDGFRβ signaling could abrogate the growth inhibitory effects of erlotinib in GBM cells endogenously expressing EGFRvIII or high levels of wild-type EGFR, we examined the effect of PDGFRβ signaling on the proliferative capacity on patient-derived GBM neurospheres. The PDGFR kinase inhibitor AG1295 (2μM) alone had no anti-proliferative effect on GBM39 (EGFRvIII positive, PTEN intact), or HK250 (high level wild-type EGFR, PTEN deficient) cells (Fig. 6f, g). In contrast, in the presence of erlotinib, addition of the PDGFR kinase inhibitor AG1295 significantly suppressed tumor cell proliferation (Fig. 6f, g, $p<0.01$). Of note, and in contrast to our studies on U87-EGFRvIII engineered cells, the patient-derived neurosphere cultures did not require the addition of exogenous PDGFR ligand, consistent with the role of autocrine and paracrine PDGF signaling in GBM (21, 22). Taken together, these data suggest a physiologic RTK switch to the PDGFRβ to maintain the growth of EGFRvIII/EGFR-activated GBMs in response to EGFR tyrosine kinase inhibitors (Fig. 7).

Discussion

Acquired drug resistance presents a significant challenge for personalized cancer therapy. In principle, upfront sequencing may guide successful combination TKI therapy by defining both the drugable kinase mutations, and the potential "seeds" of resistance - second site mutations, downstream effector mutations and co-amplification of multiple RTKs. However, non-genetic adaptive resistance mechanisms complicate this paradigm. Identifying the ways that cancer cells "rewire" their circuitry through pathway cross talk and release of inhibitory feedback loops to evade treatment may be critical for developing more successful combination approaches. By integrating studies of cells, mice, and tumor tissue from patients treated with EGFR inhibitors in a clinical trial, we provide the first experimental evidence that EGFR TKI resistance can be mediated by transcriptional de-repression of another, physiologically regulated RTK: PDGFRβ.

TKI-mediated release of inhibitory feedback loops is emerging as a frequent, non-genetic mechanism of targeted cancer drug resistance. In colorectal cancer cells bearing the BRAF v600E mutation, resistance to the BRAF inhibitor PLX-4032 (vemurafenib) is mediated by reactivation of EGFR signaling through the MAPK pathway (23, 24), although upregulation of EGFR itself does not appear to be involved. In breast cancer cells, AKT and mTOR inhibition reactivates PI3K signaling through release of an inhibitory feedback loop in a process that appears to involve multiple RTKs (25, 26). Most recently, transcriptional upregulation of the RTK AXL has been shown to promote erlotinib resistance in non-small cell lung cancer (9), although the mechanism underlying AXL up-regulation is not known. It is interesting to note that we too observed AXL upregulation in response to erlotinib treatment (Fig. 1), suggesting that it may also play a role in mediating erlotinib resistance in GBM. However, we focused on PDGFRβ because of the dominance of its signal across all *in vitro* and *in vivo* models and in all patient samples we studied.

In contrast to the recognized importance of PDGFRα alterations (7, 27–29), the role of PDGFRβ in malignant gliomas has not been as clearly defined. PDGFRβ amplifications and/or mutations are exceedingly rare events in GBM (27). In mouse genetic models, PDGF-B ligand overexpression can promote gliomagenesis by enhancing cellular proliferation (30–34). Recently, PDGFRβ has been shown to promote glioma stem cell self-renewal, suggesting a more definitive role in tumorigenesis and/or maintenance (35). In addition, a PDGF signaling class of GBMs, characterized by PDGFRβ phosphorylation and lack of EGFR signaling, amongst other features, has been identified (36). Yet the contribution of PDGFRβ signaling to drug resistance remains incompletely understood.

Here, we provide the first demonstration that mTORC1 inhibition mediates EGFR-TKI resistance in GBM through transcriptional regulation of PDGFRβ, a mechanism which could also be active in other cancer types. PDGFRβ has been shown to mediate vemurafenib resistance through transcriptional upregulation in melanoma (37). However, the mechanism underlying this event is not known. In mouse embryonic fibroblasts, PDGFRβ was shown to be a target of mTOR-dependent negative transcriptional downregulation (19). However, its role in mediating EGFR and or mTOR TKI resistance has not previously been recognized. In addition, we identify a parallel pathway (38) by which ERK signaling also suppresses PDGFRβ. Our data definitively demonstrate that EGFR inhibitors de-repress PDGFRβ transcription, providing a potent mechanism underlying RTK switching. These findings have broad implications for understanding acquired resistance to EGFR TKIs, and potentially mTOR inhibitors as well, across multiple cancer types. Future studies will be necessary in order to address this possibility more fully. In addition, future studies will be needed to identify the transcriptional machinery linking mTOR/S6K with PDGFRβ.

EGFR amplification and mutation presents perhaps the most compelling drugable target in GBM. Genetic and/or functional PTEN loss (2, 39); co-occurrence of c-MET and PDGFRα gene amplification (7, 28, 29), and pharmacokinetic considerations (40) all contribute to EGFR TKI resistance, indicating a broad repertoire of resistance mechanisms that can be co-targeted. However, the role of non-genetic "rewiring" in mediating drug resistance remains to be defined. Here, we have identified a transcriptional repressive mechanism by which EGFRvIII regulates PDGFRβ; shown that EGFR-inhibited GBMs become PDGFRβ-dependent for survival through mTOR-dependent transcriptional de-repression; and demonstrated that abrogation of EGFRvIII and PDGFRβ stop tumor growth, providing strong rationale for combination therapy. These results provide the first clinical and biological evidence for the concept of RTK "switching" as an EGFR TKI resistance mechanism in GBM, and provide a molecular explanation of how tumors can become "addicted" to a non-amplified, non-mutated, physiologically regulated RTK to evade targeted treatment.

