

Restoration of Estrogen Receptor α Overcomes Chemoresistance in Hepatocellular Carcinoma via Bax Interaction and Mitochondrial Dysfunction

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, with a disproportionately high incidence and mortality rate in males. Although chemotherapy and targeted therapies remain central strategies for advanced HCC, the rapid emergence of chemoresistance frequently leads to treatment failure, poor prognosis, and low survival rates. Epidemiological studies demonstrate that HCC occurs much less frequently in females, indicating that estrogen and estrogen receptor alpha (ER α) signaling may exert protective, tumor-suppressive actions against liver cancer progression. However, the precise non-genomic mechanisms by which ER α modulates chemoresistance remain poorly understood.

In this study, we established a multidrug-resistant (MDR) HCC cell model using male-derived HA22T cells challenged with histone deacetylase inhibitors (HDACi) and confirmed their cross-resistance to the first-line clinical agent Sorafenib. We found that both baseline ER α mRNA and protein levels were nearly undetectable in parental and MDR cells, rendering them unresponsive to exogenous 17 β -estradiol (E2). Restoring ER α via ectopic overexpression significantly sensitized MDR cells to apoptosis and suppressed pro-survival p-Akt and cofilin signaling. Mechanistically, overexpressed ER α physically interacted with Bax to form an ER α –Bax complex that translocated to the mitochondria, triggering substantial mitochondrial ROS accumulation, Cytochrome C release, and Caspase-3 activation. Furthermore, natural phytoestrogens such as Daidzein effectively suppressed MDR cell survival via p-Akt/p-cofilin downregulation. Together, these findings highlight ER α as a potent chemosensitizer and establish targeting the ER α –Bax mitochondrial axis as a promising therapeutic strategy to overcome drug resistance in HCC.

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer, which is predicted to be the sixth most commonly diagnosed cancer and the fourth leading cause of cancer death worldwide [1–3]. Eighty percent of HCC cases are found in Asia and Africa [4], where the high rate of hepatitis B virus (HBV) and hepatitis C virus (HCV) infections is strongly associated with liver inflammation. Additionally, patients with diabetes and obesity not only have a higher risk of HCC but also show a poor survival rate after chemotherapy for HCC. On the other hand, aflatoxin B1 exposure and heavy alcohol consumption are also major risk factors for the development of HCC. Moreover, liver cirrhosis is a well-known risk factor for HCC [5–7].

At present, there are three major types of clinical liver cancer therapies, including surgery, radiation therapy, and chemotherapy. Surgery is usually used for primary tumors, while radiation therapy is utilized for locally metastatic tumors. Surgery includes hepatectomy and liver transplantation; however, the therapeutic efficacy of liver transplantation remains limited, and the survival rate and prognosis are still poor in HCC patients. The effect of radiation therapy also faces restrictions, as it is difficult to deliver a safe dosage and range of radiation to the liver tissue. Due to these limitations, surgery and radiation

therapy often require combination with chemotherapy to achieve the best therapeutic outcomes [8–10]. Chemotherapy is the most vital cancer treatment method, but its efficacy is limited because the systemic accumulation of chemical drugs results in toxicity and induces severe side effects. Targeted therapy, a modern approach to chemotherapy, utilizes drugs to inhibit cancer-specific molecules, such as vascular endothelial growth factor (VEGF) and histone deacetylases (HDAC) [11–13]. Additionally, many clinical patients experience treatment failure and drug resistance after receiving therapy for a period of time, indicating that tumors have developed chemoresistance. Consequently, the clinical prognosis remains unsatisfactory, and relapse-related mortality remains a critical issue.

Previous studies from our group utilized apicidin and SAHA to treat the liver cancer cell line HA22T, successfully establishing HDAC inhibitor (HDACi)-resistant HA22T cell lines [14–16]. Moreover, our previous findings indicated that HDACi-resistant cells exhibited enhanced proliferative and pro-survival abilities compared to parental cells through the activation of the IGF-1R/PI3K/Akt signaling pathway. Up-regulated expression of B-cell lymphoma 2 (Bcl-2) and suppressed release of cytochrome c further contributed to this chemoresistance. Importantly, we discovered that the expression of the tumor-suppressor gene phosphatase and tensin homolog (PTEN) was significantly decreased in these HDACi-resistant cells [15]. Additionally, our research showed that HDACi-resistant cells activated the Wnt/ β -catenin signaling pathway and elevated matrix metalloproteinase-2 (MMP-2) expression, thereby enhancing cell metastatic potential [14]. These resistant cells also displayed increased Snail expression and EMT-like phenotypes via the I κ B kinase α/β (IKK α/β) and nuclear factor-kappa B (NF- κ B) pathways [16]. Furthermore, we discovered that these HDACi-resistant HCC cells also conferred cross-resistance to Sorafenib, a standard first-line clinical drug for HCC patients [17]. Sorafenib targets receptor tyrosine kinases, including vascular endothelial growth factor receptor 2 (VEGFR2) and platelet-derived growth factor receptor (PDGFR), thereby inhibiting tumor cell proliferation and angiogenesis while inducing apoptosis in hepatocellular carcinoma [18–20]. Accordingly, we identified our resistant cells as a multidrug resistance (MDR) cell model. These findings highlight the key mechanisms and molecular pathways underlying drug resistance when treating liver cancer with HDAC inhibitors and Sorafenib.

Accordingly, since chemoresistance leads to poor prognosis, alternative or supportive therapeutic strategies, such as hormone therapy, are urgently needed for HCC patients. Previous research has shown a striking gender disparity in hepatocellular carcinoma, with a significantly higher prevalence in males. The male-to-female ratio ranges from 2:1 to 4:1 in most published clinical studies [5, 21]. Male patients not only develop HCC more frequently but also experience worse clinical outcomes and lower survival rates compared to females [22–24]. Elevated androgen levels are thought to promote liver tumorigenesis [25, 26], whereas studies over the past few decades have indicated that estrogen plays an opposing, protective role against HCC progression [27, 28].

Estrogen mediates its biological actions by interacting with estrogen receptors (ERs), which belong to the nuclear receptor superfamily. ERs function both as transcription factors to regulate target gene expression and as mediators of non-genomic cellular responses [29–31]. Furthermore, estrogen can activate intracellular signaling cascades, such as the PI3K/Akt and MAPK pathways [32]. There are two

primary estrogen receptor subtypes: ER α and ER β . Although derived from different chromosomes, they share structural similarities. Human ER α consists of 595 amino acid residues with a molecular mass of 66 kDa, whereas ER β contains 530 amino acid residues with a molecular weight of 54 kDa [33, 34]. The tissue distribution of these receptors varies significantly: ER α is predominantly expressed in the liver, uterus, kidney, and heart, while ER β is primarily expressed in the ovary, prostate, lung, bladder, and central nervous system [29, 35]. Upon stimulation by estrogen, ER α regulates cellular signaling through four major distinct functional modes[36]. While ER α has been extensively characterized as a diagnostic and prognostic biomarker in breast cancer [37, 38], accumulating evidence underscores its critical role in liver cancer. The striking gender disparity observed in HCC incidence with a male to female ratio typically ranging between 2:1 and 4:1 strongly points to a protective effect mediated by estrogen and ER α signaling [5, 21]. Clinically, chronic liver diseases progress to cirrhosis more rapidly in males than in females, rendering liver cirrhosis and subsequent HCC predominantly diseases affecting men and postmenopausal women, both of which correlate with poor prognosis [39, 40]. Furthermore, mutated or dysfunctional ER α in male HCC patients has been shown to drive tumor cell proliferation [40]. Conversely, intact wild-type ER α expression serves as a key clinical indicator of prolonged survival and favorable post-surgical prognosis in patients with HCC.

Accordingly, to address the critical challenge of chemoresistance, our previous studies identified ER α as a potential tumor suppressor in hepatocellular carcinoma. However, the precise mechanisms underlying ER α -mediated tumor suppression and the reversal of drug resistance in HCC remain to be fully elucidated. In the present study, we aimed to investigate the functional role of ER α in tumor suppression and explore whether ER α can restore or enhance chemosensitivity in MDR HCC cells. Accumulating evidence indicates that ER α exists not only in the nucleus but also in the mitochondria and cell membrane. Based on these observations, we hypothesized that overexpressed ER α might regulate the interaction between cofilin and Bax, promoting their translocation to the mitochondria, which subsequently induces ROS accumulation and triggers cancer cell apoptosis. On the other hand, given that classical ER α functions as a ligand-dependent transcription factor that translocates into the nucleus to modulate target gene expression, we also sought to determine whether nuclear ER α upregulates specific tumor suppressor genes or alters gene regulatory networks to overcome chemoresistance.

Materials and methods

Cell culture

Human hepatocellular carcinoma HA22T cells (RRID:CVCL_7046) were purchased from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). The multidrug-resistant (MDR) HA22T cell line was established in our laboratory by continuous exposure to HDAC inhibitors (apicidin and SAHA) and displayed cross-resistance to Sorafenib as described previously [2]. Both parental HA22T and MDR cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; D2209, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% charcoal-stripped fetal bovine serum (FBS; HyClone, Logan, UT, USA) and

1% penicillin-streptomycin (P/S; Invitrogen Corp., Carlsbad, CA, USA). Cells were cultured at 37°C in a humidified incubator containing 5% CO₂.

Whole cell extraction

Cells were harvested and lysed in ice-cold cell lysis buffer containing 50 mM Tris-base (pH 7.5), 0.5 M NaCl, 1.0 mM EDTA, 1% NP-40, 1% glycerol, 1 mM β -mercaptoethanol, and a protease inhibitor cocktail. The cell lysates were cleared by centrifugation at 12,000 rpm for 15 min at 4°C, and the supernatants were collected and stored at -80°C for further analysis.

MTT assay

Cell viability was estimated using a colorimetric assay based on the conversion of tetrazolium dye (MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) into a purple formazan product. After harvesting and washing twice with PBS, the cells were cultured in phenol red-free DMEM (1 mL) with MTT (0.5 mg/mL) at 37°C for 4 h. The medium was then removed, and 250 μ L DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured using an ELISA reader at 570 nm. Cell viability was expressed as a percentage relative to the untreated control.

