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Involvement of estrogen receptor β in the progression of glioma

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Abstract

Emerging evidence suggests a decline of ER β expression in various peripheral cancers. ER β has been proposed as a cancer brake that inhibits tumor proliferation. In the current study, we have identified ER β 5 as the predominant isoform of ER β in human glioma and its expression was significantly increased in human glioma as compared with non-neoplastic brain tissue. Hypoxia and activation of hypoxia inducible factor (HIF) increased ER β transcription in U87 cells, suggesting elevated ER β expression in glioma might be induced by the hypoxic stress in the tumor. Over-expression of either ER β 1 or ER β 5 increased PTEN expression and inhibited activation of the PI3K/AKT/mTOR pathway. In addition, ER β 5 inhibited the MAPK/ERK pathway. In U87 cells, ER β 1 and ER β 5 inhibit cell proliferation and reduced cells in the S+G2/M phase. Our findings suggest hypoxia induced ER β 5 expression in glioma as a self-protective mechanism against tumor proliferation and that ER β 5 might serve as a therapeutic target for the treatment of glioma.

Keywords

Estrogen receptor β ; Glioblastoma multiforme; Hypoxia inducible factor; PTEN

1. Introduction

Loss of ER β expression has been suggested as an important step in estrogen-dependent tumor progression (Bardin et al., 2004; Burns and Korach, 2012). In breast cancer, decline of ER β expression has been repeatedly reported (Bardin et al., 2004; Murphy and Leygue, 2012). High levels of ER β are associated with lower tumor grade (Jarvinen et al., 2000), longer survival of patients (Honma et al., 2008), and higher sensitivity to tamoxifen treatment (Esslimani-Sahla et al., 2004; Hopp et al., 2004). DNA methylation in the ER β promoter region has been proposed to contribute to the decline of ER β expression in tumor cells (Zhao et al., 2003). Both *in vitro* and *in vivo* studies indicate that ER β inhibits proliferation and invasion of breast cancer cells (Lazennec et al., 2001; Paruthiyil et al.,

2004). In addition, the anti-proliferative role of ER β has been demonstrated in hormone-independent cancers of the colon and lung (Hartman et al., 2009; Skov et al., 2008). Different mechanisms have been proposed for the anti-proliferative action of ER β (Bardin et al., 2004), including inhibition of ER α transcriptional activity (Hall and McDonnell, 1999), reduction of S+G2/M phase (Liu et al., 2002; Strom et al., 2004), and inhibition of HIF1 transcriptional activity (Lim et al., 2011).

At least 5 different isoforms of human ER β have been identified which have identical N-terminal sequence but diverge from amino acid 469 to the C-terminus (Moore et al., 1998). In vitro analysis has found that each ER β isoform has distinct transcriptional activity (Leung et al., 2006; Moore et al., 1998). In breast cancer, expression levels and functions of different ER β isoforms have been studied (Leygue et al., 1999; Omoto et al., 2003; Shaaban et al., 2008). Most studies on ER β expression in cancer used antibodies that did not discriminate between different ER β isoforms, and functional analysis of ER β in cancer has mainly focused on ER β 1. Two recent studies indicated that ER β expression declined in human glioma as tumor grade increased (Batistatou et al., 2006; Sareddy et al., 2012) and that an ER β agonist inhibited proliferation of glioblastoma multiforme (GBM) cell lines (Sareddy et al., 2012). However, these studies used only immunohistochemistry to evaluate ER β expression. It was not clear which isoforms are expressed in human glioma and the distinct function of the each ER β isoform is unknown. In the present study, we evaluated the expression of ER β isoforms in human glioma using immunohistochemistry, Western blot, and real time PCR. In addition, the function of ER β 1 and ER β 5 in glioma progression was determined using human GBM cell lines.