Methods

Cell lines and media

U87 and isogenic U87-EGFRvIII, U87-EGFRvIII kinase dead, U87-EGFRvIII/shPDGFRB, U87-EGFRvIII kinase dead shPDGFRB cell lines were cultured in Dulbecco's modified Eagle's medium (Cellgro) supplemented with 10% FBS (Omega Scientific) and PSQ (Invitrogen) in a humidified atmosphere of 5% CO_2 at 37°C. U87-EGFRvIII kinase dead cells were a gift of W. Cavenee. U87-EGFRvIII-shPDGFRβ cells were generated by plasmid-mediated transfection of shPDGFRβ into U87-EGFRvIII cells followed by selection for stable clones. Neurosphere cell lines (GBM6, GBM12, GBM39, HK296, HK242, HK250) were cultured in Dulbecco's modified Eagle's medium F12 (Cellgro) supplemented with EGF, FGF, Heparin (Sigma), Glutamax, and PSQ (Invitrogen). Long-term erlotinib-resistant GBM39 neurospheres were cultured in the presence of erlotinib for 30 days until cells were resistant. U87 cells were obtained from ATCC, and engineered to express EGFRvIII in the Mischel laboratory. GBM6, GBM12, GBM39 were obtained from coauthor Dr. David James at UCSF and authenticated by DNA fingerprinting. HK296, HK242, HK250 were obtained by Dr. Harley Kornblum and authenticated by immunoblot studies.

Xenograft models

Isogenic human malignant glioma cells were implanted into immunodeficient SCID/Beige mice for subcutaneous xenograft studies as follows. SCID/Beige mice were bred and kept under defined-flora pathogen-free conditions at the Association for Assessment of Laboratory Animal Care–approved Animal Facility of the Division of Experimental Radiation Oncology, UCLA. For subcutaneous implantation of U87 tumor cells (or indicated isogenic U87-EGFRvIII, U87-EGFRvIII kinase dead, U87-EGFRvIII/shPDGFRB, U87-EGFRvIII kinase dead shPDGFRB) or GBM39 xenografts, Single cell suspensions and injected subcutaneously at 600 thousand cells/150ul in a solution of Dulbecco's phosphate-buffered saline (dPBS) and Matrigel (BD Biosciences). Tumor growth was monitored with calipers by measuring the perpendicular diameter of each subcutaneous tumor. For intracranial xenograft studies, U251 cells were injected intracranially in rats as described previously (41). Rapamycin was administered for three days at 2mg/kg/day. All experiments were conducted in accordance with the Animal Research Committee of UCLA.

Cell proliferation assays

Absolute viable cell counts were determined by trypan blue exclusion and counted on a hemocytometer. Relative cell proliferation was determined using Cell Proliferation Assay

Kit (Chemicon), per manufacturer's specifications. Briefly, cells were incubated 1.5 hours (5% CO_2, 37degrees) after addition of tetrazolium salt WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium, monosodium salt] and the absorbance was then measured in a microplate reader (Bio-Rad) at 450 nm with a background reading at 650nm subtracted. For assays using erlotinib or AG1295, small molecules or DMSO vehicle were added at the indicated doses on day 0 of assays. In assays with PDGFbb ligand, cultures were stimulated daily with PDGFbb ligand at 20ng/ml for the indicated days. Neurospheres were plated in laminin-coated 96-well dishes in neurobasal media supplemented with 5% charcoal-stripped FBS (Omega scientific) and treated with indicated drug as above. For transient siRNA knockdown of EGFRvIII, cells were incubated overnight with transient siRNA of EGFRvIII (Ambion) or scrambled SiRNA (Ambion) at 10nM with RNAiMax Lipofectamine reagent (Invitrogen) and Optimem (Invitrogen). Cells were then plated in 12 well plates and were stimulated with PDGF-bb ligand or vehicle in medium containing 2% charcoal-stripped FBS (Omega scientific) and counted as above.

Knockdown studies

Transient knockdown of EGFRvIII, S6K1, Raptor, and Rictor (Ambion) were performed as follows. siRNA was diluted to a final concentration of 10nM in Optimem and 7.5 ul Lipofectamine RNAi-max, in serum-containing, PSQ-free media overnight in a final volume of six mLs in a 60mm dish. Media was changed the following morning, and cells were incubated for 24 hours before lysate collection. For the *generation of stable knockdown cell lines,* cells (5×10^4) were seeded in 12-well plates and maintained for 24 hours, after which the medium was replaced with fresh 5% FBS medium including polybrene (5 μg/ml; Sigma), and shRNA lentivirus was added to cells followed by incubation for 24 hours.

Transient transfection

Plasmids used were pcDNA control, wildtype mTOR, mTOR S2215Y, and AKT E17K (mTOR constructs were a gift from Fuyu Tamanoi, E17K was a gift from Ingo Mellinghoff, pcDNA control was from Addgene). Empty vector, mTOR constructs, and AKT E17K were diluted to 2500ng in optimem and incubated with Lipofectamine PLUS and LTX reagents (Invitrogen) according to the manufacturer's instructions. Cells were plated in 5 mL of 5% FBS and 1mL of optimem/plasmid/Lipofectamine was added to each plate. Media was changed the next day to serum-free media and cells were incubated with erlotinib for 24 hours before lysates were collected. For S6 kinase wildtype and constitutively active forms, plasmids were diluted to 16ug in optimem and incubated with Fugene6 (Roche) according to the manufacturer's instructions. Cells were plated in 5mLs 10% FBS and 1mL of optimem/plasmid/Fugene6 was added to each plate. The media was changed to serum-free media the following morning and cells were incubated with U0126 for 24 hours. Lysates were then collected for immunoblotting. Plasmids used were pcDNA control, EES6K1 and T412D/T401D S6K1/2 (S6 kinase plasmids were a gift from Ivan Gout)

Immunoblots

Cultured cells or snap-frozen tissue samples were lysed and homogenized with RIPA buffer (buffer, Boston Bioproducts; protease and phosphatase inhibitor, Thermoscientific). Protein concentration was determined via BCA Assay (reagents A and B, Thermoscientific; standards, Biorad) and samples were subjected to 4–12% gradient SDS–polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane (Bio-Rad Laboratories). The membrane was then probed with indicated primary antibodies, followed by secondary antibodies conjugated to horseradish peroxidase (HRP). The immunoreactivity was revealed by use of an ECL kit (Thermoscientific). Antibodies used in the study include p-EGFR^{1086} (Epitomics), p-EGFR^{1068}, p-AKT^{473}, p-AKT^{T308}, AKT, p-S6$^{Ser235/236}$, p-PDGFRβ^{Y751}, p-

ERK$^{T202/Y204}$, ERK, p-Met^{Y1234}, p-S6K1^{T389}, S6K1, PathScan cocktail, p-NDRG1^{T346}, β-actin, PDGFRβ, PDGFRα (Cell Signaling), α-tubulin (Sigma); EGFR (Upstate).

Immunohistochemistry

Xenografts were excised from mice treated with vehicle or erlotinib as described above. A portion of the tumor was fixed in paraformaldehyde and ethanol and sent to the Department of Pathology and Laboratory Medicine at UCLA tissue core for slicing and staining as required.

