Cyclooxygenase-2 Is a Novel Transcriptional Target of the Nuclear EGFR-STAT3 and EGFRvIII-STAT3 Signaling Axes

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Abstract

Emerging evidence indicates a novel mode of epidermal growth factor receptor (EGFR) signaling, notably, one involves EGFR nuclear translocalization and subsequent gene activation. To date, however, the significance of the nuclear EGFR pathway in glioblastoma (GBM) is unknown. Here, we report that EGFR and its constitutively activated variant EGFRvIII undergo nuclear translocalization in GBM cells, in which the former event requires EGF stimulation and the latter is constitutive. To gain insights into the effect of nuclear EGFR on gene expression in GBM, we created isogenic GBM cell lines, namely, U87MG-vector, U87MG-EGFR, and U87MG-EGFRdNLS that, respectively, express the control vector, EGFR, and nuclear entry-defective EGFR with a deletion of the nuclear localization signal (NLS). Microarray analysis shows that 19 genes, including cyclooxygenase-2 (COX-2), to be activated in U87MG-EGFR cells but not in U87MG-EGFRdNLS and U87MG-vector cells. Subsequent validation studies indicate that COX-2 gene is expressed at higher levels in cells with EGFR and EGFRvIII than those with EGFRdNLS and EGFRvIIIIdNLS. Nuclear EGFR and its transcriptional cofactor signal transducer and activator of transcription 3 (STAT3) associate with the COX-2 promoter. Increased expression of EGFR/EGFRvIII and activated STAT3 leads to the synergistic activation of the COX-2 promoter. Promoter mutational analysis identified a proximal STAT3-binding site that is required for EGFR/EGFRvIII-STAT3–mediated COX-2 gene activation. In GBM tumors, an association exists between levels of COX-2, EGFR/EGFRvIII, and activated STAT3. Together, these findings indicate the existence of the nuclear EGFR/EGFRvIII signaling pathway in GBM and its functional interaction with STAT3 to activate COX-2 gene expression, thus linking EGFR-STAT3 and EGFRvIII-STAT3 signaling axes to proinflammatory COX-2 mediated pathway.

Introduction

Overexpression of epidermal growth factor receptor (EGFR) and/or its constitutively activated variant EGFRvIII is frequently found in glioblastoma (GBM), the most common and most malignant form of brain cancer, and is associated with tumorigenesis and more aggressive tumor phenotypes, such as invasiveness and therapeutic resistance (1-3). EGFRvIII, a product of rearrangement of the EGFR gene with a deletion within the extracellular domain that is predominantly found in GBM, is more tumorigenic than wild-type EGFR (4, 5). Importantly, both EGFR- and EGFRvIII-mediated pathways are of a high biological complexity, including the existence of two major signaling modes, at the cell surface and the other, in the nucleus (2, 6, 7).

In the cell-surface signaling mode, EGFR and EGFRvIII function as receptor tyrosine kinases that activate signaling modules, such as those mediated by PLC-γ, Ras, phosphoinositide 3-kinase, and Janus-activated kinase 2, which can contribute to tumorigenesis and more aggressive tumor behaviors (8, 9). On the other hand, signals such as EGFR ligands, oxidative stress, and DNA damage stimulate EGFR nuclear transport (2). Nuclear EGFR is localized on the inner nuclear membrane (10, 11) and in the nucleoplasm (12-15). In the nuclear signaling mode, EGFR has three key functions: (a) gene transactivation, (b) tyrosine kinase, and (c) protein-protein interactions (13, 15-19). Importantly, the level of nuclear EGFR predicts poor prognosis in patients with breast carcinomas (19), oropharyngeal and esophageal squamous cell carcinomas (19, 20), and ovarian cancer (21). Although nuclear detection of EGFR has been reported in highly proliferative normal tissues, such as regenerating livers (22) and placenta (13), and in cancerous tissues (13, 19-21), its presence in GBM remains uninvestigated. A previous report showed that EGFRvIII is detected in the nucleus of normal glial cells and primary GBM specimens (23).
Nuclear EGFR, similar to HER2, activates gene expression through its transactivation domain (13, 24). Although EGFR lacks a DNA-binding domain, it has been shown to interact with DNA-binding transcription factors, such as signal transducer and activator of transcription 3 (STAT3), E2F1, and STAT5, to induce expression of genes, including inducible nitric oxide synthase (iNOS), B-Myb, and aurora A, respectively, in breast cancer (15, 17, 25). A systemic approach has, however, yet been conducted to identify nuclear EGFR target genes. Knowledge of such genes will advance our understanding of the nature and effect of nuclear EGFR on cell physiology, provide novel insights into the role of nuclear EGFR in human cancers, and help clarify some of the molecular mechanisms underlying the observed association between nuclear EGFR and poor clinical outcome (19-21).