2. Results

2.1. Increased ER β 5 expression in glioma

We evaluated ER β expression in human GBM specimens and non-neoplastic brain specimens by Western blot using three different antibodies (H150, 1531 and 3576). With each antibody, we detected an increase of ER β expression in a human GBM patient as compared with a non-neoplastic brain tissue from female patients (Fig. 1A). The immunoreactive band we detected in Western blots was of a smaller molecular weight than that of full length ER β 1 (59 kDa). We used PCR primers for ER β isoforms 1, 2, 3, 4 and 5 to determine the expression of each ER β isoform in human GBM specimens (Moore et al., 1998). In human GBM cDNA from the same patient as in Fig. 1A, only ER β 5 was detected by PCR (Fig. 1B). This was confirmed by PCR using an alternative set of primers for ER β 1, ER β 2, ER β 4 and ER β 5 (Leung et al., 2006) (Supplement Fig. 1C). We evaluated the expression of ER β isoforms using isoform specific antibodies for ER β 1, ER β 2 and ER β 5. Consistent with the PCR results, higher level of ER β 5 was detected in human GBM by Western blot and immunohistochemistry as compared with non-neoplastic brain tissue (Fig. 1C and D). No positive staining was detected in either non-neoplastic brain tissue or GBM using ER β 1 or ER β 2 specific antibodies with immunohistochemistry. Double immunofluorescence staining demonstrated that ER β 5 positive cancer cells expressed astrocyte marker glial fibrillary acidic protein (GFAP), and that ER β 5 was mainly localized in the nucleus (Fig. 1E).

We further investigated ER β expression in a different set of non-neoplastic human brain and GBM specimens. Western blot analysis indicated an increase of ER β 5 expression in human GBM from both male and female patients as compared with non-neoplastic brain tissue (Fig. 2A and B). Consistently, real-time PCR demonstrated increased mRNA levels of ER β 5 in GBM tissue from female patients (control: $n=3$; GBM: $n=8$) (Fig. 2C). We evaluated ER β 5 expression in different grades (II, III and IV) of human glioma by immunohistochemistry using commercial human brain tissue array samples. Immunohistochemistry scoring

indicated a significant increase of ER β 5 expression in gliomas as compared with non-neoplastic brain tissues (Fig. 2D and E). ER β 5 expression was not significantly different between different grades of glioma, although a trend of ER β 5 increase was observed in high grade glioma.

We evaluated the expression of ER β in human GBM cell lines U87 and A172, which originated from female and male GBM patients, respectively. Using antibodies specific for ER β 1, ER β 2 or ER β 5, we identified the expression of all the three isoforms in both U87 (Fig. 3A) and A172 (Fig. 3B) cells. Expression of ER β 1, ER β 2 and ER β 5 in U87 and A172 was also confirmed by PCR using two different sets of primers (Supplements Fig. 1A and B). All three isoforms were localized in the nucleus as indicated by their co-staining with DAPI. We evaluated expression of ER β by immunocytochemistry in human primary astrocytes. No positive staining was observed (Supplement Fig. 2A).

2.2. Hypoxia increased ER β 1, ER β 2, and ER β 5 expression in U87 cells

Gliomas, like many other solid tumors, are under extensive hypoxic stress given their rapid proliferation (Cruickshank et al., 1994). It has been demonstrated that hypoxia increased ER β mRNA levels in rat hippocampus neuronal cultures (Heyer et al., 2005). We predicted that hypoxia in glioma might contribute to the increase of ER β expression. U87 cells were subjected to hypoxic condition (1% O₂) for 24 h and the expression of ER β 1, ER β 2, and ER β 5 were evaluated by real-time PCR. We found that hypoxia significantly increased mRNA levels of all three ER β isoforms (Fig. 4A).

In the promoter region of ER β , there is an E-box which overlaps with the HIF binding sequence (5'-RCGTG-3') (Li et al., 2000). We predicted that the hypoxia-induced increase of ER β transcription is mediated by the activation of HIF. We transfected U87 cells with plasmids P1P2N HIF1 α and P1P2N HIF2 α to express constitutively active HIF1 α or HIF2 α , respectively. At 48 h after transfection, RNA was isolated and real-time PCR was conducted to evaluate mRNA levels of ER β 1, ER β 2 and ER β 5. Compared with vector transfection, both P1P2N HIF1 α and P1P2N HIF2 α transfection significantly increased mRNA levels of all the three ER β isoforms (Fig. 4B).