Phospho-RTK array

U87-EGFRvIII tumors from vehicle or erlotinib treated mice were harvested, and homogenized in NP40 containing lysis buffer, then loaded at 2000 micrograms per RTK array membrane, according to the manufacture's instructions (R&D systems). For U87-EGFRvIII cells in culture, lysates were generated and loaded on RTK array as described above.

Pilot study of lapatinib

North American Brain Tumor Consortium (NABTC) trial 04-01 titled "A biomarker and Phase II study of GW 572016 (lapatinib) in recurrent malignant glioma" enrolled consented patients from University of California at Los Angeles (UCLA), University of California at San Francisco, Dana-Farber Cancer Center, Memorial Sloan Kettering Cancer Center, University of Pittsburgh, Neuro-oncology branch of National Institutes of Health, University of Wisconsin, and Duke University (12). Adult patients who had a Karnofsky performance score (KPS) equal to or greater than 60, who were not on enzyme-inducing antiepileptic agents and who had normal hematologic, metabolic, and cardiac function were eligible for this study. In addition, patients must have been candidates for surgical re-resection at the time of enrollment. Patients were administered 750 mg of lapatinib orally twice a day (BID) for 7 to10 days (depending on whether treatment interval fell over a weekend) before surgery, the time to steady state. Blood and tissue samples were obtained at the time of resection. After recovery from surgery, patients resumed lapatinib treatment at the neoadjuvant dose of 750 mg BID until clinical or radiographic evidence for tumor progression was found. The first cohort of patients for whom tissue was available before and after lapatinib (n = 10) were included in this study.

Tissue Microarrays

Tissue microarrays (TMAs) were used to analyze PDGFRβ and p-EGFR Tyr^{1086} immunohistochemical staining in 140 GBM patient samples. Two GBM TMAs were constructed with a 0.6-mm needle to extract 252 representative tumor tissue cores and 91 adjacent normal brain tissue cores from the paraffin-embedded tissue blocks of 140 primary GBM patients (12). These cores were placed in a grid pattern into two recipient paraffin blocks, from which tissue sections were cut for immunohistochemical analysis of p-EGFR Y1086 and PDGFRβ. EGFR and PDGFRβ staining was scored and tabulated in Chi-square.

Realtime-PCR

In vitro, U87EGFRvIII cells were incubated in serum-free media with and without erlotinib for 32 hours, as well as in 10% serum with and without rapamycin for 24 hours. At each time point, cells were lysed in Trizol for RNA extraction. RT-PCR analysis was conducted using primers designed to amplify either the primary transcript of PDGFRβ as well as the messenger RNA transcript. Primer sequences for PDGFRB mRNA are forward AGGACACGCAGGAGGTCAT and reverse TTCTGCCAAAGCATGATGAG. Primer sequences for PDGFRB primary transcripts are forward CATCTGCAAAACCACCATTG

and reverse ACTTGCCTCTGCTGAGCATC. For the washout, U87EGFRvIII cells were plated in 10%FBS containing media. The media was changed to serum-free media and erlotinib (5uM) was added at t = 0h. Media was changed at 24 h, and erlotinib (5uM) was added again at 48h. At each time point, cells were lysed in Trizol for RNA extraction as described above. mRNA and primary transcripts were normalized against 36B4.

Luciferase Assay

U87-EGFRvIII cells were transfected with Switchgear genomics PDGFβR promoter plasmid concurrently with control CMV plasmid promoter Luciferase and Renilla and Firefly Luciferase control. Luciferase assay was conducted using Promega Dual Luciferase Reporter Assay system.

Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) assays were performed in U87-EGFRvIII cells with or without rapamycin (5nM) for 24 hours. Cells in two 15-cm plates were pooled for each replicate. ChIP was performed as previously described (42) with minor modifications. Briefly, cells were cross-linked for 5 min in 1% formaldehyde in PBS. After sonication (15 min total sonication time in 30-s pulses), soluble chromatin from each replicate was split four ways for overnight immunoprecipitations with 2 ug of the following antibodies: mouse immunoglobulin G (Millipore cat #12-371) antibody against polymerase II (Millipore clone CTD4H3, cat #05-623, positive control). 5ul of chromatin was used as control. DNA-protein complexes were pulled down by incubation for 2 hours with protein G-Sepharose, washed, and eluted with 1% SDS buffer. Resulting chromatin was de-crosslinked with heat and protein digested with Proteinase K, along with input controls. Genomic DNA (gDNA) was assayed by quantitative polymerase chain reaction (qPCR) with primers amplifying PDGFR TSS and a fragment upstream of the TSS. qPCR values were normalized against the input gDNA content for each replicate. qPCR primers are available upon request.

ERK and Met studies

For treatment with erlotinib and the MEK inhibitor U0126 or the Met inhibitor PHA 665752, adherent cells were plated in 10% FBS. GBM39 cells were plated on plates coated with laminin (Sigma) as described above in complete neurosphere media. The following day, media was changed to serum-free media (U0126), 2% FBS-containing media (U87EGFRvIII, PHA 665752), or DMEM/F12 (GBM39, PHA 665752). Cells were then incubated with drug for 24 hours before being lysed for immunoblot analysis.

For HGF stimulation, U87-EGFRvIII cells were plated as described above. Media was changed to serum-free media, and HGF was added at 50 or 100ng/mL concurrently with DMSO vehicle or erlotinib (5μM) 24 hours prior to collection. Cells were stimulated again 4 hours prior to collection.

Statistical Analysis

Fisher's exact test was used to assess correlation between EGFR and PDGFRβ in clinical samples. All other comparisons of cell proliferation, transcript level, and tumor volume were performed using one-way or two way ANOVA with a Tukey HSD test as required. All results are shown as means +/− SD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Significance

These results provide the first clinical and biological evidence for receptor tyrosine kinase “switching” as a mechanism of resistance to EGFR inhibitors in glioblastoma, and provide a molecular explanation of how tumors can become “addicted” to a non-amplified, non-mutated, physiologically regulated receptor tyrosine kinase to evade targeted treatment.

