Original Article
Suppression of estrogen receptor-beta promotes gastric cancer cell apoptosis with induction of autophagy

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Abstract: Estrogen Receptor 2 (ESR2) is the protein-coding gene of estrogen receptor β (ERβ) and has been shown to be abundantly expressed in gastric carcinoma (GC), suggesting that it plays a role in GC pathogenesis. However, the underlying molecular mechanism remains elusive. In the present *in vitro* study, GC cell growth was found to be estrogen-dependent, and the expression level of ERβ was higher than that of ERα. Knocking down the endogenous expression of ESR2 in GC cells increased the apoptosis rate and the level of cleaved caspase-3, caspase-7 and poly ADP-ribose polymerase. The induced apoptosis was primarily related to GC cell growth arrest, accompanied with activation of DNA damage-inducible protein 45 alpha (GADD45α) in a p53-independent manner. Importantly, down-regulation of ESR2 also promoted autophagy. The autophagy inhibitor 3-MA or silencing ATG7 rescued the apoptosis by knocking down ESR2 via activation of the MAPK signaling pathway in AGS cells, leading to increased apoptosis. In conclusion, these results demonstrated that suppression of ESR2 gene expression could promote GC cell apoptosis, suggesting that it may prove to be a potential therapeutic target for GC.

Introduction

Gastric cancer (GC) is one of the most commonly diagnosed malignancies with high morbidity and mortality [1]. Statistically, GC is responsible for over 1,000,000 new cancer cases worldwide in 2018, making it the fifth most frequently diagnosed cancer after lung, breast, prostate and colon cancers [2]. In China, GC ranked the second most common cancer in terms of morbidity and mortality in 2015 [3]. At present, surgical resection and adjuvant chemotherapy are the standard treatment for patients with stage I and II GC [4]. Although many novel chemotherapeutic drugs and molecular targeted agents have been developed, their clinical efficacy for advanced GC remains unsatisfactory [5, 6]. Therefore, it is urgent to discover novel and more effective

therapeutic targets to increase the survival and prognosis of GC patients.

Hormone therapy [7, 8] has been successfully applied in the treatment of hormone-dependent tumors in recent years. For example, the estrogen receptor (ER) antagonist Tamoxifen (TAM) has been successfully used for the treatment of ER-positive breast cancer patients [9, 10]. Emerging evidence has shown that GC is also a hormone-associated malignancy. The results of some epidemiological studies have demonstrated that excessive exposure to estrogen may increase the risk of GC [11]. In contrast, blocking ER may increase the risk of GC. Chandanos et al [12] reported that the use of TAM shortened GC development from 13 years to 4 years. The association between ER and GC at the molecular level was first described by To-

kunaga et al in 1983 [13]. Subsequent studies [14-18] demonstrated that the expression of ERβ was substantially higher than that of ERα, although GC cells expressed both ERα and ERβ genes. However, ERβ expression inhibited GC progression and was significantly lower in advanced GC than that in early GC [19]. It was found in our previous study [20] that ERα and ERβ expression in GC tumor tissues was significantly higher than that in uninvolved mucosa, but ERβ expression was more frequently detected in young patients with advanced GC. However, the underlying mechanisms are unknown. A recent study has also shown that genetic variation in the ESR2 gene is associated with a higher survival rate of patients with locally advanced GC [21], but the specific mechanisms by which ERβ participates in GC development and progression are still unclear. Thus, we hypothesized that ERβ may play a critical role in GC tumorigenesis by interfering with apoptosis pathways. The present *in vitro* study investigated the effect of ERβ gene suppression on GC cell survival to see whether ERβ is a promising target for GC treatment.

Materials and methods

Cell lines and culture

Five GC cell lines (AGS, HGC27, MKN45, NCI-N87 and KATO-III), an eternalized human gastric mucosa epithelium cell line GES-1, a human breast cancer cell line (MCF-7) and a cervical cancer cell line HELA were obtained from the Cell Bank of the Chinese Academy of Medical Sciences (Shanghai, China). MCF-7 cells were grown in DMEM (BI 01-080-1, Bioworld, China), and all the other cells were cultured in Roswell Park Memorial Institute 1640 medium (BI 01-101-1) supplemented with 10% fetal bovine serum (FBS) (BI 04-201-1A/B) at 37°C in a humidified atmosphere containing 5% CO_2.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from GC cell lines was isolated using TRIzol reagent (Invitrogen, CA, USA) and cDNA was obtained using the PrimerScript RT Master Mix RealTime Kit (TAKARA, Japan). qRT-PCR was performed using SYBR Green Real-time PCR Master Mix (TAKARA). qRT-PCR analysis was conducted using the Applied Biosystems QuantStudio 5 RT-PCR system (Applied Bio-

systems, MA, USA). The following primer sequences were synthesized by Sangon Biotech Co., Ltd (Shanghai, China): ESR2 (5'-TGGGC-ACCTTTCTCCTTTAG-3' and 5'-TGAGCATCCCTC-TTTGAACC-3'), β-actin (forward-5'-CTGGGAC-GACATGGAGAAAA-3' and reverse-5'-AAGGAAG-GCTGGAAGAGTGC-3'). β-actin was used as an internal control. The relative expression levels were calculated according to the $2^{-\Delta\Delta Ct}$ method.

Cell transfection

To downregulate the expression of ESR2, GA-DD45α, and autophagy related 5 (ATG5) in GC cells, small interfering RNA (siRNA) was transfected with Lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA). ESR2 siRNA was synthesized by Thermo Fisher (HSS103378, HS103380 and HS-176622). GADD45α siRNA and ATG5 siRNA were purchased from Nanjing Ruizhen Co., Ltd. GC cells (3×10^5) were plated one day before transfection. Knockdown efficiency was determined by qRT-PCR and Western blot analysis.

