Combined Targeting of PDK1 and EGFR Triggers Regression of Glioblastoma by Reversing the Warburg Effect

Kiran Kumar Velpula1, Arnima Bhasin1, Swapna Asuthkar1, and Andrew J. Tsung1,2,3

Abstract

Glioblastoma multiforme is the most aggressive primary brain tumor in adults. Overexpression of the EGFR receptor (EGFR) is recognized as a widespread oncogenic signature in glioblastoma multiforme, but the complexity of its contributions is not fully understood, nor the most effective ways to leverage anti-EGFR therapy in this setting. Hypoxia is known to drive the aggressive character of glioblastoma multiforme by promoting aerobic glycolysis rather than pyruvate oxidation carried out in mitochondria (OXPHOS), a phenomenon termed the Warburg effect, which is a general feature of oncogenesis. In this study, we report that hypoxia drives expression of the pyruvate dehydrogenase kinase (PDK1) and EGFR along with the hypoxia-inducing factor (HIF)-1α in human glioblastoma multiforme cells. PDK1 is a HIF-1regulated gene and our findings indicated that hypoxia-induced PDK1 expression may promote EGFR activation, initiating a feed-forward loop that can sustain malignant progression. RNAi-mediated attenuation of PDK1 and EGFR lowered PDK1-EGFR activation and decreased HIF-1α expression, shifting the Warburg phenotype to OXPHOS and inhibiting glioblastoma multiforme growth and proliferation. In clinical specimens of glioblastoma multiforme, we found that immunohistochemical expression of PDK1, EGFR, and HIF-1α were elevated in glioblastoma multiforme specimens when compared with normal brain tissues. Collectively, our studies establish PDK1 as a key driver and candidate therapeutic target in glioblastoma multiforme.

Introduction

Glioblastoma multiforme is a metabolically active, primary adult brain tumor that evades multimodal therapy, leading to short survival times. Patients with glioblastoma multiforme have poor prognosis despite surgery and adjuvant chemotherapy with the majority dying within two years. This is commonly attributed to the diverse and heterogeneous signaling mechanisms present in glioblastoma multiforme that overcome pharmacotherapy. On the contrary, pervasive metabolic signatures have been identified, particularly the dependence upon glycolysis rather than oxidative phosphorylation (OXPHOS) with elevated lactate production regardless of oxygen availability. This bioenergetic state, when associated with increased glucose metabolism, results in tumorigenesis and tumor progression and a phenomenon termed the Warburg effect (1-6). The end result is such that despite functionally complex pathways, convergence occurs to some degree at the mitochondrial level. These metabolically active pathways in tumor cells are regulated by an upstream cascade of signaling mechanisms involving known growth factor receptor tyrosine kinases (RTK) implicated in tumorigenesis and in regulating glucose homeostasis (7, 8). Aberrant receptor kinase signaling is the primary mode contributing to malignant phenotypes with defined oncogenic signatures in human cancers, including glioblastoma multiforme. We propose that the ubiquitous EGFR activation observed in glioblastoma multiforme is associated with dysregulated metabolic responses and cell proliferation, making it an ideal target for therapy. However, clinical trials targeting EGFR alone using RTK inhibitors have been met with failure, emphasizing the fact that downstream pathways may serve as the primary modulator of tumor behavior.

Several studies have reported that pyruvate dehydrogenase along with its inhibitor pyruvate dehydrogenase kinase (PDK), a gatekeeper of glucose oxidation, can be targeted for regulating glucose oxidation and OXPHOS (9, 10). Interestingly, PDK1 is involved in cancer cell-associated glycolysis and mitochondrial dysfunction (11, 12). In this report, we demonstrated that oncogenic tyrosine kinases, when activated, orchestrate oncogenic signals to the mitochondria to activate PDK1, thereby promoting cancer cell metabolism and tumor growth. Although our knowledge of upstream signaling events in glioblastoma multiforme tumorigenesis has increased in recent years, the mechanisms and metabolic changes contributing to the aggressive nature of these tumors remain elusive. We further hypothesize that identification of EGFR-protein complexes regulating cell metabolism with which EGFR associates are critical for understanding control of cellular

Authors’ Affiliations: Departments of 1Cancer Biology and Pharmacology, and 2Neurosurgery, University of Illinois College of Medicine at Peoria; and 3Illinois Neurological Institute, Peoria, Illinois

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

Corresponding Author: Andrew J. Tsung, Illinois Neurological Institute, 530 NE Glen Oak Avenue, Peoria, IL 61637. Phone: 309-655-2700; Fax: 309-655-7696; E-mail: Andrew.J.Tsung@INI.org

doi: 10.1158/0008-5472.CAN-13-1868

©2013 American Association for Cancer Research.

www.aacajournals.org

Published OnlineFirst October 22, 2013; DOI: 10.1158/0008-5472.CAN-13-1868
proliferation, growth, and survival. Here, we present evidence of the functional significance and interaction of EGFR with mitochondrial PDK1. This mitochondrial interaction was found to be diminished after treatment with their respective inhibitors, silencing, or using human umbilical cord blood stem cells (hUCBSC). Similarly, we found evidence that dichloroacetate (DCA), a specific inhibitor of PDK1 (13), reduced EGFR phosphorylation and thereby reduced the EGFR-induced tumor growth.

