Differential Effects of Estrogen Receptor β Isoforms on Glioblastoma Progression

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Abstract

The estrogen receptor β (ERβ) functions as a tumor suppressor in glioblastoma (GBM) cells. However, the in vivo significance of endogenous ERβ and the roles of its isoforms in GBM are incompletely understood. Using ERβ isoform-specific PCR screening, we found that GBM cells predominantly express ERβ1 and ERβ5, along with low levels of ERβ2 and ERβ4. We observed greater ERβ5 expression in higher grades of glioma than in lower grades. In CRISPR-based ERβ knockout (KO) cells and ERβ KO cells uniquely expressing ERβ1 or ERβ5 only, ERβ1 significantly reduced proliferation. Compared with parental GBM cells, ERβ KO cells exhibited high migratory and invasive potentials, and reexpression of ERβ1 resulted in the reduction of this phenotype. Interestingly, ERβ5 expression increased foci formation and anchorage-independent growth of NIH3T3 cells and increased motile structure formation, including filopodia and ruffles in GBM cells. Only ERβ1-expressing tumors resulted in longer mouse survival. RNA-Seq analysis revealed unique pathways modulated by ERβ1 and ERβ5. Compared with ERβ KO cells, ERβ1 cells exhibited lower activation of mTOR signaling molecules, including p-mTOR, p-S6K, and p-S6, and ERβ5-expressing cells had enhanced mTOR downstream signaling. Unique proteins including several that function as regulators of mTOR, immunomodulatory, and apoptosis pathways bound to ERβ1 and ERβ5 isoforms. Our work confirms the tumor-suppressive potential of ERβ1 and reveals the acquired oncogenic ability of ERβ5 in GBM cells. ERβ isoform status and their unique interactions with oncogenic pathways may have important implications in GBM progression.

Significance: These findings suggest that only ERβ isoform 1 has tumor suppressor function in GBM and that ERβ isoform switching contributes to GBM progression. Cancer Res; 78(12): 3176–89. ©2018 AACR.

Introduction

Glioblastomas (GBM) are the most common and deadliest primary brain tumors that have dismal survival rates—the 1-year survival is 34.6% and the 5-year survival is 4.75%. They affect 13,000 patients per year in the United States (1, 2). Standard treatment comprises surgical resection, external radiation therapy (XRT), and adjuvant chemotherapy with temozolomide (3, 4). However, resistance to current therapies is a major clinical problem. Delineating the molecular pathways and mechanisms that contribute to the GBM progression are clinically significant and provide novel therapeutic options for GBM.

Epidemiologic evidence suggests a tumor-suppressive role of estrogen (17 β-estradiol, E2) on GBM (5). The incidence of developing GBM is greater in men than in women, and women of reproductive age have a survival advantage over men and postmenopausal women (5–7). These correlative findings suggest that estrogen plays a significant role in suppression of GBM, but how they might do so is poorly understood.

The biological effects of estrogen are mediated through estrogen receptors α and β (ERα and ERβ; ref. 8). These two ER subtypes have distinct biological functions. Unlike ERα, ERβ exhibits antitumor activity in multiple cancer types including GBM (9–14). Recent studies showed that ERβ expression decreases as GBM progresses (13). However, it remains unknown whether loss of ERβ contributes to GBM progression.

Emerging evidence suggests that ERβ in humans is expressed as five different isoforms, ERβ1, ERβ2, ERβ3, ERβ4, and ERβ5, resulting from alternative splicing of exon 8, which is the last coding exon, and these five isoforms added another layer of complexity in ERβ functions (15). Structural analysis revealed that ERβ1 is the only full-length functional isoform with the native ligand binding domain (LBD). The other isoforms lack intact LBDs; thus, their functions are likely to be mediated by forming heterodimers with either ERβ1 or other transcription factors (15, 16).

In this study, we examined the role of ERβ isoforms in GBM progression using CRISPR/Cas9-mediated knockout (KO) in GBM cells. Further, we profiled and tested the role of ERβ isoforms in GBM. Our results demonstrate that ERβ5 is highly expressed in GBM. Using in vitro and in vivo assays, we demonstrate that, ERβ5...
Unlike ERβ1 acquires oncogenic properties in GBM cells. Using ERβKO and knock-in models of ERβ1, we provide genetic evidence for the tumor-suppressive role of ERβ1 in GBM. Mechanistic studies revealed that ERβ1 and ERβ5 differentially regulate the NFκB, mTOR, and STAT-3 pathways. Our studies also discovered that ERβ5 lacks tumor-suppressive functions and acquire oncogenic properties via its interaction with several oncogenes. Using orthotopic models of GBM, we provide evidence that shows ERβ1 increases mice survival.

Materials and Methods

Cell culture and reagents

Human GBM cell lines U87, U251, T98G, and LN229 were purchased from the ATCC and were maintained in DMEM supplemented with 10% fetal bovine serum (Sigma Chemical Co). GBM cells were passaged in our laboratory for fewer than 3 months after receipt or resuscitation. Neurobasal medium and B27 serum-free supplement were obtained from Invitrogen. All cell culture and reagents were purchased from the ATCC and were maintained in DMEM supplemented with 10% fetal bovine serum (Sigma Chemical Co).

