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Involvement of Estrogen Receptor Beta 5 in the Progression of Glioma

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INVolvEMENt oF ESTROGEN ReCEPTOR BETA 5
IN THE PROGRESSIoN oF GLIoMA

DISSERTATION

Presented to the Graduate Council of the
Graduate School of Biomedical Sciences
University of North Texas Health Science Center at Fort Worth
In Partial Fulfillment of the Requirements

For the Degree of
DOCTOR oF PHILOSOPHY

By
Wenjun Li, B.S.
Fort Worth, Texas
May 2013
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Primary brain and CNS tumors

Primary brain and central nervous system (CNS) tumors include tumors that occur in these sites: brain, meninges, spinal cord, cranial nerves, pituitary and pineal glands, nasal cavity and other CNS sites [1]. Based on the *Central Brain Tumor Registry of the United States* (CBTRUS) report [2], in the United States, the incidence rate of primary brain and CNS tumors was 25.59 per 100,000 between 2005 and 2009; about 42% of the tumors occurred in male patients and 58% in female patients. Approximately 53% - 73% of these tumors were non-malignant. In children from age 0 to 19, the incidence rate was 5.13 per 100,000. It is estimated that there are more than 66,000 new cases of primary brain and CNS tumors every year in the United States; and in 2010, more than 680,000 people were living with brain and CNS tumors. Brain and CNS tumors are also the second leading cause of death (approximately 13,000 deaths a year) from neurological diseases after stroke. The meninges is the most common site for primary brain and CNS tumors (about 35%).

Glioma

Gliomas are the primary tumors originated from glia cells in the brain; gliomas include: astrocytoma, glioblastoma, oligodendroglioma, ependymoma, mixed glioma and malignant
glioma [2]. Glioma accounts for about 30% of all primary brain and CNS tumors and about 60% of glioma occur in the four lobes of the brain.

According to degree of malignancy, glioma can be categorized to grade I, II, III and IV. Gliomas usually do not metastasize outside the brain and clinical outcomes can be determined by their grades [3]. Low grade gliomas include grade I and grade II gliomas. Grade I glioma is benign and can often be cured by surgery. Grade II glioma is a low grade malignancy; they are incurable but patients can survive for more than five years. Grade III gliomas are malignant and fatal. Glioblastoma (GBM) is a grade IV glioma and the most common type glioma, which accounts for 54% of all gliomas [2]. Glioblastoma can be developed from low grade glioma through a long period (5-10 years); it can also occur as primary glioblastoma [4]. The prognosis of GBM is very poor, the median survival time of GBM patients is 3 months without treatment [2, 5]. GBM is resistant to traditional chemotherapies. Surgery is the primary treatment of GBM, followed by radiation therapy or/and temozolomide (TMZ) based chemotherapy. However, even with these treatments, the median survival time is only 14.6 months [6].

The exact cause of glioma is not clear yet; both environmental and genetic factors have been suggested to contribute to the pathogenesis. Exposure to radiation [7] and carcinogens have been reported to be associated with higher rate of glioma occurrence [8]. Increased glioma occurrence in occupations with exposure to radiation and carcinogens, such as physicians, firefighters and farmers, has been reported [9-13]. Genetics also contributes to the occurrence of glioma. Genome-wide association study has identified five susceptibility loci for glioma: 5p15.33, 8q24.21, 9p21.3, 20q13.33 and 11q23.3 [14]. Genetic as well as functional studies have identified genes and signaling pathways that involved in the occurrence of glioma, especially glioblastoma.
Glioma genes and major signaling pathways

Alternations of multiple genes have been identified in human glioma especially in GBM. These alternations include: EGFR amplification, mutations of: PTEN, P53, IDH1, IDH2, NF1, etc [15, 16]. These alternations lead to the modification of important cell signaling pathways, such as PI3K/AKT/mTOR pathway and MAPK/ERK pathway which are the major signaling pathways involved in the pathogenesis of glioma [17] (Fig. 1).

Phosphatase and tensin homolog (PTEN) negatively regulates the PI3K/AKT pathway by dephosphorylating PIP3 to PIP2 [18]. In high grade glioma, about 40% patients demonstrated PTEN mutations [15, 19]. Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein that belongs to the receptor tyrosine kinase family (RTK). Binding of EGF leads to dimerization and autophosphorylation of the receptor, which activates downstream molecules and lead to division and proliferation of cells. Amplification of EGFR gene (EGFRvIII mutant) is present in approximately 40% of GBM patients [20]. EGFR gene amplification leads to the overexpression of EGFR, which up-regulates downstream signaling pathways (MAPK/ERK, PI3K/AKT/mTOR) and increases cell proliferation, invasion, and tumor metastasis [17, 21] (Fig. 1). Mutation of the tumor suppressor P53 is present in approximately 40% of low grade gliomas and in more than 60% secondary GBMs [22, 23]. Mutations of the isocitrate dehydrogenase 1 (IDH1) gene have been found in more than 70% of the grade II, grade III gliomas and secondary GBMs [24]. Neurofibromatosis 1 (NF1) negatively regulates RAS; mutation of NF1 leads to RAS hyperactivation [25]. NF1 mutation is present in 14% GBMs [26]. Based on the gene mutations and alternations of the signaling pathways, genetically engineered animal models for glioma have been developed [27, 28], which could serve as tools to study the origin or glioma
and test new therapeutic agents. Targeted agents for the specific mutations or signaling pathways have been developed and are being tested for treatment of glioma, such as EGFR inhibitors (gefitinib, erlotinib etc) [29-31] and mTOR inhibitors (rapamycin, temsirolimus) [32-34]. It is hoped that these targeted agents will outcome the limitations of current treatment and improve the prognosis of glioma, especially glioblastoma.

**Estrogen receptor β**

Estrogen receptors belong to the nuclear receptor family. There are two main types of estrogen receptors: estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). ERα and ERβ are coded by different genes. ERβ is localized on human chromosome 14, while ERα is on chromosome 6. Structural analysis indicated that ERβ has five domains as classic nuclear receptor family members: N-terminal domain (A/B domain), DNA binding domain (C domain), hinge domain (D domain), ligand binding domain (E domain) and the C-terminal domain (F domain) [35] (Fig. 2). The N terminal A/B domain performs the activation function 1 (AF-1), which is independent of ligand binding [36, 37]. Nuclear localization sequence is in the D domain, and this domain also interacts with AP-1 [38]. The E domain also performs ligand-dependent activation function 2 (AF-2) [39]. The DNA binding domains of ERα and ERβ are highly homogeneous, with only three amino acids being different. However, their ligand binding domains show only 59% homology [40]. Specific ligand for ERβ has been synthesized: diarylpropionitrile (DPN) has a 70-fold selectivity for ERβ against ERα in binding assays [41].

Upon binding to estrogen, estrogen receptors form dimmers which translocate into the nucleus and bind to the estrogen responsive element (ERE) in the promoter of genes to activate the transcription of genes. This is the genomic pathway of estrogen receptor function. Besides
this classic ERE-dependent genomic action, estrogen receptor can also regulate genes without ERE element. About one third of estrogen responsive genes do not contain ERE-like sequence in their promoters [42]. Estrogen receptor can interact with other transcription factors such as AP-1 to regulate transcription of genes without ERE in the promoter [42-44]. Genomic actions of ERα and ERβ seem to be different. In breast cancer, ERβ inhibited transcription of 998 genes in the 1,434 gene that were activated by ERα [45]. Besides genomic pathways, non-genomic pathways of ERα action, including activation of the MAPK pathway and PI3K pathway, have also been identified [46-51]. Activation of MAPK pathway by ERβ was also observed [52, 53]. A membrane estrogen receptor GPR30, which could also activate ERK1/2, has been identified [54]. Unlike ERα or ERβ, GPR30 belong to the classical 7-transmembrane G protein coupled receptor (GPCR) family.