EGFR physically interacts and functionally cooperates with STAT3 at both cytoplasmic and nuclear levels. At the cytoplasmic level, through the two docking autophosphorylated tyrosines (Y1068 and Y1086), cell-surface EGFR interacts with STAT3 SH2 domain (26). This interaction leads to the phosphorylation of STAT3 at Y705 and its activation. Cell-surface EGFRvIII also interacts with and phosphorylates STAT3. Importantly, we and others showed in cancers of breast, colon, and skin that cell-surface EGFR cooperates with STAT3 to induce expression of TWIST (to facilitate epithelial-mesenchymal transition), vascular endothelial growth factor (to promote angiogenesis), and Eme1 endonuclease (to reduce drug-induced DNA damage; refs. 27-29). In primary breast carcinomas, coexpression of EGFR and activated STAT3 (Y705) is frequent (39%; ref. 28). In primary GBMs, we found approximately one third of the tumors to concurrently express EGFR/EGFRvIII and activated STAT3-Y705 (30). At the nuclear level, EGFR interacts with STAT3 to activate the expression of the INOS gene in carcinomas of breast and epidermoid (15).

The objectives of this current study, therefore, are to determine whether nuclear EGFR and EGFRvIII pathways are functional in human GBM and to identify the target genes of these pathways in GBM through an unbiased comprehensive approach. Our findings provide the evidence showing that GBM cell lines and primary specimens express nuclear EGFR/EGFRvIII. We also identified cyclooxygenase-2, COX-2, as a novel transcriptional target of the EGFR-STAT3 and EGFRvIII-STAT3 signaling axes and that nuclear EGFR/EGFRvIII is essential for COX-2 gene activation in GBM cells. COX-2 catalyzes the first step in the biosynthesis of the prostaglandins from arachidonic acid and plays a central role in the regulation of pro-inflammatory signaling pathways and COX-2–mediated inflammatory pathway in GBM.

Materials and Methods

Cell Lines, Primary Gliomas, Xenografts, and Reagents

Human GBM cell lines U87MG and T98G were from the American Type Culture Collection, whereas MGR3 cells were established in our laboratory from primary specimens. These cells were maintained in DMEM with 10% FCS. U87MG-vector, U87MG-EGFR, and U87MG-EGFRvIII stable transfectant lines were previously established from the parental U87MG cells that express a very low level of EGFR (30). The stable transfectant cell lines were cultured in DMEM with 10% FCS and 1 mg/mL G418. GBM xenografts established in the flanks of nude mice were provided by the Preston Robert Tisch Brain Tumor Center at Duke University. Primary GBM specimens were obtained from Preston Robert Tisch Brain Tumor Center at Duke University and Imgenex (San Diego, CA). All chemicals were purchased from Sigma unless otherwise stated. Rabbit polyclonal anti-EGFR antibody used in Western blotting was purchased from Santa Cruz Biotech (sc-03). The EGFR, EGFRvIII, and STAT3CA expression vectors were generated in our laboratory (30) and expressed as Myc-tagged fusion proteins. Anti-Myc mouse monoclonal antibody used in immunofluorescence staining and confocal microscopy was purchased from Roche. Anti–lamin B mouse monoclonal antibody was from Calbiochem. β-Actin and α-tubulin antibodies were obtained from Sigma. Rabbit polyclonal HER2 (29D8), p-EGFR (Y1068), p-STAT3 (Y705), and COX-2 antibodies were purchased from Cell Signaling.

Detection of Nuclear EGFR/EGFRvIII through Nuclear Fractionation and Western blotting

This was performed as previously described (15). Cells treated per experimental procedures were collected, washed with PBS, and swelled in hypotonic buffer [25 mmol/L Tris-HCl (pH 7.5), 5 mmol/L KCL, 0.5 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, and 0.15 m/mL aprotinin] for 20 min on ice. Following homogenization using a Dounce homogenizer, nuclei were pelleted and washed. To extract nuclear proteins from the isolated nuclei, we used an ultrasonic disruption step and sonication buffer containing 50 mmol/L Tris-HCl and 0.15 m/mL aprotinin. To isolate nonnuclear extracts, the supernatant was exposed to 1% SDS and 0.1% NP40, centrifuged at 15,000 ×g to remove cell debris, and the resulting supernatant was collected. Western blotting was conducted as previously described (30).

Immunohistochemical Staining of Primary Malignant Glioma Specimens for EGFR, Activated STAT3, and COX-2

The immunoperoxidase staining method used in these studies was a modification of the avidin-biotin complex technique, as previously described (30). Paraffin-embedded microsections (5 μm) of GBM primary specimens and xenografts were deparaffinized, dehydrated, and subjected to an...
tigen retrieval in a microwave oven followed by incubation with 0.05% trypsin in PBS for 15 min at room temperature. Endogenous peroxidase activity was blocked by treatment of 0.3% hydrogen peroxide. The slides were incubated with 10% normal goat serum for 30 min and then with the EGFR mouse monoclonal antibody (1:50; Novocastra RTU-EGFR-384), COX-2 (1:25; Cell Signaling), and p-STAT3-Y705 (1:30; Cell Signaling) rabbit polyclonal antibodies at 4°C overnight. Following washes with PBS, the slides were incubated with biotinylated secondary antibodies and then with the avidin-biotin-horseradish peroxidase complex. Detection was conducted using 0.125% aminoethylcarbazole chromogen. After counterstaining with Mayer’s hematoxylin (Sigma), the slides were mounted.

**Determination of the Half-Life of EGFR/EGFRvIII and Their Nuclear Entry–Defective Mutants**

Tumor cells exposed to 10 μg/mL cycloheximide for 0, 30, 60, 120, and 180 min were harvested; total protein were extracted and subjected to Western blotting. A Myc-tagged mouse monoclonal antibody (Roche) was used to detect Myc-tagged EGFR, EGFRdNLS, EGFRvIII, and EGFRvIIIdNLS fusion proteins.