Protein extraction and Western blot analysis

Harvested GC cells were lysed by ice-cold RIPA buffer (Beyotime, Shanghai, China) supplemented with 1% phenylmethylsulfonyl fluoride (PMSF, Beyotime) for 30 min to obtain whole-cell lysates. The protein concentration was measured by a BCA Protein Assay Kit (Beyotime). Equal amounts of protein samples in the lysate were separated using SDS-PAGE on a 10% gel and then transferred to a polyvinylidene fluoride membrane. After blocking with 5% non-fat milk at room temperature for 1 h, the membrane was incubated with primary antibodies overnight at 4°C. The membrane was then incubated with horse-radish peroxidase (HRP)-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (1:5000, CST, MA, USA). Finally, protein bands were visualized with an ECL chromogenic substrate with HRP. The results were quantified with ImageJ software and processed using Adobe Photoshop CS5. The following primary antibodies were used: anti-ER-α (ab16660, Abcam, Cambridge, UK), anti-ER-β (ab133467, Abcam, Cambridge, UK), anti-β-actin (A1978 Sigma, Merck KGaA, Darmstadt, Germany and/or its affiliates), anti-p-ERK (4370p, CST, Cell Signaling Technology, Danvers, MA, USA), anti-ERK (4695p, CST, Cell Signaling Technology, Danvers, MA, USA), anti-

PARP (sc-7150, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-caspase3 (9662s, CST, Cell Signaling Technology, Danvers, MA, USA), anti-cleaved caspase 3 (9661s, CST, Cell Signaling Technology, Danvers, MA, USA), anti-caspase 7 (9492s, CST, Cell Signaling Technology, Danvers, MA, USA), anti-cleaved caspase 7 (9491s, CST, Cell Signaling Technology, Danvers, MA, USA), anti-GADD45α (sc-797, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p-mTOR (2971s, CST, Cell Signaling Technology, Danvers, MA, USA), anti-mTOR (2983s, CST, Cell Signaling Technology, Danvers, MA, USA), anti-LC3 (ABC232, Merck KGaA, Darmstadt, Germany and/or its affiliates), anti-ATG5 (129-94P, Cell Signaling Technology, Danvers, MA, USA), anti-p-JNK (4668s, Cell Signaling Technology, Danvers, MA, USA), anti-JNK (9258p, Cell Signaling Technology, Danvers, MA, USA), anti-p-p38 (4511p, Cell Signaling Technology, Danvers, MA, USA) and anti-p38 (8690s, Cell Signaling Technology, Danvers, MA, USA). β-actin served as an internal control.

Colony formation and soft agar colony formation assays

For AGS cells, 2000 cells (siESR2 or siNC treated) in 2 mL complete medium were seeded in 6-well plates. After 7-10 days, cell colonies were washed with phosphate buffer saline (PBS), fixed using 4% paraformaldehyde, and stained with 0.4% crystal violet dye for 30 min. The images of stained cell colonies were photographed and counted under a microscope. For MKN45 cells (half adherent and half suspended), a 6-well plate was pre-layered with 0.6% agar-medium mixture, and cells were transfected with siESR2 and siNC. The transfected cells were harvested and gently mixed with 0.3% antibiotic-containing agar-medium mixture and reseeded in a prepared 6-well plate. Then, 10% FBS-containing complete medium was added into the top layer. After 4-week incubation, cell colonies were stained with 0.2% crystal violet and counted microscopically.

Cell apoptosis analysis by flow cytometry

Cell apoptosis was analyzed using the Annexin V-FITC (Fluorescein isothiocyanate)/PI (propidium iodide) Double Staining Apoptosis Assay Kit (BD Biosciences) according to the manufactur-er's protocol. The harvested cells ($1×10^6$) were washed with ice-cold binding buffer, re-suspended in 100 μl binding buffer containing 5 μl FITC and 5 μl of PI, and analyzed for apoptosis by flow cytometry (BD, New Jersey, USA).

Autophagic flux analysis

Autophagic flux was tracked by Mrfp-GFP-LC3 adenovirus (Hanbio, Shanghai, China). After 48-h adenovirus infection, cells expressing LC3 protein were labeled with mRFP-GFP. The presence of autophagosomes was indicated by yellow puncta (mRFP$^+$ and GFP$^+$), and autolysosomes were indicated by red puncta (mRFP$^+$ and GFP$^-$). Red LC3 puncta accumulation was quantified to evaluate autophagic flux. Then cells were transfected with si-NC or si-ESR2 to compare the autophagy status of the two groups. After 48-h transfection, cells were scanned with a fluorescence microscope (Olympus, Japan).

Transmission electron microscopy (TEM)

To observe subcellular ultrastructures by TEM, harvested cells were fixed in 2.5% glutaraldehyde at 4°C overnight, washed with neutral PBS for 5 min×3, post-fixed in 1% osmium tetroxide at 4°C for 1 h, dehydrated in a graded series of ethanol solutions (50%, 70%, 80%, 95% and 100%), and embedded in Epon 812 resin at 37°C for 12 h and 45°C followed by 60°C for 36 h. The Epon-embedded cell block was sliced into ultrathin 50-60 nm sections and stained with uranyl acetate and lead citrate at room temperature to observe and photograph autophagosomes and autolysosomes by TEM.

Immunofluorescence

AGS cells ($1×10^5$) were seeded into 6-well plates for 24 h, washed with PBS, fixed in 4% paraformaldehyde for 20 min, washed again with PBS (3×5 min), and then permeabilized with 0.1% Triton X-100 for 2 min. After further washing with PBS for 5 min×3, cells were treated with blocking buffer (5% goat serum (Sigma) in PBS) at 37°C for 1 h, and then incubated with 1:100 dilutions of ER antibodies in blocking buffer at 4°C overnight. Cells were then incubated with secondary antibodies (Abcam, CA, USA) at 37°C for 45 min to 1 h. Following
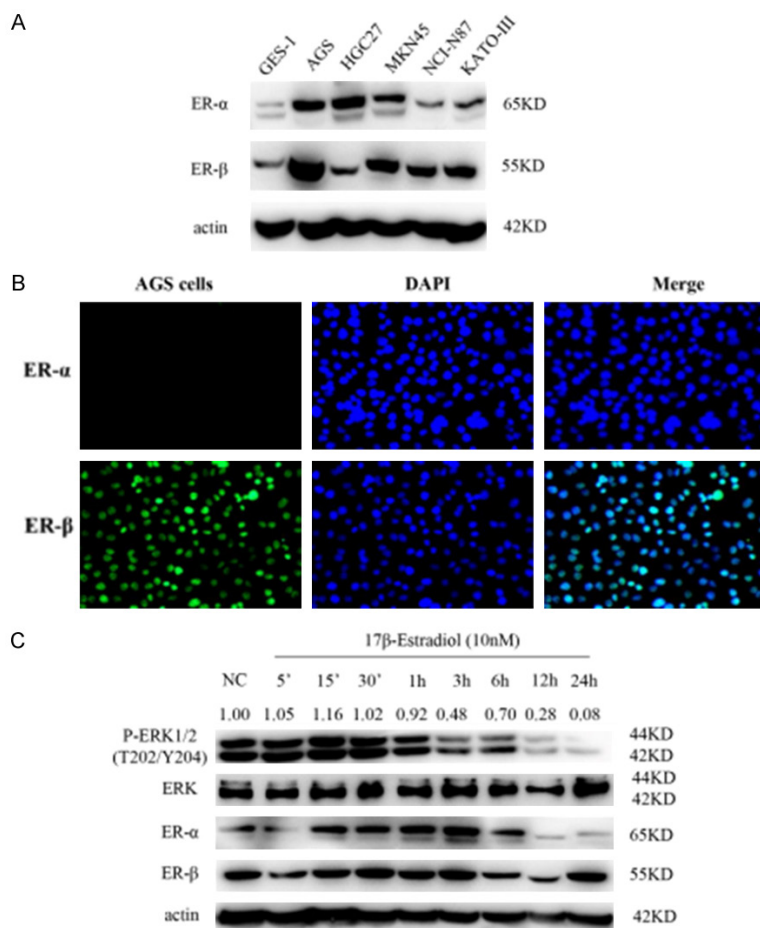
A