Human ERβ1 (705), p-STAT3(727), STAT3, p-p65 (Ser536), p65, and GAPDH antibodies were obtained from Cell Signaling Technology. The p-mTOR, mTOR, p-S6K, S6K, p-S6, S6, p-4EBP1, 4EBP1, p-STAT3, p-Akt, Akt, p-ERK1/2, ERK1/2, p-p38, p38, p-p38, p38, p-IκBα, IκBα, p-ERK, ERK, p-p90RSK, p90RSK, p-p44/42 MAPK, p44/42 MAPK, and p-ERK1/2–ERK1/2 antibodies and ERβ CRISPR/Cas9 plasmids were purchased from Santa Cruz Biotechnology. The p-ERK1/2, ERK1/2, p-Akt, Akt, p-mTOR, mTOR, p-S6K, S6K, p-S6, p-4EBP1, 4EBP1, p-STAT3, p-STAT3(727), STAT3, p-p65 (Ser536), p65, and GAPDH antibodies were obtained from Cell Signaling Technology. The Ki67 antibody was purchased from Abcam. The IL8 antibody was purchased from Gene Tex. ERβ-specific short hairpin RNA (shRNA) lentiviral plasmids and ERβ CRISPR/Cas9 plasmids were purchased from Sigma. Short tandem repeat (STR) polymorphism analysis of the cell was used to confirm the identity using UT Health San Antonio core facilities. The ERβ and TCP-1α antibodies were used to visualize the nuclei, and the images were captured by using NIH ImageJ software.

Primary GBM cells

Patient-derived primary GBM cells were isolated from discarded specimens obtained from patients undergoing surgery using an UT Health San Antonio Institutional Review Board–approved protocol and their characterization was earlier described (13, 17, 18). All patients gave written informed consent for use of tissues in research. All the studies were conducted in accordance with the declaration of Helsinki and the standards defined by UTIISA Institutional Review Board. Primary GBM lines GBM-082209, GBM-101310, GBM-110110, GBM-040815, GBM-043014, and GBM-012015 were cultured in neurobasal medium supplemented with B27 serum-free supplement, EGF (20 ng/mL), bFGF (20 ng/mL), LIF (10 ng/mL), and heparin (5 μg/mL) as described (17, 18).

Generation of ERβ knockout and ERβ shRNA cells

ERβ shRNA cells were generated by infecting cells with human-specific ERβ-shRNA lentivirus as described (13). U87-ERβ KO model cells were generated using SantaCruz CRISPR guide RNA plasmids (Cat# SC-400213). The gRNA sequences include (i) 5’TGTATATGGAGCCGTGCTCC-3′; (ii) 5’GTGTGACCCGTATACCGAT-3′; (iii) 5’CGTGCAGCGACCGTTGAC-3′. U251-ERβ KO cells were generated using Horizon CRISPR guide RNA plasmid. The gRNA sequence is 5’-GATGAGATGACTGACTTTG-3′. CRISPR/Cas9 plasmids were transfected using Turbofect transfection reagent using the manufacturer’s protocol (Thermo Fisher Scientific). The cells were sorted using flow cytometry with GFP for Cas9 transfection, and the single clones were isolated. Individual clones were screened for ERβ gene deletions using genomic PCR, and the positive clones were confirmed by genomic sequencing of the targeted region and by Western blotting and RT-qPCR. Isoform-specific ERβ expressing cells were generated by transfecting ERβ KO cells with pCDH-EF1-ERβ-GFP-T2A-Puro and pCDH-EF1-ERβ5-GFP-T2A-Puro lentiviral vectors. Vector alone was used to generate control cells. The CRISPR-resistant pCDH-EF1-ERβ1-GFP-T2A-Puro and pCDH-EF1-ERβ5-GFP-T2A-Puro vectors were generated by QuikChange Lightning Site-Directed Mutagenesis Kit (Cat# 210518, Agilent Technologies).

Cell viability and colony formation assay

Cell proliferation and viability rates of the control and the ERβ-specific isoform–expressing cells were assessed by using MTT or MTS assays as described (13, 17). For colony formation assays, U251 model cells (500 cells/well) were seeded in 6-well plates and allowed to grow for 14 days. The cells were fixed in ice-cold methanol and stained with 0.5% crystal violet solution. The colony area percentage was calculated using ImageJ software.

Cell migration and invasion assays

The cell migration rates of the ERβ-specific isoform–expressing cells were determined by using a wound healing assay and quantified using NIH ImageJ software. The invasion of GBM model cells was determined by using the Coming BioCoat Growth Factor Reduced Matrigel Invasion Chamber assay according to the manufacturer’s protocols.

Focus formation and immunofluorescence assays

The oncogenic potential of ERβ5 was determined by focus formation assay as described previously (19). For monitoring cytoskeleton changes, model cells were grown on glass cover slips and subjected to phalloidin staining as described (19). DAPI was used to visualize the nuclei, and the images were captured by using confocal microscopy.