**Identification of Nuclear EGFR Target Genes by GeneChip DNA Microarray**

Total RNAs extracted from the three U87MG stable transfectant lines, U87MG-vector, U87MG-EGFR, and U87MG-EGFRdNLS, were used to examine their gene expression profile. This was conducted in the DNA Microarray Core Facility of Duke Institute of Genome Science & Policy using the human Genome U133 Plus 2.0 Array genchips (Affymetrix) containing over 47,000 gene transcripts. ANOVA was conducted to determine statistical significance.

**Reverse Transcription-PCR and Quantitative PCR**

Total RNA isolation and reverse transcription (RT) were conducted using the SV Total RNA Isolation System (Promega). RT-quantitative polymerase chain reaction (qPCR) was done in the Mx3005P qPCR System (Stratagene) using the SuperScript III platinum SYBR green one-step qRT-PCR system (Invitrogen), in which the GAPDH gene was used as normalization controls. All experiments were in triplicate. Primer sequences were 5′-TATACTAGAAGCTGAGGCTGCTG-3′ (forward primer with the Xba I site) and 5′-TAGGCTGAGGCTGCTCACCATAATTCTACT-3′ (reverse primer with the Xho I site). To enable subcloning, a Ser codon was inserted at the 5′ end of the COOH-terminal fragments. The COOH-terminal fragments were restricted using Xho I and Xho I endonucleases and subcloned into the pCMV-Tag5A plasmid. The resulting plasmids were designated as pCMV-Tag5A-EGFRdNLS and pCMV-Tag5A-EGFRvIIIIdNLS, which encode EGFR and EGFRvIII receptors with a deletion of eight amino acids (aa 645-652 in EGFR) within the NLS region (see Fig. 2A). All transfections were conducted with the cells in exponential growth using FuGENE HD (Roche). Generation of stable transfectants was conducted as previously described (30).

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and 5'-GTGTGCGAGTTCAGAGATCA-3' (GAPDH). For standard RT-PCR, SuperScript II First-Strand cDNA synthesis system (Invitrogen) was used and PCR performed with forward and reverse primers of 5'-TGTGTTGATGGATAGGC-3' and 5'-GTATGCTGGATGAC-3' (COX-2), and 5'-GCGCGGACCACCATTTAT-3' and 5'-AGGGCGCCGCTGTCATCTACT-3' (β-Actin).

**Determination of COX-2 Promoter Activity by Mammalian Transfection of Reporter Vector and Luciferase Assay**

The COX-2 reporter construct, pCOX-2-Luc was purchased from Panomics and contains a 1 kb COX-2 promoter 5' of the firefly luciferase reporter gene. A Renilla luciferase expression vector pRL-CMV, whose expression is controlled by the CMV promoter, was used to control for transfection efficiency. Forty-eight hours after transfection, the cells were lysed and luciferase activity was measured using the Dual Luciferase Assay kit in a TD 20/20 luminometer (Promega) as previously described (15). Relative luciferase activity was calculated by normalization of the firefly luciferase activity against that of the Renilla luciferase.

**Chromatin Immunoprecipitation Assay to Measure the Binding of Nuclear EGFR-STAT3 to the COX-2 Promoter**

Chromatin immunoprecipitation (ChiP) was conducted using a ChiP assay kit (Upstate) as we previously described (15). An anti-EGFR mouse monoclonal antibody (NeoMarkers) and an anti-STAT3 rabbit polyclonal antibody (Santa Cruz, C-20) were used in these experiments. Sequences of the primers for amplifying the COX-2 promoter are 5'-GAACCTTCC-3' (forward) and 5'-TGGTCGCTAACC GAGA-3' (reverse). Control IgGs were used as negative controls for immunoprecipitation. Chromatin input was used as loading control for PCR.

**Generation of Mutant COX-2 Promoter Reporters through Site-Directed Mutagenesis**

Site-directed mutagenesis was conducted using the QuikChange Mutagenesis kit (Stratagene), according to the manufacturer’s instructions. The pCOX-2-Luc luciferase reporter construct was used as the template. Sequences of the primers used to mutate the COX-2 promoter are 5'-GGCTTACGCAATTTTTTTAAGGGGA-3' (forward) and 5'-TGCTCGTAACC GAGA-GAACCTTCC-3' (reverse). Control IgGs were used as negative controls for immunoprecipitation. Chromatin input was used as loading control for PCR.

**Determination of p-STAT3 and COX-2 Coexpression through Double Immunofluorescence Staining–Coupled IHC**

Paraffin-embedded tumor sections were deparaffinized and subjected to epitope retrieval. The sections were blocked with 5% normal goat serum and 5% normal horse serum, and incubated with a mixture of the anti-p-STAT3 rabbit polyclonal antibody (1:50, Cell Signaling) and anti-COX-2 mouse monoclonal antibody (1:50, Santa Cruz) overnight at 4°C. After several washes, the sections were incubated with Texas red–conjugated goat anti-rabbit and fluorescin-conjugated horse anti-mouse secondary antibodies (1:200, Vector Laboratories). After being mounted with VECTASHIELD Mounting Medium with 4',6-diamidino-2-phenylindole (Vector Laboratories), the sections were examined under a Zeiss Axio Observer fluorescence microscope.