From genes knockout studies, it is clear that biological functions of ERβ are distinct from ERα. For example, both female and male ERα knockout mice are infertile [55], while both female and male ERβ knockout mice are fertile [56]. Several different ERβ knockout mouse models with partial or complete ERβ deletion have been generated, although there is disagreement in their phenotypes [56-59]. ERβ was first cloned from rat prostate cDNA [60]. In human, ERβ is expressed in various tissues such as breast, ovary, prostate, brain and muscle [35]. Recently, reduction of ERβ expression has been connected to tumor progression and its functions in tumors have been extensively investigated.

**Estrogen receptor β and tumor**

The roles of estrogen receptor beta (ERβ) in tumor formation and progression have gained much attention. Loss of ERβ expression has been suggested as an important step in
estrogen-dependent tumor progression [61]. In breast cancer, decline of ERβ expression has been repeatedly reported [61, 62]. Furthermore, in breast tumor patients, high levels of ERβ were associated with lower tumor grade [63], longer survival of patients [64] and higher sensitivity to tamoxifen treatment [65, 66]. DNA methylation in the ERβ promoter region has been proposed to contribute to the decline of ERβ expression in tumor cells [67-69]. Both in vitro and in vivo studies indicate that ERβ inhibits proliferation and invasion of breast cancer cells and prevents tumor formation [70, 71]. The anti-proliferative role of ERβ has also been found in hormone-independent cancers of the colon and lung [72, 73].

Different mechanisms have been proposed for the anti-proliferative action of ERβ [61]. ERβ can repress the transcriptional activity of ERα by forming heterodimer with ERα and competing with ERα for binding site in the promoter of genes [74]. Over-expression of ERβ modified cell cycle and reduced cells at the proliferating phase [75-77]. ERβ could inhibit HIF1 transcriptional activity, which has been proposed to contribute to tumor progression due to hypoxia condition [78].

Different isoforms of human ERβ have been identified which have identical N-terminal sequences but diverge at C-terminus [79] (Table 1, Fig. 3). Furthermore, human ERβ isoforms are different from ERβ isoforms in mouse. In vitro analysis has identified distinct transcriptional activities for the isoforms [35, 79]. In vitro binding assay indicated that: ERβ1 binds to estradiol (E2) with high affinity \((K_d = 0.48 \text{ nM})\), ERβ2 does not bind to E2, ERβ4 and ERβ5 bind to E2 with moderate affinities [35, 80, 81]. Only ERβ1 is the fully functional isoform, the other isoforms do not have intact ligand binding domain and cannot form homodimers, so they do not have innate activities of their own [35].
In breast cancer, mRNA levels of ERβ2 and ERβ5 were higher than ERβ1 [82]. ERβ1 and ERβ2 inhibited ERα function differently in MCF7 cells [83]. In breast cancer patients, ERβ1, ERβ2 and ERβ5 were associated with different prognosis of the patients [84]. These studies clearly indicated the importance to differentiate different ERβ isoforms when investigating their roles in tumor progression. However, most studies on ERβ expression in cancer used antibodies that did not discriminate between different ERβ isoforms, and functional analysis of ERβ in cancer has mainly focused on ERβ1.

**ERβ in the brain**

ERβ expression is present throughout the brain in both neurons and glia cells [85, 86]. ERβ knockout mice presented attenuation of hippocampus long term potentiation (LTP) and cognitive deficits [87, 88], indicating the importance of ERβ in maintain the normal cognitive function of the brain. Neuron degeneration, and glia cell proliferation were observed in ERβ knockout mice [89]. ERβ knockout female mice also developed pituitary tumors at 2 years old [90].

Two recent studies indicated that ERβ expression declined in human glioma [91, 92]. One of the studies further reported that ERβ agonists inhibited proliferation of glioma cell lines T98G, U87, LN229, and U138. One of the agonists liquiritigenin also suppressed growth of subcutaneous glioma xenograft tumor [92]. However, both of these studies used only immunohistochemistry to evaluate ERβ expression in human glioma and neither of them indicated which isoforms were expressed. Furthermore, recent study also indicated that DPN increased proliferation of medulloblastoma cells [93]. The function of ERβ in glioma needs
further investigation, and the effects of ERβ agonist on glioma should be further tested in glioma cell and animal models.

As discussed in this chapter, in breast tumor, expression of ERβ was associated with longer survival and sensitivity to tamoxifen treatment. Clinical trials of tamoxifen treatment for GBM have been conducted and some of the patients responded to the treatment [94]. It would be very interesting to investigate the association between ERβ expression and the patients’ response to the treatment.

**Objectives of current study**

(a) To investigate ERβ expression in human glioma and its regulation mechanism;

(b) To investigate the function of ERβ in glioma progression.
Figure 1. Major cell signaling pathways involved in the pathogenesis of glioma.
Figure 2. Structure of ERβ. There are 530 amino acids in full length human ERβ protein. From 1 to 148 is the N-terminal domain (A/B); 148 to 214 is the DNA binding domain (C); 214 to 304 is the hinge domain (D); 304 to 500 is the ligand binding domain (E); 500 to 530 is the C-terminal domain.
<table>
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<th>148</th>
<th>214</th>
<th>304</th>
<th>500</th>
<th>530aa</th>
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<tbody>
<tr>
<td>A/B</td>
<td>C</td>
<td>D</td>
<td>E</td>
<td>F</td>
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MDIKNSPSSLNSPSSYNCQSILPLEHGSIYIPSSYVDSHHEYPAMTFYSVPAVMNYSIPS
NVTNELGGPGRQTTSVPNVLWPTPGHLSPLVLVHRQLSHLYAEPQKSPWCEARSLEHTL
PVNRETLRKRVSNGRASCAPVTGPGSKRDHAHFCAVCSDYASGYHYGVWSECGCKAFFK
RSIQQHNDYICPATNQCTIDKNRRKSCQACRLRKCYEVGMVCKGSGRRERCYRVLVRQ
RSADNEQLHCAGKAKRSGGHAPRVRELLDALSPEQLVLTLLEAEPHPHLISRPSAPFTEA
SMMMSSLTKLADKELVHMSWAKKIPGFEVLSLFDFQVRLLESCWMEVLMMGMLMWRS
IDHPGKLIFAPDLVLDRDEGKCVEGILEIFDMILLATTSRFRELKLQHKEYLCVKAMILLNSS
MYPLVTATQADSSRKLAHHLLNAVTADLVWVIKSGISSQQQSMRLANTLMLSHVRH
ASNKGMEHLLNMKCKNVVPVYDLLLEMLNAMVRGCKSSITGECSPAEDSKSKEGSQ
NPQSQ
Table 1. Protein length and molecular weight (MW) of ERβ isoforms in the database UniProt. Five mouse ERβ isoforms and eight human ERβ isoforms were included in the database UniProt. Names of the human ERβ isoforms appeared in literatures were listed in the brackets.
<table>
<thead>
<tr>
<th>Mouse ERβ</th>
<th>Isoforms</th>
<th>Length</th>
<th>MW (Da)</th>
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<tbody>
<tr>
<td>Isoform 1</td>
<td></td>
<td>530</td>
<td>59,070</td>
</tr>
<tr>
<td>Isoform 2</td>
<td></td>
<td>548</td>
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<tr>
<td>Isoform 3</td>
<td></td>
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<td>48,550</td>
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<tr>
<td>Isoform 4</td>
<td></td>
<td>343</td>
<td>38,185</td>
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<table>
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<th>Isoforms</th>
<th>Length</th>
<th>MW (Da)</th>
</tr>
</thead>
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<td></td>
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<td>Isoform 2 (Beta-2) (CX)</td>
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<tr>
<td>Isoform 7 (Beta-5A)</td>
<td></td>
<td>439</td>
<td>48,614</td>
</tr>
<tr>
<td>Isoform 8 (Beta-6)</td>
<td></td>
<td>375</td>
<td>41,948</td>
</tr>
</tbody>
</table>
Figure 3. Alignment of ERβ isoforms. Alignment was displayed using align tool of the database UniProt.
A  
Mouse ERβ isoforms

B  
Human ERβ isoforms
CHAPTER II
MATERIALS AND METHODS

Tissue specimens

Non-neoplastic human brain tissues (control) and human GBM specimens were collected in Department of Pathology, University of Texas Southwestern Medical Center (Dallas, TX) and Beijing Tiantan Hospital (Beijing, China). Non-neoplastic brain tissues from 9 patients and glioma specimens from 22 patients were used for Western blot analysis and cDNA synthesis. Paraffin-embedded GBM specimens from 2 patients and 2 non-neoplastic brain specimens were obtained from Beijing Tiantan Hospital. Brain tissue arrays were purchased from US Biomax (Rockville, MD). After immunohistochemistry of the tissue arrays, 7 non-neoplastic control tissues, 36 grade II gliomas, 14 grade III gliomas and 6 grade IV gliomas were included into quantitative analysis.