Materials and Methods

Reagents and plasmids
EGFR, pEGFR, PDK1, GFP, COX-2, caspase-3, caspase-9, cytochrome c, SMAC, and GAPDH antibodies were purchased from Santa Cruz Biotechnology along with erlotinib hydrochloride, gefitinib hydrochloride, HIF-1α was purchased from Abcam; PDK inhibitor DCA and Cobalt Chloride were from Sigma; si-RNA constructs for PDK1 and PDK4 knockdown were purchased from Santa Cruz Biotechnology Inc. The short hairpin RNA (shRNA) construct for EGFR was discussed previously (14). Plasmid over-expressing EGFR (Cat# 32751) and HIF-1α (Cat#18955) were obtained from Addgene. 1-Lactate assay kit (Cat# 1200011002) was obtained from Eton Biosciences. Trans-AM HIF-1 kit (47596) was purchased from Active Motif. Isolation of intact mitochondria from cultured glioblastoma multiforme cells and surgical biopsies was performed using Mitochondria Isolation Kit (Pierce Biotechnology).

Cell culture
U251 cells were obtained from the American Type Culture Collection and were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS (HyClone) and 1% penicillin–streptomycin (Invitrogen). U251 cell lines were tested and authenticated according to the providers’ instructions. Xenograft cell line 5310 (a kind gift from Dr. David James) were grown in Dulbecco’s Modified Eagle Medium and were grown in Dulbecco’s Modified Eagle Medium (DME).

Preparation and culture of hUCBSCs
Human umbilical cord blood samples were collected from the umbilical vein of newborns with informed maternal consent according to the protocol approved by the Institutional Review Board at the University of Illinois College of Medicine at Peoria (Peoria, IL), and hUCBSC harvests were processed within 24 hours of collection, with viability of more than 90%. Isolation, culture, and expansion of hUCBSCs were performed as described previously (14).

Quantitative real-time PCR
The mRNA expression levels of EGFR and PDK1 were detected by quantitative real-time PCR (qRT-PCR) using the iCycler iQ (Bio-Rad) in the cDNA isolated from the hGBM biopsies using the primer sequences listed in Supplementary Table S1. The fold changes obtained are normalized to the normal brain control. Each sample was run in triplicate for the target gene and the internal control gene GAPDH.

Western blot analysis, immunoprecipitation, and immunofluorescence labeling
Proteins extracted from cultured cells and tissue lysates were followed by immunoprecipitation and immunoblotting with corresponding antibodies, as described previously (14). Denaturation quantitation was determined using the Image J software (NIH, Bethesda, MD). Equal loading was confirmed by stripping and reprobing the membranes with GAPDH/COX2 antibodies. For immunofluorescence analysis, cells were fixed and incubated with primary antibodies, Alexa Fluor dye-conjugated secondary antibodies, and 4’, 6-diamidino-2-phenylindole (DAPI) according to standard protocols (14). Cells were examined using Olympus BX61 confocal microscope with a 60-Å oil immersion objective. SPOT basic and SPOT advanced software was used to deconvolute Olympus BX61 images (Diagnostic Instruments).

Immunohistochemistry of hGBM specimens and in vivo tissue sections
hGBM surgical biopsy specimens were obtained from Saint Francis Medical Center (Peoria, IL) and processed in accordance with the UICOMP Institutional Review Board–approved protocols. Serial sections of six human specimens (normal human brain, hGBM-1, hGBM-2, hGBM-3, hGBM-4, and hGBM-5) and mouse xenograft tissue sections were stained with the EGFR, PDK1, HIF-1α, and COX2 antibodies as described previously (14). Images were acquired by using an Olympus BX61 fluorescence microscope and processed using SPOT advance software.

Transfections and generation of stable cell lines
Cells were plated at a density of 4 × 10⁵/60 mm dish 18 hours before transfection. Transfection was carried out using XtremeGENE (Roche) reagents according to the vendor’s instructions. Transfected cultures were selected with G418 (400 μg mL⁻¹) for 10 to 14 days. At that time, antibiotic-resistant colonies were picked, pooled, and expanded for further analysis under selective conditions.

1-Lactate assay from cell culture media and tissue extracts
To measure lactate content, cells and tissues were extracted with cold 80% ethanol (tissue/solvent ratio 1:8) and centrifuged at 10,000 × g at 4 °C for 30 minutes. The supernatants were collected and diluted with water at different dilutions. The lactate contents were then assayed using the lactate assay kit (Eton Bioscience).

HIF-1α transcription activity assay
HIF-1α transcriptional activity was analyzed by using TransAM HIF-1 Transcription Factor Assay Kit (Active Motif) following manufacturer’s instructions. Briefly, nuclear extracts were added onto a 96-well microplate coated with oligonucleotides containing hypoxia response element (59-TACGTGCT-39) from the erythropoietin (EPO) gene. HIF dimers present in
nuclear extracts bind with high specificity to this response element and are subsequently detected with an antibody directed against HIF-1α. Addition of a secondary antibody conjugated to horseradish peroxidase provided a sensitive colorimetric readout and was quantified by spectrophotometry. Values are expressed as optical density (OD) at 450 nm with a reference wavelength of 655 nm.

**Mitochondria isolation and subcellular fractionation**

Mitochondrial extracts were prepared using a kit (Pierce; Cat# 89801). Tissues, around 50 to 200 mg, subjected to fractionation were washed with 2 to 4 mL of PBS and were then cut into small pieces for trypsin treatment, followed by a series of centrifugations to obtain cytosolic and mitochondrial fractions. Mitochondrial proteins (60 μg) and cytosolic proteins (30 μg) were analyzed by immunoblotting analyses. COX2 (cytochrome c oxidase subunit II) and GAPDH were used as respective loading controls.