**Statistical Analysis**

ANOVA, χ² analysis, Student’s t test, and regression analysis were conducted using STATISTICA (StatSoft, Inc.) and Microsoft Excel, as we previously described (30, 35).

**Results**

**EGFR Undergoes Nuclear Translocalization in Human GBM Cell Lines and Primary Specimens**

Using nuclear fractionation and Western blotting, we showed that following EGF treatment, EGFR undergoes nuclear translocalization in human GBM T98G cells that express high levels of endogenous EGFR (Fig. 1A). Nuclear fractionation efficiency is indicated by the lack of cytoplasmic β-Actin protein in the nuclear extracts and by the absence of the nuclear marker protein lamin B in the non-nuclear extracts.

Similarly, in U87MG-EGFR stable transfectant cells, EGFR undergoes EGF-induced nuclear transport (Fig. 1B). U87MG-EGFR cells express equivalent levels of EGFR, compared with GBM cells with endogenous EGFR. EGF-induced EGFR nuclear translocalization in T98G cells was confirmed using immunofluorescence staining/confocal microscopy (Supplementary Fig. S1). The punctate staining pattern for nuclear EGFR, shown in Supplementary Fig. S1, is consistent with what have been reported by many previous studies (18, 36). Nuclear HER2 also displays similar punctate staining rather than diffuse staining (37). To determine the presence of endogenous nuclear EGFR in GBM tumors, we analyzed 12 primary GBM specimens for EGFR expression using IHC with an antibody that is reactive to both EGFR and EGFRvIII. Six of the GBM tumors expressed EGFR/EGFRvIII with one of them showing significant levels of nuclear EGFR/EGFRvIII. In Fig. 1C, the solid arrows point to the brown-stained EGFR-positive nuclei and the dashed arrows point to the blue EGF-negative nuclei. Corroborating this observation, our earlier study found 6.9% (9 of 130) of primary breast carcinomas to express high levels of nuclear EGFR (19).

Given the facts that EGFR heterodimerizes with HER2 (8) and that HER2 undergoes nuclear translocalization (37), we examined whether nuclear EGFR and nuclear HER2 complex as heterodimers. Using T98G GBM cells know to express both EGFR and HER2, we immunoprecipitated nuclear EGFR using an EGFR antibody and subjected the immunoprecipitates to Western blotting to detect HER2. As shown in Fig. 1D (left), we did not
detect any HER2 signal even if nuclear EGFR was effectively immunoprecipitated by the EGFR antibody. As expected, IgG did not pull down EGFR or HER2, indicating specificity. Interestingly, HER2 underwent a modest level of nuclear translocalization after EGF stimulation, albeit the majority of the HER2 protein is in the nonnuclear fraction (Fig. 1D, right). Because we did not detect nuclear EGFR-HER2 heterodimers, EGF-induced HER2 nuclear transport may potentially be a result of nuclear transport of HER3. This is supported by the facts that EGFR can transactivate HER3 (38), HER3 undergoes nuclear transport (39), and that HER2 is a preferred binding partner of HER3. Together, our results show for the first time that EGFR undergoes nuclear translocalization in human GBM cells and primary specimens.

**Generation of GBM Cells Stably Expressing Mutant EGFR Defective in Nuclear Entry**

To gain insights into the nature of the nuclear EGFR pathway in GBM, we generated isogenic GBM cell lines that express wild-type EGFR and mutant EGFR, defective in its ability to translocate to the cell nucleus. For this, we engineered an EGFRΔNLS mutant construct that carries the nuclear entry–defective mutant EGFR with a deletion of eight amino acids (aa 645-652) within the NLS region (Fig. 2A). Amino acid substitutions of the basic residues within this...
NLS region have been shown to abolish the ability of EGFR to enter the nucleus (15, 40). We subsequently established a U87MG stable transfectant line, U87MG-EGFRdNLS, to express EGFRdNLS. As indicated in Fig. 2B, U87MG-EGFRdNLS and U87MG-EGFR isogenic lines express equivalent levels of the respective EGF receptors. As expected, cells expressing EGFRdNLS did not contain nuclear EGFR, as shown by immunofluorescence staining/confocal microscopy (Supplementary Fig. S2) and nuclear fractionation/Western blotting (Fig. 2C). In the nuclear fractionation/Western blotting analysis (Fig. 2C), nuclear fractionation efficiency is indicated by the absence of cytoplasmic β-Actin protein in the nuclear extract and by the lack of the nuclear protein lamin B in the nonnuclear extract.

