Loss of HAS2 Confers Acquired Antiestrogen Resistance By Upregulating Ezrin Expression In ER-Positive Breast Cancer

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Research

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Abstract

Background: Resistance to endocrine therapy is a major challenge for estrogen receptor-positive (ER+) breast cancer patients, but the underlying mechanisms remain unclear.

Methods: Loss of hyaluronan synthase 2 (Has2) in adaptive resistant cells to tamoxifen and fulvestrant was observed by immunblotting assay. CRISPR/Cas9 technology was used to knock out Has2 in MCF7 cells to verify the effect of Has2 on the expression of ER and Ezrin and Akt and MAPK/ERK signaling routes. We utilized an Ezrin small-interfering RNA and Ezrin inhibitor to inhibit Ezrin expression for evaluating Has2 and ERα expression and the Akt/MAPK signaling cascade upon tamoxifen or fulvestrant treatment.

Results: In this work, we showed that a Has2-loss state was acquired from adaptive resistance to tamoxifen and fulvestrant in luminal BrCas. Notably, the adapted loss of Has2 induced acquired resistance to antiestrogens in estrogen receptor (ER)-positive breast cancer cells through up-regulating the expression of Ezrin. Furthermore, we found that the loss of Has2 promoted while the consequent increase of Ezrin inhibited ERα expression/activity through the Akt and MAPK/ERK signaling routes, indicating an opposite effect on ERα expression during the development of antiestrogens-resistance. Inhibition of Ezrin reversed Has2 and ERα expression and the Akt/MAPK signaling cascade upon tamoxifen or fulvestrant, suggesting a Has2-Ezrin-ER negative-feedback loop in governing cellular sensitivity to tamoxifen or fulvestrant in luminal-like breast cancer cells. Finally, Knockdown or inhibition of Ezrin restored sensitivity to antiestrogens, implying that Ezrin could be a potential therapeutic target to tackle endocrine resistance.

Conclusions: Taken together, our findings provide a direct relationship between ERα and Has2 implicated in resistance to endocrine therapy and a new insight into how ERα-signaling is regulated upon antiestrogens treatment, suggesting a novel therapeutic target for ER-positive breast cancer.

Highlights

1. Acquired Has2 loss is associated with resistance to tamoxifen or fulvestrant

2. Loss of Has2 induces resistance by regulating Ezrin expression and localization through the Akt and MAPK/ERK signaling routes in ER+ cancer cells

3. A Has2-Ezrin-ER negative-feedback loop modulates cellular sensitivity to tamoxifen or fulvestrant

4. Knockdown or inhibition of Ezrin restores sensitivity to tamoxifen or fulvestrant

Background

Acquired resistance to endocrine therapy is a major challenge for estrogen receptor-positive (ER+) breast cancer and leads to unfavorable clinical outcome [1]. Despite the overall success of endocrine therapy,

acquired resistance occurs in nearly half of ER-positive breast cancer patients with advanced disease[2], even in those who initially respond to endocrine therapy[3]. However, up to now, the precise mechanisms for the acquisition of adaptive endocrine resistance in patients with ER+ breast cancer are still poorly understood.

It has been reported that, when ER was inhibited, reduction of ER expression at relapse sites were found from clinical retrospective studies [4, 5]. Subsequently, tumors develop hormone-independent resistant characteristics, including the occurrence of ligand-independent ER activation [6], altered functional interactions of ER with co-regulators [7–9], or genomic mutations of the ER gene ESR1 [10, 11]. However, even in the presence of acquired resistance to endocrine therapy, a sustained ER signaling is implied by evidence from pre-clinical and clinical studies [1, 12]. Indeed, only a minority (~10%) of endocrine-resistant breast cancers displayed loss of ER expression [13], and the majority of endocrine-resistant tumors still sustain ERα expression, displaying a continued role of ERα signaling in maintaining resistance [14]. The mechanism underlying endocrine resistance due to the reduction of ER expression is poorly understood, and encourages further study.

Hyaluronan synthase 2 (Has2) is the most important one of three hyaluronan (HA) synthases embedded in cell membrane [15], showing an indispensable role in embryonic development [16]. Besides synthesizing high molecular weight HA, Has2 itself is an important modulator of tissue microenvironment involved in restoring tissue homeostasis [17]. The expression of Has2 is sensitive to survival signals in cell microenvironment, such as hormone-type effectors (glucocorticoid) [18–20], prostaglandins[21], most growth factors, and cytokines [22]. Has2 also plays an important role in intracellular trafficking [23]. These studies suggested that Has2 was readily to be regulated by cell signals to maintain a fine balance of cell states [24]. More importantly, the Has2 gene is regulated in part by estrogen [25, 26]. However, in tumor survival microenvironment, its specific role is still largely unknown.

We previously reported that Has2 is an important mediator of increased breast cancer aggressiveness[27] and an important driver of M2-like tumor-associated macrophages formation [28]. Has2 induces excessive hyaluronan production that triggered phenotype plasticity and drug-resistance of breast cancer cells from studies in our laboratory[29] and other laboratories[30]. It is also well documented that Has2 might be a potential therapeutic target via Has2 inhibitor [31]. Moreover, from our recent study, luminal-like breast cancer cells preferentially express Has2 or ER with a positive association under pressure of PI3Kα-inhibitory chemotherapy [32]. Taken together, we hypothesize that there might be a potentially novel relationship of ER and Has2 during the development of endocrine therapy resistance. However, to our knowledge, it has yet to be shown the direct relationship between ER and Has2 in cell resistance to endocrine therapy, even though estrogen treatment have shown enhanced activity of hyaluronan synthases[33].

In the current study, we investigated for the first time the role of Has2 in luminal-like breast carcinomas with long-term exposure to endocrine therapy. We identified a Has2-loss state that was acquired due to endocrine therapy resistance. Moreover, the association between Has2 loss and ER was observed upon

tamoxifen or fulvestrant treatment. We demonstrated that Has2-loss could upreguate a robust increase of Ezrin and induce resistance to tamoxifen or fulvestrant. Also, feedback loops consisting of Has2-Ezrin-ER was found to regulate the transition from a sensitive to a resistant cell state. Overall, our study reveals a novel mechanism that links the modification of Has2 expression with ER signaling.

Material And Methods

1. Therapeutic agents

17β-Estradiol (E2), Tamoxifen, Fulvestrant and Ezrin-inhibitor (NSC668394) were purchased from MCE (MedChem Express).

2. Cell culture

The human breast cancer cell lines MCF7 (NCI-DTP Cat# MCF7, RRID: CVCL_0031) and T47D (BCRC Cat# 60250, RRID:CVCL_0553) were purchased from the American Type Culture Collection (ATCC, Manassas, Virginia). MCF7 were cultured in MEM (Gibco, Thermo Fisher Scientific, USA), supplemented with 10% fetal calf serum, 1×MEM non-essential amino acid (NEAA), 0.01 mg/ml bovine insulin, 100 U/ml penicillin and 100mg/ml streptomycin. T47D were cultured in RPMI-1640 medium, supplemented with 10% fetal calf serum, 0.0075 mg/ml bovine insulin ,100 U/ml penicillin and 100mg/ml streptomycin. All cells were grown to 85% confluence for the experiments. All the cell lines were authenticated using SNP profiling. All the cell lines were tested negative for mycoplasma. All cell lines were cultured in a humidified incubator with 5% CO_2 at 37℃.

3. Generation of resistant cell lines

MCF7 and T47D cells were maintained in phenol-red-free MEM (Gibco, Thermo Fisher Scientific, USA) and RPMI-1640 medium (Gibco, Thermo Fisher Scientific, USA) respectively for 3 days, and the media was supplemented with 5% charcoal/dextran-treated FBS. The resistant cell lines were generated by long-term treatment with 1 μM tamoxifen or fulvestrant as previous reports [34, 35].

4. Cell proliferation

The cell survival of cancer cell lines (sensitive or their resistant counterparts) was determined by CCK-8 assay (Promega, America). Equal numbers of cells (5000 cells/well) were seeded into 96-well plates for the proliferation experiment. After increasing doses of inhibitor exposure for 3 days, the CCK-8 reagent (20ul) was added to each well and incubated at 37°C for 2 h. The optical density at 490 nm was measured using an automatic microplate reader (Epoch; BioTek, USA).

5. 5-Ethynyl-20-deoxyuridine (EdU) assay

Cell proliferation was analyzed using EdU assays with a Cell-Light EdU DNA Cell Proliferation kit (Beyotime Institute of Biotechnology,China). Naive and endocrine-resistant breast cancer cells (1×10^4) were seeded in 96-well plates separately .After incubation for 48 h, 10 mM EdU was added and incubated

at 37˚C for another 2 h. Cells were fixed with 4% paraformaldehyde and stained with azide (30 min, for proliferating cells) and Hoechst 33342 (10 min, for all cells) at room temperature. Images were captured by fluorescence microscope (Nikon). The percentage of proliferating cells was calculated using ImageJ V1.50 software (ImageJ, RRID:SCR_003070, National Institutes of Health).