**Hypoxia and other treatments**

U251 and 5310 cells were exposed to 1 to 3 cycles of hypoxia and normoxia. Each hypoxic cycle consisted of a period of 24 hours in 1% oxygen followed by 24-hour recovery under normoxic conditions. During this reoxygenation period, cells were provided with fresh medium. In another experiment, hypoxic conditions were obtained by incubating cells in 150 μmol/L CoCl₂, at different time points as described previously (16). In another experiment, U251 and 5310 cells were treated with either 5 μmol/L erlotinib or gefitinib hydrochloride for 9 hours; 1 mmol/L DCA for 1 hour.

**MTT and TUNEL assay**

Cell growth from exponentially growing U251 and 5310 cells and their respective treatments including si-PDK1, sh-EGFR, DCA (1 mmol/L), Fl-EGFR (full-length EGFR), Fl-EGFR+DCA, Fl-EGFR+siPDK1 was measured using the MTT assay according to the manufacturer’s instructions (Invitrogen).

To evaluate cell death, TUNEL assay was performed in both U251 and 5310 cells treated with si-PDK1 following manufactures instructions (Roche).

**EGFR phosphorylation antibody array**

Around 500 μg of total cell lysates from the 5310- control and treated cells were subjected to EGFR phosphorylation antibody array (Ray Biotech) by following the manufacturer’s instructions.

**Subcutaneous tumors**

Female nude mice of 6- to 8-week-old (Harlan Labs) were subcutaneously injected on the right flanks with 1 × 10⁶ Fl-EGFR-3310 cells. Tumor formation was assessed every 4 to 5 days. Tumor growth was recorded by measuring two perpendicular diameters of the tumors over a 5-week time course using the formula 4π/3 × (width/2) × (length/2). Dichloro-acetate (DCA), an inhibitor for PDK1 was injected on the site of tumor at a concentration of 100 mg/kg body weight on the 12th and 15th day after tumor implantation. The tumors were harvested and weighed at the experimental endpoint and the tumor volume (mm³) of control, Fl-EGFR, or Fl-EGFR+DCA–treated 5310 cells were compared.

**Intracranial injections**

Fl-EGFR-5310 cells (5 × 10⁶) were injected intracranially into four mice per group on the right side of 4-week-old female athymic nude mice under isofluorane anesthesia with the aid of a stereotactic frame. The calvarium of each anesthetized mouse was exposed through a midline incision and a burr hole was drilled 1 mm lateral (right) and 2 mm anterior to the bregma. Two weeks after tumor implantation, the mice were intravenously injected with 100 mg/kg body weight of DCA. Following tumor cell injection and treatments, mice were observed daily until they reached a moribund state, at which time they were euthanized and their brains removed and processed for histopathologic analysis. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Illinois College of Medicine at Peoria.

**Statistical analysis**

Statistical analysis and graphical presentation was done using quantitative data from Western blot analysis and other assays were evaluated for statistical significance using GraphPad Prism 4.0. Data for each treatment group were represented as means ± SEM and compared with other groups for significance by one-way ANOVA followed by Bonferroni post hoc test (multiple comparison tests) using GraphPad Prism version 3.02. Results presented in this study are the representative images of three independent experiments (n = 3) and results are expressed as mean ± SE (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

**Results**

**EGFR and PDK1 coexpress in surgical biopsies of glioblastoma multiforme patients**

Activation of EGFR in glioblastoma multiforme earlier has shown to increase glucose uptake and lactate production through NF-xB–dependent signaling cascade, promoting tumor development (17). Studies conducted on aberrant FGFR1 revealed its common existence in tumors regulating the Warburg effect (18). Recent studies revealed that targeting PDK by DCA shifts cancer cell metabolism from glycolysis to OXPHOS by dephosphorylating mitochondrial pyruvate dehydrogenase (13). Furthermore, to better understand how tyrosine kinase signaling in conjunction with PDK1 regulates the Warburg effect, we first analyzed the expression of EGFR and PDK1 in five different human glioblastoma multiforme (hGBM) surgical specimens along with the normal brain. Interestingly, hGBMs 1, 3, and 4 showed high levels of EGFR and PDK1, whereas hGBMs 2 and 5 showed moderate expression when compared with the normal brain. Consistent with these results, immunohistochemical analysis of all the hGBMs showed similar increased levels of pEGFR and PDK1. However, reduced EGFR, pEGFR, and PDK1 levels were observed in normal control brain sections (Fig. 1A and Supplementary Fig. S1). The hGBM specimens were then tested for EGFR and PDK1 mRNA expression by semi-quantitative RT-PCR and were shown to express the increased transcripts when compared...
with the normal brain (Fig. 1B). Similarly, Western blot analysis confirmed our immunohistochemistry and RT-PCR findings (Fig. 1C). Furthermore, immunoprecipitation (IP) studies using PDK1 antibody demonstrated pEGFR expression in hGBMs 1, 2, 4 and 5; whereas using pEGFR antibody, we observed PDK1 expression in all the hGBM specimens tested in the present study. Of note is the normal brain, which showed no or minimal expression in both experimental conditions (Fig. 1D). These observations show a novel link between EGFR and PDK1 signaling and suggest that this association might play a critical role in regulating glioma cell metabolism.