2.3. ER β 1 and ER β 5 increased PTEN expression and inhibited PI3K/AKT/mTOR pathway

Previous studies demonstrated that ER β 1 can increase PTEN expression and suppress activation of the PI3K/AKT pathway (Lindberg et al., 2011). PTEN is an important tumor suppressor and somatic mutation of PTEN is common in human glioma (Wang et al., 1997). To investigate the effect of ER β on PTEN expression, we cloned ER β 1 and ER β 5 into a pCDNA vector with a flag tag at the N-terminal. The plasmids were transfected into HEK 293 cells, which do not have endogenous ER β expression. Nuclear localization of ER β 1 and ER β 5 was observed by immunofluorescent staining using anti-flag antibody in the cells with transient ER β 1 or ER β 5 transfection. In addition, the ER β 1/5 localization was not affected by the treatment of 17 β -estradiol (E2) (Fig. 5A). In the ER β 1 and ER β 5 stably transfected HEK 293 cell lines, an increase of PTEN expression was observed (Fig. 5B and D). Consistently, we detected decreased phosphorylation of AKT and P70S6K, a component of the mTOR pathway, in both cell lines (Fig. 5B). These data suggest that ER β 1 and ER β 5 could inhibit the PI3K/AKT/mTOR pathway through upregulation of PTEN expression in an estrogen-independent manner. We further tested the effects of ER β 1 and ER β 5 on the activation of the MAPK/ERK pathway. In ER β 5, but not ER β 1, expressing HEK 293 cells, we observed decreased phosphorylation of c-Raf, MEK and ERK in a ligand-independent manner (Fig. 5C).

A recent study indicated that PTEN elevation can increase energy expenditure and mitochondrial oxidative phosphorylation to reduce the Warburg effect of tumor cells (Garcia-Cao et al., 2012). We investigated the oxygen consumption rate of HEK 293 cells with stable expression of either ER β 1 or ER β 5. A significant increase of oxygen consumption rate was observed in ER β 1 and ER β 5 expressing cells as compared with vector control (Fig. 5D).

2.4. ER β 1 and ER β 5 inhibited proliferation of U87 cells

The effects of ER β agonist diarylpropionitrile (DPN) on tumor growth have been contradictory. While DPN has been reported to inhibit growth of breast cancer, colon cancer and GBM cells, it has also been demonstrated that DPN could increase proliferation of medulloblastoma cells (Belcher et al., 2009). We treated U87 cells (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. 6A). Since our data indicated that ER β 1/5 could inhibit PI3K/AKT/mTOR and MAPK/ERK pathways in a ligand-independent manner, we hypothesize that ER β 1/5 inhibits GBM proliferation in a similar fashion. To determine the effects of ER β on glioma cell proliferation, we over-expressed ER β 1 or ER β 5 in U87 cells. U87 cells with over expression of either ER β 1 or ER β 5 showed a significantly lower growth rate as compared to vector control cells ($p < 0.01$) (Fig. 6B). Furthermore, ER β 5 expressing U87 cells had an even lower growth rate than ER β 1 expressing cells ($p < 0.01$) (Fig. 6C). Western blot analysis indicated that ER β 5, but not ER β 1, decreased activation of ERK in U87 cells (Fig. 6C). In addition, we observed decreased MMP2 activation in ER β 1 and ER β 5 expressing U87 cells, which was independent of E2 treatment (Fig. 6C).

We evaluated the role of ER β 1 and ER β 5 on cell cycle in U87 cells. Cell cycle analysis indicated that, at 12 h after transfection, ER β 1 or ER β 5 over-expression lead to a reduction of cell number in the proliferative S+G2/M phase (Fig. 6D). At 24 h, a significant reduction of cell number in the S+G2/M phase was found in the ER β 1 or ER β 5 over-expression U87 cells (Fig. 6D).