6. Western blot and Antibodies

Cell lysis buffer (RIPA) (Beyotime, China) was used for protein extraction. Then, total protein concentration was determined by a bicinchoninic acid protein assay kit (Sigma, USA). Total cell lysates were collected, and equal quantities of protein were separated via 10**%** sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a polyvinylidene difluoride (PVDF) membrane. PVDF membranes were blocked with Tris-buffered saline (TBS) containing 5% skimmed milk powder for 1 h, and incubated with Has2 antibody (Abcam, ab131364) at 4°C overnight. Then, the membranes were washed with TBST buffer for three times (10 min each time) and incubated with horseradish peroxidase (HRP)-conjugated polyclonal secondary antibody for 1 h. The membranes were developed using the enhanced plus chemiluminescence assay (Thermo, USA) according to the manufacturer's instructions. Images were analyzed using Image Pro-Plus 6.0. Primary antibodies were pAKT308 (D25E6) XP Rabbit antibody (Cell Signal, 13038T), pAKT473 (D9E) XP Rabbit antibody (Cell Signal, 4060T), pERK (D13.14.4E) XP Rabbit antibody (Cell Signal, 4370P), ERK (C67E7) Rabbit antibody (Cell Signal, 4691P), Ezrin (Cell Signal, 3145s), ERα (Abcam, ab32063, RRID:AB_732249), pERα (s118) (16J4) Mouse antibody (Cell Signal, 2511S).

7. Quantitative real-time PCR

Real-time PCR was performed to verify the expression of HAS2 genes to the response of MCF7 cells and T47D cells with the treatment of ER inhibitors. According to the manufacturer's instructions, total RNA was extracted from cultured cells using RNAiso Plus (Takara, Japan). The RNA concentration was measured using NanoDrop system (Thermo Fisher Scientific, USA). Then, 1 μg mRNA was reverse transcribed using the PrimeScript™ RT Reagent kit with gDNA Eraser (Takara, Japan). qPCR assays were performed with SYBR-Green mix (Takara, Japan) according to the manufacturer's protocol. The relative RNA expression was analyzed by the change-in-threshold ($2^{-\Delta\Delta CT}$) method of the specific gene over the housekeeping genes, GAPDH. Sequences of primers used are as follow:

Has2: Forward: 5'-TTATGGGCAGCCAATGTA-3'

Reverse: 5'-ACTTGCTCCAACGGGTCT-3'

Ezrin: Forward: 5'-ACCAATCAATGTCCGAGTTACC-3'

Reverse: 5'-GCCGATAGTCTTTACCACCTGA-3'

FOS: Forward: 5'-GACTGATACACTCCAAGCGG-3'

Reverse: 5'-CATCAGGGATCTTGCAGGC-3'

PBX1: Forward: 5'-CAGTGAGGAAGCCAAAGAGG-3'

Reverse: 5'-CAGCTGTTTTGGCAGCATAA-3'

c-MYC: Forward: 5'-CCTCCACTCGGAAGGACTATC-3'

Reverse: 5'-TGTTCGCCTCTTGACATTCTC-3'

GAPDH: Forward: 5'-AACGGATTTGGTCGTATTGGG-3'

Reverse: 5'-TCGCTCCTGGAAGA TGGTGAT-3'

8. CRISPR-Cas9 knockout of Has2 in MCF7 cells

MCF7 was subjected to CRISPR/Cas9-mediated knockout of Has2 by plasmid transduction using particles from Santa Cruz Biotechnology (sc-401032). The guide RNA vector was cloned into a pCas-Guide vector which expresses Cas9 behind CBh and U6 promoters. To target the Has2, guide RNA vector including target sequence (CTCGCAACACGTAACGCAAT; TCCAGTGATAATCGCTTCGT; ACCTACGAAGCGATTATCAC) was prepared by following the depositor's instruction. This vector was co-transfected with the donor template including homologous arms and a functional GFP-puromycin cassette using UltraCruz® Transfection Reagent (sc-395739) as the delivery reagent. MCF7 cells were passaged at 48 h post-transfection for 3 passages. Cells were treated with 2μg/mL puromycin daily until pooled population of all puromycin resistant cells was expanded. The pooled population of CAS9/Control cells and Has2 KO cells were then used for functional in vitro.

9. Cell transfection

In this study, namely Lipofectamine 3000, a cationic lipid-based transfection reagent was used (Thermo Fisher Scientific, USA). The confluency of 5×10^4 tamoxifen- or fulvestrant-resistant cells (TamR or FulR) was reached in 24-well cell culture plates. There were also non-transfected cells for each cell line as a negative control. Lipofectamine 3000 was initiated by adding separately 0.5 μg of HAS2 plasmid vector (RRID:Addgene_106837)and 1.5 μl of Lipofectamine 3000 to 25 μl of serum-free medium, Opti-MEM (Gibco, Thermo Fisher Scientific, USA). Finally, 1 μl of P3000 reagent (provided in Lipofectamine 3000 kit) was added to each mixture and mixed gently by pipetting. The plates were incubated at 37°C and 5% CO_2 for 72h in the case of removing the inhibitor.

10. Human breast cancer samples

This study was approved by the ethics committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital. Written informed consent was obtained from all individual participants in accordance with the Declaration of Helsinki of the World) Medical Association. We analyzed tissue specimens (n=18) that were obtained from Shanghai Jiao Tong University Affiliated Sixth People's Hospital between November 1, 2020, and July 31, 2021. TNM staging system was used to stage tumors.

11. Immunohistochemistry

Tissue samples from breast cancer patients were fixed in 4% buffered neutral formalin, embedded in paraffin, and were mounted on glass slides. After dewaxing, dehydration, antigen retrieval, and inhibition of endogenous peroxidase activity and nonspecific binding. Then, mouse anti-human has2 (1: 100; Abcam) antibodies, rabbit anti-human ERα (1: 1000; Abcam) antibodies or rabbit anti-human Ezrin (1: 100; CST) antibodies were added, and the slides were incubated at 4°C overnight. After washing with PBS, the slides were incubated with or without biotinylated secondary antibodies for 1 h, followed by streptavidin-ABC at room temperature. Then, the slides were developed with a 2,4-diaminobutyric acid Substrate Kit and counterstained with hematoxylin. The intensities of Has2, ERα, and Ezrin were quantitatively analyzed using IMAGE-PRO PLUS 6.0 software (ImageJ, RRID:SCR_003070) (Media Cybernetics, Bethesda, MD, USA).

12. TCGA Data Analysis

As required by experiment, the mRNA expression of HAS2 and ER were analyzed in 543 cases of primary luminal-like breast tumors. Analyses of TCGA data are performed on primary luminal-like breast cancers samples (n=543) with RNA-sequencing data. A total analysis of 543 luminal-like samples with RNA-sequencing data was obtained. The same method is used for other TCGA data.

13. Generation of stably ER-expressing cell lines through lentiviral transduction

Full-length human ESR1 plasmid (NM_001291230) was purchased from Shanghai Jikai Biotechnology. ESR1 lentivirus was overexpressed in MCF7 cells. Stable ER-expressing cells were chosen post transfection using puromycin. The stably Has2-expressing cell lines were prepared through lentiviral transduction as previously described [28]. The pCMVIE-IRES-HAS2 vector encoding Has2 were purchased from Hanbio (Shanghai, China). Stable Has2-expressing cells were chosen using puromycin. Has2-shRNA-targeting lentiviral particles were obtained from Santa Cruz Biotechnology (sc-45328-V). Stablely transfected cells were also purified by using puromycin (5μg/ml).

14. Immunofluorescence

The expression of ER and Ezrin was determined by immunofluorescence assay. Cells were fixed in 4% paraformaldehyde, permeabilized with Triton X-100/phosphate-buffered saline (PBS), and blocked in 1% BSA. Cells were incubated with primary antibodies overnight at 4°C in incubation fluid (Dako, Denmark). On the second day, cells were washed in PBST for 3 times (10 min each time) and then incubated with secondary antibodies conjugated with Alexa Fluor 647 (Abcam, ab150083) for 1 h at room temperature. Then cells were incubated with Phalloidin-iFluor 488 reagent(abcam,ab176573)for 90 minutes at room temperature. Cells were washed in PBST three times (10 min each time). Images were analyzed under confocal microscopy (Nikon A1, Tokyo, Japan). Primary antibodies against ERα and Ezrin were used.