To indicate that EGFRdNLS retains its receptor tyrosine kinase function, we showed that EGFRdNLS undergoes EGF-induced autophosphorylation similar to EGFR. Isogenic U87MG-EGFR and U87MG-EGFRdNLS cells were serum starved, stimulated with EGF for 0 and 15 min, and subjected to Western blotting to determine levels of p-EGFR (Y1068; indicator of receptor activation), EGFR, and β-Actin. E, EGFRdNLS has similar stability relative to EGFR. Isogenic U87MG-EGFR and U87MG-EGFRdNLS cells were treated with protein synthesis inhibitor cycloheximide (10 μg/mL) for 0 to 180 min and subjected to Western blotting to determine levels of EGFR and β-Actin. Intensity of the band signals were quantified using the ImageJ software and protein half-life was subsequently computed. F, nuclear EGFR is important for the clonogenic growth of EGFR-expressing GBM cells. Colony formation assays were done in the absence (left two panels) and presence (right two panels) of soft agar. U87MG-EGFRdNLS cells formed significantly less colonies than U87MG-EGFR cells. As shown by the 5× images in the second panel, the colony from U87MG-EGFR cells contained more cells and was with intensive staining, whereas the one from U87MG-EGFRdNLS cells contained significantly fewer cells. As shown by the high-resolution images (fourth panel), U87MG-EGFR cells formed larger colonies compared with U87MG-EGFRdNLS cells in the presence of soft agar, suggesting that nuclear EGFR is important for the anchorage-independent growth of U87MG cells. U87MG-vector cells did not form colonies in either assay.
EGF-induced autophosphorylation similar to EGFR (Fig. 2D). Using cells treated with the protein synthesis inhibitor, cycloheximide, we showed that the deletion within the NLS region did not alter the half-life/stability of EGFR (Fig. 2E). Importantly, results of colony formation assays (Fig. 2F) indicate that EGFRdNLS renders U87MG cells significantly less efficient in forming colonies in the absence (first and second panels) and presence of soft agar (third and fourth panels). U87MG-vector cells formed loose colonies in these assays. It is also noticeable that U87MG-EGFR cells formed larger colonies compared with U87MG-EGFRdNLS cells in the presence of soft agar (high-resolution images in Fig. 2F, fourth panel), suggesting that nuclear EGFR is important for the anchorage-independent growth of EGFR-expressing GBM cells. Taken together, these results show the utility of the isogenic U87MG-vector, U87MG-EGFR, and U87MG-EGFRdNLS cell lines for subsequent gene expression profiling to identify nuclear EGFR target genes in human GBM cells.

The Human COX-2 Gene Is a Candidate Nuclear EGFR Target Gene

We reason that nuclear EGFR target genes are regulated by EGFR but not by EGFRdNLS. Thus, microarray was conducted to determine the expression levels of human gene transcripts in the three isogenic U87MG-EGFR, U87MG-EGFRdNLS, and U87MG-vector cell lines. In each of the three experiments, tumor cells were serum starved for 24 hours, treated without and with EGF for 4 hours, and total RNAs were extracted and submitted for microarray analysis. Data were analyzed by a data analyst in the Duke Microarray Facility. Here, we identified 19 genes that were significantly induced by EGF in U87MG-EGFR cells (P < 0.05; Supplementary Table S1; Fig. 3A), but not in the U87MG-vector or U87MG-EGFRdNLS cells. Interestingly, COX-2 (arrow) is one of the potential nuclear EGFR target genes (Fig. 3A). Given the longstanding interest of our laboratory in proinflammatory pathways, we directed our validation effort at COX-2. The EGF-induced expression of COX-2 transcripts in U87MG-EGFR cells but not in U87MG-EGFRdNLS or U87MG-vector cells was confirmed by quantitative RT-PCR (Fig. 3B) and by regular RT-PCR and Western blotting (Fig. 3C, left). Consistent with the microarray results, EGF significantly induced the expression of COX-2 transcripts and protein in U87MG-EGFR cells but not in U87MG-vector or U87MG-EGFRdNLS cells (Fig. 3C, right). Consistent with the elevated transcript and protein levels, we showed the COX-2 promoter to be significantly activated by EGF in U87MG-EGFR cells but not in U87MG-vector or U87MG-EGFRdNLS cells (Fig. 3D). Conversely, EGFR kinase inhibitor Iressa suppressed the ability of EGF to induce COX-2 expression, as shown by RT-PCR (Fig. 3E, left) and to activate the COX-2 promoter (right) in U87MG-EGFR cells. These results indicate that the human COX-2 gene is a candidate nuclear EGFR target gene.

EGFRvIII Undergoes Nuclear Translocalization and Activates the Human COX-2 Gene in GBM Cells

Because endogenous EGFRvIII expression is not maintained in vitro, we used U87MG-EGFRvIII stable transfectants in these studies. Here, we found that EGFRvIII is constitutively detected in the nucleus of U87MG-EGFRvIII cells (Fig. 4A, left). Importantly, U87MG-EGFRvIII xenografts expressed similar levels of EGFRvIII compared with GBM xenografts with endogenous EGFRvIII (D-256 MG and D-270 MG; Fig. 4A, right), indicating that U87MG-EGFRvIII cells express physiologic levels of EGFRvIII. To examine the role of nuclear EGFRvIII in COX-2 gene regulation, we created a EGFRvIIIIdNLS mutant that is defective in nuclear entry and established stable U87MG transfectants that express this mutant. As shown in Fig. 4B, U87MG-EGFRvIII and U87MG-EGFRvIIIIdNLS cells expressed equivalent levels of EGFRvIII (left), and importantly, EGFRvIIIIdNLS was absent from the nucleus (right). Using cycloheximide-treated cells, we showed that EGFRvIII and EGFRvIIIIdNLS proteins have similar half-lives (Fig. 4C), indicating that the deletion of the NLS sequence did not affect receptor stability. In contrast to the observation in Fig. 2F, U87MG-EGFRvIIIIdNLS cells formed colonies to the extent similar to U87MG-EGFRvIII cells (Supplementary Data; Fig. S3). We further showed, using RT-PCR (Fig. 4D, top) and Western blotting (Fig. 4D, bottom), that COX-2 gene expression is significantly higher in U87MG-EGFRvIII cells than U87MG-EGFRvIIIIdNLS and U87MG-vector cells.