Immunohistochemistry

Immunohistochemistry was done as described previously [95]. Paraffin-embedded slides were deparaffinized in xylene for 10 minutes, and subsequently hydrated in 100%, 95% and 70% ethanol for 10 minute each. Slides were heated in sodium citrate buffer (10 mM Citric Acid, 0.05% Tween 20, pH 6.0) for 30 minutes. After 1-hour blocking using 5% goat serum in superblock buffer (Thermo Scientific), slides were incubated with primary antibody overnight. The second day, the tissue slides were washed 3 times using PBST for 5 minutes each time.
Poly-HRP conjugated secondary antibody were then added to the slides and incubated in a humid chamber for 3 hours. After incubation, the slides were washed using PBST for 3 times again. Diaminobenzidine (DAB) stock (20 x) was diluted in PBS with 0.03% H$_2$O$_2$. Slides were washed in PBST when the color change was obvious. Slides were then subsequently dehydrated in 70%, 95% and 100% ethanol, for 10 minute each. Slides were then merged in xylene and sealed with Permount. For immunofluorescent staining, immunofluorescent labeled secondary antibodies were used. Slides were mounted with DAPI (Invitrogen) and sealed with nail polish.

Images of the tissues on the arrays were captured under the same exposure condition using a Zeiss Microscope (Carl Zeiss). Images were randomly numbered and scored by a researcher (Ali Winters) who did not have information of the specimens.

For immunocytochemistry, human GBM cells were seeded on cover glass and cultured overnight. Cells were washed with PBS for twice and fixed in 10% formalin for 10 minutes. Then cells were permeabilized using 0.1% Triton X-100 in PBS. After washing with PBS for 3 times, cells were incubated with primary antibody for 2 hours. Cells were then washed 3 times using PBST. Cells were incubated with fluorescent labeled secondary antibody for 1 hour. After washing with PBST for 3 times, cover glass was mounted to a slide with DAPI and sealed using nail polish.

**Scoring of immunohistochemistry staining**

To quantitatively analyze the immunohistochemistry staining data, there images were scored. Scoring of the images was conducted as described previously [96]. First, a proportion score was assigned to represent the estimated proportion of positively stained cells (0: none; 1: $<1/100$; 2: 1/100 to 1/10; 3: 1/10 to 1/3; 4: 1/3 to 2/3; 5: > 2/3); then, an intensity score was assigned to represent the average intensity of positive cells (0: no staining; 1: weak, 2:
intermediate; 3: strong). The proportion score and intensity score were added to get a final score (from 0 to 8).

**ERβ antibodies**

Three antibodies for ERβ were used for Western blot and immunohistochemistry: ab H150 (polyclonal, Santa Cruz), ab 1531 (monoclonal, Santa Cruz), ab 3576 (polyclonal, Abcam). Isoform specific antibodies for ERβ1, ERβ2 and ERβ5 were purchased from AbD Serotec (Oxford, UK).

**Cell lines**

Human glioblastoma cell lines U87 and A172 were purchased from ATCC. Human primary astrocytes were kind gifts from Dr. Anuja Ghorpade in the department of Cell Biology & Anatomy in University of North Texas Health Science Center and were prepared as described previously [97].

**Reverse-transcription PCR and Real-time PCR**

RNA was extracted (brain tissue and cultured cells) using Trizol (Invitrogen) following manufacturer’s protocol and quantitated using Nanodrop (Thermo Scientific). One μg of RNA was used for cDNA synthesis. Isoform specific primers for ERβ were designed as described in the previous publication by Moore et al for PCR [79] (Table. 2). PCR products were analyzed in 2% agarose gel. Another set of isoform specific primers by Leung et al [35] were also used for regular PCR and real-time PCR detection of ERβ isoforms (Table. 2).

Real-time PCR was carried out using a ABI 7300 Real-Time PCR System (Applied Biosystems) using SYBR Green PCR Master Mix (Promega): cDNA 1μl; 2 x Mix 10μl; reference dye 0.4μl; Water 8.2μl; Primers 0.4 μl (10 pm/ μl). The fold change in mRNA levels was calculated using the comparative Ct method [98]:
Fold change = 2^{ \text{-delta delta Ct} }

- delta delta Ct = (Ct gene of interest – Ct internal control)_{sample A} - (Ct gene of interest – Ct internal control)_{sample B}

**Plasmid construction, transfection and stable cell line.**

ERβ1 (530 aa) cDNA sequences with a flag tag (GAT TAC AAG GAT GAC GAC GAT AAG) at N-terminal were inserted into pCDNA3.1 (-) vector (Invitrogen) between cloning sites XhoI and HindIII. Flag-ERβ5 (ERβ5: 472 aa) expressing vector was generated by metagenesis from Flag-ERβ1 expressing vector. Plasmids were transfected using lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. To establish stable cell lines, forty-eight hours after transfection, G418 was added to the medium (2 mg/ml) for selection. Medium was changed every two days. G418 resistant clones were selected for culture in medium with 200 µg/ml G418. Expression of ERβ was validated using both Western blot and immunocytochemistry.

**LDH cell viability assay**

Live cells were washed once with PBS and treated with 1% Triton X-100 in PBS to release total LDH. LDH released was measured by a LDH assay using plate reader. Cell viability was calculated based on the amount of LDH released normalized to the standard curve.

**Seahorse XF-24 oxygen consumption analysis**

HEK 293 stable cell lines were seeded in the Seahorse XF-24 plate. After 24 hours, cells were changed to unbuffered DMEM (DMEM base medium supplemented with 25 mM glucose, 10mM sodium pyruvate, 31 mM NaCl, 2 mM Glutamine, pH 7.4) and incubated at 37 °C in a non-CO₂ incubator for 1 hour. Rotenone (100 nM), carbonyl cyanide-p- FCCP (300 nM), and oligomycin (1 µg/ml) were diluted into warm XF24 media and added to port A, B and C of the
cartridge respectively. Eight baseline measurements of oxygen consumption rate (OCR) were collected. Then, the three compounds were injected to the wells at the time points specified. After seahorse analysis, cells in the wells were lysed using protein lysis buffer and protein concentrations were measured using protein assay (Thermo Scientific). Oxygen consumption of each well was normalized to total protein amount (pmoles/min/µg).

**Hypoxia treatment**

Hypoxia condition was created in a humid chamber maintained at 37 °C with CO₂ and N₂ supply. CO₂ concentration was maintained at 5% for cell culture. A control module increased the concentration of N₂ and reduced the concentration O₂ to create a hypoxia environment in the chamber. U87 cells were seeded in culture plates; the plates were then incubated in the hypoxia chamber with 1% O₂ for 24 hours. Cells were washed with PBS once and then Trizol was used for RNA extraction. Real-time PCR was conducted using primers from Leung et al [35].

**Over-expression of constitutively active HIF1α and HIF2α**

Plasmids expressing constitutively active form of HIF1α and HIF2α, designated as P1P2N HIF1α and P1P2N HIF2α, were kind gifts from Dr. Joseph A. Garcia at University of Texas Southwestern Medical Center at Dallas [99]. The plasmids were generated by alanine substitution of the conserved proline or asparagine residues in HIF1α and HIF2α and cloned into pIRES-hrGFP vector [99].

Plasmids were transfected to U87 cells using lipofectamine 2000 (Invitrogen) following the manufacturer’s instruction. Transfection efficiency was evaluated by observing GFP expression. Forty eight hours after transfection, real-time PCR was carried out to evaluate mRNA levels of ERβ1, ERβ2 and ERβ5.