15. Small-interfering RNA (SiRNA) for ezrin

The expression of ezrin was downregulated with siRNAs. SiRNA transfection was performed using riboFECT^{TM}CP (RiboBio, Shanghai, China) according to the manufacturer's protocol using 100nM of ezrin

siRNA (pool of three: 5′- GGATTACTGCGTCGATTCA-3′, 5′- CCATGGCTTTCCCGGATAA-3′, 5′-GTCGAGGCATGGAGTTCAA-3′, RiboBio).

16. Co-immunoprecipitation

For the immunoprecipitation experiments, cells were first lysed in cell lysis buffer (RIPA) (Beyotime, China). Just before use, protease inhibitor, phosphatase inhibitor and PMSF were added to complete the buffer according to the manufacturer's protocol. Insoluble fractions were removed by centrifugation (12000rpm, 10min, 4°C). The total protein concentration was determined by a bicinchoninic acid protein assay kit (Sigma, USA) before being pre-cleared by incubation with 25μL Protein A/G Plus agarose beads (Yuekebio, Shanghai, China) and 3μg normal IgG antibody whose source is the same as primary antibody for 2-3 h at 4°C with gentle inversion. Then removed the Protein A/G Plus agarose beads by centrifugation (300*g* 3min) and subsequently transferred supernatant to new tubes. 3μg antibody of Ezrin or ERα protein was added to the tube. Then fresh Protein A/G Plus beads were added to the antibody mix and inverted gently for overnight at 4°C. The beads were pelleted by centrifugation at 3000rpm for 3 min, the supernatant was removed and 200μL iced PBS was added. This was repeated for a total of five washes. Kept 30μL iced PBS after last wash. Following the washing step, beads were resuspended in 5×SDS loading buffer and incubated in a boiling water bath for 7 min before being separated by 12% SDS–PAGE. The following work was the same as western blot assay.

Statistical analysis

Statistical analysis was performed by GraphPad Software. The significance of differences among groups was determined by one-way ANOVA, t test, or Fisher exact test accordingly. Statistically significance was considered as $p< 0.05$.

Results

1. Acquired Loss of Has2 was associated with resistance to tamoxifen and fulvestrant in ER-positive breast cancer

Although Has2 expression is sensitive to estrogen [25, 26], its contribution in endocrine resistance is poorly understood yet. To determine whether the ERα-responsive gene Has2 might be associated with endocrine resistance, endocrine-resistant MCF7 and T47D cells (hereafter referred to as MCF7R and T47DR, respectively) were generated by gradually exposing sensitive parental cells to increasing concentrations of tamoxifen or fulvestrant until resistance was achieved (Fig. 1A and B, Fig. S1A), similarly to previously reported methods [34, 35]. Notably, dramatically reduce of its protein levels were found in endocrine-resistant MCF7 and T47D breast cancer cells compared with endocrine-sensitive parental cells (Fig. 1C). These results indicated an adaptive loss of Has2 upon long-term endocrine therapy.

In resistance cells, reduced to lower level of ERα expression was observed (Fig.S1B). To address whether antiestrogens stimulation affects Has2 expression in ER-positive breast cancer cells, naive cells (MCF7, T47D, ZR-75-1) were treated with estrogen (E2), tamoxifen, or fulvestrant for 3 days. Unlike in resistant cells, the relative expression of Has2 mRNA and protein was significantly concurrent reduced upon tamoxifen or fulvestrant treatment at 1μM (Fig. 1D and E, Fig. S2A and B) in an exposure-time dependent manner (Fig. S2C). However, estradiol (E2) stimulation promoted Has2 expression in MCF7 and T47D cells (Fig. 1D and E). There might be a link between acquired loss of Has2 and endocrine resistance. To test this hypothesis, reduced Has2 exp ression in tamoxifen- and fulvestrant-resistant MCF7 cells was rescued by overexpression of Has2. Expectedly, we found that the sensitivity to tamoxifen and fulvestrant was increased in MCF7R cells (Fig. 1F and G). Together, these results indicate that loss of Has2 expression renders cells insensitive to antiestrogens, leading to acquired antiestrogen resistance.

2. Has2 loss induces resistance to antiestrogens in ER-positive breast cancer cells

To determine whether Has2 loss is a mechanism of acquired resistance to antiestrogens, we generated a model of Has2 knocked out by CRISPR in MCF7 cells (Fig. S3A), without affecting the expression of Has2-AS1 (Fig. S3B). The effects of Has2 loss on resistance to tamoxifen or fulvestrant were determined. Results showed that cancer cells with stable has2 knockout showed decreased sensitivity to tamoxifen or fulvestrant in comparison with their parental control cells (Fig. 2A). In addition, Has2-knockout cells had increased levels of phosphorylated AKT and and reduced tamoxifen- or fulvestrant-mediated inhibition of AKT and ERK (Fig. 2B and C).

3. Has2 loss drives up-regulation of ERα expression and activity

Given that changes in estrogen receptor (ER) expression may explain the development of acquired endocrine resistance in breast cancer [5], we therefore tested whether ER expression and activity affected Has2 loss in breast cancer cells. We firstly analyzed the data from The Cancer Genome Atlas (TCGA) and found that, comparing with normal tissues, Has2 expression was dramatically reduced in primary invasive breast cancers (n=1097) (Fig. S4A). Interestingly, in a small group of male breast cancers (n=12), Has2 expression was drastically lower than that of female breast cancers (n=1075) (Fig. S4B), indicating an association between Has2 and estrogen (E2) level in the patients. Furthermore, we found that triple-negative BrCas expressed a level of Has2 almost comparable with that of normal tissues whereas luminal-like and Her2-positive BrCas expressed much lower Has2 (Fig. S4C), implying an association between the expression level of Has2 and estrogen receptor (ER). More importantly, the expression of Has2 was found to be negatively correlated with the level of ER in luminal-like BrCas (n=543) from TCGA data base (Fig. 3A). To assess the associations, contiguous sections of breast cancers were stained for ER and Has2 (Supplementary Table 1). Representative images of ER and Has2 expression in primary breast tumors are presented (Fig. 3B). The ER-high expressing primary tumors have a significantly ($p<0.05$) lower Has2 expression compared with ER-negative carcinomas (Fig. 3C). Further investigation

showed that overexpression of ERα in luminal-like breast cancer cells (MCF7 cells) triggered significant reduction in the expression of Has2 (Fig. 3D), suggesting their correlations.

To further understand the relation between Has2 and ER, we established two stable cell lines by overexpressing or knocking out Has2 in MCF7 cells. Results showed that overexpression of Has2 impeded while knockout of Has2 promoted ER expression (Fig. 3E and F), demonstrating the directly negative association between Has2 and ER. An increase of ER expression and activity was observed in MCF7 cells after a short-time exposure to tamoxifen or fulvestrant (Fig. 3G). Furthermore, ERα expression and phosphorylation (Ser118) underwent a dramatic increase in MCF7 cells with stable has2 knockout (Fig. 3G and H). Subsequently, the effect of E2 was enhanced while the effects of tamoxifen and fulvestrant were inhibited by the forced loss of Has2 (Fig. 3G and H). These results suggested that the acquired loss of Has2 might play a role in maintaining the ER expression and function in resistant cells upon long-term exposure to antiestrogens.

4. Adaptive Has2 loss increases Ezrin expression in response to ER suppression in breast cancer

We next aimed to understand how Has2 loss leads to resistance to antiestrogens, though displaying the role in inducing increase of ER expression and activation. Given that both of Ezrin and ezrin-radixin-moesin (ERM) binding protein 50 (NHERF1) are rapidly up-regulated in response to estrogen [36, 37], for the first time we evaluated the alterations of Ezrin in the acquisition of resistance to tamoxifen or fulvestrant in luminal-like breast cancer cells. First, we sought to determine if there was evidence for Ezrin upregulation in ER-positive tumors from patients. Our previous report has revealed that Ezrin is over-expressed in primary invasive BrCas compared with normal mammary tissues from TCGA data [32]. Here, a higher level of Ezrin was observed in luminal-like BrCa compared to Her2-positive and triple-negative BrCa tissues (Fig. S5), suggesting a possible role of Ezrin in regulating cell behavior in ER-positive BrCa.

We found that exposure of luminal-like breast cancer cells (MCF7 and T47D cells) to 17β Estradiol (E2) for 3 days did not induce significant changes of Ezrin expression, whereas tamoxifen- or fulvestrant treatment for 3 days induced significant increase of Ezrin (Fig. 4A, B and C) and in a time-dependent manner (Fig. S6A), demonstrating the rapid response of Ezrin to estrogen or ER suppression. Then the expression of Ezrin in tamoxifen- or fulvestrant-resistant cells was analyzed. Results showed that resistant cells (MCF7R and T47DR cells) with Has2 loss presented higher expressions of Ezrin than that of parental cells (Fig. 4D and E). Moreover, immunofluorescence staining revealed that concentrated ezrin was localized at the apical cell surface, whereas in tamoxifen- or fulvestrant-resistant cells, Ezrin localized at in the cytoplasm, showing more diffuse staining (Fig. 4E).