DCA treatment and silencing of PDK1 suppresses EGFR expression in glioblastoma multiforme

Previously, we demonstrated the effectiveness of anti-EGFR–targeted therapy using EGFR inhibitors such as erlotinib, gefitinib, and AG1478 (14). Therefore, we sought to investigate the efficacy of these drugs against the gatekeeper of glycolysis, PDK1. Both gefitinib and erlotinib were able to effectively suppress PDK1 and EGFR. However, erlotinib treatment was observed to suppress them more effectively. (Fig. 2A and B). We extended our study by engineering glioma cell lines, U251 and 5310, to stably express either Fl-EGFR (3639 bp) or stably knockdown EGFR (sh-EGFR) by transfection. sh-EGFR–treated U251 and 5310 cells showed reduced expression levels of EGFR, pEGFR, and PDK1, whereas Fl-EGFR–U251 and 5310 cells demonstrated increased levels of EGFR, pEGFR, and PDK1 (Fig. 2C–F). To consolidate our hypothesis regarding EGFR and PDK1 crosstalk, we next examined whether PKD inhibitors also regulate EGFR expression. We treated U251 and 5310 cells with 1 mmol/L DCA for 1 hour and observed that DCA treatment

Figure 1. EGFR and PDK1 colocalize in hGBM patient specimens. A, representative immunohistochemical staining of EGFR and PDK1 in hGBM patient specimens and normal brain. All five of the tested specimens (hGBM1–hGBM5) showed a strong positive indication of EGFR and PDK1. Negative staining was seen in normal human brain samples (Bar, 100 μm). B, qRT-PCR analysis for EGFR and PDK1 in hGBM samples. The fold changes are presented after normalizing to the normal brain sample \( n = 3 \); results are shown as mean ± SE (\( P < 0.05; \*) , \( P < 0.01 \) ). C, Western blot analysis of EGFR and PDK1 protein expression in hGBM specimens and normal brain tissue. D, immunoprecipitation experiments were conducted on hGBM patient specimen tissue lysates and normal brain tissue lysates by using pEGFR and PDK1 antibodies. Western blotting analysis was performed on these immunoprecipitated samples using pEGFR and PDK1 antibodies. IgG probing was done to confirm equal loading.
reduced the expression levels of both EGFR and PDK1 (Fig. 2G and H). Furthermore, to verify if PDK1 silencing exerts similar effects as evidenced by DCA, we transfected U251 and 5310 cells with si-PDK1, which significantly inhibited (\(P < 0.01\)) the expression of both total and phosphorylated forms of EGFR (Fig. 2I and J). To ensure that the inhibition of EGFR with si-PDK1 is not an off target effect, we transfected glioma cells with si-PDK4 and surprisingly observed a minimal reduction of EGFR and pEGFR (Fig. 2K and L). These results showed that in glioblastoma multiforme cells, DCA or silencing of PDK1, exhibited a comparable response to erlotinib, gefitinib or sh-EGFR treatments.

**Hypoxia mediates EGFR and PDK1 interaction in mitochondria**

It was earlier identified that the gene encoding PDK1 is induced by hypoxia and is a direct target of HIF-1 (19). Furthermore, Franovic and colleagues reported a clinical correlation between tumor hypoxia and EGFR expression, resulting in tumor progression and poor clinical outcome (20). In the present study, we exposed U251 and 5310 cells to hypoxia (1% oxygen) or normoxia for 24, 48, and 72 hours. Immunoblotting analysis was performed...
to analyze the induction of HIF-1α in both experiments. Increased HIF-1α levels were observed at 72 hours in U251 cells and 48 hours in 5310 cells in hypoxic conditions, whereas in cells treated with CoCl₂, HIF-1α expression maximized at 6 hours in both the cell lines (Fig. 3A). To determine the involvement of HIF-1α in regulating EGFR and PDK1 signaling, we immunoblotted HIF-1α against the cell lysates of si-PDK1, Fl-HIF-1α, sh-EGFR, and Fl-EGFR. The si-PDK1 and sh-EGFR treatments showed decreased HIF-1α expression, whereas a substantial increase was noted in Fl-EGFR, Fl-HIF-1α, U251, and 5310 cells (Fig. 3B). We next investigated whether EGFR and PDK1 expression were regulated by HIF-1α by measuring its activity in U251 and 5310 cells subjected to various treatments using ELISA-based Trans-AM HIF-1α kit (Active Motif). Interestingly, increased HIF-1α activity was observed in Fl-EGFR cells, whereas activity was attenuated in si-PDK1 and