Western blotting, immunoprecipitation, mass spectrometry, and GST pulldown assay

Whole-cell lysates were prepared by using RIPA buffer, and Western blot analysis was done using phospho-specific antibodies as described (18). U87 ERβ-KO cells and U87 ERβ-KO cells expressing ERβ1 or ERβ5 isoforms were subjected to cell lysis using NP-40/Triton X-100 lysis buffer containing protease and phosphatase inhibitors. The lysates were precleared with protein A beads, followed by GFP-TRAP beads for 2 hours at 4°C, and ERβ1- and ERβ5-interacting proteins were analyzed by mass spectrometry (UT Health San Antonio MS core facility). To
confirm ERβ5 interactions with TCP-1α, GCN-1, Filamin A, and mTOR. U251-ERβ5 lysates of cells were prepared using NP-40/Triton X-100 lysis buffer and incubated with control beads, GFP-TRAP beads, or control GST, GST- ERβ5 beads followed by Western blot analysis.

RNA sequencing and quantitative real-time-PCR
Total RNA was isolated from U87, U87 ERβ-KO, and U87 ERβ-KO cells expressing ERβ1 or ERβ5 isoforms using the RNeasy mini kit according to the manufacturer’s protocol (Qiagen). RNA sequencing and analysis was performed as described previously (UT Health San Antonio genomics core facility; ref. 18). To validate the selected genes, quantitative real-time-PCR (RT-qPCR) was performed using gene-specific qPCR primer sequences obtained from Harvard Primer Bank (http://pga.mgh.harvard.edu/primebank/). ERβ Isoform status was analyzed using published human ERβ isoform-specific primers ERβ1F-GTCAAGGGA-TGGAGTACAA; ERβ1R-GGAGCCTTTCCTTGTTTTA; ERβ2F-TCTCCCTCAGCCAGCAATCC; ERβ2R-GGTCACTGCTCCATCG-TTGC; ERβ4F-CTGACGGATGCTTTGGTTTG; ERβ4R-ATCCTTTCA-TTGGCCACATGC; ERβ5F-GTACGTTGTTTGTTCGTTATG; ERβ5R-CCTCCGTGACGACATAATC (15). RT-qPCR was performed using SYBR Green (Thermo Fisher Scientific) on an Illumina Real-Time PCR system. Data were normalized to GAPDH and the difference in fold change was calculated using delta-delta-CT method.

Reporter gene assays
Reporter assays using an NFKB-Luc reporter were performed as described (20). After 24 hours, cells were stimulated with either vehicle or TNFα (20 ng/mL) for additional 24 hours. The pRL-TK vector (25 ng) was cotransfected and used for data normalization. For STAT3-luc assays, U251 cells were stably transfected with STAT3-firefly luciferase reporter lentivirus purchased from Cellomic Technology. STAT3-luc reporter expressing U251 cells were transiently transfected with empty vector or ERβ1 vector or ERβ5 vector and after 48 hours, reporter activity was measured. Cells were lysed in passive lysis buffer, and the luciferase activity was measured using the dual-luciferase reporter assay system (Promega) in luminometer.

Immunohistochemistry
IHC was performed as described previously (18). Briefly, tumor sections were incubated with Ki67, p-Akt, pS6, or p-S6 primary antibodies for overnight at 4°C, followed by secondary antibody incubation for 45 minutes at room temperature. Immunoreactivity was visualized by using the DAB substrate and counterstained with hematoxylin (Vector Lab).

In vivo orthotopic tumor model
All animal experiments were performed after obtaining UT Health San Antonio Institutional Animal Care and Use Committee approval. Male athymic nude mice of 8 to 10 weeks old were purchased from The Charles River. Model cells were labeled with the GFP-Luciferase reporter. U251 control, U251-ERβ-KO model cells (expt 1, n = 8), U251 control, U251-ERβ-KO, U251-ERβ1, and U251-ERβ5 model cells (expt 2, n = 7) were injected orthotopically into the right cerebrum of a mouse using established protocol (18). Tumor progression was monitored weekly using the Xenogen in vivo imaging system. At the end of the experiment, mice were euthanized, and brains were collected and processed for histological studies. Mouse survival was determined using Kaplan–Meier survival curves and log-rank test using GraphPad Prism 6 software (GraphPad Software).

Statistical analyses
Statistical differences between groups were analyzed with unpaired Student t-test and one-way ANOVA using GraphPad Prism 6 software. All the data represented in plots are shown as means ± SE. A value of P < 0.05 was considered as statistically significant.

Results
ERβ isoforms are aberrantly expressed in GBM
To determine the status of ERβ isoforms in GBM, we profiled the expression of various ERβ isoforms using patient-derived and established GBM cells. RT-qPCR assays demonstrated that ERβ5 isoform was widely expressed in the majority of GBM cells tested, while ERβ1 and ERβ2 were moderately expressed and ERβ4 was either undetected or least expressed (Fig. 1A and B). Further, we also profiled the ERβ isoforms in surgically removed GBM tissue samples (n = 18). Inconsistent with GBM cell lines, ERβ5 was highly expressed in the majority of GBM tissue samples, whereas ERβ1 and ERβ2 were moderately expressed, and ERβ4 was either undetected or least expressed (Fig. 1C). We next examined the expression of ERβ5 in tumor tissues using glioma tissue arrays that have different grades of gliomas as well as normal brain tissues. The intensity of staining and positivity was recorded as described earlier (13, 17). The representative staining for each grade and normal tissue is shown in Fig. 1D. IHC analysis revealed that the expression of ERβ5 was significantly greater in tumor tissues than in normal brain tissues, with being highly expressed in GBM (Fig. 1D).