3. Discussion

ER β has long been shown to be involved in carcinogenesis and cancer progression and recognized as a cancer brake in the ovarian, prostate, and colon cancers (Bardin et al., 2004). The identification of ER β isoforms has added further complexity to the action of ER β (Moore et al., 1998). In the current study, we determined the expression of each ER β isoform in human glioma using immunohistochemistry, Western blot, and real time PCR. We have found that ER β 5 is the main ER β isoform expressed in human glioma. Consistently, ER β 5 was also identified in two human glioma cell lines, U87 and A172, by PCR and immunohistochemistry. The identified expression of ER β 1, ER β 2 and ER β 4 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. We found that the expression level of ER β was low in non-neoplastic brain tissue as indicated by Western blot and PCR. In primary human astrocytes, no obvious positive staining for ER β was observed by immunocytochemistry. However, in human glioma specimens, we found a significant increase of ER β 5 expression as compared in non-neoplastic brain tissue. A trend of increase of ER β 5 expression was indicated in high grade glioma, although it was not statistically different probably due to the limited sample size. Our results contradict to two recent studies, which reported that ER β expression declined in human glioma as tumor grade increased (Batistatou et al., 2006; Sareddy et al., 2012). The discrepancy might be due to the different methods between our study and previous studies. In addition, the previous studies did not differentiate each ER β isoform. The present study argues that future studies should

be conducted using ER β isoform specific antibodies and real-time PCR to further investigate the expression of ER β isoform in human glioma.

ER β isoform messenger RNA (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 ER β isoforms occurs from at least two different promoters, namely 0 K and 0 N (Hirata et al., 2001). Further analysis has identified that ER β 5 is regulated exclusively by promoter 0 K, while ER β 1, ER β 2 and ER β 4 are under the control of both promoter 0 K and promoter 0 N (Yuet-Kin Leung, 2005). The reduction of ER β 1, ER β 2, and ER β 4 transcription has been attributed to the methylation of promoter 0 N in prostate, breast, and ovarian cancers, while promoter OK was not methylated (Nojima et al., 2001; Rody et al., 2005; Suzuki et al., 2008; Zhao et al., 2003). The higher level of ER β 5 in glioma demonstrated in our study indicates that the activation of promoter 0 K. While the absence of ER β 1, ER β 2, and ER β 4 expression in glioma suggests that promoter 0 N might be silenced by methylation in glioma. An E-box (5'-CACGTG-3', -94/-99) has been found in the promoter region of ER β (Li et al., 2000), which overlaps the HIF binding sequence (5'-RCGTG-3'). We postulated that the increased expression of ER β 5 in glioma was caused by hypoxia that commonly exists in glioma (Evans et al., 2004). Consistently, in U87 cells, hypoxia increased the transcription of ER β 1, ER β 2 and ER β 5. Furthermore, significant increases in ER β 1, ER β 2 and ER β 5 transcription were induced in U87 cells by transfection of constitutively active HIF1 α or HIF2 α . Both *in vitro* and *in vivo* studies have shown that ER β inhibits cancer. ER β 1 has been most extensively studied. In tamoxifen-treated breast cancer, ER β 5 expression was positively associated with better survival (Davies et al., 2004; Shaaban et al., 2008). However, in prostate cancer, ER β 5 was associated with poor prognosis, and over-expression of ER β 5 promoted cancer cell migration and invasion (Leung et al., 2010). These data suggest that the functions of ER β 5 might be cancer type specific. *In vitro* analysis indicated that ER β 5 can activate gene transcription without ligand binding (Poola et al., 2005). In the current study, we investigated the effects of ER β 5 on two important oncogenic pathways for glioma: PI3K/AKT/mTOR and MAPK/ERK. We found that ER β 5 inhibited both pathways in HEK 293 cells in ligand-independent manners.

