

Estrogen Receptor β as a Mitochondrial Vulnerability Factor*

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We recently demonstrated mitochondrial localization of estrogen receptor β (ER β). We herein confirm the mitochondrial localization of ER β by the loss of mitochondrial ER β immunoreactivity in ER β knockdown cells. A phenotype change characterized as an increase in resistance to oxidative stressors is associated with ER β knockdown. ER β knockdown results in a lower resting mitochondrial membrane potential ($\Delta\psi_m$) and increase in resistance to hydrogen peroxide-induced $\Delta\psi_m$ depolarization in both immortal hippocampal cells and primary hippocampal neurons. ER β knockdown cells maintained ATP concentrations despite insults that compromise ATP production and produce less mitochondrial superoxide under oxidative stress. Furthermore, similar mitochondrial phenotype changes were identified in primary hippocampal neurons derived from ER β knock-out mice. These data demonstrate that ER β is expressed in mitochondria and function as a mitochondrial vulnerability factor involved in $\Delta\psi_m$ maintenance, potentially through a mitochondrial transcription dependent mechanism.

Estrogens are known as the major female steroid hormones, which play a fundamental role in the female reproductive system. In recent years, estrogens have been appreciated as pleiotropic hormones that play roles in a wide variety of nonreproductive functions, such as cardiovascular function (1), memory and cognition (2), bone and mineral metabolism (3), and immune function (4). It is generally accepted that the majority of the biological effects of estrogens are mediated via two estrogen receptors (ERs)²: estrogen receptor α (ER α) and estrogen receptor β (ER β) (5–8). Consistent with their wide biological roles in a variety of systems, both ER α and ER β have been found to be widely distributed in different systems and tissues, including the reproductive system, central nervous system, cardiovascular system, gastrointestinal tract, urogenital tract, bone, and liver (9). ERs have been widely accepted as transcriptional factors that belong to the nuclear receptor superfamily. Classically, it is believed that estrogens could modulate the expression of

nuclear estrogen-responsive genes through both ERs. Also, estrogens could elicit rapid, nonnuclear action in a number of biological processes via nongenomic mechanisms mediated by ERs (10). Consistently, extranuclear localization of both ER α and ER β has been indicated (11–14). In fact, increasing evidence has demonstrated that ER β , while expressed in nuclei of some cell types, is mainly localized extranuclearly (14–18).

It became clear, not long after its identification, that ER β has biological roles distinct from those of ER α (9). ER α and ER β are encoded by separate genes found at different chromosomal locations. While sharing a high homology in both the DNA binding and ligand binding domains, ER α and ER β have very low sequence similarity in the N terminus (12%) and C terminus (9%), corresponding to the AF1 and AF2 domain, respectively (19). Hence, it is not a surprise that ER β has much lower nuclear transcriptional activity when compared with ER α (20–26).

There is accumulating evidence suggesting that mitochondria are also important targets for the actions of estrogens (18, 27). Mitochondria play a fundamental role in cellular respiration, oxidative phosphorylation, and ionic homeostasis as well as synthesis of heme, lipids, amino acids, and nucleotide. Not only is endogenous estrogen synthesized in the mitochondria by aromatase, but exogenously added estrogen is also mainly transported into this organelle (27, 28). We and several other laboratories have recently reported the localization of ER β in mitochondria in various cells, including rat primary neurons (15, 18, 29), rat primary cardiomyocytes (15), a murine hippocampal cell line (HT-22), neurons and glia in rat hippocampus (13, 14), human breast cancer cell lines (MCF-7 and MCF-10F) (16, 18), immortal human breast epithelial cells (18), human lens epithelial cell lines (nHLE and HLE-B3) (17, 30), human osteosarcoma cells (SaOS-2) (31), hepatocarcinoma cells (HepG2) (31), and human sperm (32). Similar perinuclear punctate staining of ER β has also been reported in a murine mammary epithelial cell line (HC11) and human fetal cortical neurons (33, 34). The localization of ER β in mitochondria suggests that ER β may function as a mitochondrial component.

In the current paper, we demonstrate that ER β regulates a variety of mitochondrial functions, using a murine hippocampal cell line (HT-22) with permanent knockdown of ER β by RNA interference (siRNA) and primary hippocampal neurons derived from ER β knock-out mice.

MATERIALS AND METHODS

Chemicals and Reagents

17 β -Estradiol was obtained from Steraloids, Inc. (Newport, RI). Tissue culture materials, MitoSOXTM, JC1, Slow-

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² The abbreviations used are: ER, estrogen receptor; ER α , estrogen receptor α ; ER β , estrogen receptor β ; $\Delta\psi_m$, mitochondrial membrane potential; siRNA, small interfering RNA; PBS, phosphate-buffered saline; ROS, reactive oxygen species; AAV-2, adeno-associated virus 2; GFP, green fluorescent protein.

Fade Light Antifade reagent, and Alexa Fluor 488 goat anti-rabbit IgG were obtained from Invitrogen. Charcoal-stripped fetal bovine serum was obtained from HyClone (Logan, UT). ER β (H-150) (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) is a rabbit polyclonal antibody raised against a recombinant protein corresponding to amino acids 1–150 mapping at the amino terminus of ER β of human origin. ER α (MC20) (Santa Cruz Biotechnology) is a rabbit polyclonal antibody raised against a recombinant protein corresponding to the 20 amino acids mapping at the C terminus of ER α of human origin.

Cell Culture

Murine Hippocampus Cell Line—HT-22 cells (gift from Dr. David Schubert, Salk Institute, San Diego, CA), which are an immortalized murine hippocampal cell line, were maintained in Dulbecco's modified Eagle's medium supplemented with 10% charcoal-stripped fetal bovine serum and 20 μ g/ml gentamycin at 37 °C in a humid atmosphere with 5% CO₂.

Rat Primary Hippocampal Neuronal Culture—Sprague-Dawley rat embryos (Charles River, Wilmington, MA) of 18 days were externalized under halothane anesthesia. Hippocampi were dissected and harvested in 2 ml of preparation medium (Dulbecco's modified Eagle's medium, 4.5 g/liter glucose, 100 units/ml penicillin, 100 μ g/ml streptomycin). The hippocampi were treated with trypsin. The tissue was then washed three times using washing medium (Hanks' medium, 4.5 g/liter glucose, 100 units/ml penicillin, 100 μ g/ml streptomycin) and individual cells were isolated by trituration 10 times using three different sizes of fire-polished Pasteur pipettes. The cells were harvested in seeding medium (Dulbecco's modified Eagle's medium, 4.5 g/liter glucose, 100 units/ml penicillin, 100 μ g/ml streptomycin, 2 mM glutamine, 19% horse serum) and filtered through a 40- μ m filter. The hippocampal cells were seeded on 25-mm coverslips. The cells were incubated in neurobasal medium (Dulbecco's modified Eagle's medium, 4.5 g/liter glucose, 100 units/ml penicillin, 100 μ g/ml streptomycin, 2 mM glutamine, B27) under normal cell culture conditions.