Lowry Protein Assay

Total protein concentration was quantified using the Lowry protein assay. A 0.5 mg/mL working solution was prepared by diluting the bovine serum albumin (BSA) standard (2 mg/mL) with distilled water. A series of BSA standard solutions (0, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL) was generated to construct the standard curve. Briefly, protein samples and standards were first reacted with an alkaline copper tartrate solution (2% Na-K tartrate, 1% CuSO₄·5H₂O, and 2% Na₂CO₃ in 0.1 N NaOH at a ratio of 1:1:98) and incubated for 10 min at room temperature (RT) to form a tetradentate copper-protein complex. Subsequently, Folin-Ciocalteu phenol reagent was added to the reaction mixture. Electron transfer from the copper complex to the phosphomolybdic/phosphotungstic acid complex resulted in color development. Following a 30-min incubation at RT, the absorbance (optical density, OD) was measured at 750 nm using an ELISA microplate reader, and sample protein concentrations were calculated against the BSA standard curve.

Western blotting analysis

Protein concentration from cell lysates was determined using the Lowry protein assay. Aliquots containing equal amounts of protein (30 μ g) were mixed with an appropriate volume of 5x sample buffer and boiled at 95-100 °C for 5 min. Samples were resolved by 8-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Amersham, UK). Membranes were blocked with 0.5% non-fat milk in TBST for 1 h at room temperature and incubated with specific primary antibodies overnight at 4 °C. Subsequently, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10000, GE Healthcare, Amersham, UK) for 1 h at room temperature. Protein bands were

visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA), and densitometric analysis of immunoblots was performed using the Alphamager 4000 digital imaging system (Digital Imaging System, San Diego, CA, USA).

Nuclear isolation

Nuclear and cytoplasmic fractions were separated using a Nuclear/Cytosol Fractionation Kit (#K266-100, BioVision, CA, USA) according to the manufacturer's instructions. Briefly, 2×10^7 cells were harvested, washed with phosphate-buffered saline (PBS), and collected by centrifugation. After discarding the supernatant, the cell pellet was fully suspended in 200 μ L of ice-cold CEB-A mixture, vortexed at maximum speed for 15 s, and incubated on ice for 10 min. Subsequently, 11 μ L of ice-cold CEB-B was added, and the mixture was vortexed, incubated on ice for 1 min, vortexed again for 5 s, and centrifuged at $16,000 \times g$ for 5 min at 4 °C. The supernatant containing the cytoplasmic fraction was collected and stored at -80 °C. The nuclear pellet was resuspended in 100 μ L of ice-cold Nuclear Extraction Buffer (NEB), vortexed for 15 s every 10 min for a total incubation of 40 min on ice, and then centrifuged at $16,000 \times g$ for 10 min at 4 °C. The resulting supernatant containing the nuclear fraction was transferred to a fresh tube and stored at -80 °C for further analysis.

Mitochondria isolation

Mitochondria were isolated using a Mitochondria Isolation Kit for Cultured Cells (#89874, Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. Briefly, an aliquot of 2×10^7 cells was harvested, washed with phosphate-buffered saline (PBS), and collected by centrifugation. After discarding the supernatant, Isolation Reagent A was added, vortexed, and incubated on ice for 2 min. Isolation Reagent B was then added, vortexed for 5 s, and incubated on ice for 5 min. Subsequently, Isolation Reagent C was added, and the tube was inverted several times to mix. The homogenate was first centrifuged at $700 \times g$ for 10 min at 4 °C to remove nuclei and unbroken cells. The resulting supernatant was transferred to a new tube and centrifuged at $12,000 \times g$ for 15 min at 4 °C to pellet the mitochondria. The mitochondrial pellet was resuspended and saved for further downstream analysis.

Co-immunoprecipitation assay

Cells were lysed in IP lysis buffer containing 50 mM HEPES (pH 7.6), 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM Na₃VO₄, 10 mM NaF, 10 mM β -glycerophosphate, and a protease inhibitor cocktail tablet (Roche) for 30 min on ice. Cell lysates were cleared by centrifugation at 14,000 rpm for 15 min at 4 °C, and total protein concentrations were determined using the Lowry protein assay. For immunoprecipitation, 700 μ g of whole cell lysates were incubated with 2 μ g of specific anti-ER α primary antibody (Santa Cruz Biotechnology) at 4 °C for 3 h with gentle rotation. Subsequently, the antibody-protein complexes were precipitated by incubation with Protein G PLUS-Agarose beads (GE Healthcare) overnight at 4 °C. The agarose beads were collected by centrifugation at 3,000 rpm for 5 min at 4 °C and washed thoroughly with IP lysis buffer. The precipitated proteins were eluted and denatured

by adding 1x SDS electrophoresis sample buffer and boiling at 100 °C for 10 min. The samples were then subjected to 10% SDS-PAGE followed by Western blot analysis.

Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was extracted and purified from cells using a Quick-RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. One microgram of total RNA per sample was reverse-transcribed into first-strand complementary DNA (cDNA) using a PrimeScript Reverse Transcriptase kit (Takara Bio, Otsu, Japan) in a total reaction volume of 50 µL. The reverse transcription reaction was carried out at 42 °C for 65 min, followed by inactivation at 90 °C for 5 min. PCR amplification was performed using Ex Taq Master Mix (Takara Bio, Otsu, Japan). A 1 µL aliquot of the synthesized cDNA was added to a final reaction volume of 25 µL. The specific primer sequences for ERα were as follows: forward primer, 5'-TGATGAAAGGTGGGATACGA-3' and reverse primer, 5'-AAGGTTGGCAGCTCTCATGT-3'. The thermal cycling conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 28 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. The mRNA expression levels of target genes were normalized to GAPDH as an internal control.

Immunofluorescence and MitoTracker staining

Cancer cells were seeded onto chamber slides and transfected with the indicated green fluorescent protein (GFP)-tagged plasmids. For mitochondrial staining, live cells were incubated with MitoTracker Red CMXRos (M7512, Invitrogen, Carlsbad, CA, USA) at 37 °C for 30 min prior to fixation. After washing with PBS, cells were fixed with 4% paraformaldehyde for 15 min at room temperature (RT) and permeabilized with 0.3% Triton X-100 in PBS for 10 min at RT. Nuclei were counterstained with 0.5 µg/mL 4',6-diamidino-2-phenylindole (DAPI) for 5 min in the dark. Slides were mounted, and fluorescence images were acquired using a fluorescence microscope equipped with appropriate filter sets for GFP (green, Ex/Em 488/509 nm), MitoTracker Red (red, Ex/Em 579/599 nm), and DAPI (blue, Ex/Em 358/461 nm) to evaluate spatial localization and co-localization.

Plasmid and gene transfection

The green fluorescent protein (GFP)-tagged ERα expression plasmid (HG13179-ACG) was purchased from Sino Biological (Beijing, China). When cells reached 60-80% confluence, the culture medium was refreshed, and cells were transfected with the ERα plasmid for 24 h using jetPRIME in vitro DNA & siRNA transfection reagent (Polyplus-transfection, New York, NY, USA) according to the manufacturer's protocol.

Statistical analysis

All experiments were independently repeated at least three times. Statistical analysis was performed using GraphPad Prism 7 software (San Diego, CA, USA). Data are presented as mean ± standard deviation (SD). Differences between groups were analyzed using Student's t-test (or one-way analysis of

variance [ANOVA] followed by Tukey's post-hoc test for multiple group comparisons). A value of $P < 0.05$ was considered statistically significant, whereas $P < 0.01$ and $P < 0.001$ were considered to represent higher levels of statistical significance.

Results

Establishment of Multidrug-Resistant HCC Model and Assessment of 17β -Estradiol Sensitivity

To evaluate the drug resistance profile of our established resistant cell line, we first examined its responsiveness to both HDAC inhibitors and the first-line clinical drug for HCC, Sorafenib. Treatment with SAHA for 24 h significantly reduced the viability of parental HA22T cells in a dose-dependent manner; however, MDR cells exhibited markedly higher cell viability, confirming their resistance to HDAC inhibitors (Fig. 1A). Next, we assessed whether these resistant cells displayed cross-resistance to Sorafenib using the MTT assay. Compared to parental HA22T cells, MDR cells demonstrated significantly decreased sensitivity to Sorafenib treatment at concentrations ranging from 2.5 to 7.5 μM (Fig. 1B), validating these cells as a robust multidrug-resistant (MDR) cell model.

Given that epidemiological evidence indicates a significantly lower incidence of HCC in females than in males [5], and our previous work showed that ER α signaling can induce apoptosis in liver cancer cells [41–43], we next investigated whether treatment with the endogenous ER α ligand, 17β -Estradiol (E2), could alter cell survival or downstream survival signaling in these models. Following 24 h or 48 h of E2 treatment across a wide concentration range (10^{-12} to 10^{-6} M), MTT assays showed no significant change in the viability of MDR cells, with parental HA22T cells displaying only minor reductions at higher concentrations (Fig. 1C, 1D). Furthermore, Western blot analysis revealed that baseline ER α protein was virtually undetectable in both parental and MDR cells, and exogenous E2 administration failed to induce ER α expression or alter downstream survival mediators, including CFL-1, p-CFL-1, p-ERK1/2, and p-Akt (Fig. 1E). Taken together, these findings indicate that the loss of functional ER α expression renders resistant HCC cells unresponsive to estrogen, and exogenous E2 treatment alone is insufficient to overcome chemoresistance or suppress MDR cell growth, prompting us to focus directly on the molecular mechanisms governed by reconstituted ER α overexpression.

Expression of endogenous ER α and dose-dependent ectopic overexpression in HA22T and MDR cells

The 17β -Estradiol (E2) treatment alone failed to affect cell viability or endogenous ER α expression, we reasoned that HA22T and MDR cells might express low baseline levels of estrogen receptor α . To test this hypothesis, we first performed RT-PCR to quantify endogenous ER α (ESR1) mRNA levels. As shown in Fig. 2A, ER α mRNA expression was exceptionally low in both HA22T and MDR cells compared to the internal control GAPDH. Western blot analysis further confirmed that endogenous ER α protein was virtually undetectable in both baseline cell lines (Fig. 2B).