ERβ1 has tumor suppression function, while ERβ5 promotes oncogenic function
To delineate the isoform-specific functions of ERβ, we generated ERβ-knockout cells (KO) in two genetic backgrounds (U251 and U87) using CRISPR/Cas9 technology, and deletion of genomic region was confirmed using genomic PCR (Fig. 2A and B). In both models, ERβ KO abolished the expression of ERβ protein as determined by Western blotting (Fig. 2A and B). As an additional model, we have used lentiviral-ERβ shRNA-transfected primary GBM cells. ERβ knockdown was confirmed by RT-qPCR and Western blotting (Fig. 2C). We next determined whether the ERβ KO contributes to tumor progression in vivo using orthotopic GBM model. To determine the effect of ERβ KO on mice survival, U251-WT and U251-ERβ KO cells were implanted intracranially into immunocompromised mice. As shown in Fig. 2D, compared with control mice, ERβ KO mice had significant reduction in survival. Next, we generated GBM model cells that uniquely expressed ERβ1 and ERβ5 isoforms in U87-ERβ-KO, U251-ERβ-KO, and primary GBM cells using lentiviral transduction, and their expression was confirmed by Western blotting (Fig. 2E). We next examined the effect of U251 and ERβ5 isoform expression on cell viability and survival of GBM cells. MTT assays demonstrated that CRISPR knockout of ERβ or shRNA knockdown of ERβ increases cell viability of the U251 cells (Fig. 2F) and the primary GBM cells than the control cells (Fig. 2G). Further, reintroduction of ERβ1 significantly reduced the cell viability while ERβ5 overexpression increased cell viability of GBM cells, respectively (Fig. 2F and G). Further, colony formation assays demonstrated...
Figure 1.
ERβ5 is highly expressed in GBM. A and B, Expression of ERβ isoforms was determined by isoform-specific RT-qPCR primers using patient-derived GBM cells (A) and established GBM cells (B). C, ERβ isoforms expression was examined using isoform-specific RT-qPCR primers in patient GBM tissue specimens (n = 18). D, A glioma tissue microarray containing control brain (n = 16) as well as grade 2 (n = 150), grade 3 (n = 29), and grade 4 (n = 33) glioma tissue specimens was used to determine ERβ5 expression using immunohistochemistry. Quantitation of total score in each grade was done as described in Materials and Methods. Data are represented as mean ± SE. * P < 0.05; ** P < 0.01; *** P < 0.001; ns, nonsignificant.
Figure 2.
Generation and validation of ERβ KO, ERβ1, and ERβ5 cells. U251 ERβ KO (A) and U87 ERβ KO (B) cells were generated using the CRISPR/Cas9 system, and ERβ knockout was confirmed by genomic PCR, and validated by Western blotting. C, ERβ1 was knocked down in GBM-040815 cells using lentiviral transduction of ERβ shRNA. ERβ knockdown was validated by RT-qPCR and Western blotting. D, U251-WT or U251-ERβ KO cells were injected orthotopically and the number of survival days of the mice were recorded and analyzed using the Kaplan–Meier graph. E, U87 cells and U251 cells stably expressing ERβ1 and ERβ5 isoforms were generated by lentivirus transduction in the ERβ KO background. Primary (GBM-040815) cells were transduced with ERβ1 and ERβ5 lentiviral plasmids. The expression of ERβ1 and ERβ5 was validated by Western blotting. The cell viability of U251 (F) and GBM-040815 (G) cells expressing ERβ1 and ERβ5 was measured by MTT and MTS assays, respectively. H and I, U251 WT, ERβ KO, ERβ1, and ERβ5 cells were seeded in 6-well plates (500 cells/well), and after 14 days, the colonies were stained with 0.5% crystal violet, and the percentage of colony area was determined. J, NIH3T3 cells were transfected with empty vector or ERβ5 vector, and focus formation assays were performed as described in Materials and Methods. K, NIH3T3 cells were transfected with indicated plasmids and subjected to soft-agar colony formation assay. Data are represented as mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
that expression of ERβ1 but not ERβ5 reduced the colony formation of U251 cells. Further, ERβKO- and ERβ5-expressing cells exhibited bigger size colonies compared with control (Fig. 2H and I). To examine whether ERβ5 acquired oncogenic properties, we next performed focus formation assay using NIH-3T3 cells as described previously (19). ERβ5-transfected cells had significantly more foci than either in the control or empty vector–transfected cells (Fig. 2J). To further confirm the oncogenic potential of ERβ5, soft-agony colony formation assays were performed. ERβ5-transfected cells had significantly more soft-agony colonies than the empty vector–transfected or ERβ1-transfected NIH-3T3 cells (Fig. 2K). Collectively, our results provide the genetic evidence that ERβ1 functions as a tumor suppressor in GBM cells while ERβ5 has oncogenic properties with loss of tumor suppressor functions.