Primary Hippocampal Neuronal Culture from ER β Knock-out Mice—Breeding pairs of ER β knock-out mice (homozygous male and heterozygous female) were purchased from Taconic (Hudson, NY) (35). Postnatal day 1 pups were euthanized, hippocampi were dissected separately from each pup, and neuronal cultures were produced as described above. The hippocampal cells were seeded on 25-mm coverslips and incubated in neurobasal medium. The cells were allowed to grow 4–7 days in culture, and mitochondrial membrane potential was analyzed by JC1 assay. For genotyping, total DNA was purified from the tail using a DNeasy blood and tissue kit (Qiagen, Valencia, CA). Three primers were used for PCR with the following sequences: Neo06122, 5'-GCA GCC TCT GTT CCA CAT ACA C-3'; mber-C1-2, 5'-CAT CCT TCA CAG GAC CAG ACA C-3'; mber-F2, 5'-TGG ACT CAC CAC GTA GGC TC-3'. The PCR product underwent electrophoresis on a 2% agarose gel, and the separation patterns were photographed under UV illumination. Homozygous ER β knockouts were characterized as a single 404 bp band, while heterozygous knock-out mice were seen as two bands with 404 and 356 bp.

Knockdown of ER β by RNA Interference

siRNA was used to permanently knock down ER β in HT-22 cells. An ER β siRNA gene-specific insert was designed that specified a nucleotide sequence from the ER β cDNA (nucleotides 777–797) (gi:6978816), separated by a short spacer from the reverse complement of the same nucleotide sequence. For the construction of the expression vectors, the following oligonucleotides, that have homology sequence with ER β , were designed. The selected sequences are homologous for humans, rats, and mice. Thus, the designed ER β siRNA is effective for cells derived from all three species: 5'-GATCCCCgtgaaggatgtaaggccttAGAGACATTAaggccttacatcctcacaTTTTTTGAA-TTCA-3' and 5'-AGCTTGAATTCAAAAAAtggaaggatgtaaggccttAATGTCTCTAaggccttacatcctcacaGGG-3'. The synthetic oligonucleotides for ER β siRNA were separately annealed, gel-purified, kinased, and ligated to HindIII and BglII-digested pBSH1RNApro to generate expression vectors of pBS-H1-ER β siRNA (36). The insertion of the synthetic oligonucleotides for ER β siRNA was confirmed by sequencing using the M13F promoter (SeqWright DNA Technology Services, Houston, TX). The Lipofectamine (Invitrogen) method was used to transfect HT-22 cells with pBS-H1-ER β siRNA or vector. Following transfection, HT-22 cells were selected for neomycin (G418) resistance (250 μ g/ml). G418-resistant clones were isolated and expanded for Western blotting and immunocytochemistry of ER α and ER β to confirm the knockdown of ER β .

Immunoblotting

The whole cell protein extracts were combined in Laemmli buffer with β -mercaptoethanol and boiled for 5 min. Mitochondrial samples (30 μ g) were separated by 10% Tris-glycine polyacrylamide gel (Gradipore Ltd., Australia) and then transferred to a nitrocellulose membrane (Millipore Corp., Bedford, MA). Lanes containing Kaleidoscope prestained standards (Bio-Rad) were used to evaluate the size of the bands detected with ER α and ER β . The membranes were blocked for 1 h with 5% nonfat dry milk in PBS and were incubated overnight at 4 °C with ER β , ER α , or β -actin antibody. The membranes were repeatedly washed with phosphate-buffered saline (PBS) prior to incubation with secondary horseradish peroxidase-conjugated goat anti-rabbit IgG. The blots were developed with an enhanced chemiluminescence reagent.

Immunofluorescence Staining and Confocal Microscopy

Monolayer cells were washed with PBS (pH 7.4) and fixed with cold methanol for 15 min at –20 °C. Cells were rinsed several times in PBS and incubated in ice-cold Triton X-100 for 10 min to permeabilize the cells. Nonspecific sites were blocked for 1 h at room temperature with 5% normal goat serum and 5% bovine serum albumin in PBS. Cells were then incubated with an ER β antibody overnight at 4 °C. The sections were washed three times for 10 min each in PBS and then incubated with Alexa Fluor 488 goat anti-rabbit IgG for 1 h at room temperature. After washing in PBS three times for 10 min each, the cells were stained with 100 μ M 4',6-diamidino-2-phenylindole for 5 min. Cells were mounted with SlowFade Light Antifade reagent and covered with a coverslip. Samples were analyzed with a Zeiss LSM confocal microscope.

ER β as a Mitochondrial Component

Cell Proliferation Assay

Cells were seeded into 35-mm dishes at a density of 40,000 cells/dish under normal culture conditions. The cultures were lifted at 12, 24, 48, or 60 h after seeding and stained with trypan blue. The cell numbers were calculated using a hemocytometer.

Cell Viability Assay

For viability assays, cells were plated at a density of 5000 cells/well in 96-well plates 24 h before the initiation of experiments. Cells were exposed to various treatments for 18–24 h. For Calcein AM assays, cells were rinsed with 1 \times PBS (pH 7.4) after treatment, and Calcein AM (25 μ M) was added. After an incubation of 15 min at 37 $^{\circ}$ C, Calcein AM fluorescence was determined at an excitation of 485 nm and an emission of 538 nm using a Biotek FL600 microplate reader (Highland Park, VT). Percentage viability was calculated by normalization of all values to the control group (equal to 100%).