To restore ER α expression and evaluate its biological functions, cells were transfected with increasing amounts of a GFP-tagged ER α expression plasmid (0, 1, 3, and 5 μ g). Western blot analysis demonstrated a marked upregulation of ER α -GFP fusion protein (~ 100 kDa) in both HA22T and MDR cells following plasmid transfection in a concentration-dependent manner (Fig. 2B). Densitometric quantification normalized to GAPDH confirmed a statistically significant elevation of ER α -GFP protein expression in both parental and drug-resistant cells compared to the non-transfected control (*P < 0.05; Fig. 2C). These results confirmed the successful establishment of transient ER α -overexpressing cell models for subsequent functional assays.

Ectopic overexpression of ER α suppresses p-Akt and triggers the caspase-dependent apoptosis pathway in HA22T and MDR cells

To elucidate the molecular mechanism by which overexpressed ER α influences cell survival, we evaluated downstream survival signaling pathways following ectopic ER α -GFP transfection in HA22T and MDR cells. Western blot analysis demonstrated a concentration-dependent increase in GFP expression (Fig. 3A) and confirmed robust ER α -GFP fusion protein expression (~ 100 kDa) in both cell lines following plasmid transfection (Fig. 3B). Densitometric quantification normalized to GAPDH confirmed a statistically significant elevation in ER α -GFP levels in both parental and drug-resistant cells (*P < 0.05; Fig. 3C).

We next examined downstream pro-survival and apoptotic effectors. Western blot analysis revealed that ectopic expression of ER α markedly suppressed the activation of the pro-survival kinase Akt, leading to a concentration-dependent reduction in p-Akt levels, which was statistically significant in MDR cells at the highest transfection dose (1 μ g/mL, *P < 0.05; Fig. 3A, 3D). Furthermore, ER α overexpression modulated the levels of cofilin-1 (CFL-1) and phosphorylated cofilin-1 (p-CFL-1) (Fig. 3E, 3F) without noticeably altering total ERK 1/2 and p-ERK 1/2 protein levels (Fig. 3A). Crucially, Western blot analysis demonstrated a marked elevation in cleaved caspase-3 levels along with pro-caspase-3 cleavage following ER α transfection (Fig. 3B), confirming the activation of the caspase-dependent apoptotic cascade. Our results indicate that ectopic ER α overexpression effectively attenuates p-Akt pro-survival signaling and induces apoptotic cell death in multidrug-resistant HCC cells.

Overexpression of ER α combined with 17 β -Estradiol co-treatment suppresses pro-survival signaling and modulates cofilin pathways in HA22T and MDR cells

Next, we aimed to determine the downstream molecular pathways regulated by overexpressed ER α with or without its ligand, 17 β -Estradiol (E2), in parental HA22T and drug-resistant MDR cells. Cells were transfected with ER α -GFP plasmid in the presence of increasing concentrations of E2 (10^{-10} , 10^{-8} , and 10^{-6} M). Western blot results showed that in resistant MDR cells, ER α overexpression combined with E2 treatment markedly inhibited the activation of the pro-survival kinase Akt, as shown by a clear decrease in phosphorylated Akt (p-Akt) levels (Fig. 4A). In addition, the protein levels of total cofilin-1 (CFL-1) and phosphorylated cofilin-1 (p-CFL-1) were also downregulated in MDR cells upon ER α transfection and E2

treatment (Fig. 4A). In contrast, the expression levels of total ERK 1/2, phosphorylated ERK 1/2 (p-ERK 1/2), and ERK 5 remained unchanged across both cell lines (Fig. 4A).

Densitometric quantification normalized to β -actin further confirmed these molecular alterations (Fig. 4B–4E). Successful expression of ER α -GFP fusion protein was verified in both HA22T and MDR cells after transfection and E2 treatment (* $P < 0.05$, $P < 0.01$, and *** $P < 0.001$; Fig. 4B). Quantitative analysis showed that p-Akt level exhibited a decreasing trend in MDR cells with ER α overexpression and E2 co-treatment (Fig. 4C). Importantly, total CFL-1 protein level was significantly reduced in MDR cells at higher E2 concentrations (Fig. 4D). Furthermore, p-CFL-1 expression in MDR cells showed a robust and significant reduction after ER α transfection and E2 treatment across all tested concentrations (Fig. 4E). Because dephosphorylated cofilin is the active form that translocates to mitochondria to facilitate Bax insertion and trigger apoptosis, this marked decrease in p-CFL-1 suggests that ER α /E2 signaling activates cofilin to promote mitochondrial-mediated cell death. Taken together, these data demonstrate that ectopic ER α overcomes chemoresistance in liver cancer cells mainly through suppressing p-Akt and promoting cofilin activation/downregulation, rather than through classical MAPK/ERK pathways.

ER α translocate to mitochondria and facilitates pro-apoptotic protein accumulation in HA22T and MDR cells

We want to investigate whether ER α induces apoptosis through organelle translocation, we isolated subcellular fractions to analyze the spatial distribution of ER α and its associated mitochondrial apoptotic effectors in HA22T and MDR cells. Subcellular fractionation purity was validated using COX-IV as a mitochondrial marker and GAPDH as a cytosolic marker. Western blot analysis revealed that following ectopic ER α -GFP overexpression, ER α protein levels were markedly enriched in the mitochondrial fraction (mito), particularly in multidrug-resistant MDR cells (Fig. 5A). Crucially, the mitochondrial translocation of ER α was accompanied by an increased accumulation of the pro-apoptotic proteins Bax, CFL-1, and Bad in the mitochondrial compartment, along with altered Cytochrome C distribution (Fig. 5A).

To confirm the subcellular localization of ER α , we performed confocal immunofluorescence staining combined with MitoTracker Red labeling. Confocal microscopic analysis demonstrated that transfected ER α -GFP (green) exhibited distinct spatial distribution, accumulating in both the nucleus (DAPI, blue) and the mitochondria (MitoTracker, red) (Fig. 5B). High-magnification merged views further clearly illustrated yellow co-localization signals between ER α -GFP and MitoTracker Red (Fig. 5C). Taken together, these findings demonstrate that ectopic ER α translocates to the mitochondria in MDR cells, where it promotes the mitochondrial recruitment of Bax and CFL-1 to trigger mitochondrial apoptotic signaling.

ER α induces apoptosis by interacting with Bax and promoting mitochondrial ROS accumulation

Following our results, we want to check the molecular interaction facilitating ER α mitochondrial translocation and apoptosis, we performed a co-immunoprecipitation (Co-IP) assay in parental HA22T and drug-resistant MDR cells transfected with ER α -GFP plasmid (1 μ g/mL). As shown in Fig. 6A,

immunoprecipitation targeting ER α -GFP successfully pulled down Bax protein in both cell lines, whereas no co-immunoprecipitated cofilin-1 (CFL-1) signal was detected, and normal IgG showed no non-specific binding. These findings demonstrate that ER α physically interacts with Bax rather than CFL-1 to form an ER α -Bax complex, which subsequently translocates to the mitochondria to initiate the intrinsic apoptotic pathway.

Given that mitochondrial accumulation of pro-apoptotic complexes disrupts electron transport and elevates oxidative stress, we next assessed mitochondrial reactive oxygen species (ROS) levels using the fluorogenic probe MitoSOX Red. Fluorescence microscopic imaging revealed a robust increase in MitoSOX red fluorescence signals in both HA22T and MDR cells expressing ER α -GFP compared to non-transfected control cells (Fig. 6B). These results indicate that ectopic ER α expression triggers significant mitochondrial ROS accumulation. Taken together, these data demonstrate that ER α translocates to the mitochondria via its physical interaction with Bax, promoting cell apoptosis through the elevation of mitochondrial oxidative stress in both parental and multidrug-resistant HCC cells.

Establishment of an inducible Tet-Off ER α system and evaluation of the phytoestrogen Daidzein in HA22T and MDR cells

To further clarify the role of ER α in reversing drug resistance, we used the Tet-Off ER α inducible expression system. Cells were transfected with pUHD15.1 (expressing tTA) and pUHD10.3-ER α (containing ER α gene with tetO sequence). Without tetracycline (Tet-Off ON), tTA can bind to tetO and turn on ER α expression; when tetracycline was added, it prevented tTA from binding tetO and turned off ER α expression (Fig. 7A). Western blot data showed that ER α was clearly expressed without tetracycline, but after adding tetracycline (10 μ g/mL), ER α expression was markedly inhibited in both HA22T and MDR cells (Fig. 7C), confirming the Tet-Off system worked well in our cell models.

Because 17 β -estradiol (E2) treatment alone did not show good cytotoxic effect, we next tested Daidzein, a major soy-derived phytoestrogen, as an alternative candidate for HCC treatment. MTT assay results indicated that Daidzein treatment for 24 h significantly decreased cell viability in HA22T and MDR cells in a dose-dependent manner from 10 to 100 μ M (*P < 0.05, #P < 0.05, ###P < 0.001; Fig. 7B). In addition, Western blot results showed that Daidzein (25 and 50 μ M) effectively decreased p-Akt and p-CFL-1 levels while increasing cleaved caspase-3 levels in both parental and resistant cells (Fig. 7D), suggesting that Daidzein can induce apoptosis to overcome chemoresistance in liver cancer cells.

Discussion

Chemoresistance remains a primary obstacle in cancer therapy, frequently leading to poor patient prognosis, treatment failure, and tumor recurrence. Consequently, extensive research has been devoted to unraveling the mechanisms underlying drug resistance to develop more effective therapeutic strategies [44–46]. Histone deacetylase (HDAC) inhibitors suppress cancer cell proliferation and tumor progression by inhibiting HDAC enzymes, thereby reactivating the transcription of critical tumor suppressors such as p53 and GATA-1 [12, 13, 47]. Similarly, Sorafenib, a multi-kinase inhibitor targeting

receptor tyrosine kinases including VEGFR2 and PDGFR, inhibits tumor cell proliferation and angiogenesis while inducing apoptosis in hepatocellular carcinoma (HCC) [18–20]. In the present study, we utilized a multidrug-resistant (MDR) HA22T cell line with cross-resistance to both HDAC inhibitors and Sorafenib to investigate downstream signal transduction pathways.