Both ER β 1 and ER β 5 inhibited the PI3K/AKT/mTOR pathway by increasing PTEN expression. An elevated level of PTEN, *in vivo*, has been shown to increase oxygen consumption and reduce the Warburg effect in tumor cells (Garcia-Cao et al., 2012). Consistently, in ER β 1 or ER β 5 expressing HEK 293 cells, we observed an increased cellular oxygen consumption rate. In breast cancer cells, ER β over-expression increased PTEN level in a ligand-independent manner (Lindberg et al., 2011). We predicted that up-regulating PTEN expression is a common anti-proliferative mechanism for ER β . However, in U87 cells, an in frame deletion causes the cells to be devoid of functional PTEN (Furnari et al., 1997). Further studies using GBM cell lines without a PTEN mutation are needed to support our prediction. In the promoter region of PTEN, no estrogen-responsive element (ERE) or ERE-like sequence has been identified (Kamalakaran et al., 2005). Yang et al. proposed that estrogen receptor could bind to the C terminus of PTEN and reduce protein turnover (Yang et al., He). Wickramasinghe et al. (2009) proposed that estradiol bound ER down-regulates miR-21 level and releases suppression of miR-21 on PTEN expression. A recent study indicated that ER β could bind to the promoter region of PTEN through Sp1 and increase PTEN transcription (Guido et al., 2012). The inhibitory action of ER β 1 and ER β 5 on U87 proliferation identified in the current study also suggests PTEN-independent mechanisms. Indeed, both ER β 1 and ER β 5 decreased MMP2 expression and ER β 5 inhibited the MAPK/ERK pathway. Consistent with previous studies (Liu et al., 2002; Strom et al., 2004), we found that, after over-expression of ER β 1 in U87 cells, cell number at the proliferative fraction S+G2/M phase was significantly reduced. Interestingly, ER β 5 over-expression produced more dramatic reduction of cell numbers in S+G2/M phase. A recent study

indicated that ER β agonist DPN inhibited proliferation of GBM cell lines including U87 cell line (Sareddy et al., 2012). 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 (Belcher et al., 2009). More detailed studies should be done to investigate DPN's effects on different types of brain tumors before any clinical trial in human is conducted. It has also been demonstrated that a tamoxifen metabolite, endoxifen, can induce ER α / β heterodimer formation to stabilize ER β protein (Wu et al., 2011). In breast cancer, higher ER β expression level has been associated with higher sensitivity to tamoxifen treatment (Esslimani-Sahla et al., 2004; Hopp et al., 2004). Clinical trials have indicated that a subgroup of glioma patients responded to tamoxifen treatment (Couldwell et al., 1996; Patel et al., 2012). It would be very interesting to evaluate the expression of ER β 5 in glioma and examine the correlation between ER β 5 expression and the response to tamoxifen treatment.

In summary, our current study demonstrated that ER β 5 is the major ER β isoform which expression was elevated in human glioma. The increase of ER β 5 might be induced via HIF signaling. In addition, our study indicated that ER β 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. 7).

4. Experimental procedures

4.1. Tissue specimens and immunohistochemistry

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 nine patients and glioma specimens from 22 patients were used for Western blot analysis and cDNA synthesis. Paraffin-embedded GBM specimens from two patients and two non-neoplastic brain specimens were obtained from Beijing Tiantan Hospital. Brain tissue arrays were purchased from US Biomax (Rockville, MD). Immunohistochemistry was done as described previously (Li et al., 2011). After immunohistochemistry of the tissue arrays, 7 non-neoplastic control tissues, 36 grade II glioma, 14 grade III glioma and 6 grade IV glioma were included into quantitative analysis. 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 given to a researcher who scored them blindly. Scoring of the images was conducted as described previously (Harvey et al., 1999). 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).

4.2. Antibodies and cells

Three antibodies for ER β 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 ER β 1, ER β 2 and ER β 5 were purchased from AbD Serotec (Oxford, UK). Other antibodies used are: actin (monoclonal, Santa Cruz), flag (monoclonal, Sigma), PTEN (monoclonal, Santa Cruz). Antibodies for the PI3K/AKT and MAPK/ERK pathways were purchased from Cell Signaling (Boston, USA). Human glioblastoma multiforme 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 and were prepared as describe previously (Fields et al., 2011).