Resting Mitochondrial Membrane Potential Assessment

The fluorescent potentiometric JC-1 dye exists as green monomer at low concentrations or at low $\Delta\psi$ m. However, at higher concentrations or higher $\Delta\psi$ m, JC-1 forms red fluorescent “J-aggregates” that exhibit a broad excitation spectrum and an emission at \sim 590 nm. Thus, the emission of this cyanine dye can be used as a sensitive measure of $\Delta\psi$ m. Cells were seeded on 25-mm coverslips and grown for 24 h. Resting $\Delta\psi$ m was assessed by JC1 uptake. The coverslip was washed twice and mounted in a cell chamber (ALA Scientific Instruments, Westbury, NY) in HEPES buffer (145 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES). Serial confocal images were taken every 5 min with a confocal scanning laser microscope (Zeiss LSM-410) with excitation at 490 nm and emission at 510 and 590 nm before and after JC1 (5 μ g/ml) incubation, respectively. For detection of $\Delta\psi$ m depolarization, the cells were incubated with JC1 (10 μ g/ml) in media for 0.5 h, and then the coverslip was washed twice and mounted in a cell chamber. Serial confocal images were taken every 10 min with a confocal scanning laser microscope (\times 40 or \times 100 objective) with excitation at 490 nm and emission at 510 and 590 nm. Collapse of $\Delta\psi$ m was induced by adding hydrogen peroxide to a final concentration of 1 mM. Quantitative analysis of the dynamic change of the $\Delta\psi$ m was performed by measurement of the red fluorescence intensity, using the Meta-Morph software (Carl Zeiss).

Measurement of ATP Levels

Experiments were initiated by plating siER β or vector-transfected HT-22 cells at a density of 1 \times 10⁶ cells/well in 12-well plates. Seventy-two h later, cells were exposed to 250 or 500 μ M H₂O₂ for 1 h, at which time cells were still alive and attached to the plate. Cellular ATP levels were quantified using a luciferin- and luciferase-based assay. Cells were rinsed with PBS and lysed with ATP-releasing buffer containing 100 mM potassium phosphate buffer at pH 7.8, 2 mM EDTA, 1 mM dithiothreitol, and 1% Triton X-100; 10 μ l of the lysate was taken for protein determination. Another 10 μ l of the lysate was added to a Nunc 96-well plate. ATP concentrations in lysates were quantified using an ATP determination kit (Invitrogen) according to the manufac-

turer's instructions. The 96-well plates were read using a Biotek FL600 microplate reader. A standard curve was generated using solutions of known ATP concentrations. ATP levels were calculated as nM ATP/mg of protein and normalized to levels in untreated control cultures.

Measurement of Mitochondrial Superoxide

Mitochondrial superoxide was determined by a fluorescent dye, MitoSOXTM. Cells were seeded on 25-mm coverslips and grown for 24 h before initiation of the experiment. Cells were loaded with MitoSOXTM at a final concentration of 50 μ M for 45 min. The coverslip was washed twice and mounted in a cell chamber in HEPES buffer. Mitochondrial reactive oxygen species (ROS) production was induced by adding hydrogen peroxide. Serial confocal images were taken every 10 min with a confocal scanning laser microscope (Zeiss LSM-410) with excitation at 510 nm and emission at 580 nm. Quantitative analysis of the dynamic changes in mitochondrial superoxide was performed by measurement of the red fluorescence intensity, using the Meta-Morph software.

Mitochondria Isolation and Cytochrome *c* Oxidase Activity Assay

Mitochondria were isolated by differential centrifugation from permanent ER β knockdown HT-22 cells and vector control cells (15). The absorption of cytochrome *c* at 550 nm changes with its oxidation state. Cytochrome *c* oxidase activity was determined by using a cytochrome *c* oxidase activity assay kit (CYTOC-OX1; Sigma). Briefly, 1 μ g of isolated mitochondria preparations was resuspended in enzyme dilution buffer (10 mM Tris-HCl, pH 7.0, containing 250 mM sucrose) and added to 1 \times assay buffer (10 mM Tris-HCl, pH 7.0, 120 mM KCl). Fifty μ l of ferrocytochrome *c* substrate solution was added to initiate the reaction. The absorption changes at 550 nm were measured immediately using a spectrophotometer for 45 s. The activity of enzyme was calculated using the formula, units/mg protein = (ΔA /min \times dilution factor \times 1.1 \times 1000)/(volume of enzyme \times 21.84).

Adeno-associated Virus 2 (AAV-2)-mediated Infection of Constructs into Primary Neurons

An ER β siRNA expression cassette under a PolIII promoter was cloned into a site flanked by the AAV-2 terminal repeats. Downstream of the siRNA expression cassette, a GFP expression cassette under the PolII promoter was inserted into that construct, resulting in plasmid that can be packaged in AAV-2. Packaging of the ER β plasmid in a recombinant AAV serotype 2 was followed by the method described in detail by Klein *et al.* (37). Briefly, 293 cells were transfected with the AAV-siER β -GFP plasmid in an equimolar ratio with the plasmid pDG, which provides the AAV-2 coat protein genes, and adenovirus genes necessary for helper function in packaging (38). At 48 h after transfection, cells were collected and pelleted. The pellet was resuspended in buffer containing 50 mM Tris and 150 mM NaCl (pH 8) and freeze-thawed three times. The sample was then incubated with 125 units of benzonase (EMD Chemical, Inc., Gibbstown, NJ) per ml of supernatant for 30 min at room temperature. After benzonase treatment, the sample was centrifuged, and the supernatant was applied to 15, 25, 40, and 60% step