**Cell Growth Curve**
U87 cells were cultured in phenol-red free medium (with 10% charcoal-stripped FBS) at 25,000 cells per well in 12-well plates. At indicated days after seeding, cells were trypsinized and counted using a hemocytometer. Four wells were assigned to each group. Cell counting was conducted by a researcher (Ali Winters) who was blinded to the treatment/group assignment. Each growth curve was validated by duplication or triplication.

**Cell Cycle Analysis**

U87 cells were seeded in 6-well plate and cultured overnight. Medium was replaced with DMEM without FBS and cultured for 6 hour for cell cycle synchronization. Then 10% FBS was added to the medium. After 12 hours and 24 hours, cells were harvested using trypsin and washed twice with PBS. Cells were then fixed in cold 70% ethanol for 30 minutes at 4°C. After fixation, cells were washed twice with PBS and then 50 µl RNase (stock solution 100 µg/ml) was added to treat the cells. Cells were stained using 200 µl propidium iodide (PI) (stock solution 50 µg/ml). After staining, cells were analyzed using a BD LSR II Flow Cytometer.

**Gelatin zymography**

Cells were lysed in RIPA buffer and protein concentration was measured using protein assay. Protein lysates were diluted to 1 µg/µl and mixed with non-reducing protein loading buffer. Gelatin zymography was done as described previously [95]. Briefly, 15 µl of sample was loaded to 10% polyacrylamide gel containing 0.1% gelatin. After electrophoresis, the gel was incubated at 37°C in incubation buffer (2.5% Triton X-100, 5 mM CaCl2, 1 µM ZnCl2) for overnight with gentle agitation. The gel was stained using Coomassie Blue solution (0.25% Coomassie Blue, 45% MeOH, and 10% acetic acid) for 2 hours and then destained with destaining solution (30% MeOH and 10% acetic acid) until the bands became clear. Image was taken using UVP imaging system.
Temozolomide (TMZ) treatment

U87 stable cells were treated with 50 and 100 µM TMZ for 96 hours. Cell viability was determined by measuring LDH release from live cells.

Statistical analysis

Values were expressed as mean ± standard error of mean (SEM). Multiple comparisons were performed by one-way ANOVA. When a significant difference was detected by ANOVA, a post hoc Tukey's test was performed to identify a specific difference between groups. Two-way ANOVA was performed to analyze growth curve data (days and clone/treatment). Between two groups, Student’s t-tests were used to acquire a p value. A p value of *, p < 0.05; or **, p < 0.01 was used to indicate statistical significance.
Table 2. Primer sequences for different isoforms of ERβ.
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<td>Shared forward primer for ERβ:</td>
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<tr>
<td></td>
<td>5′-AGT ATG TAC CCT CTG GTC ACA GC G-3’;</td>
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<tr>
<td></td>
<td>Reverse primers for ERβ isoforms:</td>
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<td></td>
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<td>ERβ2: 5′-GGA TTA CAA TGA TCC CAG AGG GAA ATT G-3’,</td>
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<td></td>
<td>ERβ3: 5′-GCA GTC AAG GTG TCG ACA AAG GCT GC-3’,</td>
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<td></td>
<td>ERβ4: 5′-GGA TTA CAA TGA TCC CAG AGG GAA ATT G-3’,</td>
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<tr>
<td></td>
<td>ERβ5: 5′-CTT TAG GCC ACC GAG TTG ATT AGA G-3’,</td>
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<td>actin forward: 5′-CCA ACA CAG TGC TGT CTG G-3’,</td>
</tr>
<tr>
<td></td>
<td>actin reverse: 5′-TGC TGA TCC ACA TCT GCT G-3’.</td>
</tr>
<tr>
<td>Leung, Mak et al. 2006</td>
<td>ERβ1 forward: 5′-GTC AGG CAT GCG AGT AAG AA-3’,</td>
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<tr>
<td></td>
<td>ERβ1 reverse: 5′-GGG AGC CCT CTT TGC TTT TA-3’,</td>
</tr>
<tr>
<td></td>
<td>ERβ2 forward: 5′-TCT CCT CCC AGC AGC AAT CC-3’,</td>
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<td></td>
<td>ERβ2 reverse: 5′-GTT CAC TGC TCC ATC GTT GC-3’,</td>
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<td>GAPDH reverse: 5′-GGA GGA GTG GGT TGC GCT GT-3’.</td>
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CHAPTER III
RESULTS

Increased ERβ5 expression in human glioma

First, expression of ERβ in human GBM specimens and non-neoplastic brain specimens was evaluated by Western blot using 3 different antibodies (H150, 1531 and 3576). ERβ antibodies are available from different companies but are not well characterized [100]. Three different antibodies were applied in western blot to cross-validate the results. With each antibody, an increased ERβ expression was detected in a GBM tissue from a female patient as compared with a non-neoplastic brain tissue from a female patient (Fig. 4). The immunoreactive band detected in Western blots was of a smaller molecular weight than that of full length ERβ1 (59 kDa).

To determine the expression of ERβ isoforms in human GBM, PCR primers for ERβ isoform 1, 2, 3, 4 and 5 from previous publication was applied [79]. In human GBM cDNA from the same patient as in Fig. 4, only ERβ5 was detected by PCR (Fig. 5). This was confirmed by PCR using an alternative set of primers for ERβ1, ERβ2, ERβ4 and ERβ5 [35]. This suggests ERβ5 as the main isoform expressed in human GBM.

To confirm this, isoform specific antibodies for ERβ1, ERβ2 and ERβ5 were used for Western blot and immunohistochemistry. Consistent with the PCR results, higher level of ERβ5 was detected in human GBM by Western blot (Fig. 6A) and immunohistochemistry (Fig. 6B) as
compared with non-neoplastic brain tissue. When using ERβ1 or ERβ2 specific antibodies, no band was detected in Western blot and no positive staining was obtained in immunohistochemistry for either non-neoplastic brain tissue or GBM (data not shown). Double immunofluorescence staining demonstrated that ERβ5 positive cancer cells expressed astrocyte marker glial fibrillary acidic protein (GFAP), and that ERβ5 was mainly localized in the nucleus (Fig. 7).

Another group of frozen human brain GBM tissues and non-neoplastic brain tissues from both male and female patients were collected. Frozen human brain tissues were processed for protein and RNA extraction. Western blot analysis indicated an increase of ERβ5 expression in most of the human GBM tissues from both male and female patients as compared with non-neoplastic brain tissues (Fig. 8A, B). RNA was extracted from a limited number of brain tissues from female patients, real-time PCR demonstrated increased mRNA levels of ERβ5 in GBM tissues from female patients, although not significant (p>0.05, control: n = 3; GBM: n = 8) (Fig. 8C). For ERβ1, ERβ2 and ERβ4, no amplification curve was observed in the real-time PCR experiment, indicating low expression level of these three isoforms.

To compare ERβ5 expression in different grade gliomas, immunohistochemistry was conducted on human brain tissue array with gliomas of grade II, III and IV. As indicated by the representative images (Fig. 9A), ERβ5 expression was increased in gliomas compared with non-neoplastic brain tissue and grade IV glioma (GBM) indicated higher expression than grade II and grade III. ERβ5 indicated nuclear localizations in gliomas. After scoring of all the images from immunohistochemistry, statistical analysis indicated a significant increase (p<0.05) of ERβ5 expression in gliomas as compared with non-neoplastic brain tissues (Fig. 9B). ERβ5 expression
was not significantly different between different grade gliomas, although there was a trend of increase as tumor grade increased.

ERβ expression was then examined in human GBM cell lines U87 and A172, which originated from female and male patients, respectively. Using antibodies specific for ERβ1, ERβ2 or ERβ5, we identified the expression of all the three isoforms in both U87 (Fig. 10A) and A172 (Fig. 10B) cells. All three isoforms were localized in the nucleus as indicated by their co-staining with DAPI. Expression of ERβ1, ERβ2 and ERβ5 in U87 and A172 was also confirmed by PCR using two different sets of primers (Fig. 11A, B).