ERβ1 suppresses and ERβ5 enhances migration and invasion in GBM cells

GBM are highly invasive, which contributes to poor prognosis of patients with GBM. To examine the role of ERβ1 and ERβ5 in GBM cell motility, we next performed migration and invasion assays using scratch wound healing and Matrigel invasion assays, respectively. Wound-healing assays demonstrated that ERβ KO cells were more motile than control cells, which was evident from rapid closure of the scratch while reintroduction of ERβ1 reduced the motility significantly (Fig. 3A). The motility of GBM cells was significantly greater in those cells expressing ERβ5 than in cells expressing ERβ1 (Fig. 3A). Further, Matrigel invasion assays revealed that ERβ5-expressing ERβ KO cells were more invasive than the control cells. Reintroduction of ERβ1 but not ERβ5 significantly reduced the invasive ability of GBM cells (Fig. 3B). To examine whether ERβ1 and ERβ5-mediated changes in cell migration/invasion involved alterations in the cytoskeleton, we have examined the status of filamentous actin structures, including filopodia, ruffles, and stress fibers. ERβ KO cells had significantly more motile actin structures such as filopodia, and ruffles and fewer stress fibers. ERβ1-expressing cells had fewer filopodia and ruffles with more stress fibers. In contrast, ERβ5-expressing cells had more motility-promoting structures, including ruffles and filopodia with minimal stress fibers (Fig. 3C). Further, we also examined whether reintroduction of ERβ1 into ERβ5-expressing GBM cells could mitigate the ERβ5-induced changes in motility, colony formation, and invasion. As shown in Fig. 3C (bottom), the overexpression of ERβ1 in ERβ5-expressing cells significantly increased stress fibers and reduced filopodia and ruffles. Further expression of ERβ1 in ERβ5-expressing cells reduced the colony formation (Fig. 3D) and cell invasion compared with ERβ5 alone expressing cells (Fig. 3E). These results suggest that ERβ1 suppresses motility while ERβ5 has the potential to promote the migration and invasion of GBM cells by promoting cytoskeletal changes.

Transcriptomic analysis of ERβ1- and ERβ5-modulated genes in GBM

To understand the mechanisms by which ERβ1 and ERβ5 promote tumor-suppressive and oncogenic activities, respectively, we performed RNA-seq analysis using U87, U87-ERKO, U87-ERβ1, and U87-ERβ5 cells. Overall, 1,211 genes (1.5-fold change over control with adjusted P < 0.05) were differentially expressed in ERβ KO cells; 526 genes were downregulated and 685 genes were upregulated. The complete list is available in the GEO database under accession number GSE104296. The differentially expressed genes among the groups were shown in the Venn diagram (Fig. 4A) and heat map (Fig. 4B). The biological significance of the differentially expressed genes was determined using Ingenuity Pathway Analysis (IPA; Fig. 4C) and gene set enrichment analysis (GSEA; Fig. 4D). The IPA of differentially expressed genes between U87 and U87-ERβ KO cells revealed that the ERβ KO–modulated genes were related to cellular migration and invasion, including NFκB–regulated TNFR2 and Toll-like receptor signaling, JAK–STAT and mTOR pathways (Fig. 4C). Further, GSEA of control versus ERβ KO–modulated genes revealed positive correlation with NFκB signaling, and JAK–STAT pathway gene sets (Fig. 4D). However, ERβ KO versus ERβ1-modulated genes revealed negative correlation with NFκB signaling, and JAK–STAT pathway gene sets (Fig. 4D). In contrast, comparisons of ERβ KO versus ERβ5 and ERβ1 versus ERβ5 revealed that differentially expressed genes were positively correlated with gene signatures of the NFκB and JAK–STAT pathways (Fig. 4D). Validation studies using RT-qPCR assays confirmed that the genes related to the NFκB and JAK–STAT pathway were significantly upregulated in ERβ KO cells compared with control cells (Fig. 4E). Reintroduction of ERβ1 but not ERβ5 decreased the expression of selective NFκB target genes (Fig. 4E). To further confirm the role of isoform ERβ1 and ERβ5 on NFκB signaling, reporter gene assays were performed. Compared with control cells, ERβ KO cells had significantly higher NFκB-Luc activity (Fig. 4F). As expected, the introduction of ERβ1 but not ERβ5 decreased the NFκB-Luc activity in U87 and U251 GBM cells (Fig. 4F). Further, we also examined the activation of NFκB by examining the phosphorylation of p65. As shown in Fig. 4G, ERβ1 overexpression reduced the levels of p-p65 compared with ERβ-KO cells, whereas ERβ5 overexpression significantly increased the phosphorylation of p65. Collectively, these results suggest ERβ isoforms differentially modulate the NFκB and JAK–STAT pathways in GBM cells.

Immunoprecipitation–mass spectrometry identified novel ERβ1- and ERβ5-specific binding proteins

The tumor-suppressive functions of ERβ1 are mediated by modulation of gene expression either by direct interaction of target gene chromatin or indirectly by its interactions with protein complexes, including ERα, p53, and NFκB (12, 20–22). ERβ5 lacks the complete LBD, and its functions are thought to be mediated via interaction with other proteins. However, the precise interacome of ERβ5 that mediates its oncogenic functions is largely unknown. To identify the ERβ5 interacome, the lysates of the ERβ KO, ERβ1, and ERβ5 cells were subjected to GFP pulldown, followed by immunoprecipitation–mass spectrometry (IP-MS) analysis (Fig. 5A). Several novel proteins such as chaperonin T-complex protein subunits, FH1L2, Filamin C, Filamin A, and GCN1 uniquely bound to ERβ5 but not to ERβ1. The list of top unique binders of ERβ1 and ERβ5 is shown in Fig. 5B. We confirmed the binding of ERβ5 to TCP-1α, mTOR, Filamin A, and GCN-1 by performing GFP-ERβ5 pulldown assays (Fig. 5C) and GST-ERβ5 pulldown assay in U251 cells (Fig. 5D).