We next attempted to determine whether Has2 plays a role in modulating Ezrin upon tamoxifen or fulvestrant treatment. Results showed that overexpression of Has2 inhibits while knockout of Has2 promotes Ezrin expression (Fig. 4F), which subsequently impedes or enhances the response to tamoxifen or fulvestrant treatment (Fig. 4G), displaying the influence of Has2 and Ezrin. Moreover, the expression of

Ezrin upon tamoxifen or fulvestrant treatment in resistant cells is partly reduced by the rescue of Has2 expression (Fig. 4H), further demonstrating the regulation of Ezrin by acquired Has2 loss.

5. Inhibition of Ezrin reversed Has2 and ERα expression

To determine whether the increase of Ezrin regulates the acquisition of antiestrogens resistance induced by Has2 loss in breast cancer cells, we examined the influence of Ezrin knockdown on resistance to tamoxifen and fulvestrant in Has2- knockdown cells (Fig. S6B). Results revealed that the increase of antiestrogens resistance in Has2-knockout MCF7 cells was suppressed by Ezrin knockdown (Fig. 5A), indicating the contribution of Ezrin to Has2 loss induced antiestrogens resistance.

Further, to understand the mechanisms by which Ezrin regulates antiestrogens resistance, we examined the changes of ERα and Has2 expression after Ezrin- knockdown in naïve and resistant BrCa cells (Fig. 5B). Our results showed that both of ERα and Has2 in Ezrin-knockdown cells were markedly increased, compared to the parental cells (Fig. 5B and C), indicating contribution of Ezrin in ERα-loss during the generation of acquired resistance. Further, immunohistochemical assay in primary breast tumor indicated the negative correlation of ER and Ezrin expression (Fig. S7 and Supplementary Table 1). In ER-positive BrCas, the expression of Ezrin in Has2-high expressing tumors was significantly lower than that of Has2-low (Fig. S7). And the co-localization of Ezrin and ER from co-immunoprecipitation assay (Fig. S8) indicated a direct link between Ezrin and ER. These results, together with the reversal of Has2 and ERα expression by Ezrin-knockdown, suggested negative-feedback loops consisting of Has2, Ezrin, and ERα occur in response to antiestrogens treatment (Fig. 5D).

6. Inhibition of Ezrin reversed the Akt/MAPK signaling cascade

In addition, prior studies have implicated the AKT/mTOR signaling pathway [38] or the MAPK pathway [39] as potential modulators of endocrine resistance in breast cancers. We further sought to explore whether Has2 loss regulates ERα and Ezrin in breast cancer cells via either of these pathways. We examined components of the AKT and MAPK signaling pathway and found suppression of Akt and MAPK/ERK signaling (loss of Akt and ERK1/2 phosphorylation) upon tamoxifen or fulvestrant treatment in Ezrin knockdown cells (Fig. 5E), suggesting that active signaling of the Akt and ERK1/2 pathway and low Has2 levels may be an additional basis for acquired endocrine resistance in many patients with ER-positive breast cancers.

To further confirm the association of Ezrin with activation of ER, we examined ER pathway targets utilizing real-time PCR. As expected, we found knockdown of Ezrin partially suppresses Has2-loss induced activation of ER-dependent transcription, including FOS, PBX1, and Myc (Fig. 5F), further supporting the role of Ezrin in regulating of Has2 loss and inducing the acquired resistance.

7. Inhibition of Ezrin restores sensitivity to antiestrogens

To determine whether the increase of Ezrin induced by Has2 loss might be sufficient to promote resistance to tamoxifen or fulvestrant, the Ezrin level was knocked down in resistant and sensitive breast cancer cells by siRNA targeting (Fig. S6B). We observed that, in resistant cells, knocking out of Ezrin restored the sensitivity of ER inhibitors (Fig. 6A) and the capacity of ER inhibitors to reduce Akt and ERK1/2 phosphorylation (Fig. 6B), indicating that the increased Ezrin is a critical mediator of the endocrine resistance induced by Has2 loss. Accordingly, the ER-dependent transcription was influenced by Ezrin silence in resistant cells. The candidate target genes FOS and PBX1 were reduced while c-MYC were increased upon tamoxifen or fulvestrant stimulation after Ezrin silence (Fig. 6C), which might indicate the restoring of the sensitivity to ER inhibitors. Also, similar effects on cell response were noted in naïve MCF7 cells treated with tamoxifen or fulvestrant Fig. 6D-F). Additionally, Ezrin inhibitor (NSC668394) was used to evaluate the change of cell sensitivity to tamoxifen or fulvestrant. Results showed that treatment of Ezrin inhibitor at 0.5μM showed no obvious influence on cell viability (Fig. 6G). However, combination of Ezrin inhibitor (0.5μM) and tamoxifen or fulvestrant resulted in obviously decreased cell proliferation (Fig. 5H) and a reversal response of ER-dependent transcription in MCF7 cells (Fig. 6I).

Results showed that, although treatment of Ezrin inhibitor at 0.5μM showed no obvious influence on cell viability (Fig. 6G), combination of Ezrin inhibitor (0.5μM) and tamoxifen or fulvestrant resulted in decreased cell proliferation (Fig. 5H). However, no obvious changes in ER-dependent transcription were found in MCF7 cells (Fig. 6I) with combination of Ezrin inhibitor (0.5μM) and tamoxifen or fulvestrant. Overall, inhibiting Ezrin effectively reverses the effect of Has2 loss on resistance to tamoxifen or fulvestrant and restores their capacity to suppress cell proliferation and ER-dependent signaling.

Discussion

Endocrine therapy has demonstrated remarkable antitumor activity in clinical ER+ breast cancer. Despite this impact, acquired endocrine resistance is widespread and poorly understood. Through resistant cancer cells treated with these drugs, we have identified loss of the Has2 as mechanisms of resistance development to antiestrogens. Although enhanced activity of hyaluronan synthases was shown after estrogen treatment[33], the link between Has2 and endocrine resistance was not previously anticipated. In this report, we show that loss of Has2 is an important endocrine resistance driver in ER-positive breast cancer and may be involved in a Has2-Ezrin-ER negative-feedback loop. We showed the coordination of Has2 loss and subsequent increase of Ezrin expression, resulted in a fine balance of ER-signaling and endocrine resistance through the Akt and MAPK/ERK signaling routes. The effectiveness of Ezrin knockdown or inhibition in treating the Has2-loss endocrine-resistant cells, and subsequently reversal of ERα signaling in endocrine-resistant cancer cells, implies the potential role of Ezrin as a valuable target for overcoming endocrine resistance.

Based on previous observations related to Has2 gene where it is readily to be regulated by hormone-type effectors [18–20], and related to cell plasticity [30, 32], its role in development of endocrine resistance is

likely to be emerging. The specific association between Has2 and ER were in our cohort is consistent with the analysis of TCGA data broadly.

This work reveals that a Has2-loss state was acquired upon antiestrogens treatment in luminal BrCas, which mediates adaptive endocrine resistance. The acquired loss of Has2 might play a role in sustaining ER expression in resistant cells upon long-term treatment with antiestrogens. Indeed, the patients who receive endocrine therapy do sustain ERα expression [14] and, based upon our findings, are also likely to acquire a Has2-loss state. Previous studies have indicated that the expression of Has2 was easily influenced by hormones, including estrogen, and contributed to changes of cell state [25, 40]. Moreover, our recent reports identified Has2 as a rapid responsive gene upon drug therapy in breast cancer [32]. For the first time, we demonstrated that Has2 loss was a rapid response to endocrine therapy and played an important role in the acquisition of endocrine resistance.

Further, we provide evidence for the first time that Ezrin was up-regulated by Has2 loss upon antiestrogens treatment. Although Ezrin and its binding protein (ezrin-radixin-moesin binding protein 50, NHERF1) have been shown to be promptly upregulated in response to estrogen in breast and ovarian cancer [36, 37, 41], how this happens and its association with the antiestrogens treatment in breast cancer remains largely unexplored. In this study, we demonstrated that Ezrin is primarily increased in the acquisition of resistance by the adapted Has2 loss. In addition, the translocation of Ezrin from apical cell surface into the cytoplasm in endocrine-resistant cells suggested a positive association with endocrine resistance. These results are consistent with previous reports that the switch of ezrin localization is correlated with adverse features in breast cancer [42]. Moreover, knockout of endogenous Has2 promoted the expression of Ezrin while overexpression of Has2 inhibited Ezrin protein levels. Collectively, these results demonstrated that the increase of Ezrin and Has2 loss were involved in antiestrogens resistance.