To compare with the human GBM cell line, expression of ERβ in human primary astrocytes was evaluated by immunocytochemistry using ERβ antibody 1531; however, no positive staining was observed (Fig. 12A). Monoclonal antibody ab1531 was applied because it was more specific for ERβ compared with abH150 or ab3576 from our experience in Western blot analysis. As a positive control, 24 hours after transient transfection of with Flag-ERβ1 plasmid, positive staining for ERβ was detected using the same antibody under the same experimental condition (Fig. 12B).
**Figure 4.** Increased expression of ERβ in a female GBM. Western blot analysis using 3 different ERβ antibodies indicated increased expression of ERβ in human GBM tissue compared with non-neoplastic brain tissue (CTL). ERβ expressed in these tissues indicated a molecular weight between 46 kDa and 58 kDa indicated by the protein marker.
CTL  GBM

58kDa —  
46kDa —  
58kDa —  
46kDa —  
58kDa —  
46kDa —  

ab H150  
ab 1531  
ab 3576  
actin
Figure 5. Expression of ERβ5 in human GBM cDNA. PCR using two different sets of isoform specific primers was applied to detect different ERβ isoforms in human GBM. Both sets of primers indicated existence of ERβ5 in human GBM cDNA.
**Figure 6.** Detection of ERβ5 in human GBM using ERβ5 specific antibody.  
**A.** Representative Western blots of ERβ5 expression in a human GBM specimen and a non-neoplastic brain tissue.  
**B.** Immunohistochemistry of human GBM specimen using ERβ5 specific antibody.
**Figure 7.** Nuclear localization of ERβ5 and co-localization with GFAP in human GBM. Double-staining of ERβ5 (green) and GFAP (Red) in human GBM specimen. Nucleus was stained by DAPI (blue).
Figure 8. Increased expression of ERβ5 in human glioma. A. Western blot analysis of ERβ5 expression in non-neoplastic (control) and GBM specimens from female patients. B. ERβ5 expression in non-neoplastic and GBM specimens from male patients. C. Real-time PCR for ERβ5 mRNA in female patients (CTL: n = 3; GBM: n = 8).
**Figure 9.** ERβ5 immunohistochemistry in human brain tissue arrays. **A.** Representative staining of non-neoplastic brain tissues and different grade (II, III and IV) glioma specimens. **B.** Quantitative analysis of ERβ5 immunohistochemistry in human brain tissue arrays (CTL: n = 7; Grade II: n = 36; Grade III: n = 14; Grade IV: n = 6; **, p<0.01, *, p<0.05).**
Figure 10. ERβ expression in GBM cell lines. U87 (A) and A172 (B) were originated from a female patient and a male patient, respectively. Immunocytochemistry was conducted using monoclonal antibody 1531 and isoform specific antibodies for ERβ1, ERβ2 and ERβ5. Nucleus was stained by DAPI (blue).
Figure 11. PCR using ERβ isoform specific primers in GBM cell lines. **A.** PCR using primers by Moore et al. in cDNA of U87 and A172 cells. **B.** PCR using primers by Leung et al. in cDNA of U87 and A172 cells.
Figure 12. Negative ERβ expression in human primary astrocytes. A. Immunocytochemistry of ERβ using 1531 antibody in human primary astrocytes. B. Immunocytochemistry of ERβ using 1531 antibody in human primary astrocytes at 24 hours after transient transfection of Flag-ERβ1 plasmid for positive control.
**Hypoxia increased ERβ expression in U87 cells**

Hypoxia increased ERβ mRNA levels in rat hippocampus neuronal cultures [101]. Gliomas, like many other solid tumors, are under extensive hypoxic stress given their rapid proliferation [102]. We proposed that increased expression of ERβ5 in human glioma could be caused by the hypoxia condition in the tumor.

To test this, U87 cells were treated with hypoxia in a humid chamber with 1% O₂ supply. After 24 hours’ hypoxia treatment, U87 cells were collected for RNA extraction and cDNA synthesis. Expression of ERβ1, ERβ2, and ERβ5 were evaluated by real-time PCR. Statistical analysis indicated that hypoxia significantly increased mRNA levels of ERβ1 (p<0.05), ERβ2 (p<0.05), and ERβ5 (p<0.01) (Fig. 13). ERβ1, ERβ2, and ERβ5 mRNA levels were increased by 31, 14 and 13-fold respectively.

Activation of HIF is one of the major cellular events when cells are exposed to hypoxia. HIF is an αβ heterodimer belong to the basic-helix-loop-helix transcription factors. Both HIF-α and HIF-β subunits have a series of isoforms. HIF1β is a constitutive nuclear protein. HIF1α and HIF2α are inducible by hypoxia. In oxygenated conditions, hydroxylation at prolyl residues (Pro402 and Pro564 in human HIF1α) lead to degradation of HIF-α subunits [103, 104]. During hypoxia, HIF hydroxylases are inactivated, allowing HIF to escape degradation [105]. In the promoter region of ERβ, there is an E-box which overlaps with the HIF binding sequence (5’-RCGTG-3’) [106]. This is a potential HIF binding site (Fig. 14A). We hypothesized that the hypoxia-induced increase of ERβ expression is mediated by the activation of HIF.

U87 cells were transfected with plasmids P1P2N HIF1α and P1P2N HIF2α to express constitutively active HIF1α or HIF2α. Transfection efficiency was evaluated by the green fluorescence. Trials with more than 60% cells transfected were used for following experiments.
At 48 hours after transfection, RNA was extracted and real-time PCR was conducted to evaluate mRNA levels of ERβ1, ERβ2 and ERβ5. Compared with vector transfection, both P1P2N HIF1α and P1P2N HIF2α transfection significantly increased mRNA levels of ERβ1, ERβ2, and ERβ5 (p<0.05) (Fig. 14B), although, the fold increase was not as significant as the increase after hypoxia treatment.
**Figure 13.** Hypoxia increased ERβ transcription in U87 cells. U87 cells were treated with hypoxia condition (1% O₂) for 24 hours. Real-time PCR analysis was conducted to evaluate ERβ1, ERβ2 and ERβ5 mRNA after hypoxia (n = 4; **, p<0.01, *, p<0.05).
**Figure 14.** Activation of HIF increased transcription of ERβ in U87 cells. **A.** Potential HIF binding site (CACGTG) in the promoter of human ERβ. **B.** Real-time PCR analysis of ERβ1, ERβ2 and ERβ5 mRNA in U87 cells 48 hours after transfection with plasmids (P1P2N HIF1α, P1P2N HIF2α) expressing constitutively active form of HIF1α or HIF2α (n = 6; *, p<0.05).
A

Potential HIF binding site

GCATATTCTCAGCCCTACTCCAAGACCTCTTAAAATCTCAGACTGCTGGGCTCGGGGAGCGCATACTGTGCGCCACCTGTTGTTGGAAGACAGTCCGTGAGTGATGCACCTGCTCTCATTAGAGTCAAGTCCAGGCGGGGTCTGCG
CCTCACTTGTTGAGGGTGGAAGACAGTCCGTGAGTGATGCACCTGCTCTCATTAGAGTCAAGTCCAGGCGGGGTCTGCG

TCTCAGACTGCTGGGCTCGGGGAGCGCATACTGTGCGCCACCTGTTGTTGGAAGACAGTCCGTGAGTGATGCACCTGCTCTCATTAGAGTCAAGTCCAGGCGGGGTCTGCG
CCTCACTTGTTGAGGGTGGAAGACAGTCCGTGAGTGATGCACCTGCTCTCATTAGAGTCAAGTCCAGGCGGGGTCTGCG

Start codon

B

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<th>ERβ5</th>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>HIF2α</td>
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</table>

mRNA fold Change

* significant difference
ERβ inhibited PI3K/AKT/mTOR pathway and MAPK/ERK pathway

ERβ1 and ERβ5 have identical sequence from N-terminal to amino acid 468 (Fig. 15). To investigate their functions, expression vectors were constructed. First, ERβ1 was inserted to a pCDNA3.1(-) vector with a flag tag at N-terminal. Then a site-directed mutagenesis kit was used to generate a vector for expression of flag-ERβ5 (Fig. 15). To test the vector, the plasmids were transfected into HEK 293 cells, which do not have endogenous ERβ expression. After transient transfection, expression of ERβ1 and ERβ5 was detected using anti-flag antibody (Fig. 16A). Nuclear localization of ERβ1 and ERβ5 was observed by immunofluorescent staining using anti-flag antibody in the cells with transient ERβ1 or ERβ5 transfection. The protein localization was not affected by the treatment of 17β-estradiol (E2, 100nM) (Fig. 16B).