ERβ5 enhances mTOR and its downstream signaling in GBM cells

IP-MS studies demonstrated that ERβ5 interacts with filamin and GCN-1, which are shown to enhance cellular migration by activating the Akt/mTOR pathway (23, 24). Further, p90 ribosomal S6 kinase and p70 ribosomal S6 kinase were shown to
ERβ1 suppresses and ERβ5 promotes migration and invasion of GBM cells. A, The migration ability of U251 model cells was determined by using the scratch wound-healing assay. Representative images of the wound-healing assay at various time points are shown. Comparisons of migration distances in the U251 model cells were determined using NIH ImageJ software. B, Cell invasion of U87, U251, and primary GBM-040815 model cells was determined by using Matrigel invasion chamber assays. Representative images of model cells are shown. C, The alterations in cytoskeleton of U251 model cells were determined using phalloidin staining for filamentous actin. Images were captured using confocal microscopy and representative images are shown. DAPI was used for nuclei staining. D, Colony formation ability of U251 cells that express ERβ1, ERβ5, and ERβ1+ERβ5 was determined. E, Cell invasion of U251 cells that express ERβ1, ERβ5, and ERβ1+ERβ5 was determined by Matrigel invasion assay. Data are represented as mean ± SE. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 4.
Analysis of global transcriptional changes modulated by ERβ isoforms in GBM cells. Total RNA was isolated from the U87-WT, -KO, -ERβ1, and ERβ5 cells and subjected to RNA sequencing. The Venn diagram (A) and heat map (B) show differentially expressed genes among the groups. Heat map shows the clustering of all samples and genes with RPKM > 1. C, Top IPA pathways modulated in ERβ KO cells compared with control. D, GSEA testing correlation of isoform-modulated genes with signatures of the NFκB signaling gene set and the JAK–STAT3 gene set. E, The selective genes were validated through RT-qPCR in U251 cells. F, U87 and U251 model cells were transfected with NFκB-luc reporter plasmid, and after 24 hours, cells were stimulated with human TNFα, and the reporter activity was measured after 24 hours. G, The status of phospho-p65 and p65 in ERβ-KO, ERβ1, and ERβ5 cells was determined by Western blotting. Data are represented as mean ± SE. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
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**Unique ERβ5-interacting Proteins**

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**Graphs and Figures**

- **Figure A**: Western blot analysis showing protein expression patterns for ERβ5, ERβ1, and KO in A549 cells.
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- **Figure H**: Bar graph showing relative STAT3-Luc activity in A549 cells.
- **Figure I**: Western blot analysis of mTOR, p-S6K, S6K, p-S6, S6, and p-4EBP1 expression in A549 cells.
- **Figure J**: Graph showing the effect of AZD8055 on p-4EBP1 expression in A549 cells.
phosphorylate TCP-1α (25). To understand the significance of ERβ5 interaction with TCP-1α, FHL-2, and GCN-1, we studied the activation of the Akt/mTOR pathway using Western blotting. Overexpression of ERβ1 reduced the phosphorylation of ERK1/2, Akt, and mTOR, while overexpression of ERβ5 significantly increased their phosphorylation (Fig. SE and F). Previous studies demonstrated that mTOR phosphorylates STAT3 at ser727 for maximal activation of STAT3 (26–28). We therefore examined mTOR-mediated phosphorylation of STAT3 (727) in ERβ1- and ERβ5-expressing cells. As shown in Fig. 5G, ERβ1 reduced the ser727 phosphorylation of STAT3; however, ERβ5 activated its phosphorylation. Accordingly, tyrosine phosphorylation of STAT3 (705), which is negatively regulated by ser727 STAT3, was increased in ERβ1 cells, while it was reduced in ERβ5-overexpressing cells. Further, we examined the activation of STAT3 using STAT3-luciferase reporter assay following ERβ1 and ERβ5 overexpression. As shown in Fig. 5H, ERβ1 overexpression in U251 cells decreased the STAT3-luc activity; however, ERβ5 overexpression increased its activity compared with control. We next studied the magnitude of activation of the mTOR pathway in response to serum stimulation. Compared with ERK KO cells, ERβ1 expression suppressed and ERβ5 expression increased serum-induced phosphorylation of mTOR, S6K, S6, and 4EBP-1, confirming that the mTOR pathway is suppressed by ERβ1 and activated by ERβ5 (Fig. 5I). We next determined whether ERβ5 mediated increase in mTOR signaling essential for cell viability by treating the cells with the mTOR inhibitor AZD8055. Treatment with AZD8055 significantly reduced the cell viability advantage seen in ERβ5-overexpressed cells compared with control cells suggesting that ERβ5-mediated mTOR signaling is responsible for increased cell viability in ERβ5 cells (Fig. 5J). Collectively, these results indicate that ERβ1 and ERβ5 differentially regulate mTOR signaling and ERβ5-mediated oncogenic potential may involve the activation of mTOR downstream signaling.