Figure 3. HIF-1α expression increases PDK1 and EGFR interplay under hypoxic conditions. A, U251 and 5310 cells were exposed to hypoxia for various time points. In another experiment, hypoxic conditions were induced using 150 μmol/L CoCl₂. Increased HIF-1α expression was observed at 72 hours for U251 cells and 48 hours for 5310 cells. For cells treated with CoCl₂, both cell lines showed an increase at the time point of 6 hours. B, downregulation of both PDK1 and EGFR using their respective shRNAs resulted in decreased expression of HIF-1α in U251 and 5310 cell lysates. Stably expressing Fl-EGFR in both U251 and 5310 cells showed increased expression of HIF-1α as compared with the control. GAPDH served as the loading control. C, HIF-1α activity was measured using TransAM HIF-1 Transcription Factor Assay Kit. Increased HIF-1α was noted with EGFR overexpressing cells, whereas downregulation of both PDK1 and EGFR showed a decrease in HIF-1α activity. D, representative immunohistochemical staining of HIF-1α in human glioblastoma multiforme patient specimens and normal brain. All five tissues showed a strong positivity of HIF-1α (bar, 100 μm). E, mitochondrial fractions of U251 and 5310 cells were immunoblotted with anti-PDK1 and anti-pEGFR antibodies, with COX2 serving as the loading control. Results showed decreased mitochondrial expression for siPDK1 and shEGFR treatments, whereas increased expression was noted for PDK1 and pEGFR in Fl-EGFR-treated cells. These aforementioned samples were immunoprecipitated with pEGFR and immunoblotted against PDK1. The same blots were stripped and reprobed with anti-pEGFR antibody. F, dual immunohistochemical staining for colocalization was conducted on the control brain and hGBM specimens with anti-PDK1, anti-pEGFR, and anti-COX2 antibodies followed by the secondary antibodies conjugated with fluorophores for red (PDK1), green (pEGFR), and blue (COX2) fluorescence, respectively. Representative merged images show the cells expressing PDK1, pEGFR, and COX2 (bar, 100 μm). G, furthermore, we carried out immunocytochemistry on normoxic and 48-hour-induced hypoxic-treated U251 and 5310 cells to study the expression of PDK1-pEGFR. PDK1 is conjugated with Alexa Fluor-594 (red), pEGFR was conjugated with Alexa Fluor-488 (green), and COX2 is conjugated with Alexa-Flour-647 (blue; bar, 200 μm). All experiments were performed in triplicate (n = 3). Results from three independent experiments are shown as mean ± SE (*, P < 0.05; **, P < 0.01).
sh-EGFR cells when compared with their respective controls. These results demonstrated a positive feed-forward loop between HIF-1α, PDK1, and EGFR (Fig. 3C). It is understood that a majority of glioblastoma multiforme tumors are hypoxic and that this hypoxic setting induces HIF expression in glioblastoma multiforme cells lines and primary cultures (21, 22). Immunohistochemical analysis revealed abundant expression of HIF-1α in five hGBM specimens used in this study compared with the normal brain control (Fig. 3D), suggesting that HIF-1α expression plausibly controls the RTK coupled glioma metabolic pathway. Furthermore, PDK1 is listed as one of the HIF-regulated mitochondrial target genes that mediate functional changes observed in hypoxia (23). In addition, we conducted immunoblot analysis on the mitochondrial isolates and observed reduced mitochondrial levels of PDK1 and pEGFR in si-PDK1, sh-EGFR–treated U251 and 5310 cells, whereas increased levels were observed in Fl-EGFR–treated cells compared with their respective controls. To further confirm PDK1 association with EGFR in the mitochondria, we conducted immunoprecipitation analysis on the mitochondrial lysates obtained from the aforementioned treatments using pEGFR antibody. We observed increased pEGFR-PDK1 interaction in Fl-EGFR treatments, whereas si-PDK1 and sh-EGFR treatments showed a suppressed interaction (Fig. 3E). To verify the existence of pEGFR-PDK1 interaction in the mitochondria in hGBM specimens, we performed immunocolocalization studies with PDK1, pEGFR, and COX2 antibodies. We recorded a significant pEGFR(Green)-PDK1(Red)-COX2(Blue) interaction in five hGBM specimens as indicated in white, when compared with the normal counterpart (Fig. 3F), highlighting

Figure 4. hUCBSC influences the expression levels of EGFR and PDK1 in vivo. A, nude mice with pre-established intracranial human glioma tumors (U251 or 5310) were treated with hUCBSC by intracranial injection (2 × 10⁶). The hUCBSC-treated and control brain sections were immunoprobed for the presence of PDK1/pEGFR complex using appropriate secondary antibodies. Each experiment was performed in duplicate with each sample (n = 2). PDK1 (red) and pEGFR (green) were observed to be colocalized on COX2 (blue) that was used to stain the mitochondria (bar, 100 μm). B, equal amounts of proteins (40 μg) from untreated (control) and hUCBSC-treated tissue lysates obtained from orthotopic intracranial tumors of U251 and 5310 were loaded onto 8%–14% gels and transferred onto nitrocellulose membranes, which were then probed with respective antibodies. GAPDH was used a positive loading control. D and F, immunoblot analysis of mitochondrial (D) and cytoplasmic (F) fractions of hUCBSC–treated U251 and 5310 cells showed decreased levels of EGFR, pEGFR, and PDK1 when compared with control cells (C, E, and G). The relative band intensities were measured by densitometry and normalized against the respective GAPDH signals. Results from three independent experiments are shown as mean ± SE (*, P < 0.05; ***, P < 0.01).
the fact that targeting this interaction occurring at the mitochondrial membrane might be effective in modulating cell metabolism. Finally, we examined the role of hypoxia in EGFR and PDK1 mitochondrial interaction by immunofluorescence analysis on 48-hour-induced hypoxic samples. Increased pEGFR and PDK1 mitochondrial localization was observed in both U251 and 5310 hypoxic cells, when compared with their respective normoxic controls (Fig. 3G). In both the experiments (Fig. 3F and G and Supplementary Fig. S2A and S2B), we used COX2 to identify mitochondria. These data provide evidence that EGFR–PDK1 interaction at the mitochondria is upregulated under hypoxia, uncovering a previously uncharacterized mechanism of HIF1-EGFR-PDK1 activation in both glioblastoma multiforme cell lines and patient specimens.