B

C

Figure 1. Expression of estrogen receptors in gastric cancer cell lines and the proliferative effect of low-dose estrogen on AGS cells *in vitro*. A. Western blot analysis of expression of ER-α and ER-β in GES-1 and 5 gastric cancer cell lines showed both ER-α and ER-β was highly expressed in gastric cancer cell lines than normal immortalized GES-1 cells. B. Immunocytofluorescense staining of ER-α and ER-β in AGS cells shows ER-β was the dominant type of estrogen receptor. C. Treatment of AGS cells with 17β-estradiol (10 nmol/L) for different time periods indicated AGS cells were hormone-dependent. Data represent three independent experiments with similar results.

immunostaining, cells were washed 3 times with PBS (10 min each) and treated with 5 μg/mL DAPI for nuclear staining. Cells were imaged using an Olympus fluorescence microscope. The following secondary antibodies were used: FITC goat anti-mouse IgM+IgG (ab47830, Abcam, Cambridge, UK) and FITC goat anti-rabbit IgG (ab6717, Abcam, Cambridge, UK).

Dual-luciferase reporter assay

For reporter gene experiments, siESR2-treated cells or siNC-treated AGS cells (5×10^4) were seeded into 24-well plates and cotransfected with a Renilla (Rluc) luciferase reporter (0.5 μg)

with either a p53 response element-luciferase construct (1 μg) or a control construct (1 μg) using Lipofectamine 2000, according to the manufacturer's protocol. Cells were harvested 24 h after transfection for subsequent analysis of luciferase activity using a Promega Luciferase Assay Kit. Renilla luciferase activity was used as the reference.

Statistical analysis

All data with normal distribution are presented as the mean ± standard deviation (SD). Student's *t*-test (two-tailed) was conducted to analyze differences between groups. Statistical significance was calculated using SPSS version 20.0 software (SPSS, Inc., Chicago, IL, USA). A *P* value < 0.05 was considered statistically significant.

Results

Expression of ERs in GC cell lines and effects of low-dose estrogen on AGS cells in vitro

ER protein levels were assessed in eternalized human *gastric mucosa* epithelium GES-1 *cells and 5 GC cell lines* by Western blot analyses. As shown in **Figure 1A**, the levels of both ERα and ERβ in GC cells were higher than those in GES-1. The expression levels of ERs in GC cells were compared to those in MCF-7 breast cancer and HeLa cervical cancer cell lines (Figure S1). To assess the cellular location of ERα and ERβ proteins in AGS cells, an immunofluorescence assay was performed, showing that ERβ was primarily expressed in the nucleus (**Figure 1B**). The expression of ERβ was higher than that of ERα in AGS cells. To evaluate the effect of estradiol on GC cells, AGS cells were treated with low-dose 17β-estradiol (10 nM) for different time periods, and protein expression levels of p-ERK1/2, ERK, ERα and ERβ were detected by Western blot analysis.
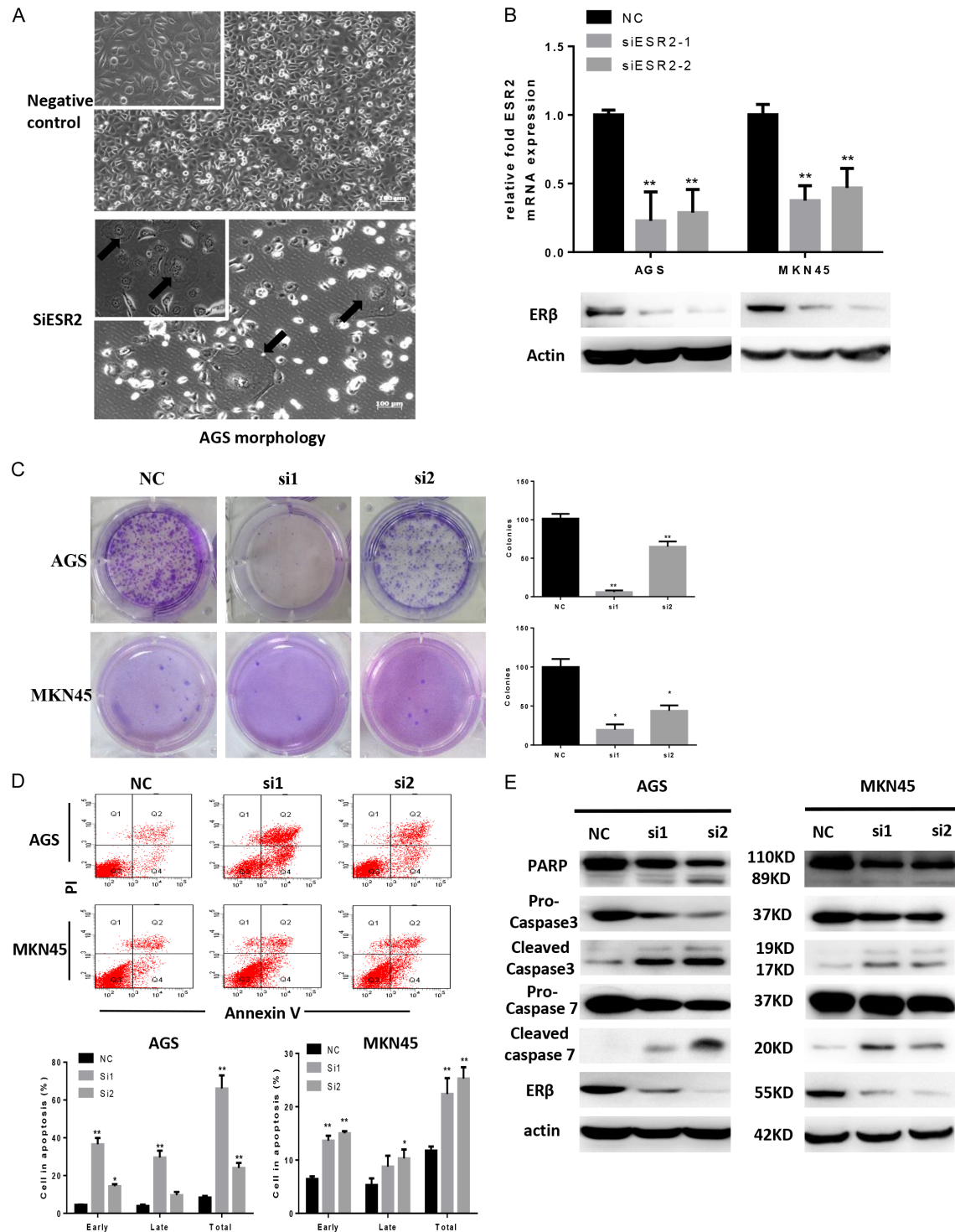
Figure 2. Knockdown of ESR2 expression in AGS and MKN45 GC cells induces apoptosis. A. Cells were transfected with siRNAs targeting ESR2 or with nonsense sequence for 48 h. Morphological changes were visualized using a light microscope at 200× magnification. Cell blebbing was observed in siESR2 cells. B. The mRNA and protein expression levels of ESR2 after transfection in AGS and MKN45 cells were detected by qRT-PCR and Western blotting, using β-actin as an internal control. The transfection efficiency was about 60-80%. C. Colony formation assays were performed in AGS and MKN45 cells. The number of colonies was decreased in both transfected AGS and MKN45 cells. D. Annexin V/PI staining was used to detect cell apoptosis in AGS and MKN45 cells. The percentage of positive Annexin V cells is sigficantly higher in knockdown cells. E. The protein levels of total PARP, pro-caspase-3 and pro-