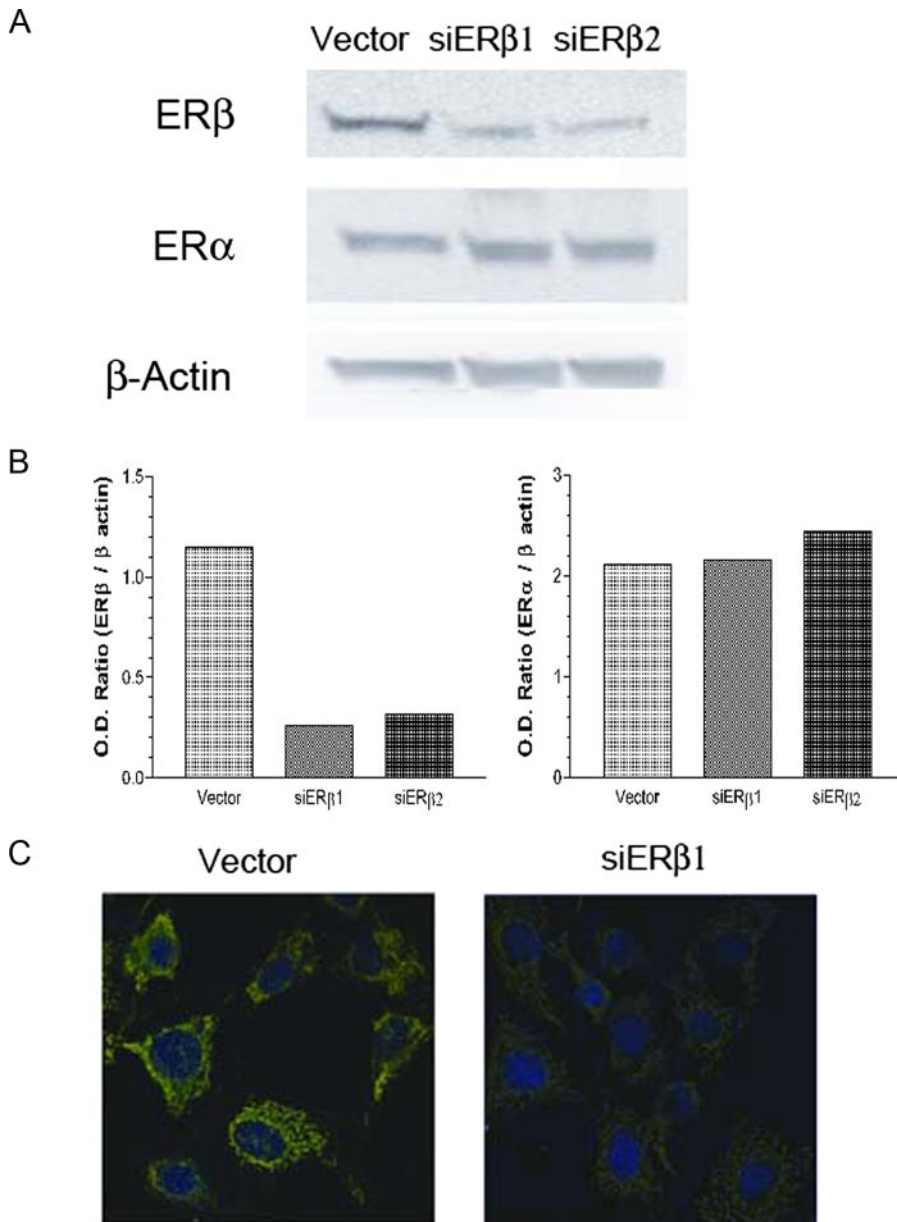


FIGURE 1. *A*, Western blots of ER β and ER α in ER β knockdown and vector-transfected HT-22 cells. *Lane 1*, vector-transfected cells; *lane 2*, siER β 1 knock-down cells, clone 1; *lane 3*, siER β 2 knock-down cells, clone 2. *B*, quantitative analysis of the ER α and ER β Western blots. *C*, immunocytochemistry staining of ER β in vector-transfected (*Vector*) and siER β 1-transfected HT-22 cells. The merged images are presented, and ER β is shown in green, and the nucleus is shown in blue (4',6-diamidino-2-phenylindole; DAPI).

gradients of iodixinol. The gradient was run at 65,000 rpm in a 70.1 Ti rotor (Beckman, Fullerton, CA) for 1 h at room temperature. The AAV-2 was removed and added to a heparin affinity column (Sigma), and the eluent was washed and concentrated using Amicon Ultra 24 units. The packaging of the AAV-siER β -GFP construct into virus was confirmed by electron microscopy. The titers of the viral stocks were $\sim 10^{12}$ for siRNA and 10^{13} for GFP. For AAV-2 infection, primary neurons were treated with 9×10^7 viral particles/50,000 neurons on day 7 in culture. Four days later, cultures were assessed for $\Delta\psi_m$ and immunocytochemistry.

Statistical Analysis

All data are presented as mean \pm S.E. Comparisons between two groups were performed using Student's *t* test. Comparisons

of multiple groups were analyzed using a two-way analysis of variance followed by Tukey's multiple comparison test. For all tests, $p < 0.05$ was considered significant. All statistical analyses were performed using Prism software (GraphPad Software, San Diego, CA).

RESULTS

Knockdown of ER β by RNA Interference in HT-22 Cells—In order to investigate the role of ER β , we established two permanent siER β clones together with a vector control clone. The clones were selected against and maintained in G418 medium. All the described experiments were conducted using these permanent cell lines within four passages. In the two permanent ER β knockdown clones, more than 80% reductions in ER β expression were achieved without significant changes of ER α expression, as indicated by Western blots (Fig. 1, *A* and *B*). To further confirm the immunoblotting results, we conducted immunocytochemistry on these siER β and vector clones. In vector clones, ER β was nearly exclusively localized in mitochondria, whereas the mitochondrial immunoreactivity for ER β was substantially reduced in ER β knockdown cells (Fig. 1*C*). siER β 1 was used to investigate the effects of ER β knockdown in HT-22 cells, siER β 2 had similar effects in all experiments performed (data not shown).

Effect of ER β Knockdown on Cell Vulnerability—We studied the effects of ER β knockdown on cell proliferation and cell vulnerability to

various insults. There was no significant difference in the proliferation rates between the vector and siER β clones (Fig. 2*A*) or between siER β clones and wild type cells. However, ER β knockdown significantly reduced cell death induced by iodoacetic acid treatment (Fig. 2*B*). Even more profound phenotype changes, characterized as an increase of resistance to glutamate and H₂O₂ toxicity, were demonstrated in ER β knockdown cells (Fig. 2, *C* and *D*). Similar results were observed in repeated experiments in both permanent clones of the ER β knockdown cell lines.

Effect of ER β Knockdown on Mitochondrial Function—We studied the effect of ER β knockdown on mitochondrial cytochrome *c* oxidase activity. As is evident from Fig. 3*A*, a significant increase of cytochrome *c* oxidase activity was seen in the

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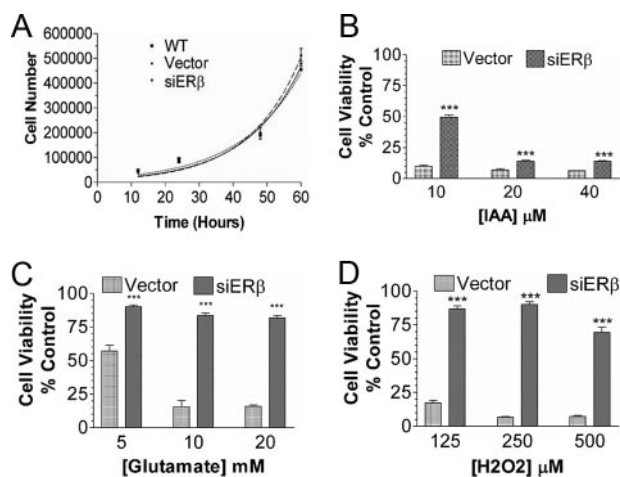


FIGURE 2. A, effect of ER β knockdown on HT-22 cell proliferation. No significant difference in cell proliferation was seen between wild type HT-22 cells (WT), vector-transfected HT-22 cells (Vector), and ER β knockdown HT-22 cells (siER β). B, effect of ER β knockdown on cell vulnerability to various oxidative insults, including iodoacetic acid (IAA), glutamate (C), and H₂O₂ (D). Substantial cell death was induced by iodoacetic acid, glutamate, and H₂O₂ in vector cells, which was significantly diminished in ER β knockdown cells. Results are shown as mean \pm S.E. from 6–8 experiments/group. ***, $p < 0.001$ versus vector.