Stem cell treatment inhibits EGFR-PDK1 interaction in vivo

Previously, we showed that hUCBSCs reduced EGFR translocation to the mitochondria in glioblastoma multiforme cells, highlighting the existence of hUCBSC-mediated antagonism of EGFR in mitochondria (24). In light of our findings that EGFR colocalizes with mitochondrial PDK1 (Fig. 3F and G), we asked whether hUCBSC could regulate their localization. To study this further, we conducted immunohistochemical analysis on mice brain tissue sections of control and hUCBSC-treated U251 and 5310 cells. Interestingly, we observed a great degree of pEGFR-PDK1 localization on COX2 in both U251 and 5310 control sections, indicating the interaction, whereas hUCBSC treatment reduced pEGFR–PDK1 interaction (Fig. 4A and Supplementary Fig. S3), suggesting that this interaction might be required to modulate PDK1-EGFR-mediated cell metabolism. Furthermore, Western blotting conducted on the in vivo U251 and 5310 controls and their respective treatments with hUCBSC revealed a marked decrease in the individual expression levels of PDK1, EGFR, and pEGFR. However, minimal expression of pEGFR and PDK1 was observed in hUCBSC controls (Fig. 4B and C). We next fractionated control and hUCBSC-treated tissues of U251 and 5310 to investigate the expression of PDK1 and EGFR. hUCBSC treatment reduced both the mitochondrial (Fig. 4D and E) and cytoplasmic levels of (Fig. 4F and G) EGFR, pEGFR, and PDK1 when compared with the controls, thereby suggesting that hUCBSC treatment may be effective in regulating cancer cell metabolism by reducing the expression of signature molecules PDK1 and EGFR and their mitochondrial association.

si-PDK1 induces apoptosis, decreases cell proliferation and lactate release in glioblastoma multiforme

Several articles suggested that targeting metabolically important key enzymes such as pyruvate dehydrogenase kinase (9), lactate dehydrogenase (9, 25), or pyruvate kinase (26), increase apoptosis and decrease proliferation and tumor growth. Similarly, our Western blot analysis conducted on cell lysates obtained from si-PDK1–treated U251 and 5310 cells probed with caspase-3, caspase-9, cytochrome c, and SMAC showed their increased expression (Fig. 5A) compared with their respective controls, suggesting the induction of apoptosis. In addition, apoptosis in response to si-PDK1 treatment showed 75% and 82% TUNEL positivity in U251 and 5310 cells respectively, when compared with the controls (Fig. 5B). We next sought to study whether si-PDK1 contributed to deregulated cell survival using a MTT assay. U251 and 5310 cells were stimulated with si-PDK1, sh-EGFR, 1 mmol/L DCA, Fl-EGFR, Fl-EGFR+DCA, and Fl-EGFR+si-PDK1. Reduced proliferation was observed in si-PDK1, DCA, and sh-EGFR–treated U251 and 5310 cells; however, cells stimulated with Fl-EGFR demonstrated increased proliferation. Notably, both si-PDK1 and DCA treatments were effective in reducing the cell proliferation in U251 and 5310 cells expressing Fl-EGFR (Fig. 5C). We further examined potential metabolic changes initiated by si-PDK1 treatments in U251 and 5310 cells. Both the control glioblastoma multiforme cells showed increased aerobic glycolysis as indicated by increased lactate production; however, si-PDK1, sh-EGFR, and DCA treatments maximally reduced the production of lactate. Conversely, Fl-EGFR treatment increased the lactate production, indicating that EGFR overexpression facilitated the induction of aerobic glycolysis. Reduced lactate release in the Fl-EGFR cells exposed to DCA underscores that silencing PDK1 targeting EGFR might effectively reduce lactate production, mitigating cells towards oxidative phosphorylation (Fig. 5D). Similarly, hUCBSC treatment reduced lactate release in treated extracts compared with their respective controls. Interestingly, hUCBSC control samples alone showed minimal lactate production (Fig. 5E). Experiments conducted using hGBM samples showed a relative abundance of lactate compared with the normal brain (Fig. 5F), indicating that a greater portion of lactate was derived through glycolysis. Taken together, our data show that si-PDK1 not only induced apoptosis, but also reduced EGFR-mediated cell proliferation and lactate production in glioblastoma multiforme.

DCA treatment abrogates EGFR phosphorylation and suppresses the tumorigenic ability of glioblastoma multiforme cells in vivo