Nuclear EGFR–Mediated Activation of the Human COX-2 Gene Is Enhanced by STAT3

Although nuclear EGFR is known to have transactivational activity, it does not directly associate with DNA and thus requires cooperation with DNA-binding transcription factors to regulate gene expression (13, 15). In previous studies, we showed that nuclear EGFR associates with STAT3 to induce iNOS gene expression (15). Similarly, nuclear EGFR–vIII–STAT3 complex was shown to be present in glial cells and to be involved in their malignant transformation (23). Against this background, we examined whether nuclear EGFR and EGFRvIII cooperate with STAT3 to induce COX-2 gene expression in GBM cells. Using intracellular protein–DNA binding ChIP assay, we showed that nuclear EGFR and nuclear STAT3 associate with the COX-2 gene promoter and that the association was dependent on EGF stimulation (Fig. 5A). Figure 5B shows that COX-2 gene expression is modestly enhanced by EGFRvIII alone and STAT3CA alone but significantly increased by the combination of EGFR and STAT3CA. STAT3CA is a constitutively activated STAT3 variant with two Cys substitutions that enable STAT3 molecules to dimerize spontaneously without phosphorylation at Y705 (41). Because STAT3CA is constitutively activated independent of EGF stimulation, we found EGF to modestly enhance (1.3-fold) the ability of EGFR and STAT3CA coexpression to activate the COX-2 promoter (data not shown). Similar to wild-type EGFR, the COX-2

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promoter was also activated by EGFRvIII (Fig. 5C). Unlike the robust activation by the EGFR-STAT3CA combination, STAT3CA only modestly enhanced EGFRvIII-mediated activation of the COX-2 gene promoter. Consistent with these observations, EGFRdNLS-STAT3CA cotransfection had lower effects on the COX-2 promoter than the EGFR-STAT3CA combination (Fig. 5D), further providing evidence of an important role for nuclear EGFR in the activation of the COX-2 promoter by STAT3. EGFRvIIIIdNLS-STAT3CA cotransfection activated the COX-2 promoter

FIGURE 3. Gene expression analysis identified the human COX-2 gene as a candidate nuclear EGFR target gene. A, microarray shows 19 genes to be significantly induced by EGF in U87MG-EGFR cells, but not in U87MG-vector or U87MG-EGFRdNLS cells. Cells were treated with and without EGF, and the levels of expression of over 47,000 human gene transcripts were determined. Data represent results of three independent experiments. ANOVA was conducted to derive P values. Clustering analysis included a total of 22 transcripts, with 19 known genes, which were significantly activated by EGF in U87MG-EGFR cells, but not in U87MG-vector or U87MG-EGFRdNLS cells (P < 0.05). Arrow, COX-2. B, COX-2 transcripts are significantly upregulated by EGF in U87MG-EGFR cells, but not in U87MG-EGFRdNLS or EGFR-vector cells. Microarray results was validated using RT-qPCR and the results show that the COX-2 transcription is significantly increased by EGF in U87MG-EGFR cells, but not in U87MG-EGFRdNLS or EGFR-vector cells. C, U87MG-EGFR cells express higher levels of COX-2 transcripts and protein than U87MG-EGFRdNLS or EGFR-vector cells. Three U87MG stable transfectants were subjected to RT-PCR (top) and Western blotting (bottom) to determine levels of COX-2 expression. Left, cells were grown under regular growth conditions with 10% FCS. Right, cells were serum starved for 24 h and treated with EGF (100 ng/mL) for 0 and 4 h. D, the human COX-2 promoter is significantly activated by EGF in U87MG-EGFR cells but not in U87MG-EGFRdNLS or U87MG-vector cells. A firefly luciferase reporter construct under the control of a 1-kb COX-2 promoter was transfected into the three U87MG isogenic cell lines. Twenty-four hours later, the transfected cells were serum-starved for 24 h and stimulated with 100 ng/mL EGF for 0 and 4 h. E, EGFR kinase inhibitor Iressa reduced EGF-induced COX-2 expression and promoter activation. U87MG-EGFR cells were serum starved for 24 h in the presence of Iressa (25 μmol/L) or 1% DMSO for 24 h, treated with EGF for 0 and 1 h, and analyzed for COX-2 expression through RT-PCR (left). Furthermore, aliquots of cells were transfected with pCOX-2-Luc and pRL-TK. Twenty-four hours after transfections, the cells were similarly treated as described for the RT-PCR experiments and subjected to luciferase assay.
similar to that observed for the EGFRvIII-STAT3CA combination. These findings are in line with several studies reporting that EGFRvIII preferentially activates the phosphoinositide 3-kinase/Akt signaling axis over the STAT3 and Ras/mitogen-activated protein kinase downstream signaling modules (42, 43).