4.3. Reverse-transcription PCR and real-time PCR

RNA was extracted using Trizol (Invitrogen) following manufacturer's protocol and quantitated using Nanodrop (Thermo Scientific). One μ g of RNA was used for cDNA synthesis. Isoform specific primers for ER β were designed as described in the previous publication by Moore et al. (1998) for PCR. Shared forward primer: 5'-AGT ATG TAC CCT CTG GTC ACA GC G-3'; Reverse primers: ER β 1: 5'-CCA AAT GAG GGA CCA CAC AGC AG-3', ER β 2: 5'-GGA TTA CAA TGA TCC CAG AGG GAA ATT G-3', ER β 3: 5'-GCA GTC AAG GTG TCG ACA AAG GCT GC-3', ER β 4: 5'-GGA TTA CAA TGA TCC CAG AGG GAA ATT G-3', ER β 5: 5'-CTT TAG GCC ACC GAG TTG ATT AGA G-3'; actin forward: 5'-CCA ACA CAG TGC TGT CTG G-3', actin reverse: 5'-TGC TGA TCC ACA TCT GCT G-3'. PCR products were analyzed in 2% agarose gel. Another set of isoform specific primers by Leung et al. (2006) were also used for regular PCR and real-time PCR detection of ER β isoforms: ER β 1 forward: 5'-GTC AGG CAT GCG AGT AAC AA-3'; ER β 1 reverse: 5'-GGG AGC CCT CTT TGC TTT TA-3'; ER β 2 forward: 5'-TCT CCT CCC AGC AGC AAT CC-3'; ER β 2 reverse: 5'-GGT CAC TGC TCC ATC GTT GC-3'; ER β 4 forward: 5'-GTG ACC GAT GCT TTG GTT TG-3'; ER β 4 reverse: 5'-ATC TTT CAT TGC CCA CAT GC-3'; ER β 5 forward: 5'-GAT GCT TTG GTT TGG GTG AT-3'; ER β 5 reverse: 5'-CCT CCG TGG AGC ACA TAA TC-3'; GAPDH forward: 5'-TCC CTG AGC TGA ACG GGA AG-3'; GAPDH reverse: 5'-GGA GGA GTG GGT GTC GCT GT-3'. Real-time PCR was carried out by a 7300 Real-Time PCR System (Applied Biosystems) using SYBR Green PCR Master Mix (Promega).

4.4. Plasmid constructs, transfection and stable cell line

ER β 1 (530 aa) and ER β 5 (472 aa) cDNA sequences with a flag tag (GAT TAC AAG GAT GAC GAC GAT AAG) at N-terminal were inserted into pCDNA3.1 (Invitrogen) between cloning sites XhoI and HindIII. Plasmids were transfected using lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Forty-eight hours after transfection, G418 was added to the medium (2 mg/ml) for selection of stable clones. Medium was changed every two days. G418 resistant clones were selected for culture in medium with 200 μ g/ml G418. Expression of ER β was validated using both Western blot and immunocytochemistry.

Plasmids expressing constitutively active form of HIF1 α and HIF2 α , designated as P1P2N HIF1 α and P1P2N HIF2 α , were kind gifts from Dr. Joseph A. Garcia at University of Texas Southwestern Medical Center at Dallas (Dioum et al., 2008). The plasmids were generated by alanine substitution of the conserved proline or asparagine residues in HIF1 α and HIF2 α and cloned into pIRES-hrGFP vector (Dioum et al., 2008). Forty eight hours after transfection, real-time PCR was carried out to evaluate mRNA levels of ER β 1, ER β 2 and ER β 5.

4.5. Seahorse XF-24 oxygen consumption analysis

HEK 293 stable clones were seeded in the Seahorse XF-24 plates. After 24 h, cells were changed to unbuffered DMEM (DMEM base medium supplemented with 25 mM glucose, 10 mM sodium pyruvate, 31 mM NaCl, 2 mM Glutamine, pH 7.4) and incubated at 37 °C in a non-CO₂ incubator for 1 h. Eight baseline measurements of oxygen consumption rate (OCR) were collected. 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 (pmol/min/ μ g).

4.6. 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 who was blinded to the treatment/group assignment. Each growth curve was validated by duplication or triplication.