ER β knockdown cell line, compared with the vector-transfected cell line. We further determined the effect of estrogen treatment on mitochondrial cytochrome *c* oxidase activity in wild type HT-22 cells. HT-22 cells were treated with 17 β -estradiol at the concentration of 100 nM or vehicle (DMSO) for 24 h, mitochondria were separated, and cytochrome *c* oxidase activity was determined. Compared with vehicle, a significant increase of cytochrome *c* oxidase activity was indicated in wild type HT-22 cells upon estrogen treatment (Fig. 3A).

We studied the effect of ER β on mitochondrial membrane potential ($\Delta\psi_m$), using a lipophilic cationic dye, JC-1. Initially, the permanent ER β knockdown HT-22 or permanent vector control cells were incubated with JC-1 (5 μ g/ml), and the uptake of JC-1 into the mitochondria was followed. As is evident from Fig. 3B, the JC-1 uptake rate was much slower in ER β knockdown HT-22 cells than that in the vector clone, suggesting that these mitochondria in ER β knockdown cells have a lower resting $\Delta\psi_m$. We further assessed ATP production under oxidative stress in siER β and vector clones. Vector clone exhibited a robust decline in ATP production in response to H₂O₂ treatment, whereas siER β cells showed an attenuated decline in ATP levels in response to H₂O₂ insult (Fig. 3C). Mitochondria are well known as a predominant cellular source of ROS under both physiological and pathological conditions. We assessed the role of ER β on mitochondrial superoxide production following oxidative insults. ER β knockdown HT-22 cells were more resistant to H₂O₂-induced increases in superoxide production (Fig. 3D). We also examined the effects of ER β knockdown on oxidative stress-induced $\Delta\psi_m$ collapse in HT-22 cells. ER β knockdown HT-22 or vector control cells were first labeled with JC-1 probe (10 μ g/ml) for 0.5 h, and then $\Delta\psi_m$ collapse was induced by the treatment of H₂O₂ (1 mM). After exposure to H₂O₂, aggregated JC1 (in red) became monomeric and fluoresced green upon mitochondrial depolarization, as

evidenced by the increase of yellow (merging of red and green) in the time series images. The oxidative stress-induced mitochondrial depolarization was minimized in ER β knockdown HT-22 cells (Fig. 3E).

Knockdown of ER β in Primary Hippocampal Neurons and Its Effect on Mitochondrial Membrane Potential—We assessed the effect of ER β on $\Delta\psi_m$ in rat primary hippocampal neurons. Primary hippocampal neurons were infected with AAV-2, containing the ER β siRNA-GFP construct or a control GFP construct. The effectiveness of infection and ER β knockdown was confirmed by double staining of neurons with GFP and ER β . GFP immunoreactivity was identified in both AAV2-GFP and AAV-2 ER β primary hippocampal neurons. In AAV-2 GFP-infected neurons, a perinuclear staining of ER β was observed, whereas the staining was absent in AAV-2 ER β siRNA-GFP-infected neurons, which demonstrated a high effectiveness of infection and loss of ER β immunoreactivity in the ER β siRNA-GFP construct-infected neurons (Fig. 4A). We further determined the effect of ER β knockdown on mitochondrial membrane potential in the primary hippocampal neurons. Infected neurons were loaded with JC-1 and were then treated with H₂O₂ to induce a prompt $\Delta\psi_m$ collapse. As shown in Fig. 5B, H₂O₂ caused the expected prompt and progressive decline in $\Delta\psi_m$ in control-infected primary neurons. In contrast, no significant effects of the high concentration of H₂O₂ were seen through 30 min in the ER β siRNA-GFP construct-infected primary neurons (Fig. 4B). These data indicate that infection of primary neurons with a construct that reduces mitochondrial ER β causes a resistance of mitochondria to prooxidant insults, which is consistent with the phenotype change in ER β knockdown HT-22 cells.

Change of Mitochondrial Membrane Potential after Oxidative Insult in Primary Hippocampal Neurons Derived from ER β Knock-out Mice—We assessed the effect of ER β knock-out on $\Delta\psi_m$ in primary hippocampal neurons derived from homozygous or heterozygous ER β knock-out mice. Primary hippocampal neurons were first labeled with JC-1 probe (10 μ g/ml) for 0.5 h, and then $\Delta\psi_m$ collapse was induced with H₂O₂ (1 mM). Primary neurons derived from the homozygous ER β knock-out mice were significantly more resistant to pro-oxidant insult-induced $\Delta\psi_m$ collapse than neurons from littermate heterozygous ER β knock-out mice (Fig. 5A). Representative confocal images are shown in Fig. 5B.

DISCUSSION

The role of estrogens in growth and in the physiology of reproductive tract tissues and organs in females has been recognized for more than half a century (19). Great efforts have been invested to decipher the mechanisms underlying estrogen's physiological function. Two decades ago, the cloning of the first ER, now known as ER α (5), simplified estrogen action to one hormone, one receptor, and one system. However, a decade ago, the identification of the second ER (7), known as ER β , has fundamentally changed the concept of estrogens and opened a new chapter in estrogen functions. Estrogens are now recognized as affecting systems beyond female reproduction. Estrogens have been found to modulate growth of tissues, bone integrity, cardiovascular tissue, the immune system, and nerv-