Moreover, we found that loss of Has2 and subsequent increase of Ezrin displayed opposite effect on ERα expression in the acquirement of antiestrogens-resistance of BrCa cells. Changes in ER expression/activity may explain the development of acquired endocrine resistance in breast cancer [43, 44]. During the development of endocrine resistance, patients with breast cancers relapse due to the loss of ER [45, 46]. Although loss of ERα expression/function has been documented leading to rapid resistant to multiple antiestrogens [5, 43, 44], it only occurred in less than 25% of tamoxifen-resistant tumors [47, 48]. Indeed, the majority of the treatment-refractory breast tumors still express ER [49–51]. The transition from ERα positive to negative with antiestrogens treatment is thought to reflect emergence of a resistant phenotype [52]. However, how to coordinate loss of ERα and maintenance of ER-signaling is largely unknown. Here, we showed that the rapid loss of Has2 following antiestrogens treatment promoted while increase of Ezrin inhibited ERα expression during the development of antiestrogens-resistance. These opposite effects might be the mechanism contributing to explain the increase of ER expression upon short-time exposure to antiestrogens and the maintaining low level of ER expression in endocrine-resistant cells after long-term stimulation of antiestrogens. Indeed, there does exist a group of patients untreated with endocrine therapy who harbor low level of Has2 and high level of Ezrin who might be readily to develop endocrine resistance.

Conclusions

In summary, we uncovered a novel mechanism for endocrine resistance that a rapid loss of Has2 and a following Has2-Ezrin-ER feedback loop govern cellular sensitivity to tamoxifen or fulvestrant in luminal-like breast cancer cells. We characterized the acquired loss of Has2 and that the subsequent increase in Ezrin expression in endocrine resistance. As Ezrin expression is upregulated in luminal breast tumors from TCGA data, we found that knockdown or inhibition of Ezrin could reverse resistance of breast cancer cells and restore cellular sensitivity to endocrine therapy. Discovery of novel drugs targeting Ezrin could be a potential therapeutic strategy to tackle endocrine resistance. These emerging findings provide a new insight into how the ERα-signaling is regulated and drives alteration of antiestrogen response, suggesting a novel therapeutic target for breast cancer.

Abbreviations

BrCa: Breast carcinomas; ER: Estrogen receptor; Has2: Hyaluronan synthase 2; TCGA: The Cancer Genome Atlas

Declarations

Acknowledgment

Not applicable.

Authors' contributions

C. Y., F. G. conceived and designed the overall project. X. S. performed the in vitro experiments. F. T. performed the clinical data analyses. X. S., C. Y. initially drafted the manuscript. Y.L., Y.H. performed the technical assistance of the immunohistochemistry assays. Y.D., G.Z. reviewed the manuscript and provided critical input into the manuscript. All authors read and approved the final manuscript.

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Compliance with Ethical Standards

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Consent for publication

All authors have agreed to publish this manuscript.

Availability of supporting data

Not applicable.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Figures

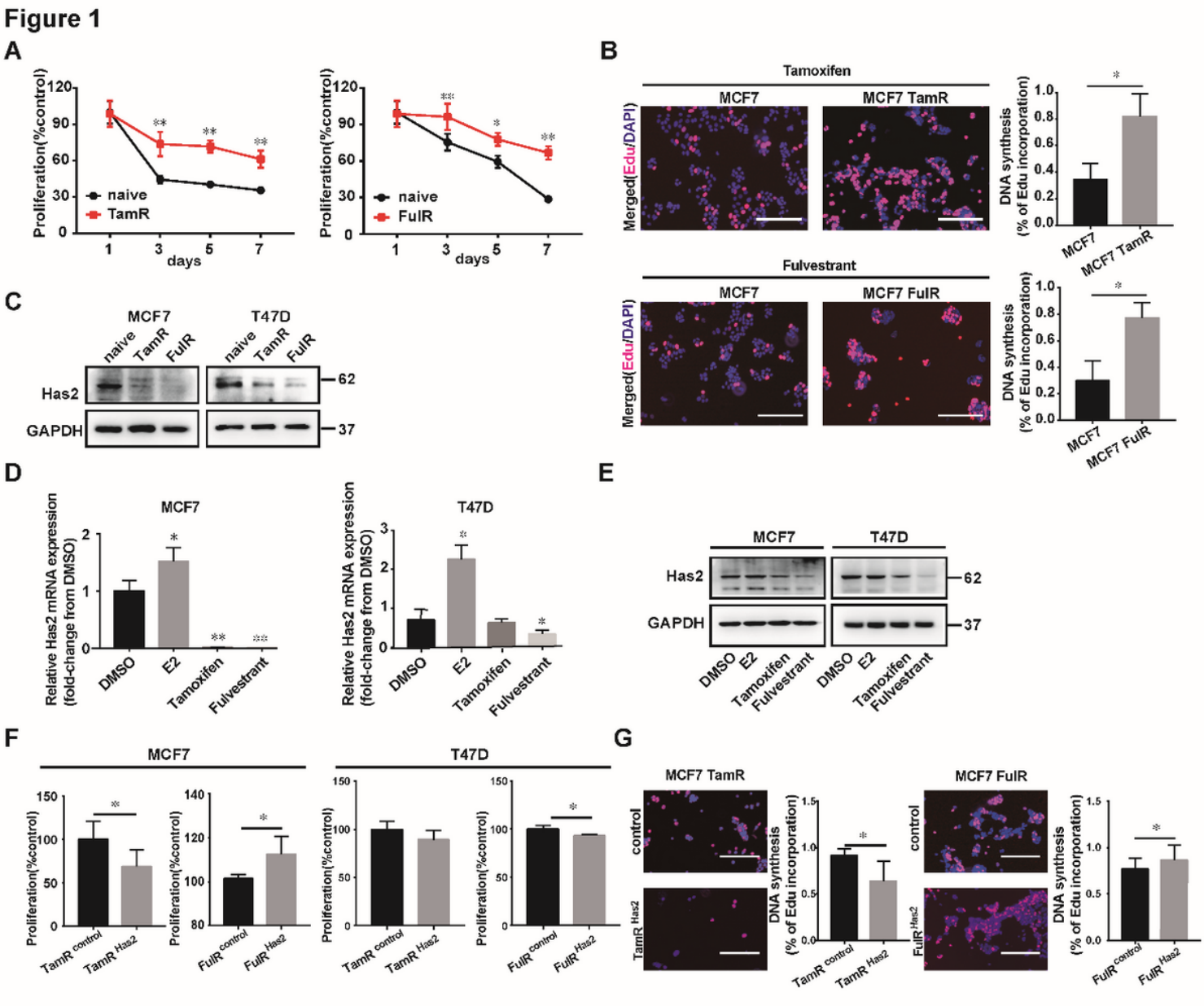

Figure 1

Acquired Loss of Has2 was associated with endocrine resistance in ER-positive breast cancer

A. Proliferation of resistant cells was compared with their sensitive counterparts upon ER inhibitors(1μM) at 1, 3, 5, or 7 days. Each value is the mean (±SD), n=5 (Statistical analysis: T-test, *p< 0.05, **p< 0.01, vs. DMSO).

B. An EdU (5-ethynyl-2'-deoxyuridine) assay was performed to assess DNA synthesis in resistant cells. EdU (red) and DAPI (blue) were used to stain cells and nuclei. Represent image of at least three experiments were showed. Scale bar, 200 μm. Quantitative assay data were shown. *p < 0.05 (Student's t-test).

C. The expression of Has2 in naive breast cancer cells and their resistant counterparts was determined by western blot.

D. The expression of Has2 genes in naive MCF7 and T47D cells treated with DMSO, E2 (1μM), tamoxifen (1μM), or fulvestrant (1μM) for 3 days were determined by RT-qPCR. Values are presented as the mean (±SD). All data represent at least three biological repeats. *p< 0.05, **p< 0.01, vs. DMSO treatment group, n=3.

E. Western blot analysis of Has2 expression in naïve MCF7 and T47D cells treated with DMSO, E2 (1μM), tamoxifen (1μM), or fulvestrant (1μM) for 3 days.

F. Proliferation of resistant cells (MCF7R and T47DR) transfected with Has2 plasmid for overexpression upon tamoxifen (1μM) or fulvestrant(1μM) treatment for 3 days. Data are represented as the mean (±SD). *p< 0.05 vs. control, n=5.

G. An EdU (5-ethynyl-2'-deoxyuridine) assay of resistant cells (MCF7R and T47DR), in which Has2 plasmid were transfected for overexpression. Represent image of at least three experiments were showed. Scale bar, 200 μm. Quantitative assay data were shown. *p < 0.05 (Student's t-test).