caspase-7 decreased, while cleaved PARP, caspase-3 and caspase-7 increased in knockdown cells. ER-β expression was significantly decreased in siESR2 group. Data represent three independent experiments. Data are presented as the mean ± SD. *$P < 0.05$ and **$P < 0.01$ compared to the control group.

Estrogen treatment led to a general trend of decreased expression of these genes after the initial increase in p-ERK1/2, ERα and ERβ (**Figure 1C**). In addition, CCK-8 assay was utilized to assess the growth of AGC cells after 17β-estradiol treatment. It was found that cell viability increased slightly in the first hour, and then began decreasing in the next 24 h (data not shown), suggesting that GC was a hormone-dependent malignancy.

Down-regulation of ESR2 induces apoptosis in AGS and MKN45 GC cells

Seeing that ERβ protein levels in AGS and MKN45 cells were higher than those in the other GC cell lines, AGS and MKN45 cells were used for subsequent experiments. To evaluate the specific roles of ESR2 in GC progression, ESR2 expression was knocked down using small interfering RNA (siRNA) targeting regions of ESR2. qRT-PCR and Western blotting showed that ESR2 expression effectively decreased by 60-80% (**Figure 2B**). Compared to NC group, ESR2 suppression exerted a significant inhibitory effect on AGS cell growth as indicated by cell blebbing (**Figure 2A**). The impact of ESR2 on cell proliferation was assessed using a colony formation assay. It was found that ESR2 down-regulation effectively inhibited the colony formation ability of AGS and MKN45 cells as compared to the control group (**Figure 2C**). To determine whether the reduced proliferation was attributed to cell apoptosis, flow cytometry was performed using Annexin V/PI double labeling. ESR2 suppression resulted in a significant increase in the apoptosis rate compared to NC group ($P < 0.01$) (**Figure 2D**). The levels of apoptosis-related proteins were also examined by Western blotting. As compared to the control group, the levels of full-length PARP, procaspase-3 and procaspase-7 were decreased, while the pro-apoptosis markers, cleaved PARP, caspase-3 and caspase-7 were increased (**Figure 2E**). All these results demonstrated that ESR2 played an important role in inducing apoptosis of AGS and MKN45 GC cells.

ESR2 knockdown-induced apoptosis is independent of the p53 signaling pathway and occurs mainly through GADD45α activation

Knowing that p53 is an important transcription factor that regulates the expression of many pro-apoptotic target genes, we investigated the ability of p53 in mediating early- and late-stage apoptosis in GC cells. The p53-deficient KATO-III GC cell line was used for the following experiments. KATO-III cells were treated with siESR2 as described above, and the knockdown efficiency is shown in **Figure 3A**. Cellular apoptosis was detected using the Annexin V/PI double staining method (**Figure 3B**) by flow cytometry. The apoptosis rate of KATO-III cells in the knockdown group was significantly higher than that of cells in NC group ($P < 0.01$). The effect of siESR2 on the caspase pathway was analyzed by Western blotting (**Figure 3C**). In addition, the dual luciferase reporter system showed that the p53 response element was not activated (**Figure 3D**), directly suggesting that siESR2-induced apoptosis was not mediated by the p53 pathway in GC cells.