ERβ1 but not ERβ5 increased the survival of tumor-bearing mice

We next determined whether ERβ1 and ERβ5 overexpression could affect the survival of the mice using in vivo orthotopic GBM models. U251-WT, U251-ERβ-KO, U251-ERβ1, and U251-ERβ5 cells were implanted intracranially into immunocompromised mice. The knockout of ERβ resulted in significant decrease in the survival of tumor bearing mice compared with wild-type tumors. Reintroduction of ERβ1 significantly improved the mice survival compared with U251-ERβ-KO and U251-WT tumor bearing mice (Fig. 6A). However, survival was significantly reduced in the mice with ERβ5 tumors than in the U251-ERβ1 and U251-WT tumor-bearing mice (Fig. 6A). Further, IHC analysis of tumor sections from mice with U251-WT, U251-ERβ-KO, U251-ERβ1, and U251-ERβ5 revealed that ERβ-KO tumors had more Ki-67–positive cells than U251 WT tumors. ERβ1 but not ERβ5 tumors exhibited significantly less Ki-67–positive cells compared with ERβ-KO and wild-type tumors (Fig. 6B and C). Further, to confirm the in vitro mechanistic observations, we determined the status of phosphorylation of Akt/m-TOR signaling molecules p-Akt and p-S6 and NFkB target gene IL8 in tumors. IHC analysis revealed that ERβ-KO significantly increased the levels of p-Akt, p-S6, and IL8 compared with wild-type tumors, whereas ERβ1 but not ERβ5-expressing cells had low levels of p-Akt, p-S6, and IL8 (Fig. 6D). These results demonstrated that ERβ1 but not ERβ5 possess tumor-suppressing functions in GBM.

Discussion

Most studies have shown that ERβ functions as a tumor suppressor, and that its expression is reduced in many human malignancies (9, 29–31). A few studies have showed that ERβ could be oncogenic in some cancers (32–34). However, the tumor-suppressive or oncogenic functions of ERβ in these studies have been attributed to the presence of total ERβ in the cells. The recent discovery of various isoforms of ERβ has complicated the interpretation of these results, and now a better understanding of the functions of the isoforms is needed.

Our results using the CRISPR/Cas9 knockout of ERβ GBM model cells provided evidence that the genetic deletion of ERβ leads to an aggressive GBM phenotype. Reintroduction of ERβ1 mitigated these effects in ERβ-KO cells, whereas reintroduction of ERβ5 promoted oncogenicity via modulation of the mTOR and NFκB pathways. Further, our results suggested that introduction of ERβ1 but not ERβ5 into GBM cells improved mice survival. ERβ reduces cell proliferation and induces apoptosis in several cancer cell types, and its expression declines during tumor progression (9, 14, 31, 35, 36). Recent studies including ours demonstrated that ERβ exhibits tumor-suppressive functions in GBM cells, and that high expression of ERβ was an independent favorable prognostic factor (13, 37–40). Further, the natural and synthetic ligands of ERβ exhibit antitumor activities in GBM models. These findings imply that ERβ may play a role in the suppression of GBM.

Our work established the significance of endogenous ERβ in GBM progression using ERβ KO GBM cells. ERβ KO cells had more cell viability and colony formation ability as well as had enhanced migration and invasion properties. These findings corroborate previous findings that knockdown of ERβ leads to increased cell viability, migration, and invasion (30, 41–43). The ERβ KO cells were highly enriched with motile structures such as filopodia and...
Figure 6. Differential effects of ERβ1 and ERβ5 isoforms on the progression of GBM in vivo. A, Athymic nude mice were implanted with U251-WT, U251-ERβ-KO, U251-ERβ1, or U251-ERβ5 cells orthotopically into the right cerebrum. Survival of the mice was plotted using the Kaplan–Meier curve. B, Mouse brains collected from the WT, KO, ERβ1, and ERβ5 groups were fixed in formalin and processed for immunohistochemical staining for Ki-67. C, The number of Ki-67–positive cells from five different images was counted and plotted as histogram. D, Tumor sections were subjected to immunohistochemical staining for the detection of p-Akt, p-S6, and IL8. Data are represented as mean ± SE. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
nuffles, while control cells had more of the less motile structures, such as stress fibers. Compared with implanted WT cells, ERβ KO cells reduced mice survival. Thus, by knocking out endogenous ERβ in GBM cells, we provide the genetic evidence for the role of ERβ in GBM suppression.

Multiple isoforms of ERβ exist and may have distinct roles in various cancers (15, 44). The ERβ2 isomorph is overexpressed in chronic lymphocytic leukemia, prostate cancer, non-small cell lung cancer, breast cancer, and ovarian cancer (9, 45–51). ERβ2 expression associated with worse disease-free survival and overall survival of patients and disease-free survival of tamoxifen-treated patients (52). Further, ERβ2 is implicated in prostate cancer metastasis (46, 48). ERβ3 has limited tissue distribution restricted to testis (53). ERβ5 is overexpressed in ovarian cancer and prostate cancer and is associated with poor prognosis (48, 54, 55), while ERβ5 expression is associated with good prognosis in non–small cell lung cancer and confers sensitivity to chemotherapeutic agent-induced apoptosis in breast cancer cells (48, 49, 54, 56). Our results suggested that ERβ5 was highly expressed in the majority of primary and established GBM cells compared with ERβ1 and ERβ2, with ERβ4 being the least expressed.