4.7. Cell cycle analysis

U87 cells were seeded in 6-well plate and cultured overnight. Medium was replaced with DMEM without FBS and culture for 6 h for cell cycle synchronization. Then 10% FBS was added to the medium. After 12 h and 24 h, cells were collected for propidium iodide (PI) staining. After staining, cells were analyzed using a BD LSR II Flow Cytometer.

4.8. 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 (Li et al., 2011). 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 h and then destained with destain solution (30% MeOH and 10% acetic acid) until the bands became clear. Image was taken using UVP imaging system.

4.9. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Increased expression of ERβ in human GBM. (A) Western blot analysis using three different ERβ antibodies in human GBM and non-neoplastic brain tissue (CTL). (B) PCR using isoform specific primers for ERβ in human GBM cDNA. (C) Representative Western blots of ERβ expression in human GBM specimen and non-neoplastic brain tissue, using ERβ5 specific antibody. (D) Immunohistochemistry of human GBM specimen using ERβ5 specific antibody. (E) Double-staining of ERβ5 (green) and GFAP (red) in human GBM specimen. Nucleus was stained by DAPI (blue). (Fig. 1(A)–(C) are from the same female control and the same female GBM patient. Fig. 1(D) and (E) are from the same female GBM patients). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Increased expression of ERβ5 in human glioma. (A) Western blots 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: $n=8$). (D) Representative microscopy of ERβ5 immunohistochemistry in tissue arrays of non-neoplastic brain tissues and different grade (II, III and IV) glioma specimens. (E) Quantitative analysis of ERβ5 immunohistochemistry of human brain tissue arrays (CTL: $n=7$; grade II: $n=36$; grade III: $n=14$; grade IV: $n=6$).

Fig. 3. ERβ expression in GBM cell lines U87 and A172, which was derived from female and male patient, respectively. Immunocytochemistry of ERβ1, ERβ2 and ERβ5 in U87 cell line (A) and A172 cell line (B).

Fig. 4.

Hypoxia increased ERβ transcription through activation of HIF. (A) Real-time PCR analysis of ERβ1, ERβ2 and ERβ5 mRNA in U87 cells after 24-h hypoxia (1% O₂) ($n=4$). (B) Real-time PCR analysis of ERβ1, ERβ2 and ERβ5 mRNA in U87 cells 48 h after transfection with plasmids expressing constitutively active form of HIF1α or HIF2α ($n=6$).

Fig. 5.

Over-expression of ERβ1 and ERβ5 decreased activation of PI3K/AKT pathway and MAPK/ERK pathway. (A) Immunocytochemistry using anti-flag antibody in HEK 293 cells 24 h after transfection of plasmids expressing flag-ERβ1 and flag-ERβ5. (B) Western blot of ERβ1 and ERβ5 expression in HEK 293 stable clones using anti-flag antibody. PTEN expression and activity of PI3K/ AKT pathway in the clones with and without E2 treatment (100 nM for 48 h). (C) Western blot analysis of MPAK/ERK pathway in HEK 293 cells stably expressing ERβ1 or ERβ5 with and without E2 treatment (100 nM for 48 h). (D) Quantitative analysis of Western blot ($n=3$) of PTEN expression in the ERβ expressing HEK 293 clones and oxygen consumption rates (OCR) of the clones ($n=8$).

Fig. 6.

ERβ inhibited U87 proliferation in a ligand-independent manner. (A) U87 growth curves in the presence of 100 nM E2, 100 nM DPN, or vehicle control (CTL) ($n=4$). (B) Growth curves of U87 clones expressing ERβ1, ERβ5, or vector ($n=4$). (C) Western blot analysis of ERβ1, ERβ5, pERK, and total ERK in U87 clones. Gelatin zymography of MMP2 activity of U87 clones. (D) PI staining and cell cycle analysis of the U87 clones using flow cytometry at 12 h and 24 h after transfection ($n=3$).

Fig. 7.

Schematic presentation of the potential mechanisms underlying the action of ERβ5 on glioma progression. 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).