Given the difficulty of overcoming chemoresistance, supportive therapeutic strategies, such as hormone therapy, have emerged as alternative options for cancer management. Accumulating evidence indicates that sex steroids significantly influence tumor growth and progression [29–31]. Previous studies have reported that estrogen signaling exerts a protective role in the liver, suppressing HCC progression and encouraging tumor cell apoptosis [27, 28]. However, our data revealed that treatment with 17 β -estradiol (E2) alone failed to induce cell death or attenuate tumor progression signaling in either parental HA22T or MDR cells (Fig. 1C–1E). Clinical observations have shown that ER α expression is markedly downregulated in HCC tissues compared to normal liver tissues [41]. Consistent with these reports, we observed that both baseline ER α mRNA and protein levels were exceptionally low in both cell lines (Fig. 2A, 2B), suggesting that the absence of sufficient endogenous ER α accounts for the loss of estrogen-mediated tumor-suppressive functions. Furthermore, our laboratory's previous work demonstrated that estrogen receptor signaling is a critical determinant in modulating liver cancer risk [41–43], and that restoring ER α expression combined with pharmacological intervention may offer clinical efficacy in preventing or treating HCC. Supporting this concept, our current findings confirm that ectopic overexpression of ER α successfully reinstates the apoptotic cascade in liver cancer cells (Fig. 3A, 3B).

Following these findings, we overexpressed ER α and confirmed that ectopic ER α expression successfully restored apoptotic susceptibility in drug-resistant liver cancer cells (Fig. 3A, 3B). Classical estrogen receptor signaling predominantly operates through genomic mechanisms, wherein ligand-activated ER α or ER β translocates to the nucleus to regulate target gene transcription [48]. However, emerging evidence highlights non-genomic actions of estrogen receptors, with several studies demonstrating that both ER α and ER β can localize to the mitochondria in various cell types, including HepG2 human hepatoma cells and rat primary hepatocytes [49, 50]. Cytoplasmic and mitochondrial pools of ERs suggest that these receptors may exert direct organelle-specific functions independent of traditional nuclear transcription.

Consistent with these reports, our immunofluorescence and subcellular fractionation analyses demonstrated that overexpressed ER α translocates to both the nucleus and the mitochondria in HA22T and MDR cells (Fig. 5A, 5B). Intrigued by this organelle targeting, we further investigated how mitochondrial ER α governs the apoptotic cascade. Previous literature has suggested that E2/ER signaling can influence mitochondrial respiratory function and reactive oxygen species (ROS) generation [51, 52]. Our co-immunoprecipitation assays revealed that ectopic ER α physically interacts with the pro-apoptotic effector Bax to form an ER α –Bax complex in both parental and drug-resistant cells (Fig. 6A). This complex translocates to the mitochondria, promoting mitochondrial outer membrane permeabilization, triggering a robust accumulation of mitochondrial ROS (Fig. 6B), and ultimately

activating Caspase-3-dependent apoptosis. These results establish a novel, non-genomic pathway by which overexpressed ER α interacts with Bax to induce mitochondrial oxidative stress and cell death in chemoresistant HCC cells.

Following the evidence from previous studies and our present findings demonstrates that ER α functions as a pivotal regulator of tumorigenesis, promoting cell death in drug-resistant hepatocellular carcinoma through its physical interaction with Bax and subsequent mitochondrial translocation. Tetracycline-controllable (Tet-based) gene expression systems serve as robust tools for precise, externally regulated transgene expression in both cultured cell lines and animal models, owing to the high specificity, efficiency, and low toxicity of tetracycline [57].

To understand the downstream mechanisms more clearly, future studies will use Flow cytometry to check cell apoptosis, TUNEL assay to confirm DNA damage, and JC-1 staining to detect mitochondrial membrane potential loss. In addition, we will use these Tet-Off ER α stable clones to continuously overexpress ER α to confirm the translocation of ER α and Bax. We will also perform mitochondrial isolation and Co-IP assays to confirm whether ER α interacts with Bax and translocates to mitochondria together. Through these experiments, we hope to prove that restoring ER α expression can damage mitochondria and overcome drug resistance in liver cancer.

Conclusion

In conclusion (Fig. 8), our study demonstrates that overexpressed ER α plays a crucial tumor-suppressive role and effectively sensitizes multidrug-resistant hepatocellular carcinoma (MDR-HCC) cells to cell death. Although exogenous 17 β -estradiol (E2) treatment alone exerted negligible cytotoxic effects due to exceptionally low baseline endogenous ER α expression in HA22T and MDR cells, ectopic overexpression of ER α successfully reinstated apoptotic pathways. Mechanistically, overexpressed ER α suppresses pro-survival p-Akt signaling and physically interacts with Bax in the cytoplasm. This ER α -Bax complex translocates to the mitochondria, leading to mitochondrial dysfunction, marked accumulation of mitochondrial ROS, Cytochrome C release, and subsequent activation of Caspase-3-dependent apoptosis. Furthermore, plant-derived phytoestrogens, such as Daidzein, effectively suppress p-Akt/p-cofilin signaling and inhibit MDR cell growth, providing valuable therapeutic strategies. Overall, targeting ER α mitochondrial translocation and its interaction with Bax represents a highly promising candidate strategy to overcome chemoresistance in liver cancer therapy.

Declarations

Data Availability Statement

All data generated or analyzed during this study are included in this published article and its supplementary information files

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Authors' Contributions

Conceptualization: Po-Hsiang Liao, Wei-Wen Kuo, Yao-Chun Hsu, and Chih-Yang Huang; methodology: Po-Hsiang Liao, Wei-Wen Kuo, Yo-Wei Wu, Dennis Jine-Yuan Hsieh, and Yueh-Min Lin; investigation: Po-Hsiang Liao, Yo-Wei Wu, and Kuan-Ho Lin; validation and formal analysis: Po-Hsiang Liao, Chia-Hua Kuo, Dennis Jine-Yuan Hsieh, and Kuan-Ho Lin; data curation: Po-Hsiang Liao, Wei-Wen Kuo, and Dennis Jine-Yuan Hsieh; funding acquisition and resources: Chih-Yang Huang, Yao-Chun Hsu, and Shinn-Zong Lin; writing – original draft preparation: Po-Hsiang Liao; writing – review and editing: Po-Hsiang Liao, Wei-Wen Kuo, Chia-Hua Kuo, Yueh-Min Lin, Yao-Chun Hsu, and Chih-Yang Huang; supervision and project administration: Chih-Yang Huang, Yao-Chun Hsu, and Shinn-Zong Lin. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics Statement

Ethical approval was not required for this study as it exclusively utilized established commercial cell lines (HA22T) and derived resistant cell lines, with no human participants, clinical tissues, or animal subjects involved.

References

1. Villanueva, A. & Hepatocellular Carcinoma *N Engl. J. Med.* **380**, 1450–1462, doi:10.1056/NEJMr1713263 (2019).
2. Liao, P. H. et al. Phosphorylation of cofilin-1 by ERK confers HDAC inhibitor resistance in hepatocellular carcinoma cells via decreased ROS-mediated mitochondria injury. *Oncogene* **36**, 1978–1990. 10.1038/onc.2016.357 (2017).
3. Bray, F. et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *Cancer J. Clin.* **68**, 394–424 (2018).
4. Vizoso, F. et al. Liver expression of steroid hormones and Apolipoprotein D receptors in hepatocellular carcinoma. *World J. gastroenterology: WJG.* **13**, 3221 (2007).
5. Hsu, H. T. & Chi, C. W. Emerging role of the peroxisome proliferator-activated receptor-gamma in hepatocellular carcinoma. *J. Hepatocell Carcinoma.* **1**, 127–135. 10.2147/JHC.S48512 (2014).

6. El-Serag, H. B. Epidemiology of viral hepatitis and hepatocellular carcinoma. *Gastroenterology* **142**, 1264–1273 e1261, (2012). 10.1053/j.gastro.2011.12.061
7. Uhl, P., Fricker, G., Haberkorn, U. & Mier, W. Current status in the therapy of liver diseases. *Int. J. Mol. Sci.* **15**, 7500–7512. 10.3390/ijms15057500 (2014).
8. Befeler, A. S. & Di Bisceglie, A. M. Hepatocellular carcinoma: diagnosis and treatment. *Gastroenterology* **122**, 1609–1619. 10.1053/gast.2002.33411 (2002).
9. Nowak, A. K., Chow, P. K. & Findlay, M. Systemic therapy for advanced hepatocellular carcinoma: a review. *Eur. J. Cancer.* **40**, 1474–1484. 10.1016/j.ejca.2004.02.027 (2004).
10. Baylin, S. B. Resistance, epigenetics and the cancer ecosystem. *Nat. Med.* **17**, 288–289. 10.1038/nm0311-288 (2011).
11. Shen, Y. C., Hsu, C. & Cheng, A. L. Molecular targeted therapy for advanced hepatocellular carcinoma: current status and future perspectives. *J. Gastroenterol.* **45**, 794–807. 10.1007/s00535-010-0270-0 (2010).
12. Dokmanovic, M., Clarke, C. & Marks, P. A. Histone deacetylase inhibitors: overview and perspectives. *Mol. Cancer Res.* **5**, 981–989. 10.1158/1541-7786.MCR-07-0324 (2007).
13. Eckschlager, T., Plch, J., Stiborova, M. & Hrabeta, J. Histone Deacetylase Inhibitors as Anticancer Drugs. *Int. J. Mol. Sci.* **18** 10.3390/ijms18071414 (2017).
14. Hsieh, C. H. et al. Apicidin-resistant HA22T hepatocellular carcinoma cells strongly activated the Wnt/beta-catenin signaling pathway and MMP-2 expression via the IGF-IR/PI3K/Akt signaling pathway enhancing cell metastatic effect. *Biosci. Biotechnol. Biochem.* **77**, 2397–2404. 10.1271/bbb.130503 (2013).
15. Hsu, H. H. et al. Apicidin-resistant HA22T hepatocellular carcinoma cells massively promote pro-survival capability via IGF-IR/PI3K/Akt signaling pathway activation. *Tumour Biol.* **35**, 303–313. 10.1007/s13277-013-1041-3 (2014).
16. Tu, C. C. et al. Activation of snail and EMT-like signaling via the IKK α /NF- κ B pathway in Apicidin-resistant HA22T hepatocellular carcinoma cells. *Chin. J. Physiol.* **56**, 326–333. 10.4077/CJP.2013.BAB158 (2013).
17. Kudo, M. et al. Lenvatinib versus sorafenib in first-line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. *Lancet* **391**, 1163–1173. 10.1016/s0140-6736(18)30207-1 (2018).
18. Kim, D. W., Talati, C. & Kim, R. Hepatocellular carcinoma (HCC): beyond sorafenib-chemotherapy. *J. Gastrointest. Oncol.* **8**, 256–265. 10.21037/jgo.2016.09.07 (2017).
19. Liu, L. et al. Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. *Cancer Res.* **66**, 11851–11858. 10.1158/0008-5472.CAN-06-1377 (2006).
20. Wang, L. et al. Combined anti-tumor effects of IFN- α and sorafenib on hepatocellular carcinoma in vitro and in vivo. *Biochem. Biophys. Res. Commun.* **422**, 687–692 (2012).