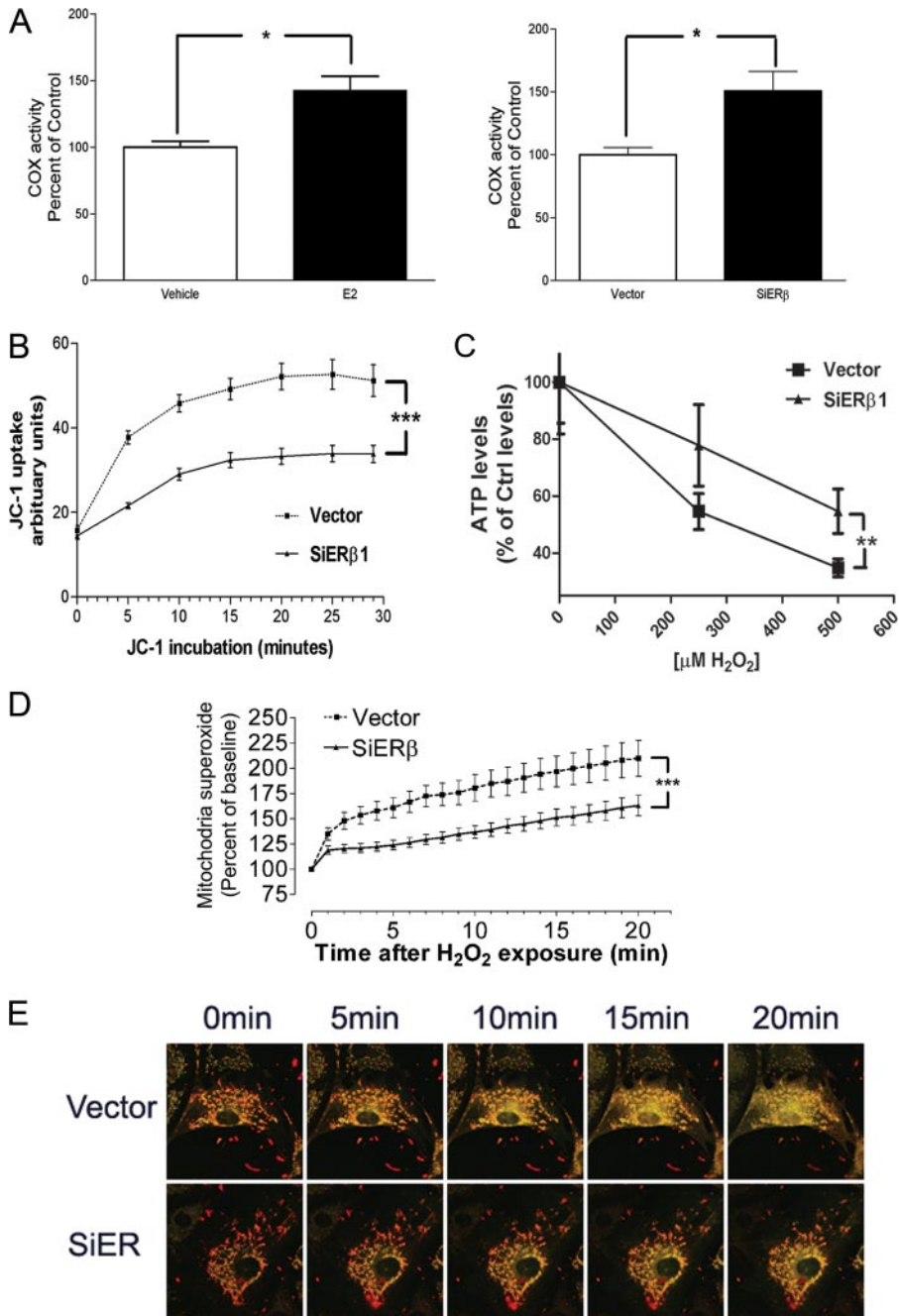


FIGURE 3. *A*, right panel, 17 β -estradiol (E2) treatment increases cytochrome c oxidase activity in HT-22 cells. *, $p < 0.05$ versus vehicle. Left panel, quantitative analysis of cytochrome c oxidase activity in vector and siER β cells. *, $p < 0.05$ versus vector. *B*, quantitative analysis of dynamic change of JC-1 uptake in vector-transfected (Vector) and ER β knockdown HT-22 cells (siER β). $n = 16$. ***, $p < 0.001$ versus vector. *C*, H $_2$ O $_2$ -induced decrease of ATP production in vector-transfected and ER β knockdown HT-22 cells. $n = 6$. **, $p < 0.01$ versus vector. *D*, quantitative analysis of dynamic changes in H $_2$ O $_2$ -induced mitochondrial superoxide production, measured by MitoSox fluorescence, in vector and siER β HT-22 cells. $n = 16$. ***, $p < 0.001$ versus vector. *E*, effect of ER β on H $_2$ O $_2$ -induced $\Delta\psi$ m collapse in HT-22 cells. Confocal microscopy images show the same field of cells viewed before (0 min) and at 5, 10, 15, and 20 min after H $_2$ O $_2$ (1 mM) exposure in vector and siER β HT-22 cells.

ous system physiology as well as male physiology (19, 39). Further, given the widespread role of estrogen in human physiology, it is not surprising that estrogen is also implicated in numerous diseases, including but not limited to various types of cancer, osteoporosis, neurodegenerative diseases, cardiovascular disease, insulin resistance, lupus erythematosus, endometriosis, and obesity (19).

In addition to exerting classical genomic actions on the expression of nuclear estrogen-responsive genes, estrogens have substantial effects on mitochondrial structure, biogenesis, and function, which were documented decades ago and recently reassessed (for reviews, see Refs. 18, 27, and 40). Four years ago, we and several other groups independently identified the localization of ER β in the mitochondria in various types of cells, suggesting that ER β may function as a mitochondrial component. In the present study, we investigated the role of ER β in mitochondrial functions via the knockdown of ER β in a murine hippocampal cell line (HT-22) through RNA interference technique. The knockdown of ER β was confirmed by both immunoblotting and immunocytochemistry. Given the high specificity of RNA interference, the substantial reduction of ER β immunoreactivity in the mitochondria of HT-22 cell provides further evidence to support that ER β is localized in mitochondria. In the current studies, the phenotype change of mitochondria functions of HT-22 cells with permanent ER β knockdown was investigated. We observed a substantial reduction in cellular vulnerability to various oxidative insults. This phenotype change was repeatedly demonstrated in two permanent clones of ER β knockdown HT-22 cell when compared with both permanent vector-transfected and wild type HT-22 clones. We believe that the phenotype change can be attributed to the modification of mitochondrial function induced by the knockdown of ER β .

Mitochondria produce the majority of cellular energy in the form of ATP by the process of oxidative phosphorylation. Furthermore, mitochondrial oxidative phosphorylation generates the majority of ROS.