To further investigate the structural requirement in the COX-2 promoter for EGFR/STAT3-mediated activation, we analyzed the human COX-2 promoter for consensus STAT3 binding motifs, 5′-TT-N(4-6)-AA-3′, using a Web-based search engine, TFSearch, as we previously described (15, 44). The results of the search showed two putative STAT3-binding sites within the human COX-2 promoter (Fig. 5E, top). We mutated each motif by substituting two nucleotides and such that the TT-AA palindrome was disrupted. Using these mutant reporter constructs, we showed that mutations within the proximal STAT3-binding motif (motif A), but not in motif B, significantly decreased the ability of EGFR-STAT3CA and EGFRvIII-STAT3CA cotransfections to activate the COX-2 promoter (Fig. 5E). Collectively, the results summarized in Fig. 5 indicate that STAT3 cooperates with both EGFR and EGFRvIII to activate the COX-2 gene promoter, with the former being more active than the latter.

**Increased COX-2 Expression Is Associated with EGFR/EGFRvIII and Activated STAT3 in Primary GBM Specimens and Xenografts**

To determine the potential clinical relevance of the association between COX-2 and EGFR/EGFRvIII-STAT3,
we examined 12 primary specimens of malignant gliomas and three GBM xenografts for the expression of COX-2, EGFR, and p-STAT3 (Y705) through IHC. The results showed that 58.3% (7 of 12) of the primary malignant gliomas coexpressed EGFR and COX-2 and that levels of EGFR correlate with those of COX-2, as indicated by χ² analysis (P = 0.03). In the three GBM xenografts we analyzed, we found COX-2 to be expressed at a higher level in the EGFR/EGFRVIII-positive, p-STAT3–positive U87MG-EGFR (data not

![Figure 5](image.png)

**FIGURE 5.** STAT3 significantly enhances the nuclear EGFR–mediated activation of the human COX-2 gene. A, nuclear EGFR and nuclear STAT3 associate with the COX-2 gene promoter in an EGF-dependent fashion. In intracellular protein-DNA binding ChIP assay, we used an anti-EGFR antibody (Ab) and an anti-STAT3 Ab in immunoprecipitation, whereas normal IgGs were used as negative controls. Input chromatin was used as loading controls for PCR. B, COX-2 promoter activity is enhanced by EGFR alone and STAT3CA alone but is significantly elevated by the combination of both. STAT3CA, constitutively activatedSTAT3 with two Cys substitutions that enable STAT3 molecules to dimerize spontaneously without phosphorylation. U87MG cells were transfected with pCOX-2-Luc and EGFR plasmid alone, STAT3CA plasmid alone, or in combination for 48 h and then luciferase activity was determined. pRL-TK vector was cotransfected as transfection efficiency control. Relative COX-2 reporter activity was determined by normalizing the firefly luciferase activity against that of the Renilla luciferase. Columns, mean of three independent experiments; bars, SD. C, the COX-2 promoter is activated by EGFRvIII. The experiment was conducted as described in B. Although the COX-2 promoter is activated by EGFRvIII, unlike the potent activation by EGFR-STAT3CA combination, STAT3CA only modestly enhances the EGFRvIII-mediated activation of COX-2 promoter. D, nuclear EGFR is important for the EGFR-STAT3CA–mediated activation of the COX-2 promoter. This was conducted as described in B. EGFRdNLS-STAT3CA cotransfection yields significantly lower activity than the EGFR-STAT3CA combination. In contrast, EGFRvIII-NLS-STAT3CA cotransfection similarly activates the COX-2 promoter compared with EGFRvIII-STAT3CA combination. E, the EGFR-STAT3 signaling axis activates the COX-2 promoter through the STAT3-binding site proximal to the TATAAA Box. Top, consensus STAT3-binding site. The Web-based search engine, TFSearch, identifies two putative STAT3-binding sites in the human COX-2 promoter, namely the proximal motif (motif A; nt -134 to -127) and the distant motif (motif B; nt -759 to -751). pCOX-2-A-Luc construct carries the mutant COX-2 promoter with two nucleotide substitutions with the proximal motif. pCOX-2-B-Luc construct contains the mutant COX-2 promoter with two nucleotide substitutions with the distant motif. The results show that mutations within the proximal motif, but not motif B, significantly decreased the ability of EGFR-STAT3CA and EGFRvIII-STAT3CA to activate the COX-2 promoter.
shown), and D-256 MG GBM xenografts but not in the U87MG xenografts, which have very low levels of EGFR and p-STAT3 (Fig. 6A). We further examined whether p-STAT3–expressing GBM cells express COX-2 using double fluorescence staining–coupled IHC. In these studies, p-STAT3 is indicated by red fluorescence, and COX-2 is indicated by green fluorescence. Tumor nuclei were stained by 4′,6-diamidino-2-phenylindole (blue). As indicated by Fig. 6B, the majority of p-STAT3–positive nuclei (pink signals as merged products of red and blue fluorescence) in the D-256 MG xenograft expressed high levels of COX-2. Those cells with p-STAT3–positive nuclei but did not express COX-2 can potentially be due to the expression of a transcription repressor of COX-2. Noticeably, COX-2 is also expressed in cells without significant p-STAT3 expression, suggesting that p-STAT3–independent mechanisms may account for the transcriptional activation of the COX-2 gene in these cells.