Reduced levels of EGFR in si-PDK1- or DCA-treated U251 and 5310 cells prompted us to further study the associated mechanism by using an EGFR phosphorylation antibody array. We subjected 5310–control, Fl-EGFR, and Fl-EGFR+1 mmol/L DCA for analysis. EGFR phosphorylation array revealed substantial inhibition of EGFR (Tyr845) by 75%, EGFR (Tyr1045) by 50%, EGFR (Tyr1086) by 90%, EGFR (Tyr1148; Tyr1173) by 60%, EGFR (Ser1046/1047; Ser1070) by 50.2%, ErbB2 (Tyr1112) by 82.4%, ErbB2 (Tyr1221/1222) by 40.4%, ErbB2 (Ser1113) by 50.33%, and ErbB3 (Tyr1289) by 68.9% in DCA-treated Fl-EGFR 5310 cells, thus confirming the existence of functional cooperativity and interaction between these proteins (Fig. 6A). Having demonstrated that a diminution of EGFR by DCA treatment had substantial in vitro effects, we next sought to determine its effects on in vivo behavior. We first implanted the Fl-EGFR-S310 cells into the right flanks of athymic nude mice. The data in Fig. 6B show that cells from Fl-EGFR-5310 cells formed tumors with increased kinetics, whereas tumor growth was remarkably suppressed when the same numbers of cells injected were treated with two
intravenous doses of DCA. The latter cells formed tumors at the same time as the Fl-EGFR-5310 cells, but the tumors further, did not progress in size (Fig. 6B and C). We next examined whether the tumor growth suppression observed in heterotopic subcutaneous inoculations was also apparent with ectopic stereotactic implantation to the brain. Fl-EGFR-5310 cells were injected intracerebrally into the right side of the brains of nude mice in both control and test animals. DCA administered intravenously suppressed intracranially implanted 5310-FL-EGFR–induced tumor by 60% (insets of Fig. 6F). Survival curves...
plotted revealed that DCA treatment reduced the tumor growth and increased the survival by more than 3 weeks (Fig. 6D). Immunofluorescence experiments conducted to study whether DCA treatment reduced the PDK1 and EGFR interactions revealed concurrent results (Fig. 6E). Immunohistochemical analysis of the intracerebral tumors using anti-EGFR or anti-PDK1 antibody showed substantial expression in the tumors formed by Fl-EGFR-5310 cells, whereas minimal immunoreactivity was apparent in the DCA-treated sections (Fig. 6F).

Figure 6. Modulation of EGFR phosphorylation levels after DCA treatment in 5310 cells; DCA treatment suppressed EGFR-mediated glioblastoma growth. A, whole-cell lysates of 5310 control, 5310-Fl-EGFR, and 5310-Fl-EGFR + DCA-treated cells were subjected to EGFR phosphorylation array and representative array images from three independent experiments were presented. B and C, effects of DCA on glioblastoma cancer growth in the nude mouse model of subcutaneous xenograft. 5310-Fl-EGFR (1 × 10⁶) was subcutaneously inoculated into the right flank of nude mice (n = 3 per treatment group). One hundred mg/kg body weight DCA was injected on the tumor regions of the right flanks on 13th and 15th day of tumor implantations. A representative subcutaneous tumor mass from each group was shown in the insets. The subcutaneous tumors were removed and processed for immunohistochemistry analysis as represented along with representative hematoxylin and eosin (H&E) staining. Tumor size was measured weekly for nearly 7 weeks. Tumor volume was calculated by the formula as mentioned in Materials and Methods. D, Kaplan-Meier survival curves from mice bearing intracranial 5310-Fl-EGFR tumors. Mice were treated with 5310-Fl-EGFR [heavy solid line]; 5310-Fl-EGFR + 1 mM DCA (heavy dotted line). Analysis showed the treatment group had an increased survival compared with the 5310-Fl-EGFR group with a median survival 19 days. E, immunofluorescent staining for PDK1 (red), EGFR (green), and nuclei (blue), demonstrating decreased PDK1-EGFR assembly in the DCA-treated sections compared with control (bar, 100 μm). F, immunohistochemical analysis of EGFR and PDK1 expression in 5310-Fl-EGFR tumors (left) and 5310-Fl-EGFR + DCA (right; n = 3). Representative H&E staining is seen in the insets of left panel (bar, 100 μm).
Discussion

Cell metabolism in cancer is a diverse network of dynamic pathways utilized by the cells to synthesize and obtain energy for an altered "cellular goal"- one that represents exponential growth rather than homeostatic mechanisms. To support growth and proliferation, cancer cells have to significantly transform cellular metabolism by deregulating the expression of several key proteins. A well-known oncogenic metabolic signature in glioblastoma multiforme is increased glycolysis despite the presence of oxygen, termed the Warburg effect. Several important oncogenes involved in the progression of common human cancers including glioblastoma multiforme, have been found to be involved in the regulation of glycolysis. For example, Yang and colleagues showed an important mechanistic interplay between the EGFR and NF-κB pathways in cancer metabolism during tumor development (17). Hitosugi and colleagues reported that oncogenic tyrosine kinases such as FGFR1, localized in the cancer cell mitochondria, phosphorylate and activated mitochondrial PDK1 to promote cancer cell metabolism and tumor progression (18). Typically, EGFR shares extensive sequence homology with other receptor tyrosine kinase subfamilies such as FGFR, VEGFR, and PDGFR, and clinical studies with specific EGFR inhibitors such as gefitinib and erlotinib revealed that FGFR can also be targeted to patients with lung cancer bearing active FGFR signaling pathways (27). In view of these findings, we hypothesized that EGFR phosphorylation might interact and activate PDK1 in glioblastoma multiforme. Our findings therefore emphasize the importance of studying the mechanisms linking EGFR with other potential targets, eventually translating to novel treatment strategies.