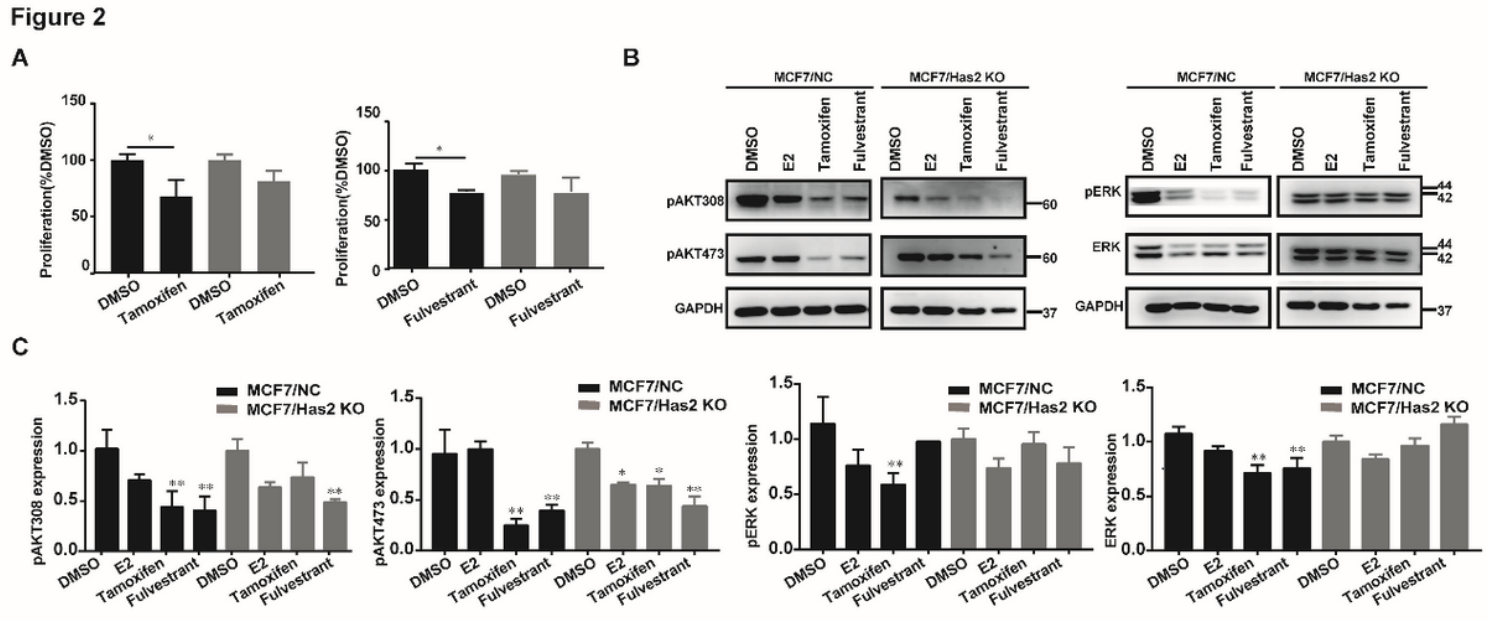

Figure 2

Has2 loss induces resistance to antiestrogens in ER-positive breast cancer cells

A. The effects of Has2 loss on resistance upon tamoxifen or fulvestrant treatment were determined by a CCK8 kit. Each value is the mean (±SD), n=5. (Statistical analysis: T-test, *p < 0.05 vs. DMSO).

B. The effects of Has2 loss on Akt and MAPK signaling pathway upon tamoxifen or fulvestrant treatment were determined by western blot.

C. Densitometry analysis was performed comparing Has2 loss and control MCF7 cells based on three biological repeats. Each value is the mean (±SD), n=3. (Statistical analysis: One way-ANOVA, *p< 0.05, **p<0.01 vs. DMSO).

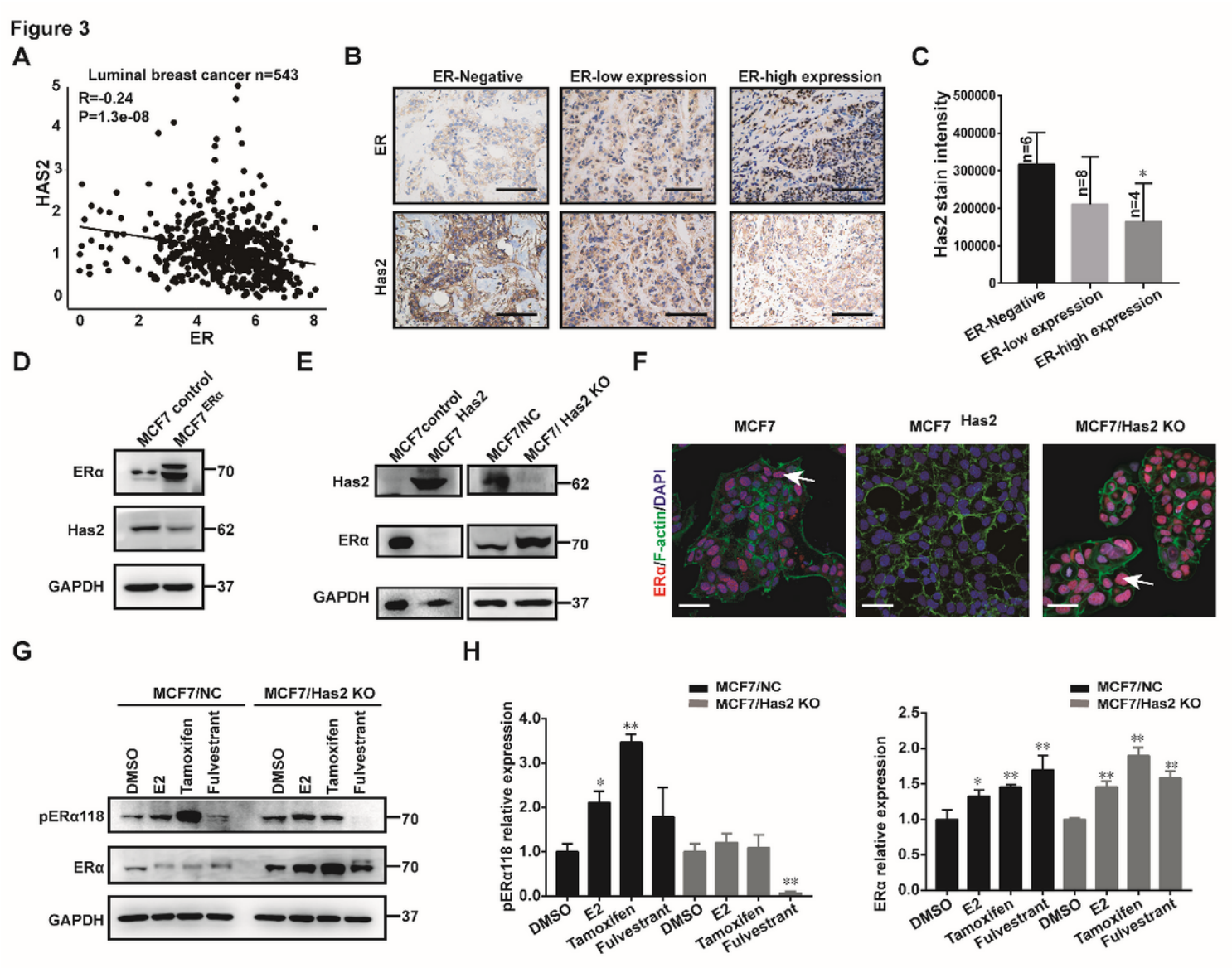

Figure 3

Has2 was associated with ER in breast cancer.

A. Correlations between Has2 and ER expression in primary luminal-like breast cancers (TCGA) are shown. The mRNA expression of Has2 and ER were analyzed in 543 cases of primary luminal-like breast tumors in TCGA dataset and the correlation of expression was analyzed. The Spearman correlation coefficient (R) and corresponding p-value were shown.

B. Immunohistochemical expression of ER and Has2 in human mammary tumor contiguous sections. Representative sections are presented.

C. Patient primary tumors were evaluated for ER and Has2 staining. A significant (*p < 0.05) decrease in Has2 staining was observed in those patients with ER-high expressing compared with those with ER-negative. Scale bars, 100 μm.

D. The effect of ER overexpression on the level of Has2 in MCF7 cells was measured by western blot.

E. The effect of Has2 overexpression or knockout on the expression of ER in MCF7 cells was measured by immunoblotting assay.

F. The influence of Has2 overexpression or knockout on the level of ER in MCF7 cells was measured by immunofluorescence assay. Cells stained with ER antibodies (red), Phalloidin-iFluor 488 reagent (green) and DAPI for nuclear staining (blue). Scale bars, 50 μm.