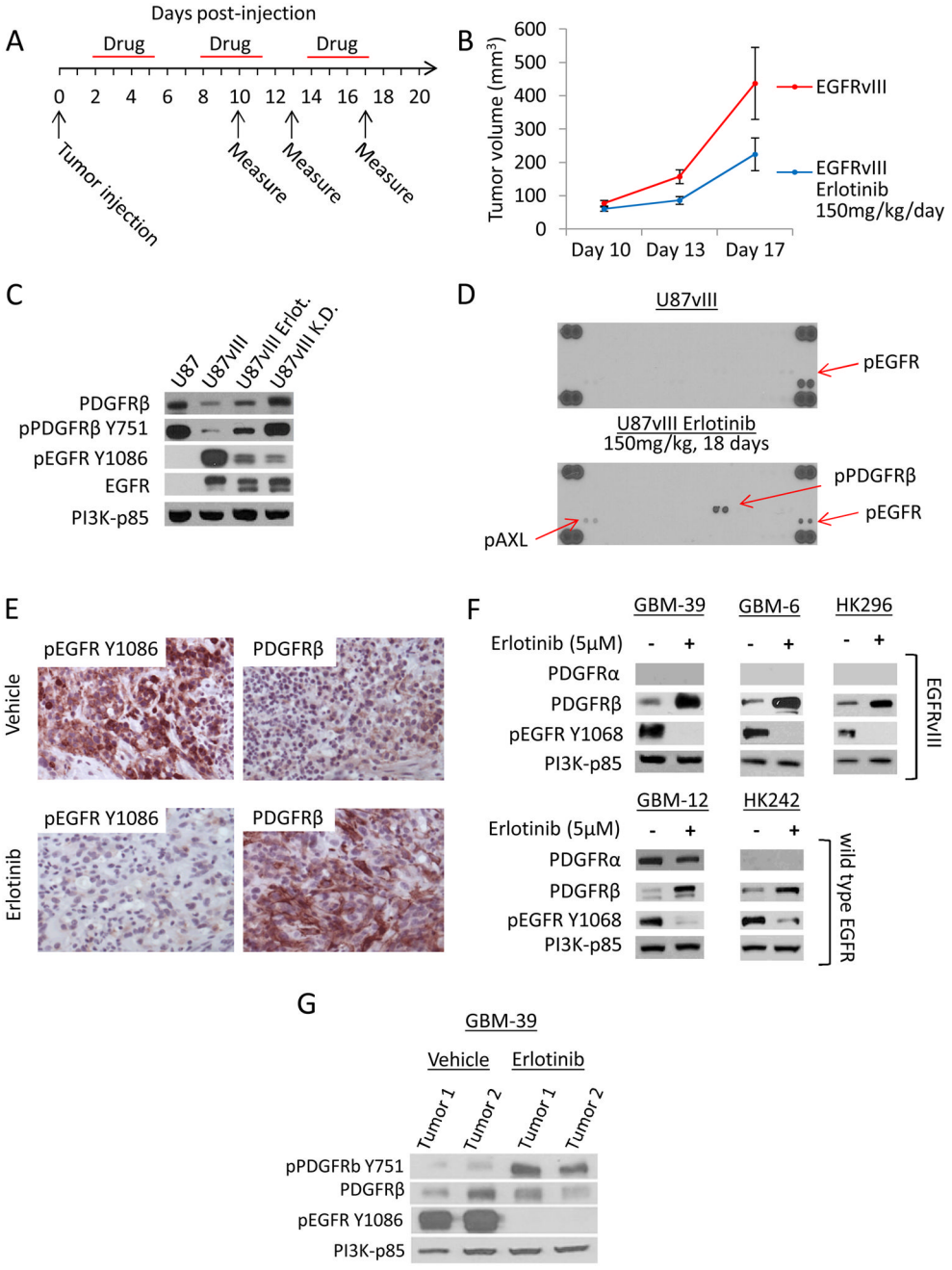
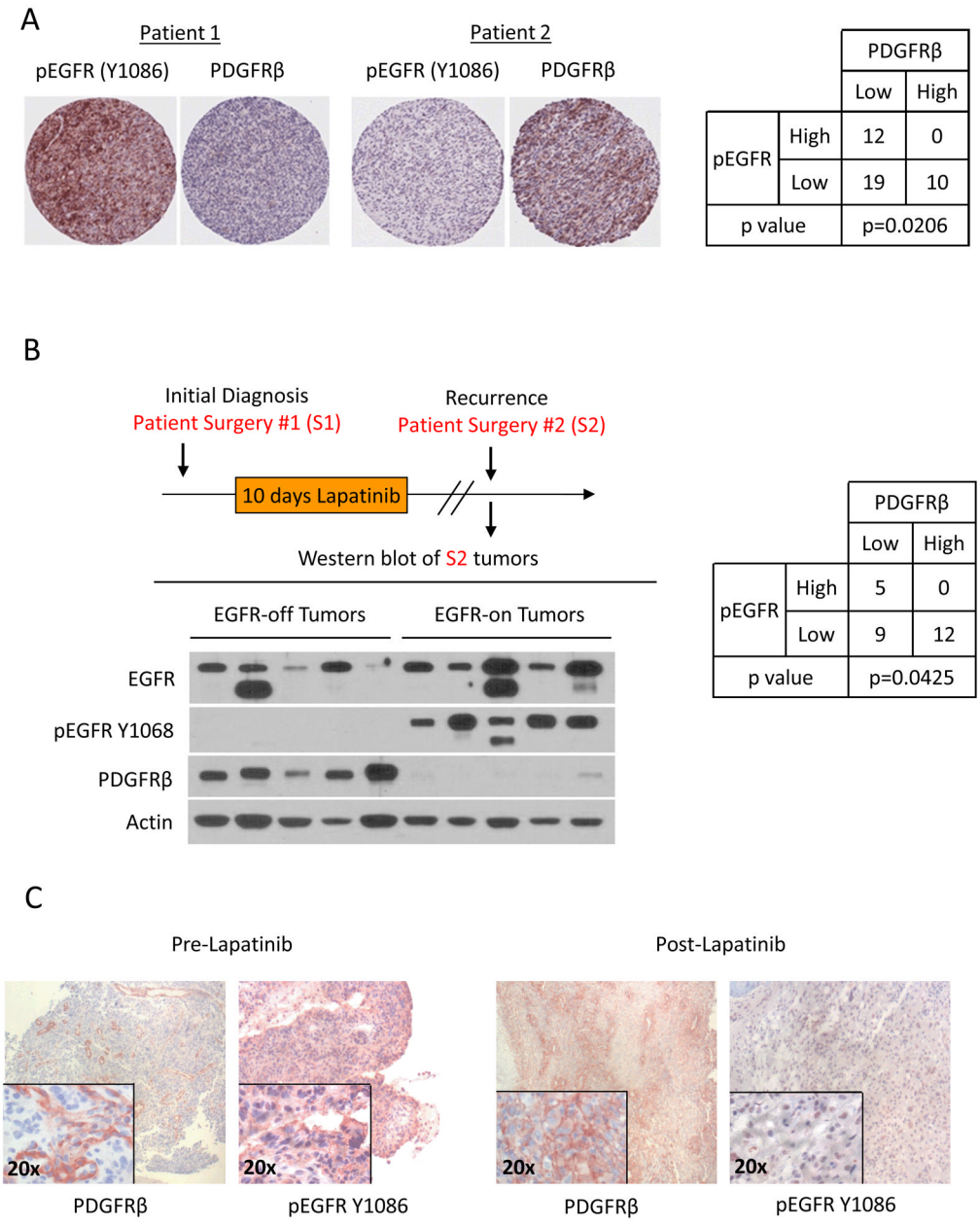