In high necrotic core areas in any given tumor, the amount of oxygen observed is extremely low, thereby accelerating the cells to adapt to hypoxia mediated by HIF-1 stabilization. Furthermore, HIF-1 is known to transcribe and express several glycolytic genes that critically regulate cell death and apoptosis including PDK1 and EGFR (20, 28, 29). In this study, we confirmed that both PDK1 and EGFR expression were significantly correlated with HIF-1α expression in both in vitro and in vivo experiments. As PDK1 and EGFR reside in different subcellular compartments, we questioned their location of intracellular interaction. PDK1 is known to function in the mitochondria and a few studies have already confirmed that EGFR translocates to the mitochondria (24, 30). To begin addressing this question, we conducted immunofluorescence studies and found that PDK1 colocalizes with EGFR in the mitochondrial matrix. Our question whether hypoxia mediated by HIF-1α activation affects EGFR-PDK1 localization was addressed by observing increased EGFR–PDK1–COX2 interaction in hypoxic conditions, when compared with decreased or minimal interaction in normoxic conditions, suggesting that hypoxia mediated by HIF-1α stabilization may be required for PDK1 and EGFR interaction (Fig. 5G). Although this mechanism appears speculative, PDK1-EGFR interaction might provide starting points in targeting glioma cell metabolism.

Furthermore, after examining the expression of EGFR and PDK1 involved in human glioblastoma multiforme glycolytic pathway, we then evaluated potential therapeutic modalities by correlating in vitro prognosis with in vivo test results. We have earlier reported that stem cells derived from human umbilical cord blood can be effectively used as an alternative therapy in targeting glioblastoma multiforme (14). Here, immunohistochemical analysis revealed that hUCBSC treatment reduced EGFR-PDK1 interaction and subcellular fraction experiments and further showed that hUCBSC effectively reduced EGFR and PDK1 expression, both at the mitochondrial and the cytoplasmic levels. Further studies are warranted to determine how hUCBSC functionally regulates cancer cell metabolism. We also were able to demonstrate that silencing of PDK1 exhibited apoptotic and anti-proliferative effects on U251 and 5310 cells. Our results presented in this study correlated with previous reports regarding the effect of DCA (31). In addition, both si-PDK1 and DCA treatments suppressed lactate production in U251 and 5310 cell lines, and sh-EGFR treatment also demonstrated similar results. Conversely, hGBM tissues demonstrated a great fold of lactate production confirming the occurrence of typical metabolic remodeling reflective of the Warburg effect. Collectively, the effect of siPDK1, DCA, and sh-EGFR treatment shifted U251 and 5310 glioma cell metabolism towards oxidative phosphorylation. Preliminary data supporting the use of DCA in glioblastoma multiforme begs further analysis, particularly with regard to ubiquitous EGFR-mediated growth mechanisms. Here, we sought to verify whether DCA treatment reduces EGFR–PDK1 interaction, although previous reports substantiate its activity against PDK1 in glioblastoma multiforme (18).

Our observations with DCA suggested that inhibition of either PDK1 or EGFR or both may affect cancer cell metabolism in vitro. These findings compelled us to explore the existence of an uncovered mechanism by which PDK1 regulates EGFR and its activity. To explain this, we used an EGFR phosphorylation antibody array. It was interesting to find that DCA-treated 5310-EGFR expressing cells demonstrated a reduced expression of multiple EGFR phosphorylation sites, including Tyr845, suggesting that 5310-EGFR expressing cells might in part rely on PDK1 expression for EGFR phosphorylation. In contrast, 5310 cells alone demonstrated basal EGFR phosphorylation levels. Earlier findings by Demory and colleagues that phosphorylation of EGFR at Tyr845 is essential for mitochondrial translocation (29), provides strong support to our conclusions regarding reduced EGFR phosphorylation with DCA treatment. Further evidence substantiates that DCA treatment decreased tumor growth in some xenograft models (13, 32, 33) and in human patients (34). In the present study, we generated a subcutaneous and intracranial tumor model by injecting 5310-Fl-EGFR cells. DCA treatment reduced the EGFR-induced subcutaneous tumor by 80% and intracranial treatment decreased tumor growth in some xenograft models (13, 32, 33) and in human patients (34). In the present study, we generated a subcutaneous and intracranial tumor model by injecting 5310-Fl-EGFR cells. DCA treatment reduced the EGFR-induced subcutaneous tumor by 80% and intracranial tumor by 80%. Our current findings reveal for the first time that DCA treatment reduces EGFR-induced glioblastoma tumor growth, highlighting the distinct functions of PDK1/DCA relative to tumor development. The illustrated mitochondrial functions of EGFR, its role in regulating mechanisms for glioblastoma metabolism, and its interaction with PDK1 provide important insights into glioblastoma tumor progression.
and targets for treating glioblastoma. In summary, our work suggests that PDK1 may serve as a novel therapeutic target in treating glioblastoma along with EGFR. Targeting this protein complex may result in further treatment avenues in the metabolic modulation of glioblastoma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: K.K. Velpula, A.J. Tsung
Development of methodology: K.K. Velpula, A. Bhasin, S. Asuthkar
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.K. Velpula, A. Bhasin, A.J. Tsung
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.K. Velpula, A. Bhasin, A.J. Tsung
Writing, review, and/or revision of the manuscript: K.K. Velpula, A. Bhasin, S. Asuthkar

References


Combined Targeting of PDK1 and EGFR Triggers Regression of Glioblastoma by Reversing the Warburg Effect

Kiran Kumar Velpula, Arnima Bhasin, Swapna Asuthkar, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-13-1868

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2013/10/22/0008-5472.CAN-13-1868.DC1

Cited articles
This article cites 34 articles, 9 of which you can access for free at:
http://cancerres.aacrjournals.org/content/73/24/7277.full#ref-list-1

Citing articles
This article has been cited by 5 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/73/24/7277.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.