$\Delta\psi$ m is an electrochemical gradient, which is generated by transportation of electrons, born of NAD $^+$ and succinate in the tricarboxylic acid cycle through electron transport chain components. Mitochondria also play a critical role in the initiation of both necrotic and apoptotic cell death (41). Collapse of the $\Delta\psi$ m is one of the early indicators of cell death. We determined the effects of ER β on resting $\Delta\psi$ m, using a lipophilic cationic

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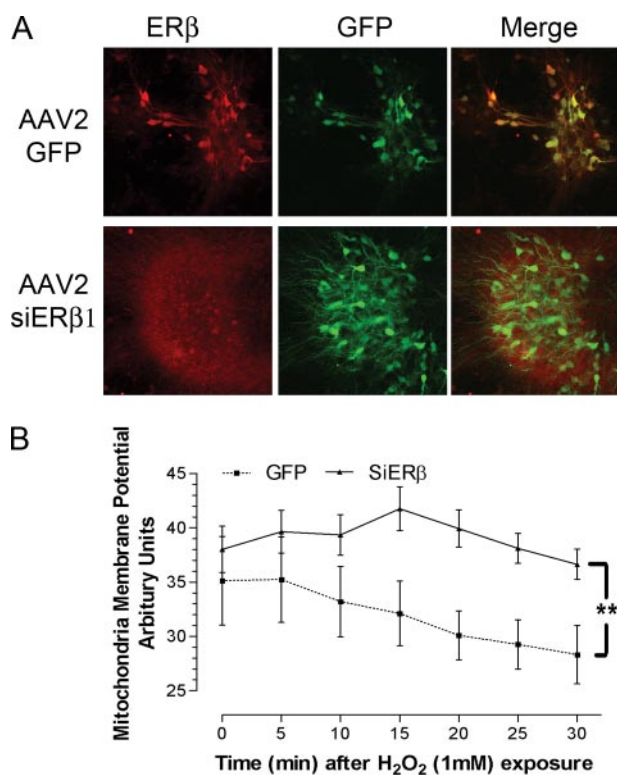


FIGURE 4. *A*, immunocytochemistry staining of ER β (red) and GFP (green) in the AAV-2 GFP construct-infected (AAV2-GFP) and AAV-2 ER β siRNA-GFP construct-infected (AAV2-siER β 1) primary hippocampal neuronal cultures, used for $\Delta\psi_m$ determination. *B*, quantitative analysis of dynamic changes in $\Delta\psi_m$ under H₂O₂ exposure in AAV-2 GFP construct-infected (GFP) and AAV-2 ER β siRNA-GFP construct-infected (SiER β) primary hippocampal neurons. A significant reduction of $\Delta\psi_m$ was observed in GFP-infected neurons after H₂O₂ insult but not in siER β -AAV2-transfected neurons. Results are shown as mean \pm S.E. for $n = 7$. *, $p < 0.05$ versus GFP.

dye, JC-1, a potentiometric dye that accumulates in mitochondria in accordance with the Nernst equation. The knockdown of ER β led to a mitochondrial phenotype change similar to mitochondrial uncoupling, a lower $\Delta\psi_m$ condition in which electron transport is disconnected from the production of ATP. Investigations of endogenous uncoupling proteins have provided evidence to support a beneficial role of mitochondrial uncoupling. Consistently, exposure of HT-22 cells to an uncoupling agent, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone, resulted in a reduction of cell death induced by glutamate (data not shown). We further determined the effect of ER β knockdown on oxidative stress-induced $\Delta\psi_m$ collapse. As predicted, oxidative insults induced a prompt collapse of $\Delta\psi_m$ in vector-transfected HT-22 cells, which was minimized in the ER β knockdown HT-22 cells. Consistently, ER β knockdown HT-22 cells were more resistant to oxidative stress-induced ATP depletion. It is noteworthy that the basal ATP levels in the ER β knockdown HT-22 cells were lower than that in vector-transfected cells (data not shown). The lower basal ATP production levels in ER β knockdown cells parallels the lower resting $\Delta\psi_m$ in these cells, since $\Delta\psi_m$ is the direct driving force for ATP production (41). Oxidative phosphorylation in mitochondria is the major endogenous source of ROS. We then determined mitochondrial superoxide production under oxidative stress. Consistent with mitochondrial uncoupling, a reduction

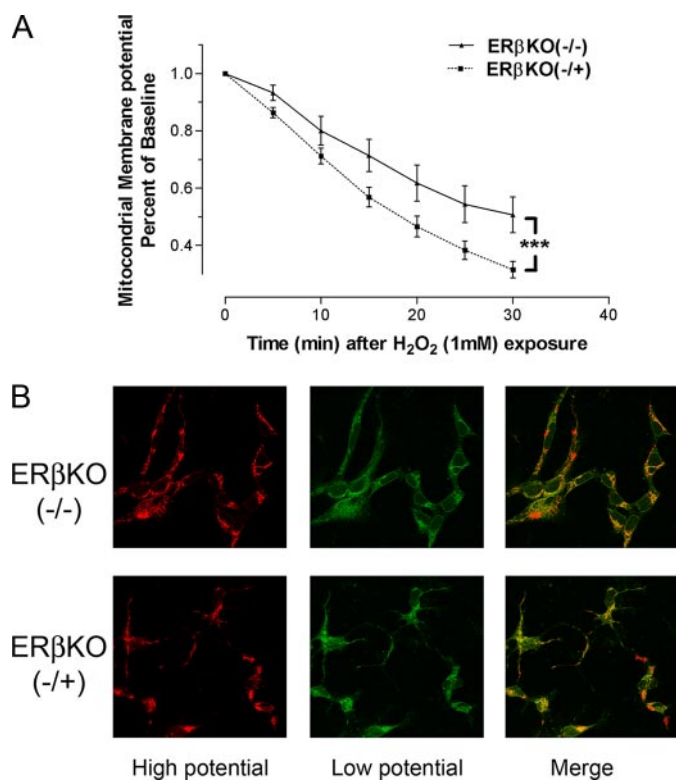


FIGURE 5. *A*, quantitative analysis of dynamic changes in mitochondrial membrane potential ($\Delta\psi_m$) under H₂O₂ exposure in hippocampal neuronal cultures derived from homozygous (ER β KO(-/-)) and heterozygous (ER β KO(-/+)) ER β knock-out mice. $\Delta\psi_m$ collapse was evaluated by JC-1 fluorescence intensity. ***, $p < 0.001$. *B*, representative confocal microscopy image after 30 min of H₂O₂ exposure.

of mitochondrial superoxide generation under oxidative stress was seen in ER β knockdown HT-22 cells.