HEK 293 stable cell lines expressing ERβ1 or ERβ5 were made by Ethan Poteet. Expression of ERβ1 and ERβ5 in the stable cell lines were confirmed by Western blot (Fig. 17) and immunocytochemistry (data not shown). Activation of the PI3K/AKT/mTOR and MAPK/ERK pathways was evaluated. In both ERβ1 and ERβ5 stable cell lines, a significant increase of PTEN expression was observed (p<0.05). Consistently, decreased phosphorylation of AKT and P70S6K, a component of the mTOR pathway, was observed in both cell lines. Estrogen treatment (100nM) did not induce further change (Fig.17). This suggests that ERβ1 and ERβ5 could inhibit the PI3K/AKT/mTOR pathway through upregulation of PTEN expression in an estrogen-independent manner.

In ERβ5, but not ERβ1 stable cell line, decreased phosphorylation of c-Raf, MEK and ERK were observed and it appeared in a ligand-independent manner (Fig. 18). Both ERβ1 and ERβ5 inhibited proliferation of HEK293 cells (p<0.05) (Fig. 19).
A recent study indicated that PTEN elevation can increase energy expenditure and mitochondrial oxidative phosphorylation to reduce the Warburg effect of tumor cells [107]. Since ERβ1 or ERβ5 increased PTEN expression in the HEK 293 stable cell lines, they might also change the metabolism of the cells. Seahorse was used to evaluate oxygen consumption rate of HEK 293 stable cell lines with expression of either ERβ1 or ERβ5. A significant increase of baseline oxygen consumption rate was observed in ERβ1 and ERβ5 expressing cells as compared with vector control (p<0.05) (Fig. 20).
**Figure 15.** Cloning ERβ1 and ERβ5. **A.** Human ERβ1 and ERβ5 proteins differ at C-terminal sequence. **B.** Primers used to add flag tag to ERβ1 and clone it into pCDNA3.1(-) vector between XhoI and HindIII restriction site. Flag-ERβ5 expressing vector was generated by metagenesis from Flag-ERβ1 expressing vector using the mutation primers.
A

361  VLDRDEGKCGEILIFDMLLATTTRFRELKLQHKEYLICVKAMILLNNSMYPLVTATQDA  420  ERβ1
361  VLDRDEGKCGEILIFDMLLATTTRFRELKLQHKEYLICVKAMILLNNSMYPLVTATQDA  420  ERβ5

------------------------------------------
421  DSSRLAKLHLLNAVTDALVWVIASGISSQQSMRLANLLMLSSHVRHASNKGMEHLLNME  480
421  DSSRLAKLHLLNAVTDALVWVIASGISSQQSMRLANLLMLSSHVRHARYAP---------  472

------------------------------------------
481  CKNVVFYDOLLLEMLNAHVRLRGCKSITGSECSPAEFSKSGEQSNPSQ  530
473  ----------------------------------------------------------  472

B

Forward Primer with Flag tag for human ERbeta (530aa), 80bp

XhoI  KOZAK  Flag tag

5’-GAC CTC GAG GCC ACC ATG GAT TAC AAG GAT GAC GAC GAT AAG ATA GATATAAA
AAAACTCACC ATCTAGCTT AATTCTCC-3’

Stop HindIII
Reverse Primer: 29bp 3’-GTCTTTGGG TGTCAAGTC ACT TTCGAA TC-5’

Mutation primers to generate Flag-ERβ5 pCDNA3.1 vector
1. hERB5(mut)R
5’ CGTTAGGGCGCGTACCTCGACGCTGTGGGACAG 3’

2. hERB5(mut)F
5’ ACCGCTGATC AGCCTCGACT G3’
**Figure 16.** Transient transfection of plasmids expressing flag-ERβ1 and flag-ERβ5. **A.** Western blot of ERβ1 and ERβ5 expression in HEK 293 cells 24 hours after transient transfection using anti-flag antibody. **B.** Immunocytochemistry using anti-flag antibody in HEK 293 cells 24 hours after transfection of plasmids expressing flag-ERβ1 and flag-ERβ5. Nucleus was stained with DAPI (blue).
Figure 17. Over-expression of ERβ1 and ERβ5 increased PTEN expression and inhibited activation of the PI3K/AKT pathway. A. Western blot analysis of ERβ1 and ERβ5 expression in HEK 293 stable cell lines using anti-flag antibody. Western blot analysis of PTEN expression and activity of PI3K/AKT pathway in the clones with and without E2 treatment (100 nM for 48 hours). B. PTEN expression normalized to actin; quantitative analysis indicated a significant increase in PTEN protein levels in ERβ1 and ERβ5 stable cell lines (n=3, *, p<0.05).
**Figure 18.** Over-expression of ERβ5 inhibited activation of the MAPK/ERK pathway. Western blot analysis of MAPK/ERK pathway activation in HEK 293 stable cell lines expressing ERβ1 or ERβ5 with and without E2 treatment (100 nM for 48 hours).
Figure 19. Over-expression of ERβ1 and ERβ5 inhibited HEK 293 cell proliferation (*, p<0.05).

Same number of cells were seeded; after 4 days’ culture, cell viability were evaluated by
detecting total LDH release (n=8).
Figure 20. Over-expression of ERβ1 and ERβ5 increased oxygen consumption. Oxygen consumption rates (OCR) of the clones were evaluated using Seahorse XF24. Oxygen consumption rate was normalized to protein amount of each well. Bar graph shows baseline oxygen consumption rate of the stable cell lines before injection of the compounds (*, p<0.05). Seahorse experiments were conducted with the help from Ethan Poteet.
Oxygen consumption

![Graph showing OCR (pMoles/min)/Protein (ug) over time with different treatments: Oligomycin, FCCP, Rotenone.]

Base line oxygen consumption

![Bar graph comparing Vector, ERβ1, and ERβ5 with asterisks indicating statistical significance.]

Vector ERβ1 ERβ5
ERβ1 and ERβ5 inhibited proliferation of U87 cells

First, effects of ERβ agonist DPN on GBM cell proliferation were determined. U87 cells were treated (in phenol red free medium with 10% charcoal-stripped FBS) with E2 or DPN at concentrations of 100 nM. A six-day growth curve indicated no obvious change in the cell growth rate (Fig. 21). Growth curve analysis of A172 cells was also conducted and no obvious change was observed with E2 or DPN treatment (data not shown).

Since our data indicated that ERβ could inhibit PI3K/AKT/mTOR and MAPK/ERK pathways in a ligand-independent manner, we hypothesized that ERβ might inhibit GBM cell proliferation in a similar ligand-independent fashion. To determine the effects of ERβ on glioma cell proliferation, ERβ1 or ERβ5 was over-expressed in U87 cells. U87 stable cell lines with over-expression of either ERβ1 or ERβ5 showed a significantly lower growth rate as compared with vector control stable cell line (p<0.01) (Fig. 22). ERβ5 expressing U87 cells had an even lower growth rate than ERβ1 expressing cells (p<0.01) even though ERβ1 expression level seemed to be a little higher than ERβ5 as indicated in Western blot (Fig. 23). Western blot analysis indicated that ERβ5 also decreased activation of ERK in U87 cells. There was decreased MMP2 activation in both ERβ1 and ERβ5 expressing U87 stable cell lines, which was independent of E2 treatment (Fig. 23). Matrix metalloproteinases (MMPs) are required for degradation of extracellular matrix for invasion of tumor cells.