GADD45α is the canonical p53 target gene that can induce apoptosis and cell cycle arrest in a p53-independent manner. Western blot analysis showed that phosphorylated p53 was not significantly increased, while GADD45α was increased after siESR2 transfection of the AGS, MKN45 and KATO-III GC cell lines (**Figure 3E**). To further assess the involvement of GADD45α activation in siESR2-induced apoptosis, a GADD45α-specific siRNA was used to determine whether GADD45α protected AGS cells against siESR2-induced apoptosis. As shown in **Figure 3F**, **3G**, GC cell apoptosis was decreased in the double knock-down group compared to siESR2-alone group. These data demonstrated that siESR2-induced apoptosis in GC cells was partially mediated through GADD45α activation. In addition, mRNA expression data in the Cancer Cell Line Encyclopedia (CCLE) database (Figure S3) showed that ESR2 and GADD45a expression in GC cell lines had an opposite trend, which is consistent with the
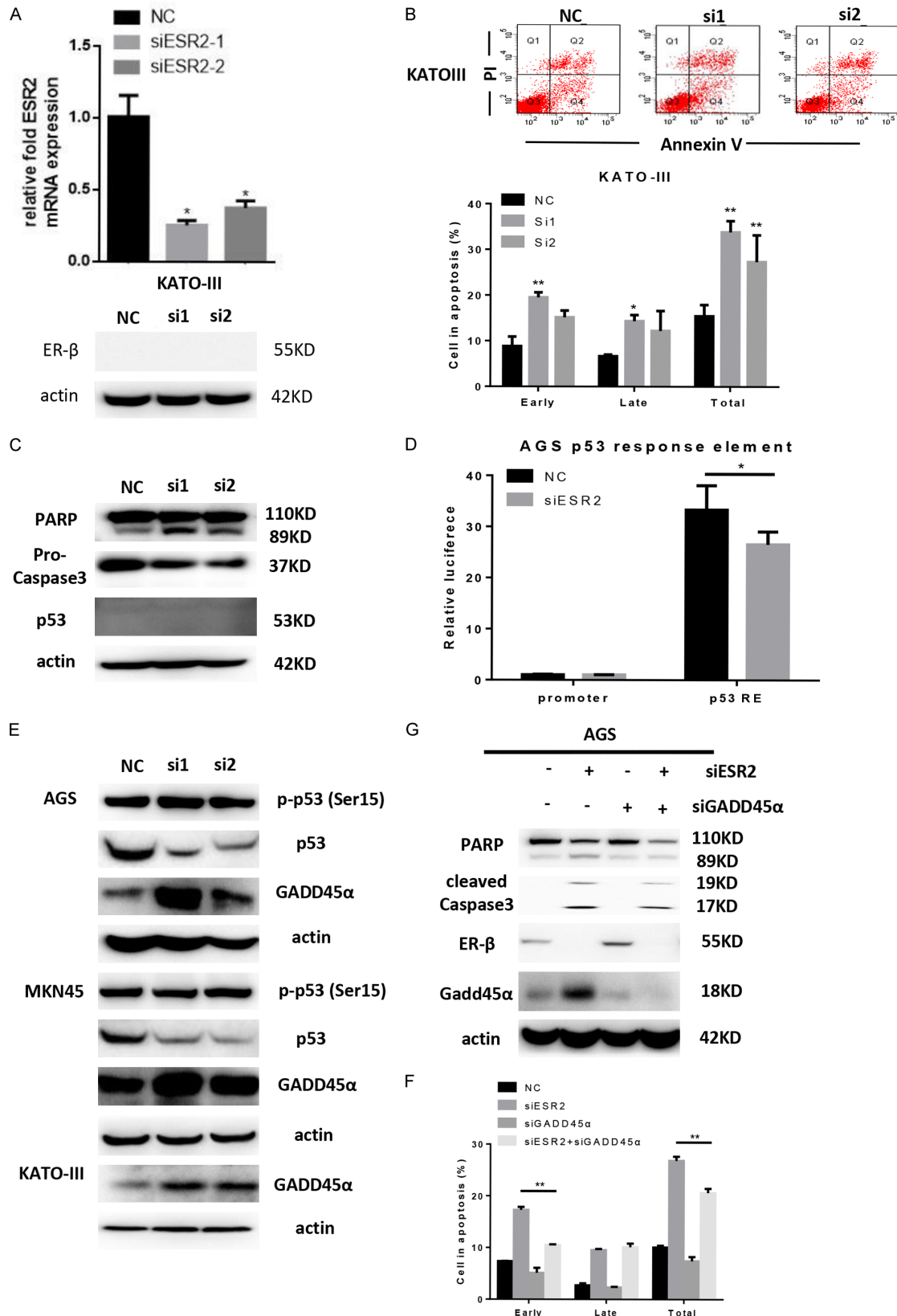
Figure 3. siESR2-induced apoptosis is independent of the p53 signaling pathway and occurs primarily by activation of GADD45α. A. p53-negative KATO-III cells were transfected with either siESR2 or siNC for 48 h. The corresponding transfection efficiency was evaluated by qRT-PCR and Western blotting. B. Annexin V/PI staining was used to detect

cell apoptosis in KATO-III cells. The percentage of early and late stage apoptotic cells is significantly increased in siESR2 cells. C. The protein levels of total PARP and pro-caspase-3 decreased, while cleaved PARP increased. D. After transfected with siESR2 or the corresponding control, dual luciferase reporter assay demonstrated no significant activation of p53 response element in AGS cells. E. The protein levels of p-p53, p53 and GADD45α were measured in three GC cell lines by Western blot analysis. As expected, p-p53 was not increased in knockdown cells, while as the classical target gene of p53, GADD45α was signicantly activated. F. AGS cells were co-transfected with siGADD45α and siESR2. Annexin V/PI staining showed cell apoptosis was decreased in co-transfected group. G. The protein levels of PARP, cleaved PARP, cleaved caspase-3, ERβ and GADD45α in AGS cells of each group were measured by Western blot analysis, which also showed less apoptotic change in AGS cells.
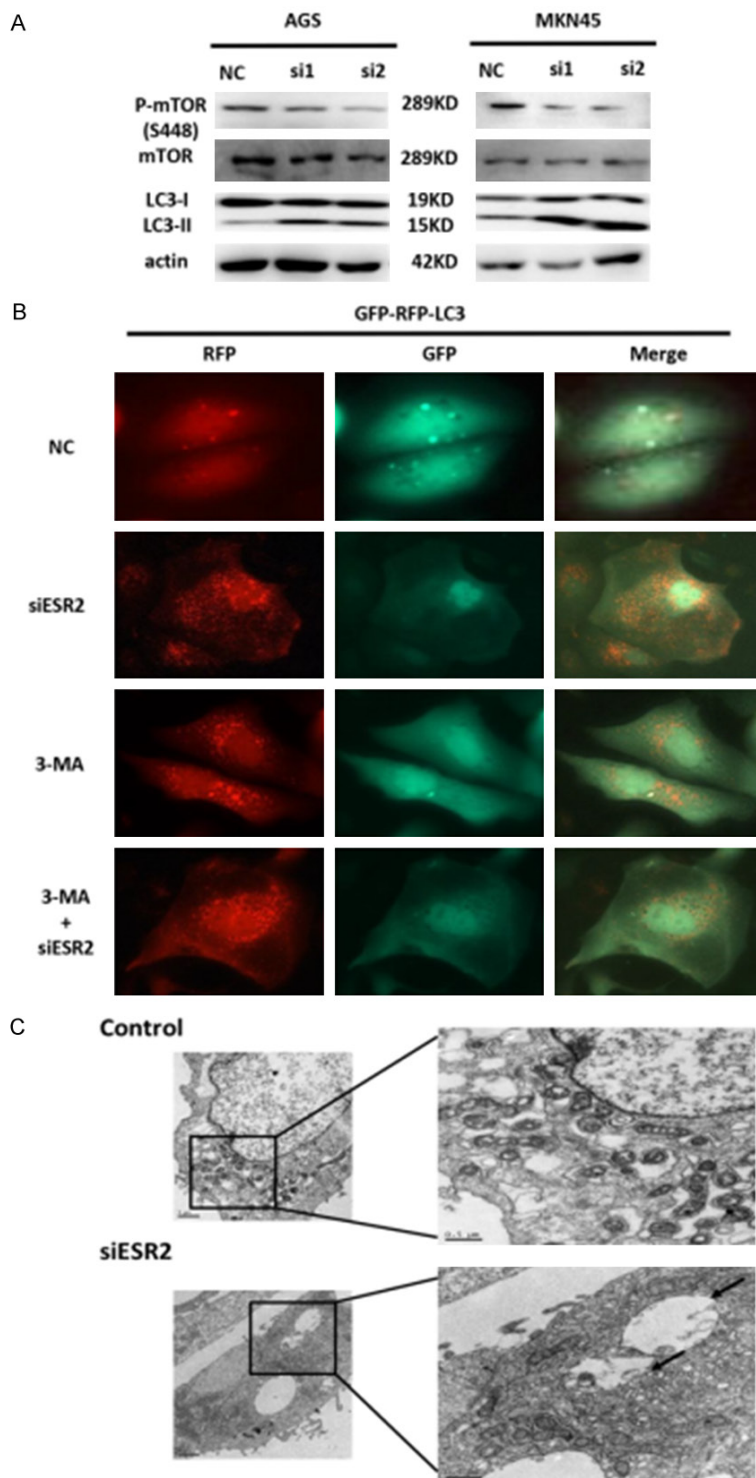