We speculated that the functions of ER β , as a mitochondrial component, were not cell-specific, since localization of ER β in mitochondria has been identified in various cell types, including but not limited to primary neurons, rat and human primary cardiomyocytes, human breast cancer cell lines, immortal human breast epithelial cells, human lens epithelial cell lines, human osteosarcoma cells, hepatocarcinoma cells, and human sperm. We knocked down ER β expression in primary hippocampal neurons by RNA interference. Consistent with the phenotype change of HT-22 cells, ER β knockdown in primary neurons rendered them resistant to oxidative stress-induced $\Delta\psi_m$ collapse. Furthermore, a similar mitochondrial phenotype change was also demonstrated in primary hippocampal neurons derived from ER β knock-out mice.

Our findings may provide important insight into the widely demonstrated neuroprotective effect of estrogens. The abrupt decrease in ovarian estrogens at the time of menopause may contribute to neuronal vulnerability (42–44), and the present data suggest that this is due to a state of unliganded ER β . The cytoprotective actions of estrogens are demonstrated in a variety of cells, including neurons, glia, vascular endothelium (42, 45), and cardiomyocytes (46). The pancellular protective effect of estrogens and the profound effects of estrogens on a wide range of cell signaling indicate that estrogens could target very upstream signals induced by a variety of insults, converging

upon regulation of mitochondrial function (40, 45, 47). Indeed, estrogens have fundamental effects on mitochondria that are similar to those seen with ER β knockdown. Estrogen has been shown to reduce mitochondrial Ca²⁺ influx in response to mitochondrial toxins, oxidative stress, or glutamate excitotoxicity (48–50). We have shown that estrogen maintains ATP production in the presence of mitochondrial toxins as well as pro-oxidants that would otherwise reduce ATP levels (48, 49). Estrogens and a variety of nonfeminizing estrogen analogues demonstrate protection of $\Delta\psi_m$ from collapse induced by mitochondrial overload, mitochondrial toxin, or prooxidants (48, 49). In the present study, we demonstrated that knockdown of ER β renders decreased vulnerability of cells to various insults, which could be achieved by estrogen treatment under normal ER β expression circumstances. In addition, our study demonstrated an increase of cytochrome *c* oxidase activity in ER β knockdown cells, which was also indicated in wild type cells upon estrogen treatment. These data suggest that estrogens cause a loss of function of ER β , which attenuates the negative function of mitochondrial ER β . As such, the depletion of estrogens could uncover the default function of mitochondrial ER β , which causes an increased vulnerability to oxidative damage in the organism.

Our findings are also significant in view of cancer development and progression. Localization of ER β has been identified in breast cancer cells (16, 18). Various studies have shown decreased expression of ER β in many cancers, including breast, ovary, colon, prostate, and glioma (51–54). Thus, ER β has been suggested as a cancer brake. The proapoptotic properties of ER β have been suggested in various cells. In the absence of ER β , there was a decrease in apoptosis in colonic epithelium (55). In both prostate cancer cells and breast cancer cells, the introduction of ER β caused a strong inhibition of cell proliferation due to the increase of apoptosis through the mitochondrial pathway (56–58). The ER β -selective agonist, 2,3-bis(4-hydroxyphenyl)-propionitrile, inhibited cell growth and induced apoptosis in a mammary epithelial cell line (34). Our study suggests that down-regulation of ER β in cancer cells could decrease vulnerability of mitochondria to various oxidative insults and hence decrease apoptosis and promote cancer progression.

In the present study, knockdown of ER β in a hippocampal cell line did not change the proliferation rate. On the other hand, signs of hyperproliferation of bladder epithelium, epithelium of the dorsal prostate, the coagulation glands, and the urethra have been identified in ER β knock-out mice, which indicates that ER β may have some kind of inhibitory effect against hyperproliferation (9). The inconsistent effect of ER β on proliferation in hippocampal cells and epithelium suggests that the antiproliferative effect of ER β could be cell type-dependent.

The crystal structure of ER β has been well demonstrated. ER β shares a highly conserved structure with other nuclear receptors, such as ER α . Although ER α and ER β have nearly identical DNA-binding domains, increasing evidence has indicated that they regulate the expression of a totally distinct set of genes (59, 60). Currently, most of the studies have been focused on the nuclear transcription regulation. Consistently, most of the genes modified in ER β knock-out mice are mitochondrial structural proteins related to oxidative phosphorylation (60).

We predict that this distinction could be partly due to different compartmentation of ER α and ER β . Indeed, recent studies demonstrated that ER β is localized in the mitochondrial matrix, hence enabled its access to the mitochondrial genome (18). Therefore, both the ER β structure and matrix localization provide ER β the capacity to regulate mitochondrial gene expression. Indeed, recently published studies have found that ER β could directly interact with mitochondrial genes to modulate cytochrome *c* oxidase subunit expression (18). Consistently, we have demonstrated that ER β is involved in the regulation of cytochrome *c* oxidase. This notion was further supported by the results generated in the primary hippocampal neurons derived from ER β knock-out mice. The ER β knock-out mice used in the present study were created by insertion of a neomycin resistance gene into exon 3, corresponding to the coding sequence for the DNA binding domain of ER β (35). Splicing variants missing sequences that encode the essential first zinc finger of the DNA binding domain have been found in these ER β knock-out mice (35). The consistency of the mitochondrial phenotype change obtained by ER β knockdown in HT-22 cells, rat hippocampal neurons, and ER β knock-out hippocampal neurons derived from these ER β knock-out mice suggests that ER β could function as a mitochondrial transcription factor and that it is involved in mitochondrial membrane potential maintenance. In addition, many other possibilities could also contribute to the phenotype change seen when ER β levels are decreased. The mitochondrial genome only codes 13 of the mitochondrial structural proteins, with all others coded by the nuclear genome. Mitochondrial function change could affect nuclear gene expression through mitochondria nucleus cross-talking. Thus, it would not be a surprise that nuclear actions could also play a role in the dramatic mitochondrial phenotype change induced by ER β knockdown.

In summary, our data demonstrate that ER β knockdown leads to a mild mitochondrial uncoupling phenotype characterized by a lower resting $\Delta\psi_m$. These cells are resistant to provocative insults, as evident by their resistance to oxidative stressor-induced $\Delta\psi_m$ depolarization, ATP depletion, and ROS generation. These data suggest that ER β could function as a mitochondrial vulnerability factor that is involved in mitochondrial membrane potential maintenance and mitochondrial vulnerability, potentially through a mitochondria transcription-dependent mechanism.

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