Cell cycle analysis indicated that at 12 hours, U87 stable cell lines with ERβ1 or ERβ5 over-expression indicated reduction of cell number in the proliferative fraction S+G2/M phase (S+G2/M phase); at 24 hours, both clones indicated significantly reduction (p<0.01) of cells in the S+G2/M phase and ERβ5 over-expression induced a larger reduction than ERβ1 (Fig. 24).
Figure 21. U87 cell proliferation in the presence of 100 nM E2, 100 nM DPN, or vehicle control (CTL) (n = 4). U87 cells were seeded in 12 well plates in phenol red-free medium with 10% charcoal-stripped FBS. Cell counting was conducted by Ali Winters.
The graph shows the variation of cell number over days for three different conditions: CTL, E2, and DPN. The x-axis represents the days, ranging from 0 to 6, and the y-axis represents the cell number, ranging from 0 to 1,000,000. The CTL condition is represented by open circles, the E2 condition by open squares, and the DPN condition by open diamonds. The graph indicates an increasing trend in cell number over time for all conditions.
Figure 22. ERβ inhibited U87 cell proliferation. Growth curves of U87 stable cell lines expressing ERβ1, ERβ5, or vector (n = 4; **, p<0.01). Cell counting was conducted by Ali Winters.
Figure 23. ERβ5 inhibited MAPK/ERK pathway in U87 cells. Western blot analysis of ERβ1, ERβ5, pERK, and total ERK in U87 stable cell lines. Gelatin zymorgraphy indicated total MMP2 activity in the stable cell lines.
**Figure 24.** ERβ reduced proliferating fraction cells at S+G2/M phase. 

**A.** Flowcytometer analysis of cell cycle of the U87 stable cell lines using PI staining after 12 hours and 24 hours culture.  

**B.** Quantitative analysis of S+G2/M phase cells after 24 hours culture (n=3; ***, p<0.01).
A

12 hours  
Count

24 hours  
Count

Vector  
29.1%

ERβ1  
23.5%

ERβ5  
17.8%

PE-Texas Red-A

B

24 hours  
S+G2/M (%)

Vector  
**

ERβ1  
**

ERβ5  

S+G2/M phase (%)
**ERβ decreased sensitivity to temozolomide (TMZ) treatment**

Temozolomide (TMZ) is an alkylating agent and current first line chemotherapeutic drug for GBM. TMZ causes DNA damage and triggers cell cycle arrest at G2/M phase [108, 109]. However, here we found that ERβ reduced cells at S+G2/M phase, which we suspect might affect sensitivity of GBM cells to TMZ treatment. To test the hypothesis, U87 stable cell lines were treated with 50 and 100µM TMZ for 96 hours, cell viability evaluation indicated that in the vector control U87 cells, 100µM TMZ significantly reduced live cell (p<0.01), while both ERβ1 and ERβ5 over-expressed U87 indicated decreased sensitivity to TMZ treatment (Fig. 25).

**Tamoxifen and 4-hydroxyl-tamoxifen (4OHT) increased ERβ protein level**

Previous studies have indicated that tamoxifen, at micro-molar concentrations, could inhibit protein kinase C (PKC) activity [110] and induce toxicity in GBM cell lines, including U87 and A172 [111]. Antiproliferative effect of 4OHT was also observed in U87 cells with an IC50 of 1.46 ± 0.16 µM [112]. A recent study demonstrated that the tamoxifen metabolite endoxifen increased protein level of ERβ [113]. In HEK 293 ERβ stable cell lines and U87 cells, both tamoxifen and 4OHT at 100 nM concentrations increased protein levels of ERβ1 and ERβ5 (Fig. 26A, B). We further tested the effects of 100nM tamoxifen and 4OHT on U87 growth. The results indicated that, at 100nM concentration, both tamoxifen and 4OHT could inhibit the proliferation of U87 cells (p<0.05) (Fig. 26C). This suggests that the suppressive effects of tamoxifen and 4OHT on U87 cell proliferation may be due in part to the upregulation of ERβ expression in the cells.
**Figure 25.** ERβ decreased sensitivity to TMZ treatment in U87 cells. U87 stable cell lines were treated for 96 hours with 50 and 100µM TMZ. Cell viability was determined using LDH assay (**, p<0.01).
Figure 26. Low concentration of tamoxifen and 4OHT increased ERβ protein level and inhibited U87 cell proliferation. A. Western blot of ERβ in HEK 293 stable cell lines expressing ERβ1 and ERβ5 after treatment with 100 nM tamoxifen or 100 nM 4OHT for 48 hours. B. Western blot of ERβ1 in U87 cell treated with tamoxifen or 4OHT for 48 hours. C. Growth curves of U87 in the presence of vehicle (medium), 100 nM tamoxifen, or 100 nM 4OHT (*p<0.05).
CHAPTER IV

DISCUSSION

ERβ has been shown to be involved in carcinogenesis and cancer progression and recognized as a cancer brake in the ovary, prostate, and colon [61]. The identification of ERβ isoforms has added further complexity to the action of ERβ [79]. In the current study, we determined the expression of each ERβ isoform in human glioma using immunohistochemistry, Western blot, and real time PCR. We have found that ERβ5 is the main ERβ isoform expressed in human glioma. Consistently, ERβ5 was also identified in two human glioma cell lines, U87 and A172, by PCR and immunohistochemistry. The identified expression of ERβ1, ERβ2 and ERβ4 in U87 and A172 cells might be due to the in vitro culture conditions which could not precisely replicate the glioma cells’ in vivo micro-environment. Expression level of ERβ was low in non-neoplastic brain tissue as indicated by Western blot and PCR. In primary human astrocytes, no obvious positive staining for ERβ was observed by immunocytochemistry. However, in human glioma specimens, there was a significant increase of ERβ5 expression as compared with non-neoplastic brain tissue. We also observed a trend of increased ERβ5 expression as the grade of glioma increased, although it was not statistically different probably due to the limited sample size. These results contradict to two recent studies, which reported that ERβ expression declined in human glioma as tumor grade increased [91, 92]. The discrepancy might be due to the different methods between current study and previous studies. In addition,
the previous studies didn’t differentiate each ERβ isoform. The present study argues that future studies should be conducted using ERβ isoform specific antibodies and real-time PCR to further investigate the expression of ERβ isoform in human glioma.

ERβ isoform mRNA sequence analysis has identified two different 5′-untranslated regions (5′UTR) composed of two distinct untranslated first exons, indicating that transcription of different human ERβ isoforms occurs from at least two different promoters, namely 0K and 0N [114]. Further analysis has identified that ERβ5 is regulated exclusively by promoter 0K, while ERβ1, ERβ2 and ERβ4 are under the control of both promoter 0K and promoter 0N [115]. The reduction of ERβ1, ERβ2, and ERβ4 transcription has been attributed to the methylation of promoter 0N in prostate, breast, and ovarian cancers, while promoter OK was not methylated [67-69, 116]. DNA methylation of promoter region CpG islands can lead to the inactivation of gene expression by changing chromatin structure [117, 118]. Extensive DNA methylation in human GBM has been reported [119]. The higher levels of ERβ5 in glioma demonstrated in current study indicates that the activation of promoter 0K, while the absence of ERβ1, ERβ2, and ERβ4 expression in glioma suggests that promoter 0N might be silenced by methylation in glioma.

An E-box (5′-CACGTG-3′, -94/-99) has been found in the promoter region of ERβ [106], which overlaps the HIF binding sequence (5′-RCGTG-3′). We postulated that the increased expression of ERβ5 in glioma was caused by hypoxia that commonly exists in glioma [120]. Consistently, in U87 cells, hypoxia increased the transcription of ERβ1, ERβ2 and ERβ5. Furthermore, under normoxia condition, significant increase in ERβ1, ERβ2 and ERβ5 transcription was induced in U87 cells by transfection of constitutively active HIF1α or HIF2α.
We hypothesize that in human glioma, DNA methylation and hypoxia together regulated expression of ERβ isoforms. When promoter 0N and 0K are not methylated, hypoxia will activate both promoters and increase transcription of all the ERβ isoforms they regulated; this might explain the increased expression of all the three ERβ isoforms in U87 cells after hypoxia treatment. However, when promoter 0N is methylated, only promoter 0K can be activated, and only ERβ5 can be expressed; this might be the cause of ERβ5 expression alone in human glioma we have reported (Fig. 27). We would suggest future studies to investigate the methylation status of ERβ promoter region in human glioma as well as the cell lines to test this hypothesis.