Figure 4. Suppression of ESR2 promotes autophagy in AGS and MKN45 cells. A. The protein levels of mTOR remained the same in both transfected or non-transfected cells, while p-mTOR, LC3-I and LC3-II levels were increased. β-actin was used as a loading control. B. AGS cells were stably transfected with mRFP-GFP-LC3 to show autophagic flux. Fluorescence microscopy showed that ESR2 siRNA-transfected AGS cells had more autolysosomes (RFP) than siNC-transfected AGS cells. The autophagy inhibitor 3-methyladenine (3-MA) decreased the number of autolysosomes. Red fluorescent puncta represent autolysosomes and yellow fluorescent puncta represent autophagosomes. C. Representative TEM images of AGS cells transfected with siESR2 and their corresponding control. Autolysosomes are indicated by black arrows.

conclusion of the present study (especially those indicated by red arrows).

Suppression of ESR2 promotes autophagy in AGS and MKN45 GC cells

Knowing that the relationship between autophagy and apoptosis is interconnected by several molecules, we next investigated the ability of siESR2 in inducing autophagy in GC cells. As the protein levels of autophagy-related proteins LC3I and LC3II are considered to be the hallmark of autophagy [22], we detected mTOR and phosphorylated mTOR by Western blot analysis. As shown in **Figure 4A**, phosphorylated mTOR protein

levels were significantly decreased after ESR2 suppression in AGS and MKN45 cells, but total mTOR protein levels remained unchanged significantly. In addition, siESR2 increased the ratio of LC3II/LC3I, indicating that ESR2 silencing promoted autophagy in an mTOR-dependent manner. In contrast, treatment of AGS cells with diarylpropionitrile (DPN), a selective ERβ agonist, significantly increased phosphorylated mTOR protein levels, and the ratio of LC3II/LC3I was markedly decreased (Figure S2), indicating that autophagy was suppressed after ERβ activation. To determine whether ESR2 gene silencing increased autophagy flux in AGS cells, mRFP-GFP-LC3 adenoviral vectors were utilized to assess the autophagic level in cells transfected with siESR2 or a control vector. As expected, after adenoviral infection, both fluorescent proteins were successfully introduced into AGS cells, and the cells transfected with siESR2 exhibited typical dense accumulation of RFP-LC3 puncta in the perinuclear region and cytoplasm. Pretreatment of siESR2-transfected cells with the autophagy inhibitor 3-methyladenine (3-MA, 10 mM) reduced the siESR2-mediated formation of LC3 puncta (red) (Figure 4B). TEM analysis of the ultrastructure of siESR2-transfected AGS cells demonstrated typical autophagosomes consisting of double membranes (black arrow), compared to NC group (Figure 4C). These data indicate that autophagy was induced by ESR2 silencing.

Development of autophagy promotes GC cell apoptosis primarily through MAPK pathway activation

Autophagy and apoptosis can occur simultaneously in cells. Although the molecular mechanisms and morphology of these two processes are significantly different, a functional relationship exists between them. Therefore, autophagy was suppressed using drugs and genetic silencing to observe apoptosis (Figure 5A). The percentage of apoptotic cells was decreased after pre-treatment of AGS cells with 3-MA, compared to siESR2-alone group (Figure 5A, 5B), indicating that inhibition of autophagy could reverse apoptosis. Knowing that binding of ATG5 and ATG12 promotes autophagosome formation and inhibits the development of autophagic flux [23], we detected apoptosis in AGS cells after treatment with ATG5 siRNA to suppress autophagy. As shown in Figure 5C, ATG5 protein levels were effectively knocked

down after siATG5 transfection. In addition, the levels of cleaved-caspase 3 and cleaved-PARP and LC3II in AGS cells were lower in siESR2 and siAtg5 treatment groups than those in siESR2-alone group, suggesting that apoptosis was partially reversed after autophagy inhibition at the gene level (Figure 5C). The signaling pathways mediated by siESR2-induced apoptosis and autophagy were further investigated. It was found that extracellular estrogen stimulation could rapidly induce activation of the MAPK family, and the binding of canonical estrogen to its receptors could activate the downstream Ras/Raf/MAPK pathway to induce gene transcription. Therefore, the JNK MAPK, p38 MAPK and ERK1/2 MAPK levels were detected. It was found that siESR2 transfection in these two cell lines activated the MAPK pathway as indicated by increased levels of p-JNK MAPK, p-p38 MAPK and p-ERK1/2 MAPK (Figure 5D). These findings demonstrated that siESR2 primarily activated the MAPK pathway and promoted autophagy via activation of GADD45α, thereby inducing apoptosis in GC cells (Figure 5E).