G. Has2 knockout in naive MCF7 cells induced an increase of ER expression upon tamoxifen or fulvestrant treatment.

H. The ERα and pER118 expression was quantified and normalized against GAPDH by densitometry analysis. Results are expressed as mean ± SD, n = 3. *p< 0.05 vs. DMSO.

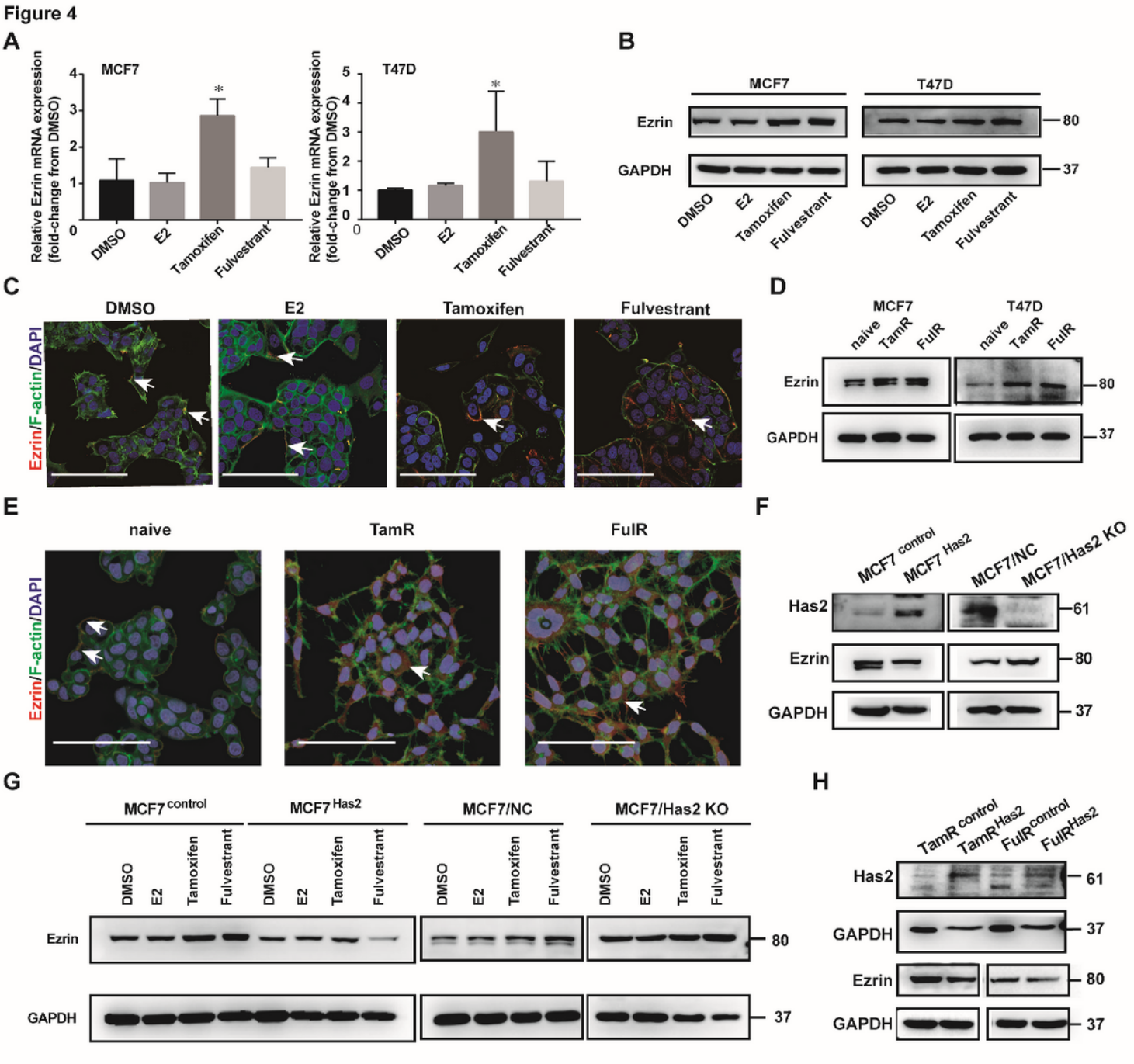

Figure 4

Has2 loss promotes the expression of Ezrin upon ER suppression.

A. The influence of ER suppression on the expression of Ezrin was determined by real-time PCR assay. Luminal-like breast cancer cells (MCF7 and T47D cells) were exposed to tamoxifen (selective ER modulator, SERM), fulvestrant (selective ER down-regulator, SERD), and estrogen (E2) at 1μM for 72 hours. Then the expression change of Ezrin was analyzed by real-time PCR assay. (Statistical analysis: One way-ANOVA, *p<0.05, vs. DMSO).

B. The effect of ER suppression on Ezrin expression was determined by western blot. Luminal-like breast cancer cells (MCF7 and T47D cells) were exposed to tamoxifen (selective ER modulator, SERM), fulvestrant (selective ER down-regulator, SERD), or estrogen (E2) at 1μM for 3 days. Then the change of Ezrin expression was analyzed by western blot.

C. The changes of Ezrin expression and distribution were observed by immunofluorescence assay. MCF7 cells were treated with DMSO, E2 (1μM), tamoxifen (1μM), or fulvestrant (1μM) for 3 days. MCF7 cells were treated as indicated, stained with Ezrin antibodies (red), Phalloidin-iFluor 488 reagent (green) and DAPI for nuclear staining (blue), and visualized by confocal microscopy. Scale bars, 50 μm.

D. Western blot analysis of Ezrin expression was performed using cell lysates from sensitive and resistant cells as indicated.

E. The localization of Ezrin in endocrine-resistant cells was observed by immunofluorescence assay. Cells were stained with Ezrin antibodies (red), Phalloidin-iFluor 488 reagent (green) and DAPI for nuclear staining (blue). Scale bars, 50 μm.

F. The changes of Ezrin expression in luminal-like MCF7 cells after stable Has2 overexpression or knockout.

G. The effect of ER activity on Has2-mediated depression of Ezrin expression was evaluated by immunoblotting. MCF7 cells with Has2 overexpression or knockout were stimulated by E2 (1μM), tamoxifen (1μM), or fulvestrant (1μM) for 3 days. Then the expression of Ezrin was analyzed by western blot.