21. El-Serag, H. B. & Rudolph, K. L. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* **132**, 2557–2576. 10.1053/j.gastro.2007.04.061 (2007).
22. De Maria, N., Manno, M. & Villa, E. Sex hormones and liver cancer. *Mol. Cell. Endocrinol.* **193**, 59–63 (2002).
23. Zhao, Y. & Li, Z. Interplay of estrogen receptors and FOXA factors in the liver cancer. *Mol. Cell. Endocrinol.* **418**, 334–339 (2015).
24. Nagasue, N. & Kohno, H. Hepatocellular carcinoma and sex hormones. *HPB Surg.* **6**, 1–6 (1992).
25. Sukocheva, O. A. Estrogen, estrogen receptors, and hepatocellular carcinoma: Are we there yet? *World J. Gastroenterol.* **24**, 1 (2018).
26. Shi, L., Feng, Y., Lin, H., Ma, R. & Cai, X. Role of estrogen in hepatocellular carcinoma: is inflammation the key? *J. translational Med.* **12**, 93 (2014).
27. Kalra, M., Mayes, J., Assefa, S., Kaul, A. K. & Kaul, R. Role of sex steroid receptors in pathobiology of hepatocellular carcinoma. *World J. gastroenterology: WJG.* **14**, 5945 (2008).
28. Bigsby, R. M. & Caperell-Grant, A. The role for estrogen receptor-alpha and prolactin receptor in sex-dependent DEN-induced liver tumorigenesis. *Carcinogenesis* **32**, 1162–1166 (2011).
29. Jia, M., Dahlman-Wright, K. & Gustafsson, J. Å. Estrogen receptor alpha and beta in health and disease. *Best Pract. Res. Clin. Endocrinol. Metab.* **29**, 557–568 (2015).
30. Riggs, B. L. & Hartmann, L. C. Selective estrogen-receptor modulators—mechanisms of action and application to clinical practice. *N. Engl. J. Med.* **348**, 618–629 (2003).
31. Wallace, O. B., Richardson, T. I. & Dodge, J. A. Estrogen receptor modulators: relationships of ligand structure, receptor affinity and functional activity. *Curr. Top. Med. Chem.* **3**, 1663–1680 (2003).
32. Moriarty, K., Kim, K. & Bender, J. Estrogen receptor-mediated rapid signaling. *Endocrinology* **147**, 5557–5563 (2006).
33. Yaşar, P., Ayaz, G., User, S. D., Güpür, G. & Muyan, M. Molecular mechanism of estrogen–estrogen receptor signaling. *Reproductive Med. biology.* **16**, 4–20 (2017).
34. Koš, M., Reid, G., Denger, S. & Gannon, F. Minireview: genomic organization of the human ERα gene promoter region. *Mol. Endocrinol.* **15**, 2057–2063 (2001).
35. Matthews, J. & Gustafsson, J. Å. Estrogen signaling: a subtle balance between ERα and ERβ. *Mol. Interv.* **3**, 281 (2003).
36. Hall, J. M., Couse, J. F. & Korach, K. The multifaceted mechanisms of estradiol and estrogen receptor signaling. *J. Biol. Chem.* **276**, 36869–36872 (2001).
37. Beelen, K., Zwart, W. & Linn, S. C. Can predictive biomarkers in breast cancer guide adjuvant endocrine therapy? *Nat. reviews Clin. Oncol.* **9**, 529–541 (2012).
38. Clarke, R., Tyson, J. J. & Dixon, J. M. Endocrine resistance in breast cancer—an overview and update. *Mol. Cell. Endocrinol.* **418**, 220–234 (2015).
39. Shen, M. & Shi, H. Estradiol and estrogen receptor agonists oppose oncogenic actions of leptin in HepG2 cells. *Plos one.* **11**, e0151455 (2016).

40. Hsu, H. T. & Chi, C. W. Emerging role of the peroxisome proliferator-activated receptor-gamma in hepatocellular carcinoma. *J. hepatocellular carcinoma*. **1**, 127 (2014).
41. Chang-Lee, S. N. et al. E 2/ER β inhibits PPAR α to regulate cell-proliferation and enhance apoptosis in Hep3b-hepatocellular carcinoma. *Pathol. Oncol. Res.* **23**, 477–485 (2017).
42. Lin, Y. M. et al. Activation of estrogen receptors with E2 downregulates peroxisome proliferator-activated receptor γ in hepatocellular carcinoma. *Oncol. Rep.* **30**, 3027–3031 (2013).
43. Chen, Y. F. et al. Estrogen and ER α enhanced β -catenin degradation and suppressed its downstream target genes to block the metastatic function of HA22T hepatocellular carcinoma cells via modulating GSK-3 β and β -TrCP expression. *Environ. Toxicol.* **32**, 519–529 (2017).
44. Dunn, B. & Cancer Solving an age-old problem. *Nature* **483**, S2–S6 (2012).
45. Zahreddine, H. & Borden, K. Mechanisms and insights into drug resistance in cancer. *Front. Pharmacol.* **4**, 28 (2013).
46. Goldie, J. H. Drug resistance in cancer: a perspective. *Cancer Metastasis Rev.* **20**, 63–68 (2001).
47. Marks, P. A., Richon, V. M., Breslow, R. & Rifkind, R. A. Histone deacetylase inhibitors as new cancer drugs. *Curr. Opin. Oncol.* **13**, 477–483 (2001).
48. Hewitt, S. C. & Korach, K. S. Estrogen receptors: structure, mechanisms and function. *Reviews Endocr. Metabolic Disorders.* **3**, 193–200 (2002).
49. Pedram, A., Razandi, M., Wallace, D. C. & Levin, E. R. Functional estrogen receptors in the mitochondria of breast cancer cells. *Mol. Biol. Cell.* **17**, 2125–2137 (2006).
50. Solakidi, S., Psarra, A. M. & Sekeris, C. E. Differential subcellular distribution of estrogen receptor isoforms: localization of ER α in the nucleoli and ER β in the mitochondria of human osteosarcoma SaOS-2 and hepatocarcinoma HepG2 cell lines. *Biochim. et Biophys. Acta (BBA)-Molecular Cell. Res.* **1745**, 382–392 (2005).
51. Sastre-Serra, J. et al. Estrogen down-regulates uncoupling proteins and increases oxidative stress in breast cancer. *Free Radic. Biol. Med.* **48**, 506–512 (2010).
52. Felty, Q. et al. Estrogen-induced mitochondrial reactive oxygen species as signal-transducing messengers. *Biochemistry* **44**, 6900–6909 (2005).
53. Zhu, Z. et al. Tetracycline-controlled transcriptional regulation systems: advances and application in transgenic animal modeling. *Semin. Cell Dev. Biol.* **13** (2), 121–128 (2002).

Figures

Figure 1

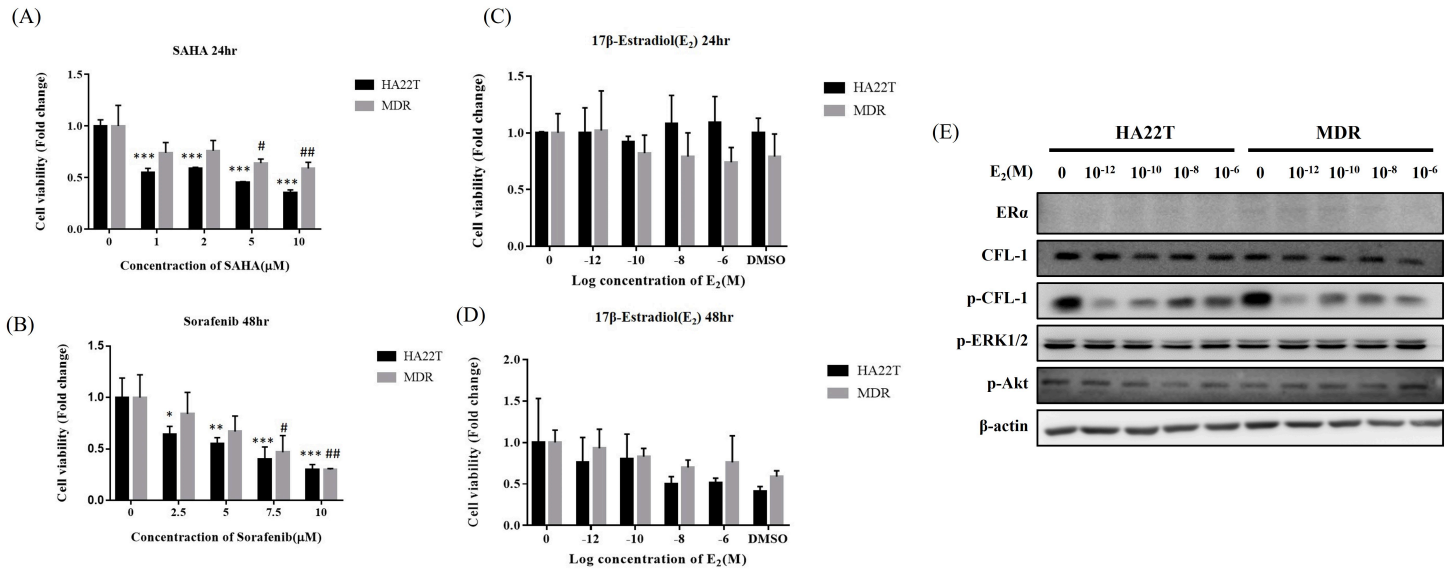


Figure 1

Evaluation of multidrug resistance and cellular responses to 17β-estradiol (E2) in parental HA22T and MDR cells.