Discussion

The present study showed that low-dose estrogen (10 nM) induced a weak proliferative effect in GC cells in addition to ERK1/2 protein activation. Moreover, the expression levels of ERα and ERβ in GC cell lines were higher than those in the normal gastric epithelial cell line. Compared to ERα, immunofluorescence showed that ERβ was more widely distributed in AGS cells, suggesting that ERβ plays an oncogenic role in GC cells. Knockdown of the ERβ expression by siRNA showed that the three GC cell lines exhibited apoptosis through GADD45α activation independent of p53 pathway. In addition to apoptosis, autophagy was increased in these cells. With the suppression of autophagy, apoptosis was reduced in both drug treatment group and at the overall level, suggesting that autophagy is a prerequisite for apoptosis, and that autophagy induces apoptosis. Finally, MAPK signaling pathway activation was also observed. The p38 and JNK signaling pathways directly induced apoptosis, while ERK1/2 induced an increase in autophagy.

Some studies [24] found that the GADD45α gene could promote growth arrest after DNA damage, and it was the first p53 downstream
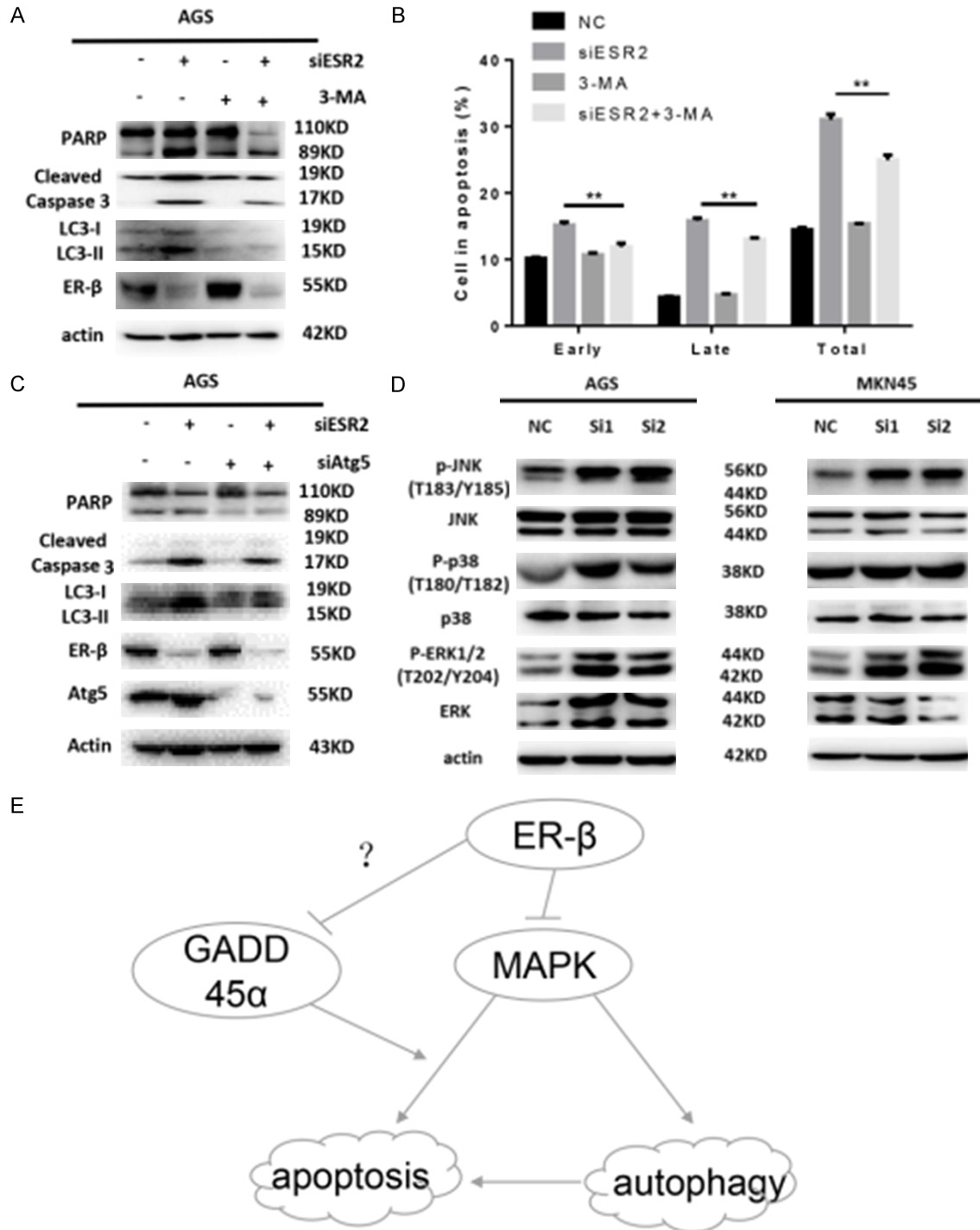
Figure 5. siESR2-induced autophagy and apoptosis are mediated by MAPK signaling pathway activation. A. AGS cells were transfected with ESR2 siRNA or treated with 3-MA. Protein levels of PARP showed a decreasing trend in both siESR2 and 3-MA group, compared to siESR2 alone, but cleaved PARP and cleaved caspase-3 levels decreased, indicating 3-MA rescued siESR2 induced apotosis. As expected, LC3-I and LC3-II was decreased in double treatment group. B. Flow cytometry analysis also showed a decreasing trend of apoptosis in double treatment group. C. AGS cells were cotransfected with siESR2 and siATG5. The protein levels of PARP, cleaved PARP, cleaved caspase-3, LC3-I and LC3-II, ERβ and ATG5 in AGS cells showed the same trend of 3-MA treatment group. D. The protein levels of p-JNK, p-p38, p-ERK were all increased in siESR2 group, while with no change in total JNK, p38 and ERK. E. The involvement of ER-β related signaling pathway and molecules in autophagy and apoptosis is shown.

target gene that had been discovered. Others [25] reported that the expression of GADD45α could be induced through both p53-dependent and p53-independent pathways, after which this protein participated in the regulation of important cellular activities such as cell cycle checkpoints, cell apoptosis, DNA damage/repair and signal transduction. Therefore, they believed that GADD45α played an important role in the maintenance of genome stability. In addition, MAPK kinases were found to bind to OCT-1 and CATT on the GADD45α promoter to regulate the transcriptional expression of GADD45α. It was found in our study that siESR2 transfection promoted GADD45α transcription by activating the MAPK pathway. Some recent studies [25, 26] reported the existence of an AP-1 binding site in the upstream of the GADD45α promoter. ERs can participate in the regulation of transcriptional activity of the AP-1 transcription factor. ERα activates transcription, while ERβ inhibits transcription. Therefore, after ESR2 silencing, factors inhibiting Gadd45α transcription could be released, which would increase its transcription