Fig 1. A reciprocal relationship between EGFR and PDGFRβ in glioma

(A) Experimental design of a mouse model of EGFR inhibitor resistance. U87-EGFRvIII cells were subcutaneously implanted in the mouse flank on day zero. Mice were treated with erlotinib (150mg/kg) on day two and as indicated thereafter. (B) Tumor growth curve of U87-EGFRvIII xenografts in mice treated with erlotinib (150mg/kg as indicated in (A)) or vehicle. (C) Immunoblot of indicated tumor lysates determining total and phospho-PDGFRβ or EGFR from U87, U87-EGFRvIII +/− eroltinib treatment and U87-EGFRvIII kinase dead xenografts harvested on day 21. PI3K-p85 is used as a loading control in this and subsequent immunoblots. (D) Receptor Tyrosine Kinase (RTK) array of 42 RTKs performed on U87-EGFRvIII xenograft lysates on day 21 from mice treated with erlotinib or vehicle as

described in (A). (E) IHC of PDGFRβ and phospho-EGFR in vehicle and erlotinib-treated U87-EGFRvIII orthotopic xenografts. (F) Immunoblot of PDGFRα, PDGFRβ and phospho-EGFR from patient-derived GBM neurospheres expressing EGFRvIII or wildtype EGFR as indicated. Whole cell lysates were collected after 24 hours of erlotinib or vehicle treatment. (G) Immunoblot of tumor lysates from EGFRvIII expressing GBM-39 xenografts following oral gavage with vehicle or erlotinib for ten days.



		PDGFRβ	
		Low	High
pEGFR	High	12	0
	Low	19	10
p value		p=0.0206	

		PDGFRβ	
		Low	High
pEGFR	High	5	0
	Low	9	12
p value		p=0.0425	

Fig. 2. PDGFRβ expression is suppressed in EGFR activated GBMs

(A) IHC staining for phospho-EGFR and PDGFRβ in clinical GBM tissues. The p value indicated was calculated using Fisher's exact test. (B) Immunoblot of PDGFRβ and EGFR in clinical GBM tumors samples treated with lapatinib. Patients were treated with lapatinib for ten days following initial diagnosis. Second tumor samples were obtained following recurrence. Tumor lysates were prepared and grouped according to phospho-EGFR status (EGFR-off/on). The p value was calculated using Fisher's exact test. (C) IHC staining for PDGFRβ and phospho-EGFR in pre and post lapatinib-treated GBM tissue.