(A) HA22T and MDR cells were treated with indicated concentrations of SAHA (0, 1, 2, 5, and 10 μM) for 24 h. Cell viability was determined by MTT assay. (B) HA22T and MDR cells were treated with indicated concentrations of Sorafenib (0, 2.5, 5, 7.5, and 10 μM) for 48 h, and cell viability was evaluated by MTT assay. (C, D) HA22T and MDR cells were treated with various concentrations of 17β-estradiol (E₂; 0, 10⁻¹², 10⁻¹⁰, 10⁻⁸, and 10⁻⁶ M) or vehicle control (DMSO) for (C) 24 h and (D) 48 h, followed by cell viability measurement using MTT assay. (E) Western blot analysis of ERα, CFL-1, p-CFL-1, p-ERK1/2, p-Akt, and β-actin in HA22T and MDR cells treated with increasing concentrations of E₂ for 24 h. β-actin served as an internal loading control. Data are expressed as mean ± SD from three independent experiments. **P* < 0.05, *P* < 0.01, ****P* < 0.001 compared with the control group in HA22T cells; #*P* < 0.05, ##*P* < 0.01 compared with the control group in MDR cells.

Figure 2

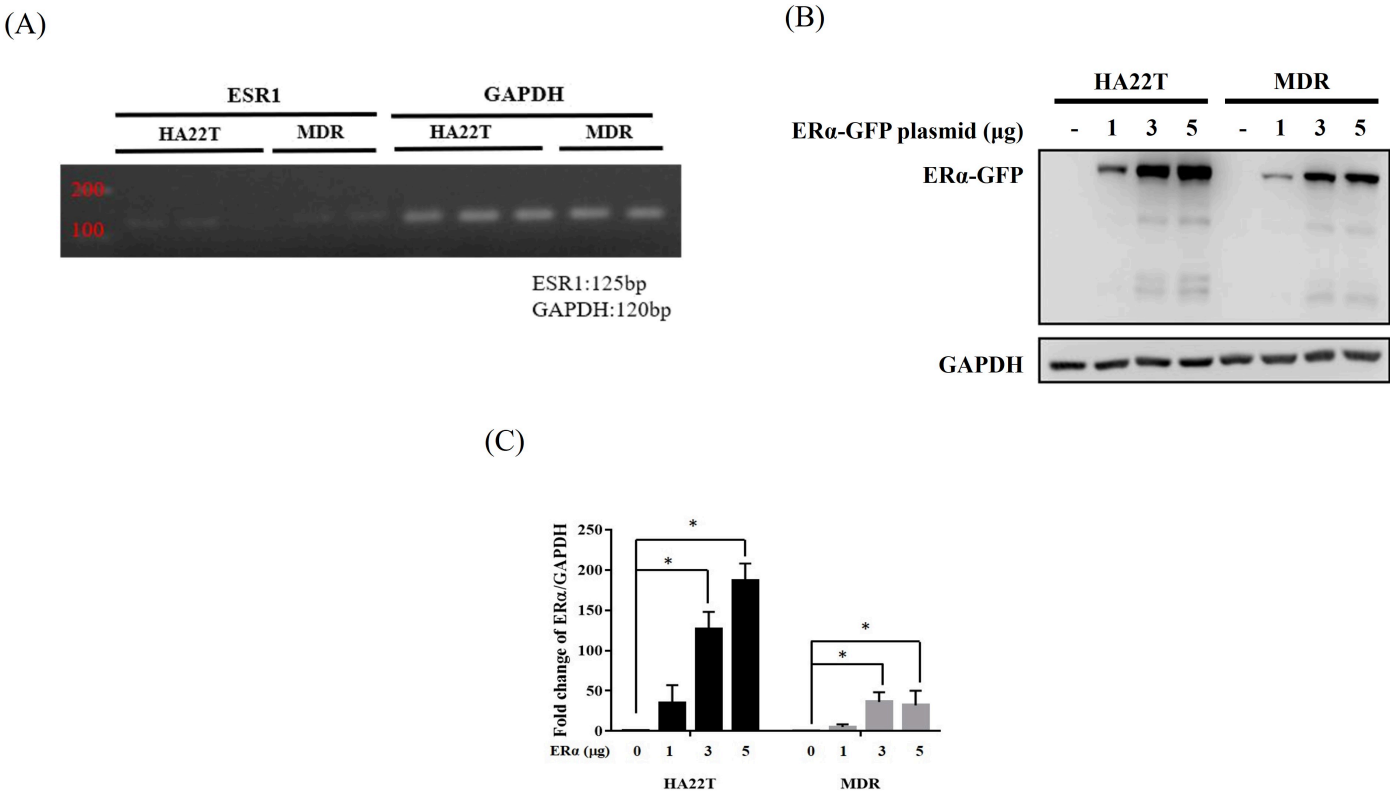


Figure 2

Endogenous ERα expression and validation of dose-dependent ectopic ERα overexpression in HA22T and MDR cells.

(A) RT-PCR analysis of endogenous ERα (ESR1; 125 bp) mRNA expression in parental HA22T and drug-resistant MDR cells. GAPDH (120 bp) was used as an internal amplification control. DNA ladder markers (100 bp and 200 bp) are indicated on the left. (B) Western blot analysis of ERα-GFP fusion protein expression in HA22T and MDR cells transfected with increasing amounts of ERα-GFP plasmid (-, 1, 3, and 5 μg) for 24 h. ERα-GFP was detected using an anti-ERα antibody. GAPDH served as an internal loading control. (C) Densitometric quantification of ERα-GFP protein levels normalized to GAPDH. Data are presented as mean ± SD from three independent experiments. *P < 0.05 compared with the corresponding control group.

Figure 3

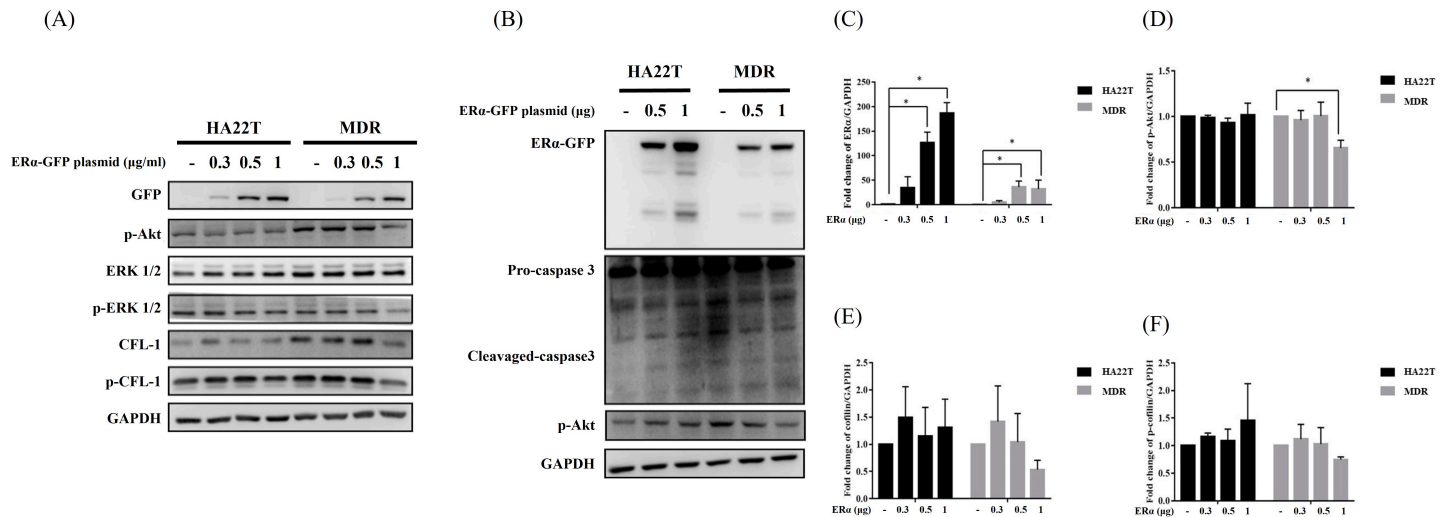


Figure 3

Ectopic ERα overexpression suppresses pro-survival signaling and activates caspase-dependent apoptosis in HA22T and MDR cells.