The functions of estrogen are categorized as canonical genomic transcriptional regulation and non-canonical regulation. Non-canonical regulation includes non-sex hormone receptor binding and plasma membrane sex hormone receptor binding [27]. The response of the non-canonical pathway is fast. First, this pathway can change the current activated pathway within a short period of time to promote activation of the MAPK pathway, especially ERK MAPK. Next, this pathway involves interactions with ERs on the cell membrane to activate Ras/Raf/MAPKs through phosphorylation. The canonical pathway is represented by the interaction of estrogen and nuclear ERs, which then form a dimer. In turn, ERs bind to co-regulatory molecules by which they recognize estrogen response elements of target genes and initiate gene transcription. After ERβ silencing, estrogen activates the MAPK pathway mainly through the non-canonical pathway. In addition, the ESR2 gene is downstream of the ERK gene [28]. When ESR2 expression is reduced, a compensatory increase in ERK pathway activation compensates for the growth inhibition caused by loss of the ESR2 gene.

Autophagy is a double-edged sword in tumor development. Several studies [29] have con-

sidered that autophagy is suppressed in tumor cells during the early stages of cancer development, which promotes tumor progression. The expression of the autophagy-related gene Beclin 1 was reported to be reduced in ovarian cancer, prostate cancer and breast cancer [30]. However, other studies [31] argued that autophagy provided an optimal environment for tumor cells under ischemic and hypoxic conditions. In addition, autophagy can occur in the extracellular matrix to promote tumor metastasis. As a protective mechanism in tumors, autophagy can suppress tumor cell apoptosis [32]. However, as type III programmed cell death, autophagy can be the initiator of apoptosis, which also furthers the induction of apoptosis [33]. The relationship between autophagy and apoptosis in GC is a popular research topic. Zhao et al [22] showed that autophagy played a protective role in toxicarioside N-induced GC cell apoptosis mainly through inhibition of the Akt/mTOR signaling pathway. Song et al [34] showed that rottlerin promoted GC cell apoptosis and this process was mediated by activation of autophagy. Xu Y et al [35] reported that 2-deoxy-D-glucose enhanced TRAIL-induced apoptosis in human GC cells by down-regulating the JNK-mediated cell protective autophagy. It was found in our study that autophagy and apoptosis occurred together after interference of the ESR2 gene expression. In addition, inhibition of autophagy attenuated apoptosis, suggesting that autophagy initiates apoptosis and induces the development of apoptosis.

In summary, the present study utilized a series of *in vitro* experiments to investigate the role of ERβ in GC. The results suggest that ESR2 may be a novel target gene in GC therapy. Unfortunately, we did not perform animal experiments to investigate GC cell lines with the potential for ovarian metastasis. In addition, ERs have many subtypes and are involved in diverse signal transduction pathways. Further studies that include mechanism experiments are still required to elucidate the relationship between GC and estrogen.

Disclosure of conflict of interest

None.

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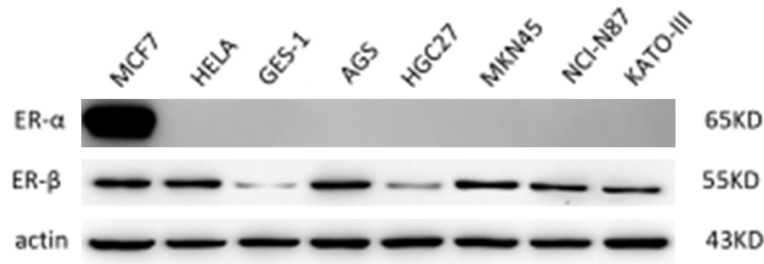
Figure S1. A comparison of the expression levels of ERs in GC cells and other cells. The expressions of ER-α and ER-β in the breast cancer cell line MCF-7, cervical cancer cell line HeLa, GES-1 cell line and five gastric cancer cell lines were determined by Western blot analysis. As compared with breast cancer cells, protein levels of ER-α and ER-β was lower, especially ER-α. But ER-β was commonly expressed in all gastric cancer cell lines. Data represent three independent experiments.
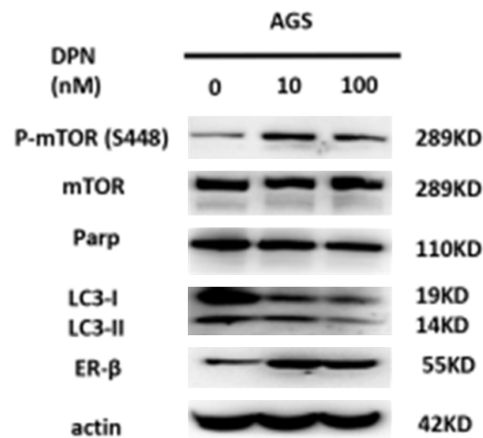

Figure S2. Autophagy is suppressed with ER-β activation. AGS cells were treated with different concentrations of the DPN-selective ER-β agonist. Western blot analysis showed, in DPN group, p-mTOR, LC3-I, LC3-II, and total PARP levels were all decreased, while mTOR expression remained the same, indicating a reverse effect compared to siESR2. Data represent three independent experiments.
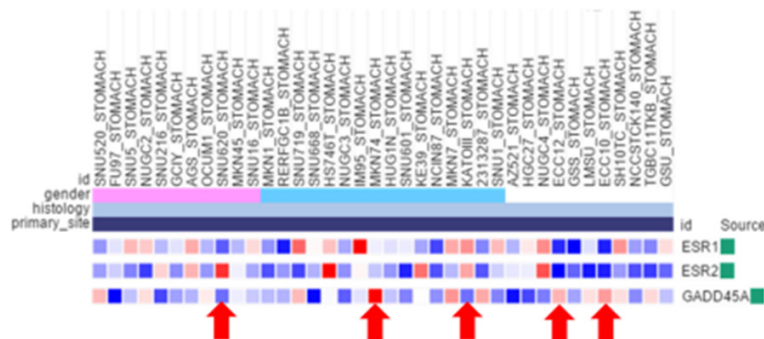

Figure S3. mRNA expression data in the Cancer Cell Line Encyclopedia (CCLE) database. mRNA expression levels of ESR1, ESR2 and GADD45A in GC cell lines according to the CCLE database. A negative correlation was seen in ESR2 and GADD45A as indicated by red arrows.