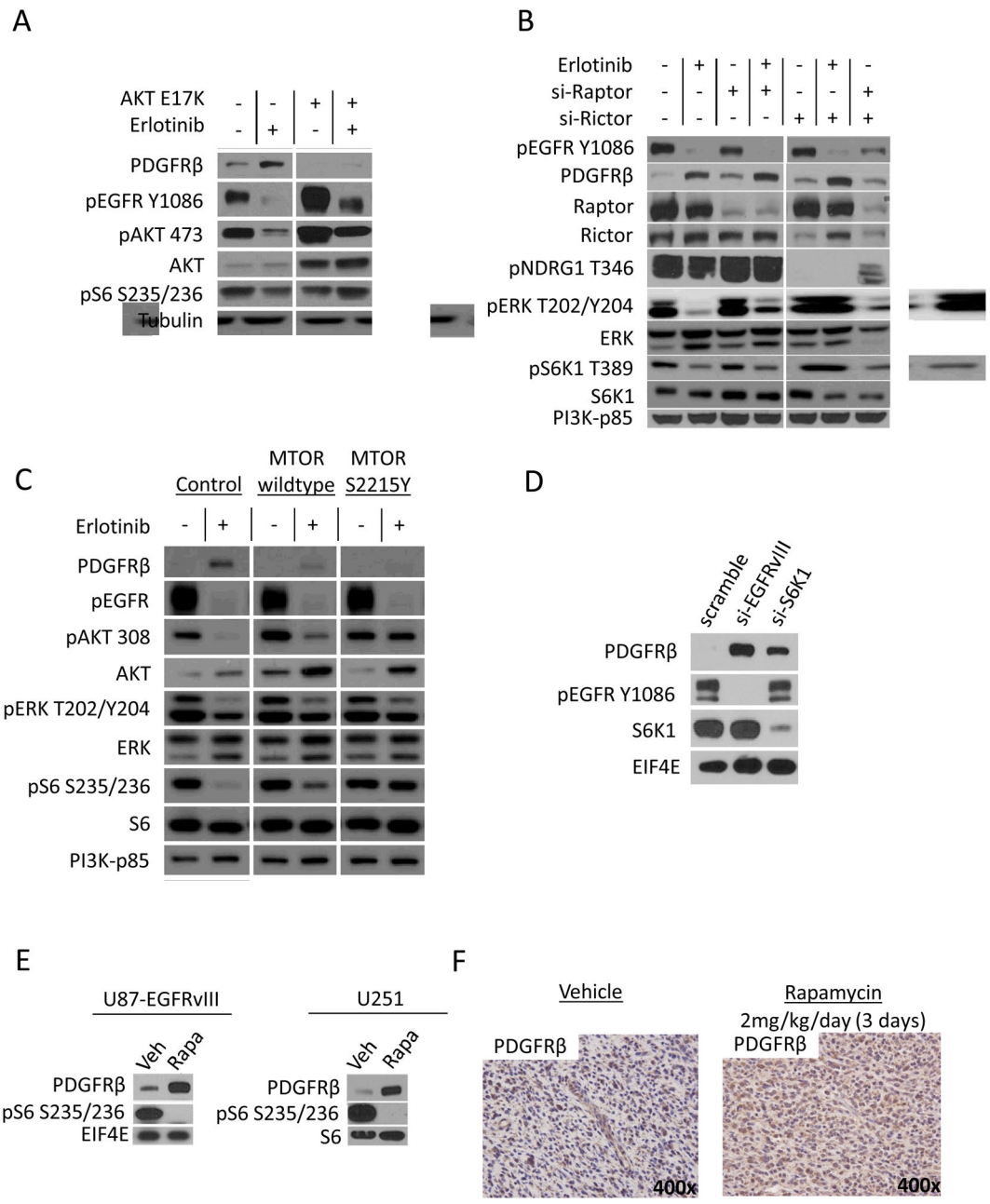


Fig. 3. EGFRvIII suppresses PDGFRβ through AKT and mTORC1 signaling

(A) Immunoblot of PDGFRβ and indicated proteins in U87-EGFRvIII cells expressing constitutively active AKT1 (E17K) treated with erlotinib (5μM) for 24 h. (B) Immunoblot of lysates from U87-EGFRvIII cells with transient knockdown of MTOR complex proteins Raptor or Rictor and treated with erlotinib (5μM) as indicated. (C) Immunoblot of U87-EGFRvIII cells expressing constitutively active (S2215Y) or wildtype mTOR and treated with erlotinib (5μM) for 24 h as indicated. (D) Immunoblot of PDGFRβ levels in response to transient knockdown of EGFRvIII, or S6 kinase 1 in U87-EGFRvIII cells. (E) PDGFRβ levels in U87-EGFRvIII and U251 cells treated with vehicle or rapamycin (5nM) for 24 hours. (F) IHC of PDGFRβ in intracranial U251 GBM tumors following 3 days of rapamycin (2 mg/kg/day) or vehicle treatment.

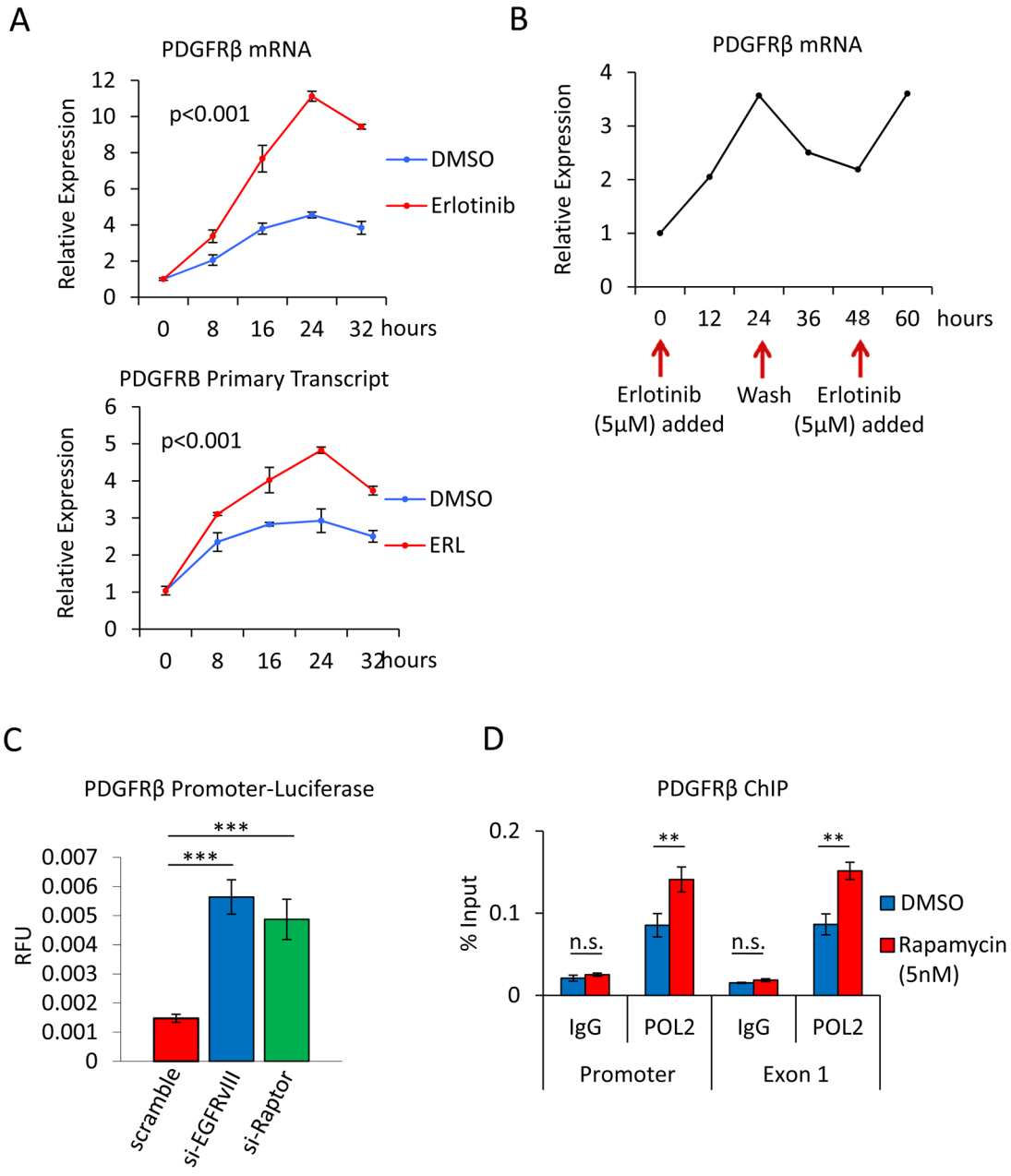


Fig. 4. EGFR signaling regulates transcription of PDGFRβ gene

(A) Time course of PDGFRβ primary transcript and mRNA expression in U87-EGFRvIII cells treated with erlotinib (5μM) or vehicle for up to 32 h. (B) Determination of PDGFRβ mRNA expression in response to erlotinib treatment or media washout over 60 h. (C) Luciferase assay comparing PDGFRβ promoter activity in U87-EGFRvIII cells transfected with scrambled siRNA to siRNA against raptor or EGFRvIII. (D) Chromatin immunoprecipitation of RNA Polymerase II at the promoter or exon 1 of PDGFRβ gene following treatment with vehicle or rapamycin (5nM) for 24 h. *p<0.05, **p<0.01, ***p<0.001.