(A) Western blot analysis of GFP, p-Akt, ERK 1/2, p-ERK 1/2, CFL-1, p-CFL-1, and GAPDH in HA22T and MDR cells transfected with increasing concentrations of ERα-GFP plasmid (-, 0.3, 0.5, and 1 µg/mL) for 24 h. GAPDH served as an internal loading control. (B) Western blot analysis of ERα-GFP, Pro-caspase 3, Cleaved-caspase3, p-Akt, and GAPDH in HA22T and MDR cells transfected with ERα-GFP plasmid (-, 0.5, and 1 µg). GAPDH was used as an internal loading control. (C–F) Densitometric quantification of (C) ERα, (D) p-Akt, (E) cofilin, and (F) p-cofilin protein levels normalized to GAPDH. Data are presented as mean ± SD from three independent experiments. *P < 0.05 compared with the corresponding non-transfected control group

Figure 4

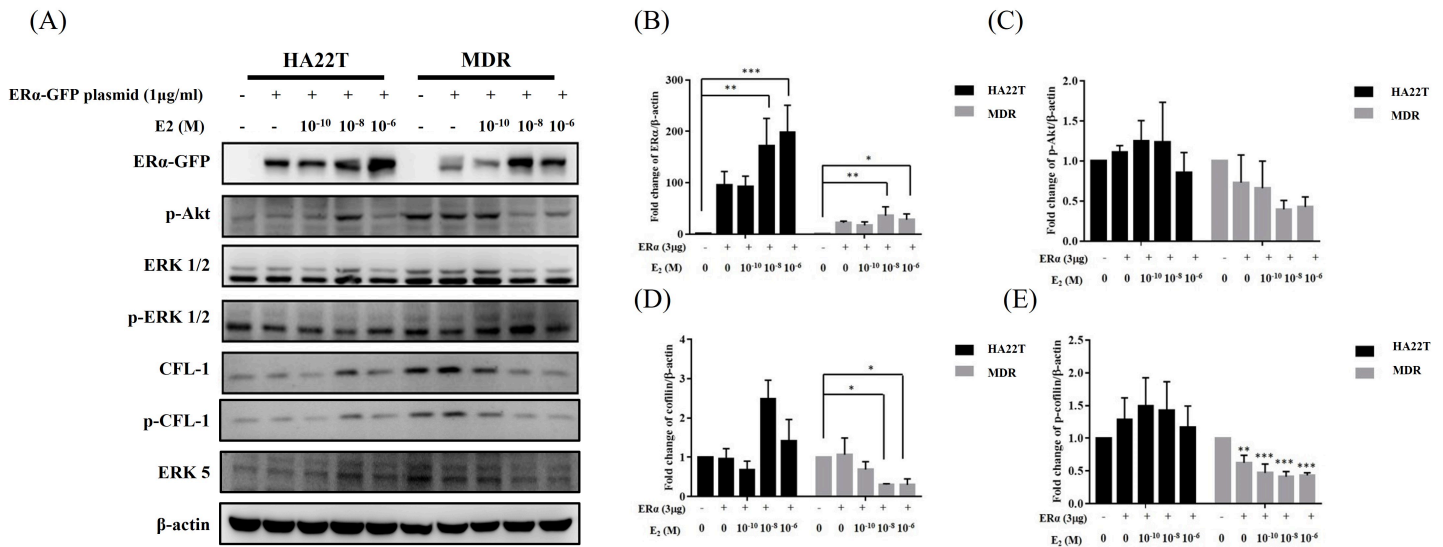


Figure 4

Targeting the p-Akt and CFL-1 Pathways in Resistant HCC via ERα Restoration Combined with E2 Treatment

(A) Western blot analysis of ERα-GFP, p-Akt, ERK 1/2, p-ERK 1/2, CFL-1, p-CFL-1, ERK 5, and β-actin in parental HA22T and drug-resistant MDR cells transfected with ERα-GFP plasmid (1 μg/mL) in the absence or presence of increasing concentrations of 17β-estradiol (E₂; 10⁻¹⁰, 10⁻⁸, and 10⁻⁶ M) for 24 h. β-actin served as an internal loading control. (B–E) Densitometric quantification of (B) ERα-GFP, (C) p-Akt, (D) CFL-1, and (E) p-CFL-1 protein levels normalized to β-actin. Data are presented as mean ± SD from three independent experiments. *P < 0.05, P < 0.01, ***P < 0.001 compared with the corresponding control or indicated comparison groups.

Figure 5

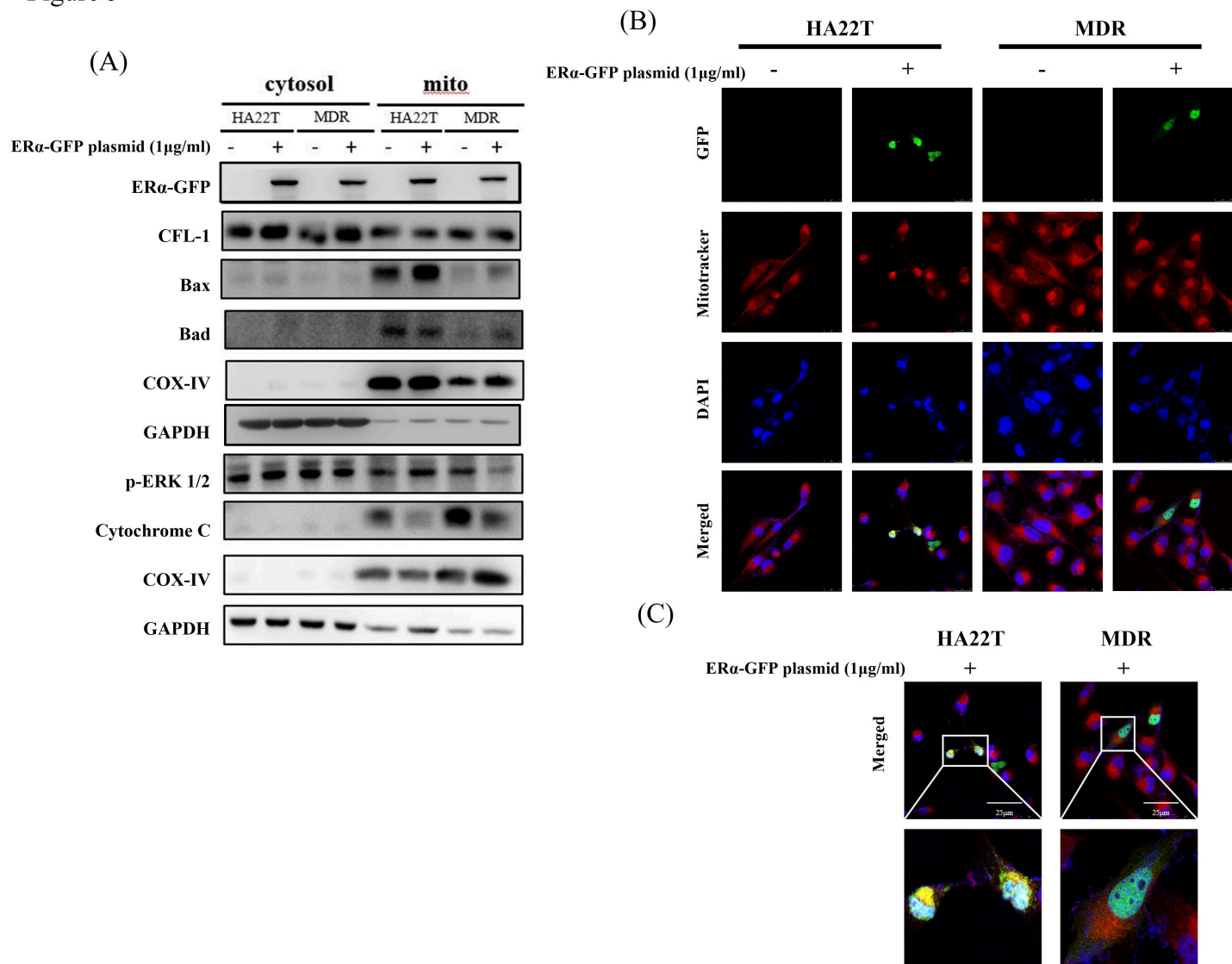


Figure 5

Mitochondrial translocation of ERα drives the organelle-specific accumulation of pro-apoptotic regulators in chemoresistant hepatocellular carcinoma.

(A) Subcellular fractionation and Western blot analysis of ERα-GFP, CFL-1, Bax, Bad, p-ERK 1/2, and Cytochrome C in cytosolic (cytosol) and mitochondrial (mito) fractions of parental HA22T and drug-resistant MDR cells transfected with or without ERα-GFP plasmid (1 μg/mL) for 24 h. COX-IV served as the mitochondrial marker, and GAPDH served as the cytosolic marker and loading control. (B) Confocal immunofluorescence microscopy of ERα subcellular localization. HA22T and MDR cells transfected with ERα-GFP plasmid (1 μg/mL) were stained with MitoTracker Red to label mitochondria and DAPI (blue) to counterstain nuclei. (C) Merged panels and high-magnification zoom-in views demonstrating the co-localization (yellow signals) of ERα-GFP (green) with mitochondria (red) and nuclei (blue). Scale bar: 25 μm.

Figure 6

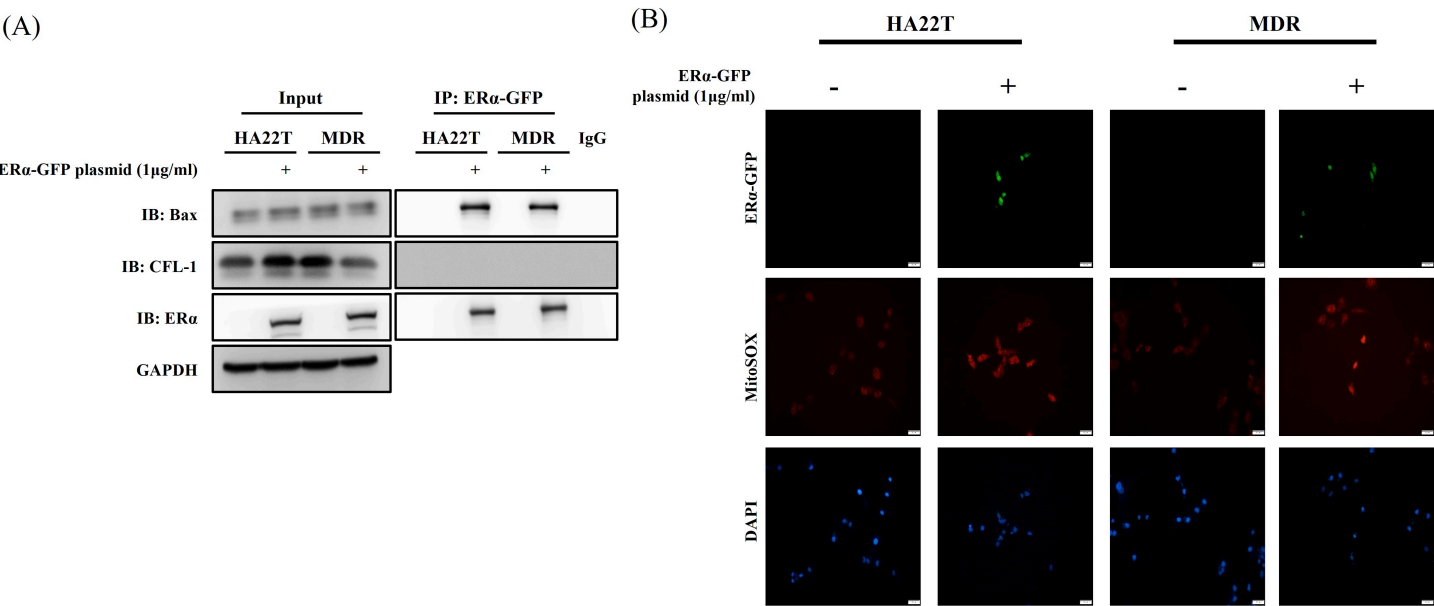


Figure 6

Physical interaction of ERα with Bax promotes mitochondrial ROS generation in chemoresistant HCC cells.