Recent studies have shown that GBM cells express ERβ1 and that its expression decreases during GBM progression. In this study, we found greater expression of ERβ5 in higher grades of glioma than in low-grade glioma and normal brain tissues. Our studies are in agreement with the recent study that also reported ERβ5 is overexpressed in GBM using a small cohort of samples (57). However, the signaling results reported in this study differ from our findings. This discrepancy could be in part due to the use of HEK-293T cells for signaling studies, low levels of expression of endogenous ERβ in their GBM models compared with ERβ1 and presence of endogenous ERβ1. The GBM models we used in this study differ from those models due to complete knockout of endogenous ERβ and its isoforms. Further, our results using coexpression of ERβ1 and ERβ5 suggested that ERβ1 presence might negate some of the effects of ERβ5. Collectively, these findings suggest the ERβ1 functions as a tumor suppressor, its suppression functions may include counteracting oncogenic functions of other ERβ isoforms and ratio of levels of ERβ1 with other ERβ isoforms may have implications in GBM progression.

To study the functions of ERβ1 and ERβ5 isoforms in GBM cells, we have established GBM cell model cells expressing individual ERβ1 and ERβ5 in the absence of endogenous ERβ. Compared with parental GBM cells, ERβ1-expressing cells had significantly less viability, lower survival, less migratory, and invasive potential. In addition, compared with control cells, ERβ5-expressing cells transformed the NIH3T3 fibroblasts and increased the focus formation and soft-agar colony formation. ERβ1 failed to transform or increase the foci formation in NIH3T3 cells. Survival in mice with ERβ1-expressing tumors was longer than in mice with ERβ5-expressing tumors. Our results suggest that ERβ1 has tumor-suppressive function in GBM whereas ERβ5 exhibits oncogenic functions in GBM. Results from cell viability, invasion, migration, and F-Actin experiments suggested that ERβ5 has the potential to promote migratory phenotype. The use of orthotopic models and aggressive nature of GBM limited our ability to quantify invasive potential in our study. However, IHC analysis of invasive marker IL8 showed that knockout of ERβ increased IL8 expression, whereas ERβ1 reduced the expression of IL8 and ERβ5 increased its expression. Even though these findings support ERβ5 in promoting cell invasion, future studies are clearly needed.

Our RNA-Seq analysis revealed unique pathways modulated by ERβ1 compared with ERβ5 such as NFκB and JAK–STAT3 pathways. Further, GSEA data also demonstrated that ERβ1-modulated genes were negatively correlated with the NFκB and JAK–STAT3 pathway gene sets, and ERβ5-modulated genes were positively correlated with NFκB and JAK–STAT3 pathways. IP-MS studies identified unique proteins that bind to each of the isoforms. ERβ5 uniquely interacted with several proteins that function as regulators of mTOR, immunomodulatory, DNA repair, and migration/invasion pathways, including TCP-1α, FHL2, and filamins. Interestingly, emerging evidence suggests that TCP-1α, FHL2, and filamins are involved in the activation of NFκB and STAT3 (23–25, 58–60). These results further corroborate with the findings from the RNA-seq studies that showed that NFκB and STAT3 pathways are attenuated by ERβ1 and, by contrast, are activated by ERβ5. Accordingly, mechanistic studies showed that ERβ1 reduced the activation of mTOR signaling molecules, including p-mTOR, p-S6K, and p-S6, in GBM cells compared with ERβ-KO cells, while ERβ5 enhanced mTOR downstream signaling. Collectively, our results discovered that ERβ5 unlike ERβ1 acquires oncogenic ability and its interactions with mTOR, DNA repair, invasion/migration, and apoptosis pathways may have important implications in GBM progression.

In conclusion, our data demonstrate isoforms of ERβ have distinct functions in GBM progression. Using ERβ KO GBM model cells, we have provided strong evidence using both in vitro and in vivo models demonstrating the tumor suppressor potential of endogenous ERβ1. Further, our studies indicate that ERβ5 is overexpressed in high-grade gliomas compared with low-grade tumors and normal brain. Our studies also discovered that ERβ5 lacks tumor suppressive functions and acquire oncogenic properties via its interaction with several oncogenic molecules. We propose that upregulation of ERβ1 expression/functions along with treatments that downregulate ERβ5 downstream signaling is an attractive therapy for GBM.

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

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Acknowledgments

This study was supported by the NIH/NCI grant NIH-CA178499 (R.K. Vadlamudi and A. Brenner), NCI Cancer Center Support Grant P30CA054174-17, and Voelcker young investigator grant (G.R. Sareddy). We thank Dr. Indra Pooja for providing ERβ isoform plasmids. Mass spectrometry analyses were conducted in the UTH San Antonio Bioinformatics and Spectrometry Laboratory, supported in part by NIH-shared instrumentation grant S10RR025111 (to S.T. Weintrab) and NIH grant CA054174 (UT Health Cancer Center–Mass Spectrometry Shared Resource). Data generated in the Genome
Sequencing Facility were supported by NIH-shared instrument grant 1S10OD021805-01 (S10 grant), NIH NCI grant CA054174 (ITT Health Cancer Center Next Generation Sequencing Shared Resources), and CIPRT Core Facility Award (RP160732).

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Received November 9, 2017; revised March 13, 2018; accepted April 11, 2018; published first April 16, 2018.

References


Differential Effects of Estrogen Receptor β Isoforms on Glioblastoma Progression

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