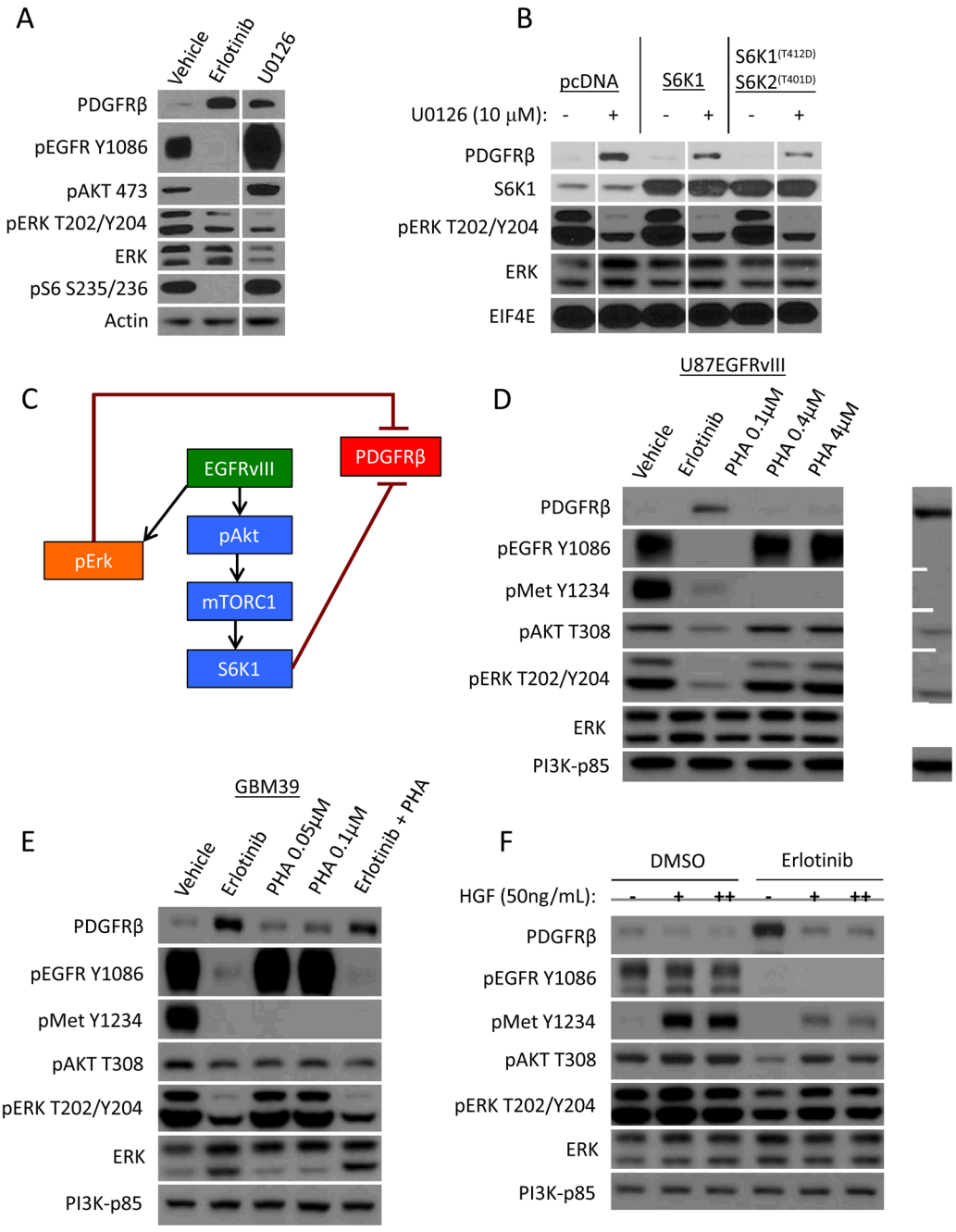


Fig. 5. ERK signaling contributes to the regulation of PDGFRβ

(A) Immunoblot of PDGFRβ and indicated proteins from whole cell lysates of U87-EGFRvIII cells treated with MEK inhibitor U0126 (5μM), erlotinib (5μM) or vehicle for 24 h. (B) Determination of PDGFRβ protein levels from U87-EGFRvIII cells transfected with empty vector (pcDNA), wild type S6K1, or constitutively active S6K1 (T412D) and S6K2 (T401D). In addition cells were treated with MEK inhibitor U0126 (10μM) or vehicle for 24 h as indicated. (C) A schematic of the signals downstream of EGFRvIII regulating PDGFRβ protein expression. (D, E) PDGFRβ protein levels from U87-EGFRvIII cells (D) or patient derived neurosphere GBM-39 (E) treated with erlotinib (5μM) or MET inhibitor PHA at the

indicated dose. (F) Immunoblot of PDGFRβ and indicated proteins from U87-EGFRvIII cells treated with erlotinib and MET ligand HGF (50–100 ng/mL) for 24 h as indicated.