(A) Co-immunoprecipitation (Co-IP) analysis demonstrating complex formation between ERα and Bax. Whole-cell lysates from parental HA22T and drug-resistant MDR cells transfected with or without ERα-GFP plasmid (1 μg/mL) were immunoprecipitated using an anti-ERα antibody (or normal IgG as a negative control), followed by immunoblotting with antibodies against Bax, CFL-1, and ERα. Whole-cell lysates (Input) were analyzed in parallel for Bax, CFL-1, ERα, and GAPDH (internal loading control). (B) Evaluation of mitochondrial superoxide levels using MitoSOX Red fluorescence staining. HA22T and MDR cells expressing ERα-GFP (green) were stained with MitoSOX Red (red) and counterstained with DAPI (blue) to assess mitochondrial ROS accumulation. Scale bar: 25 μm.

Figure 7

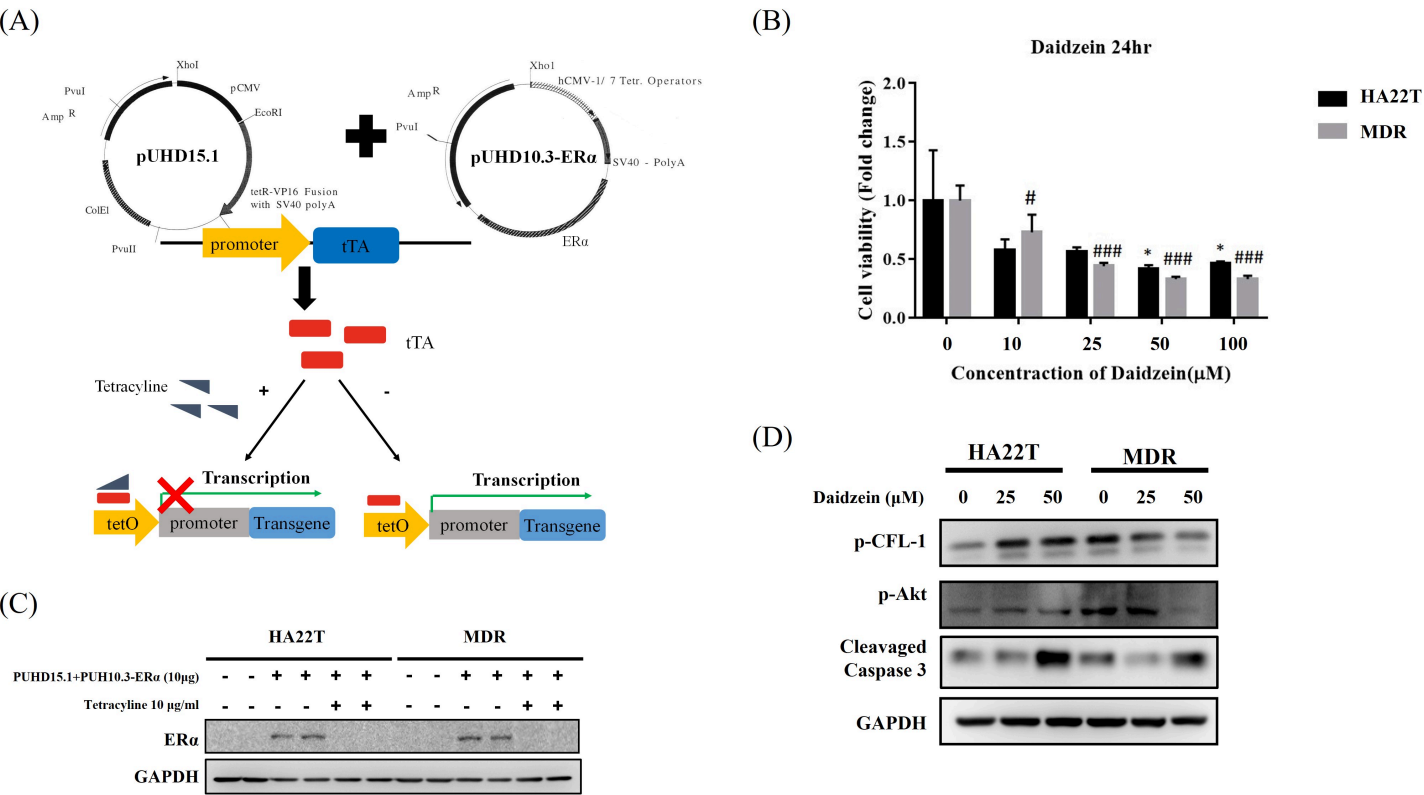


Figure 7

Characterization of the inducible Tet-Off ERα expression system and anti-tumor efficacy of the phytoestrogen Daidzein in chemoresistant HCC cells.

(A) Schematic illustration of the tetracycline-regulated Tet-Off expression system. Cells were co-transfected with pUHD15.1 encoding the tetracycline-controlled transactivator (tTA) and pUHD10.3-ERα containing the ERα cDNA downstream of tetracycline operator sequences (tetO). In the absence of tetracycline (Tet-Off ON state), tTA binds to tetO to drive ERα transcription; the addition of tetracycline prevents tTA from binding tetO, thereby shutting off ERα expression. (B) HA22T and MDR cells were treated with indicated concentrations of Daidzein (0, 10, 25, 50, and 100 μM) for 24 h, followed by the evaluation of cell viability using MTT assay. (C) Western blot analysis of ERα protein expression in HA22T and MDR cells harboring the Tet-Off system cultured with or without tetracycline (10 μg / mL). GAPDH served as an internal loading control. (D) Western blot analysis of p-CFL-1, p-Akt, Cleaved Caspase 3, and GAPDH in parental HA22T and drug-resistant MDR cells treated with indicated concentrations of Daidzein (0, 25, and 50 μM) for 24 h. GAPDH served as an internal loading control. Data in (B) are presented as mean ± SD from three independent experiments. *P < 0.05 compared with the control group in HA22T cells; #P < 0.05, ###P < 0.001 compared with the control group in MDR cells.

Figure 8

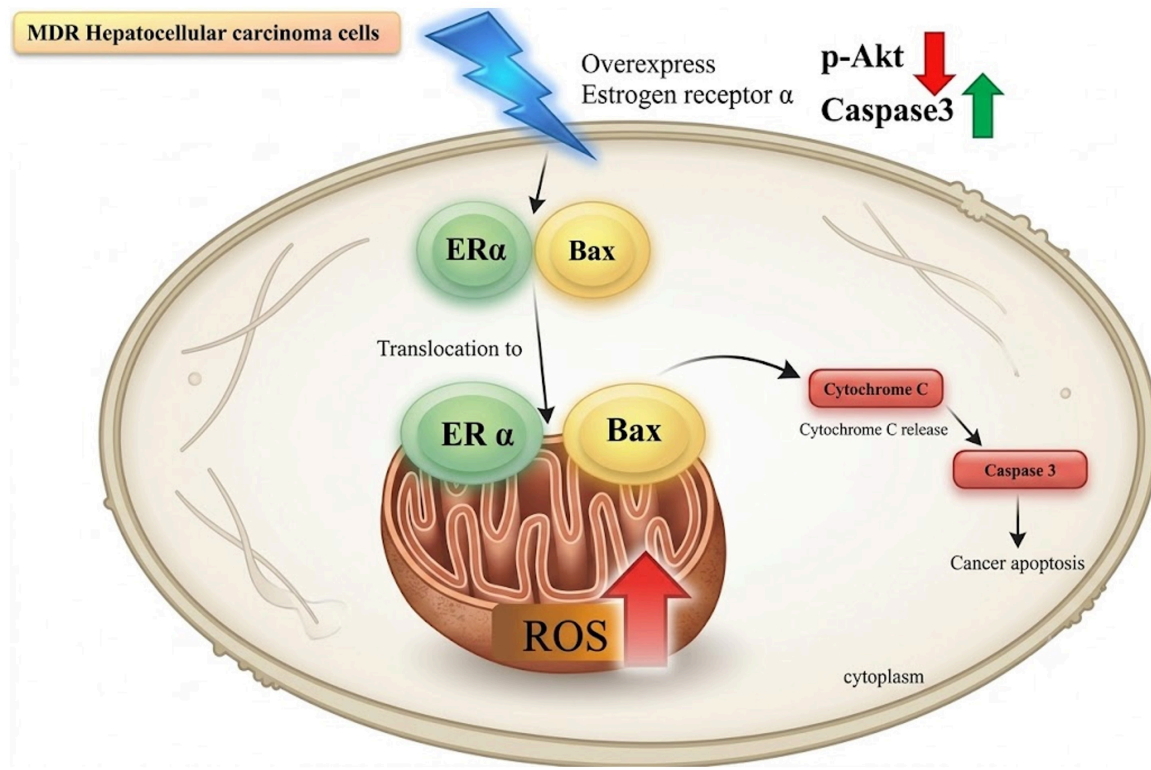


Figure 8

Schematic working model illustrating the mechanism of ERα-mediated apoptosis in multidrug-resistant hepatocellular carcinoma (MDR-HCC) cells. Ectopic overexpression of estrogen receptor α (ERα) suppresses pro-survival p-Akt signaling and facilitates the formation of a physical complex between ERα and the pro-apoptotic protein Bax in the cytoplasm. The ERα–Bax complex translocates to the mitochondria, triggering substantial accumulation of mitochondrial reactive oxygen species (ROS), Cytochrome C release into the cytoplasm, and activation of Caspase-3, thereby overcoming chemoresistance and executing apoptotic cell death in MDR-HCC cells.

Supplementary Files

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