H. Restoring the expression of Has2 in resistant cells inhibited the increase of Ezrin upon ER suppression.

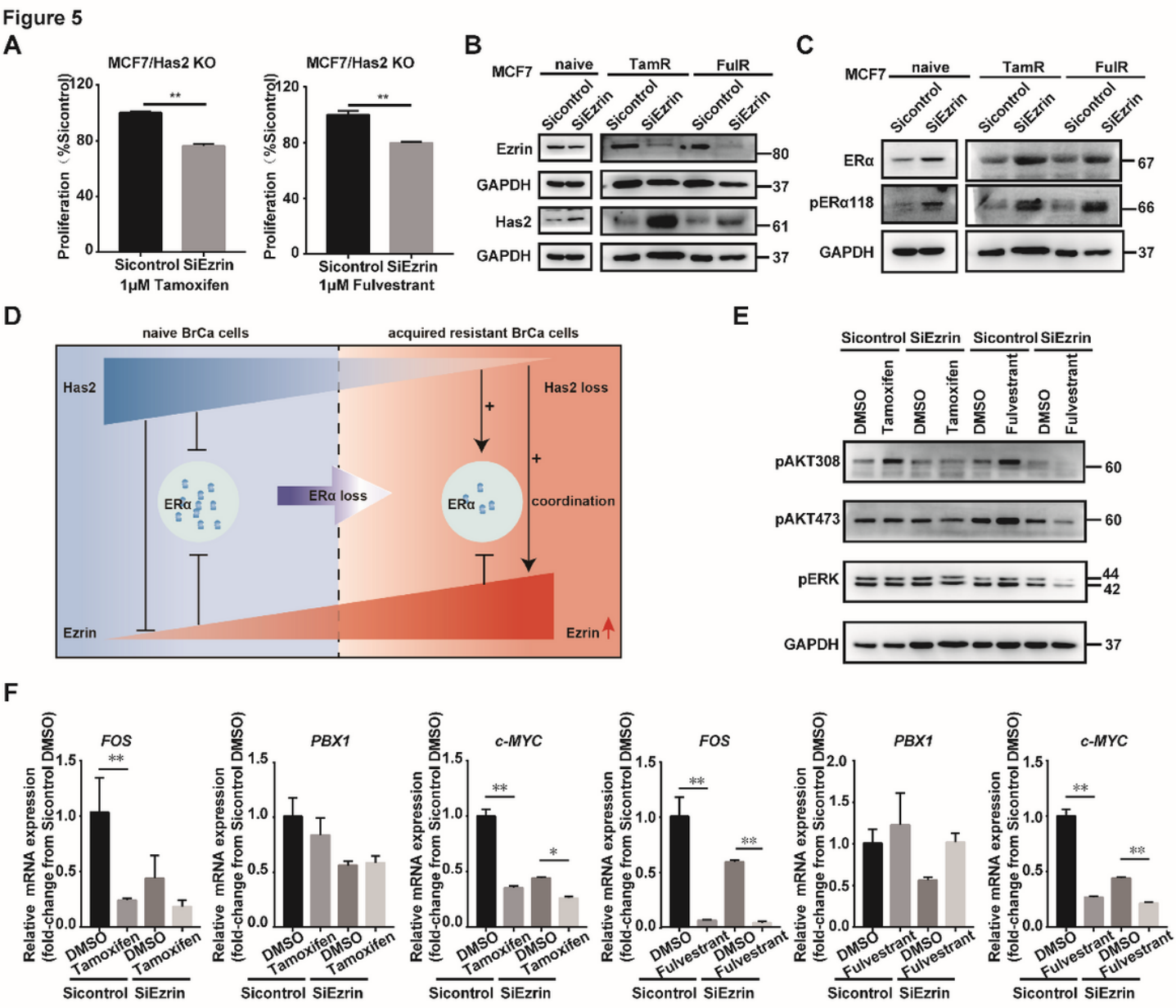

Figure 5

Inhibition of Ezrin reversed Has2 and ERα expression

A. Proliferation of parental and Ezrin-knockdown cells, constitutively expressing Has2- knockdown, exposed to 1 μM of tamoxifen or fulvestrant for 3 days. Each value is the mean (±SD), n=5. (Statistical analysis: T-test, **p< 0.01 vs. Sicontrol).

B. Ezrin knockdown rescued Has2 loss upon antiestrogens stimulation in naive/resistant cells.

C. Knockdown of Ezrin increased ER expression and activation upon antiestrogens stimulation in naïve/resistant cells.

D. Scheme summarizing the negative-feedback loops consisting of Has2, Ezrin, and ERα.
The negative feedback loops collaborate to maintain ERα in a low level in endocrine resistant cells. The adapted loss of Has2 promoted while the consequent increase of Ezrin inhibited ERα expression, showing an opposite effect on ERα expression during the development of endocrine-resistance.

E. The effect of Ezrin knockdown on phosphorylation and expression of Akt and MAPK pathway components in Has2-knockout cells upon ER suppression.

F. Ezrin knockdown reduces Has2 loss-mediated increase of ER-dependent transcription upon ER suppression. (Statistical analysis: T-test, **p<0.01 vs. DMSO).

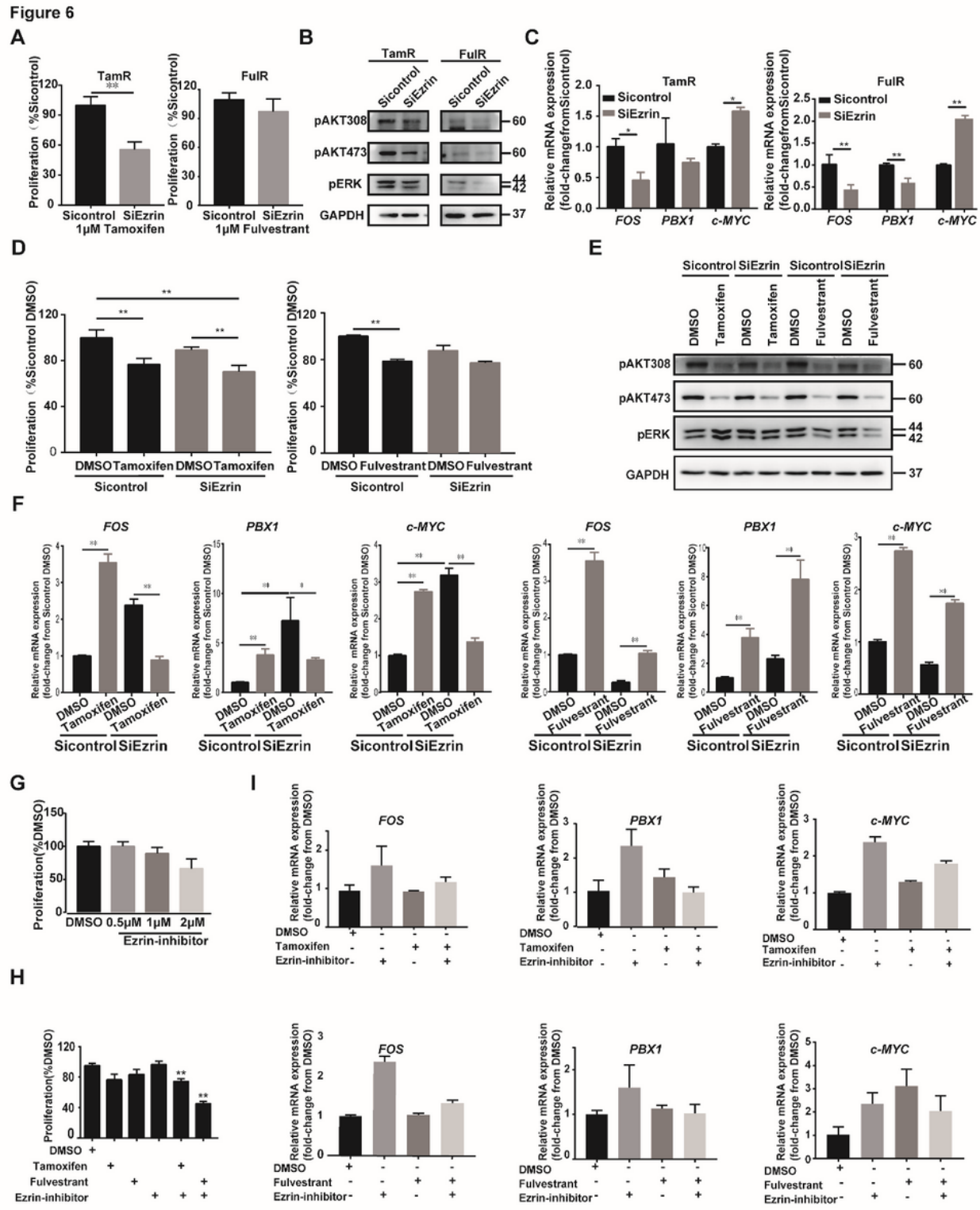

Figure 6

Ezrin inhibition restores sensitivity to tamoxifen or fulvestrant.

A. The changes of cell sensitivity to tamoxifen or fulvestrant in resistant MCF7 cells with Ezrin knocking down were determined by CCK-8 assay. Resistant cells as indicated were transfected with with siControl

or siRNA-Ezrin, after which proliferation analysis was performed by CCK-8 assay. Each value is the mean (±SD), n=5. (Statistical analysis: T-test, **p< 0.01 vs. Sicontrol).

B. The changes of cell survival signaling to tamoxifen or fulvestrant in resistant MCF7 cells with Ezrin knockdown were determined by immunoblotting assay.

C. Ezrin mediates ER-dependent transcription upon ER suppression in resistant cells.

D. The influence of Ezrin knockdown on the response of naïve luminal-like breast cancer cells to tamoxifen or fulvestrant treatment was assessed using a CCK-8 kit. Cells were exposed to tamoxifen (1μM) or fulvestrant (1μM) for 3 days. Data are the mean ± s.e.m. of three experiments. **p < 0.01, unpaired Student's t-test.

E. The effect of Ezrin knockdown on the cell survival signaling in naïve luminal-like breast cancer cells upon tamoxifen or fulvestrant treatment was assessed by immunoblotting assay.

F. Ezrin mediates ER-dependent transcription upon ER suppression in naive MCF7 cells. All data represent at least three biological repeats. *p< 0.05, **p< 0.01 vs. DMSO treatment group, n=3.

G. The effect of Ezrin inhibitor (NSC668394) at different concentrations on cell proliferation in naive MCF7 cells was detected.

H. The influence of Ezrin inhibitor (NSC668394) on the sensitivity of naïve luminal-like breast cancer cells upon tamoxifen or fulvestrant stimulation was determined using a CCK-8 kit. Data are represented as the mean (±SD). **p< 0.01 vs.DMSO, n=5

I. The effect of Ezrin inhibitor (NSC668394) on mRNA expression of ER-dependent transcription upon ER suppression in naïve MCF7 cells was analyzed.

Supplementary Files

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- 5thSupplementaryFiguresandlegends.docx