**Discussion**

Although overexpression of EGFR and EGFRvIII is a well-known characteristic of many human cancers, our understanding of EGFR- and EGFRvIII-associated tumor biology remains insufficient, which is likely due to the high degree of biological complexity that lies within these pathways (45). This is, in part, indicated by emerging evidence showing the ability of EGFR...
In this study, we provide evidence that for the first time, the nuclear EGFR and EGFRvIII pathways are functional in GBM cells. In this study, we provide evidence that for the first time, the nuclear EGFR and EGFRvIII pathways are functional in human GBM cells, the most common and deadliest brain malignancy in adults. Using an unbiased approach to identify genes that are targeted specifically by nuclear EGFR, but not by the cell-surface counterpart, we identified COX-2 as a nuclear EGFR target. Consistent with the known ability of nuclear EGFR to physically and functionally associate with the STAT3 transcription factor, we found the nuclear EGFR-STAT3 signaling axis to transcriptionally activate the COX-2 gene in GBM cells. Collectively, these data provide a novel link between nuclear EGFR/EGFRvIII-mediated mitogenic pathway and the COX-2-associated inflammatory pathways in GBM.

COX-2 plays a central role in many important cellular processes, including, inflammatory response, tumorigenesis, and tumor progression (31). Several studies have shown that EGFR and COX-2 are cooverexpressed in cutaneous squamous cell carcinoma (46) and lung adenocarcinoma (47). EGFR has been shown to induce COX-2 expression through phosphoinositide 3-kinase, p38, and protein kinase C-δ (48, 49), albeit the exact transcriptional pathway that mediates the transcriptional activation of COX-2 was not determined under these conditions. Interestingly, a previous report showed that nuclear HER2 binds to and activates the human COX-2 gene promoter, leading to its transcriptional activation in breast cancer cells (37). The identified HER2-associated sequence (HAS) is a 20-bp element located at −1,750 to −1,731 bps of the human COX-2 promoter (37). Because the COX-2 reporter construct used in this study contains up to −1 kb of the COX-2 promoter, the observed EGFR- and EGFR-induced COX-2 promoter activation (Figs. 3D-E and 5B) is independent of HER2 or HAS. Our immunoprecipitation results indicate that nuclear EGFR does not complex with nuclear HER2 in GBM cells analyzed, further supporting the notion of nuclear EGFR–dependent and nuclear HER2-independent activation of the COX-2 gene expression in GBM cells. Furthermore, our results indicate that nuclear EGFR and activated STAT3 cooperate synergistically to activate the COX-2 gene activity. This synergy may not apply to nuclear HER2-mediated COX-2 gene activation, given the fact that the HAS motif (5′-ATAAACCTT CAAAATTCAGTA-3′) does not seem to contain a putative STAT3 -binding site (TT-N(4,6)-AA-3′) and STAT3 is therefore unlikely to activate the COX-2 promoter through binding to HAS. The results of the present study combined with those of the study by Wang et al. (37), together unraveled an important link between the ErbB family of receptor tyrosine kinases, their nuclear translocalization, and COX-2–mediated proinflammatory pathway.

To date, only four nuclear EGFR-targeted genes, namely cyclin D1, iNOS, B-Myb, and Aurora A, have been reported (13, 15, 17, 25). The identification and validation of the COX-2 gene as an additional transcriptional target of the nuclear EGFR and nuclear EGFRvIII pathways and the evidence that this activation of COX-2 by nuclear EGFR/EGFRvIII occurs in GBM cells growing in vitro and in vivo are significant. Unlike these previous studies, the current study undertook an unbiased comprehensive approach to identify nuclear EGFR target genes using tumors expressing wild-type versus genetically modified EGFR molecules. Although we focused on COX-2 in this study, it is important to note that other genes have been identified in the microarray analysis. Ongoing studies are directed at these genes to gain further insights into their role in the malignant biology of tumors with active nuclear EGFR and nuclear EGFRvIII pathways.

STAT3 is highly activated in many human cancers, including malignant gliomas (30, 50) and cancers of the breast and the head and neck (51, 52). Similar to increased COX-2 expression, STAT3 activation is associated with inflammation, more malignant tumor phenotype, and poor prognosis (53, 54). Consequently, STAT3 and COX-2 both have emerged as targets for anti-GBM therapy (30, 55). The findings in this study linking STAT3 and COX-2 should contribute to a better understanding of their role in nuclear EGFR signaling–associated malignant biology.

Given that GBMs expressing EGFRvIII or activated EGFR are more refractory to therapy compared with those without EGFR activation, our findings could provide, potentially, the basis for novel combination therapies that simultaneously target EGFR/EGFRvIII and COX-2. Indeed, in preclinical and clinical studies, combination of inhibitors of EGFR and COX-2 have yielded promising results in advanced cutaneous squamous cell carcinoma, non–small cell lung carcinoma, intestinal cancer, and prostate cancer (46, 56–59). In summary, the results reported in this study define the COX-2 gene as a novel transcriptional target of the nuclear EGFR-STAT3 and EGFRvIII-STAT3 signaling axes and provide new insights into the malignant biology of a subpopulation of human tumors with concurrently activated EGFR/EGFRvIII and STAT3 pathways. They also provide a potential rationale for combination therapy targeting EGFR/EGFRvIII, STAT3, and COX-2 pathways.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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