Both *in vitro* and *in vivo* studies indicate that ERβ inhibits proliferation and invasion of breast cancer cells and prevents tumor formation. ERβ over-expression inhibited the proliferation of MDA-MB-231 breast cancer cells in a ligand independent manner; migration of the cells were also inhibited [70]. ERβ inhibited tumor formation in MCF7 cell xenograft model [71]. Different mechanisms have been proposed for the anti-proliferative action of ERβ [61]. In breast cancer, ERβ inhibited transcription of genes activated by ERα; ERβ also activated transcription of genes that inhibit cancer cell proliferation [45]. ERβ can change cell cycle and reduce S+G2/M phase cells [76]. ERβ inhibited HIF1 transcriptional activity [78]. Previous study demonstrated that ERβ can increase expression of tumor suppressor PTEN, which will suppress activation of the PI3K/AKT pathway [121].

However, functional analysis has been mainly focusing on ERβ1. Leung, et al. reported that ERβ5 cannot form homodimer and does not have innate activity because of loss of helix 12 in the ligand binding domain [35]. Emerging evidence suggests association between ERβ5 expression level and clinical outcomes in cancer patients. In tamoxifen-treated breast cancer, ERβ5 expression was positively associated with better survival [84, 122]. However, in prostate
cancer, ERβ5 was associated with poor prognosis, and over-expression of ERβ5 promoted cancer cell migration and invasion [123]. These data suggest that the functions of ERβ5 might be cancer type specific. *In vitro* analysis indicated that ERβ5 can activate gene transcription without ligand binding [124]. In the current study, we investigated the effects of ERβ5 on two important oncogenic pathways for glioma: PI3K/AKT/mTOR and MAPK/ERK. We found that ERβ5 inhibited both pathways in HEK 293 cells in ligand-independent manners.

Both ERβ1 and ERβ5 inhibited the PI3K/AKT/mTOR pathway by increasing PTEN expression. PTEN is a tumor suppressor that antagonizes the PI3K/AKT pathway by dephosphorylating phosphatidylinositol (3,4,5)-trisphosphate (PIP3) to phosphatidylinositol (4,5)-bisphosphate (PIP2) [125]. AKT has multiple targets that regulate various cellular processes, such as cell survival, growth, apoptosis proliferation and metabolism [126, 127]. An elevated level of PTEN, *in vivo*, has been shown to increase oxygen consumption, which could reduce the Warburg effect in tumor cells [107]. Warburg effect is the phenomenon that tumor cells tend to use the less efficient aerobic glycolysis to generate energy rather than using oxidative phosphorylation. This altered metabolism is important for tumor growth and survival [128]. Agents that can inhibit glycolysis have shown promising anticancer effects [129, 130]. In ERβ1 or ERβ5 expressing HEK 293 stable cell lines, we observed an increased cellular oxygen consumption rate.

In breast cancer cells, ERβ increased PTEN expression in a ligand-independent manner [121]. In the promoter region of PTEN, no estrogen-responsive element (ERE) or ERE-like sequence has been identified [131]. Yang et al. proposed that estrogen receptor could bind to the C terminus of PTEN and reduce protein turnover [132]. Wickramasinghe et al. proposed that estradiol bound estrogen receptor downregulates miR-21 level and releases suppression of miR-
21 on PTEN expression [133]. A recent study indicated that ERβ could bind to the promoter region of PTEN through Sp1 and increase PTEN transcription [134]. We predict that upregulating PTEN expression is a common anti-proliferative mechanism for ERβ. However, PTEN mutation is very common in human glioma [15, 19, 135, 136]; in U87 cells, an in frame deletion causes the cells to be devoid of functional PTEN [137].

The inhibitory action of ERβ1 and ERβ5 on U87 proliferation identified in this chapter suggests PTEN-independent mechanisms. Indeed, both ERβ1 and ERβ5 decreased MMP2 expression and ERβ5 inhibited the MAPK/ERK pathway. MMPs have been found to be involved in tumor progression; inhibition of MMP2 expression could suppress the migration and invasion of tumor cells [138].

Consistent with previous studies [75, 76], our data indicated that after over-expression of ERβ1 in U87 cells, cell number at the proliferative fraction S+G2/M phase was significantly reduced; interestingly, ERβ5 over-expression produced more dramatic reduction in S+G2/M phase cells. A recent study indicated that ERβ agonist DPN inhibited proliferation of GBM cell lines including U87 cell line [92]. However, we were not able to detect the inhibitory effect of DPN on U87 cell proliferation in the growth curve analysis. Moreover, another previous study demonstrated that DPN increased proliferation of medulloblastoma cells [93]. More detailed studies should be done to investigate DPN’s effects on different types of brain tumors before any clinical test in human is conducted.

In U87 cells, ERβ reduced S+G2/M phase cells and reduced sensitivity to TMZ treatment. TMZ is the first line drug for chemotherapy of GBM, however, some GBMs present innate resistance to TMZ or develop resistance during treatment [139, 140]. Methylation of the promoter of O-6-methylguanine-DNA methyltransferase (MGMT) has been suggested as a
predictor of sensitivity to TMZ treatment [140]. MGMT is a DNA repair protein that transfers a methyl group from the O\(^6\)-position of a guanine DNA nucleotide, while TMZ and other DNA alkylating chemotherapeutic drugs add an alkyl group to the O\(^6\)-position of guanine. Our data suggests that expression of ER\(\beta\)5 might also predict the sensitivity of GBM to TMZ treatment; we would suggest future clinical studies to investigate the correlation between ER\(\beta\) expression level and outcome of TMZ treatment; data from these studies could suggest whether ER\(\beta\) can be used as a molecular marker for personalize treatment.

The tamoxifen metabolite, endoxifen, can induce ER\(\alpha/\beta\) heterodimer formation to stabilize ER\(\beta\) protein [113]. In current study, tamoxifen and 4OHT increased ER\(\beta\) protein levels. However, no ER\(\alpha\) expression was found in HEK 293 cells stably expressing ER\(\beta\)1 and ER\(\beta\)5, suggesting alternative mechanisms might contribute to the tamoxifen and 4OHT induced reduction of ER\(\beta\) protein turnover. In breast cancer, higher ER\(\beta\) expression level has been associated with higher sensitivity to tamoxifen treatment [65, 66]. Clinical trials have indicated that a subgroup of glioma patients responded to tamoxifen treatment [141, 142]. We predict that ER\(\beta\) expression level in glioma might be correlated with the outcome of tamoxifen treatment. Future studies are warranted to evaluate the expression of ER\(\beta\) in glioma and examine the correlation between ER\(\beta\) expression and the response to tamoxifen treatment. Further, our data suggests a possibility that tamoxifen might be more effective for patients with higher expression of ER\(\beta\).

In summary, current study detected elevated expression of ER\(\beta\)5 in human glioma, which might be induced by hypoxia via HIF signaling. This study indicated that ER\(\beta\)5 functions as a potent suppressor against glioma progression through multiple mechanisms including suppression of oncogenic PI3K/AKT/mTOR and MAPK/ERK pathways in a ligand independent
manner (Fig. 28). We also reported potential association between ERβ expression and outcomes of TMZ or tamoxifen treatment, which might be of practical clinical values.

Future studies should investigate the expression of ERβ isoforms in TMZ and tamoxifen treated patients. If correlation can be built between ERβ isoform expression level and the clinical outcome of treatments, personalized treatment would be possible to increase efficacy and reduce potential side effects. Also, expression of ERβ isoforms and methylation of ERβ promoters ON and OK should be investigated, which unveil the regulation mechanism of ERβ expression in human glioma.
Figure 27. Hypothesis on the transcriptional regulation of ERβ expression in human glioma. A. When there is no methylation in promoter 0N, both promoters can be activated and all the ERβ isoforms will be transcribed. B. When promoter 0N is methylated only promoter 0K can be activated and only ERβ5 can be transcribed.
Figure 28. ERβ5 inhibits glioma progression through multiple mechanisms including suppression of oncogenic PI3K/AKT/mTOR and MAPK/ERK pathways (OCR, oxygen consumption rate; OXPHOS, oxidative phosphorylation).
**BIBLIOGRAPHY**


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