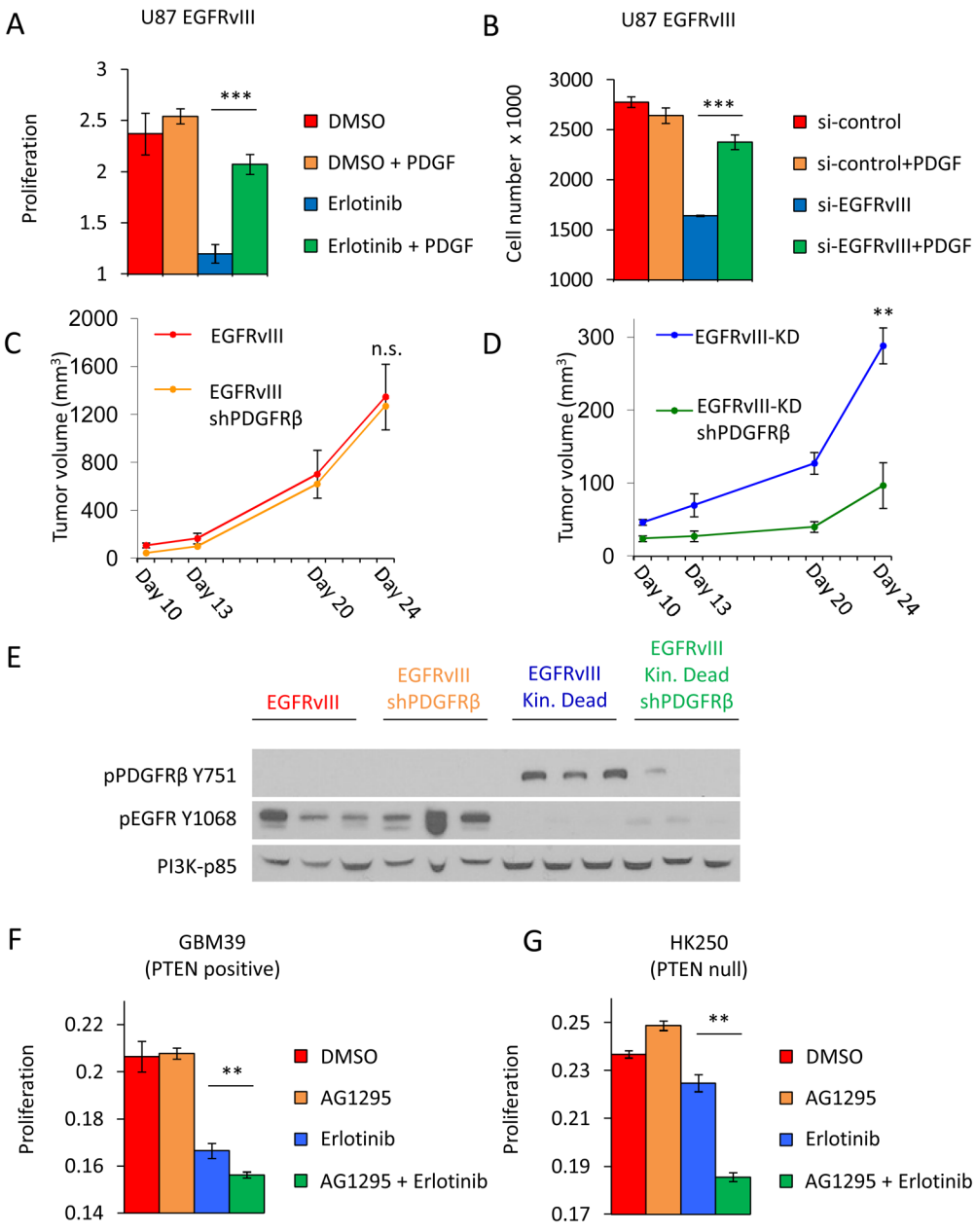


Fig. 6. PDGFRβ is dispensable for EGFRvIII-driven GBM growth, but is required for the optimal growth of EGFR-inhibited tumors

(A) Proliferation of U87-EGFRvIII cells over 4 days treated with erlotinib (5μM) or PDGF-bb ligand (20ng/mL) alone or in combination as indicated. Eroltinib was added on day zero of culture and PDGF-bb was added daily at 20ng/mL thereafter. (B) Growth of U87-EGFRvIII cells transiently transfected with control scrambled siRNA or siEGFRvIII and treated with PDGF-bb as described in (A). (C, D) Growth curve of xenografts subcutaneously implanted with U87EGFRvIII, U87EGFRvIII/shPDGFRβ, U87EGFRvIII kinase dead, or U87EGFRvIII kinase dead/shPDGFRβ cells as indicated. (E) Immunoblot of phospho-PDGFRβ and EGFR from lysates harvested on day 24 from tumors as described in C and D. (F, G) Proliferation of EGFRvIII expressing patient-derived neurospheres GBM-39

and HK-250 treated with erlotinib (5μm) and PDGFRβ inhibitor AG1295 (3μM) alone or in combination as indicated. *p<0.05, **p<0.01, ***p<0.001.

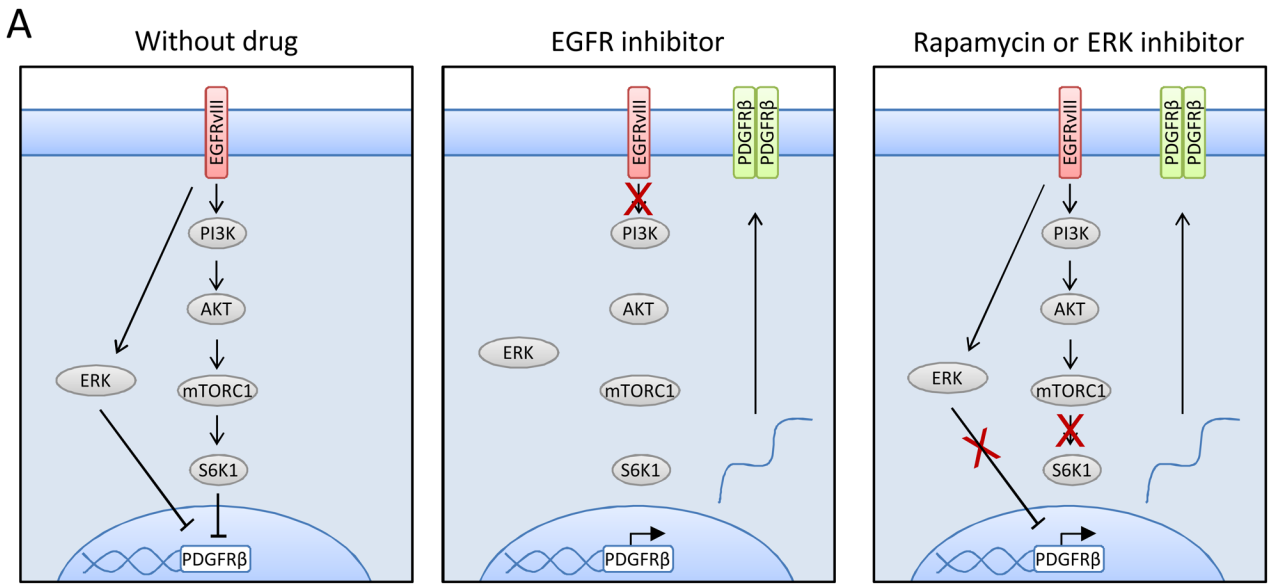


Fig. 7. Model of proposed RTK-switch

Under conditions of heightened growth receptor signaling (e.g., EGFRvIII mutation), PDGFRb expression is repressed by downstream ERK and mTOR activity. Inhibition of these growth pathways, such as EGFR- or mTOR-inhibitors results in the transcription of the *PDGFRβ* gene and the upregulation of PDGFβ receptor.