Reproductive Sciences
2019, Vol. 26(8) 1111-1120

DOI: 10.1177/1933719118808912
journals.sagepub.com/home/rsx
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Farnesoid X Receptor Agonist GW4064 Inhibits Aromatase and ERβ Expression in Human Endometriotic Stromal Cells

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Abstract

Endometriosis is an estrogen-dependent disease. Farnesoid X receptor (FXR) activation has been shown to inhibit estrogen signaling in breast cancer and testicular tumors. However, the role of FXR in endometriosis is still poorly understood. Here, we aimed to investigate whether FXR activation by its synthetic agonist GW4064 has a therapeutic effect on endometriosis and the underlying molecular mechanisms. We found that the expression of FXR (encoded by the *NR1H4* gene) in endometriotic tissues and stromal cells (ESCs) was higher than that in eutopic endometrial tissues and stromal cells. The GW4064 treatment led to a dose-dependent decrease in aromatase and estrogen receptor β (ERβ) expression and induced ERK1/2, p38, AMPK, and Stat3 activation in ESCs. In contrast, ERK1/2 inhibitor reversed the GW4064-induced reduction in aromatase expression. In addition, treatment with p38, AMPK, and Stat3 inhibitors or small interfering RNAs could also reverse the GW4064-induced reduction of ERβ expression in ESCs. The GW4064 treatment markedly increased Stat3 phosphorylation, enhancing the binding of Stat3 to the *ESR2* promoter, which resulted in the downregulation of ERβ. Coimmunoprecipitation assay and chromatin immunoprecipitation analysis revealed that FXR was able to compete with cyclic AMP response element-binding (CREB) protein for binding to a common sequence on the aromatase promoter region after GW4064 treatment in ESCs. Moreover, treatment of endometriosis xenografts with GW4064 suppressed aromatase and ERβ expression in nude mice. Our results suggest that FXR may represent a potential therapeutic target for future therapy.

Keywords

endometriosis, FXR, CREB, Stat3, aromatase, ERβ

Introduction

Endometriosis is characterized by the growth of functional endometrial tissue outside the uterine cavity. Typical symptoms are chronic pelvic pain, dyspareunia, and infertility. Endometriosis affects up to 10% of women of reproductive age.[1] Currently, the most common medical approaches for endometriosis are hormones, but their long-term use is limited due to their side effects and contraindications.[2] Therefore, there is an urgent need to develop novel and effective nonsteroidal medical therapies for endometriosis.

Endometriosis is an estrogen-dependent disease. The survival and growth of ectopic lesions are dependent on local estrogen (E2) produced by endometriotic implants.[3] Aromatase (encoded by *CYP19A1* gene) is the key enzyme for estrogen biosynthesis.[4] Several studies, including our own, have shown that *CYP19A1* is highly expressed in endometriotic stromal cells (ESCs).[5] Moreover, the promoter region of *CYP19A1* contains multiple transcription factor binding sites, including the cAMP response element (CRE), located at -211/-203 in the promoter II, which can mediate the transcriptional regulation of aromatase.[6] Estrogen works primarily through its 2 distinct nuclear estrogen receptors, ERα and estrogen receptor β (ERβ), which are encoded by different genes (*ESR1* and *ESR2*), to mediate its biological responses. Higher levels of ERβ and lower levels of ERα have been reported in human ectopic endometrial tissues and primary stromal cells than in eutopic endometrial tissues and cells.[7] Recently, Han et al revealed that ERβ drives endometriosis progression by interacting with the inflammasome complex and cytoplasmic apoptotic machinery, enhancing the proliferation and inhibiting cell death in endometriotic tissues.[8] Hence, any approaches to reduce aromatase and ERβ expression and/or their activities could be new therapeutic strategies for the treatment of endometriosis.

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The Farnesoid X receptor (FXR, encoded by *NR1H4* gene), belonging to the nuclear receptor superfamily, is a ligand-activated transcription factor.[9] Upon ligand binding, FXR will undergo a conformational change which increases its affinity for coactivator or corepressor proteins and subsequently binds to its response element (FXRE) to regulate gene transcription.[10] Recently, FXR activation was identified as a negative modulator of estrogen signaling by downregulating aromatase expression in breast cancer and testicular tumors.[11,12] Bile acids are well-known natural ligands for FXR. However, in addition to bile acid regulation, the FXR plays critical roles in maintaining many other metabolic pathways, including lipid and glucose homeostasis.[13-15] Polyunsaturated fatty acids (PUFAs), such as arachidonic acid, have also been demonstrated as ligands of FXR.[16] Moreover, in previous studies, a decreased risk of endometriosis in animal models has been demonstrated with high n-3 PUFA intake.[17,18] However, the role of FXR activation in the regulation of aromatase expression in endometriosis remains poorly understood, and studies about the effect of FXR activation on ERβ expression are still limited. In the present study, we detected higher expression of FXR in ectopic endometrium tissue than in eutopic endometrium tissue. We speculate that FXR may be an therapeutic target for drug development in endometriosis

GW4064 is a specific synthetic agonist of FXR. The aim of this study was to investigate whether FXR activation by GW4064 can work as a therapeutic strategy for endometriosis and the underlying molecular mechanisms. We proposed that FXR competes with cyclic AMP response element-binding (CREB) protein in binding the promoter region of *CYP19A1* after GW4064 treatment, which results in the downregulation of aromatase expression. GW4064 administration suppressed the expression of ERβ through the recruitment of Stat3 to the promoter region of *ESR2*.

Materials and Methods

Patients and Primary Cell Culture

Ectopic endometrial tissues from the cyst walls of ovarian endometriomas and eutopic endometrial tissues were obtained from 11 patients with endometriosis immediately after surgery, composing 11 self-controlled pairs. All patients (age range, 23-40 years old) had regular menstrual cycles, and none received hormonal therapy. All the samples were histologically confirmed, and the phase of the menstrual cycle was determined by preoperative history and histological examination. Half of the tissue samples were in the proliferative phase and the other half in the secretory phase. The experimental protocol was approved by the Institutional Review Board of Peking University (No.2014[789]), and informed consent forms were signed by each patient before samples were used. Human endometrial stromal cells (EMs) and ESCs were isolated from the collected tissues using a protocol previously described by Ryan et al with minor modifications.[19] Briefly, eutopic and ectopic endometrial tissues were washed with phosphate-buffered saline (PBS) and minced into small pieces of 1 mm^3. After the enzymatic digestion of minced tissues with collagenase (1 mg/mL; Sigma, St. Louis, Missouri) and DNase (0.04 mg/mL; Sigma) in a shaking bed for 1 hour at 37°C, they were separated by filtration through a 70-μm and then a 20-μm nylon mesh to remove epithelial cells. Stromal cells were harvested and suspended in Dulbecco modified Eagle medium (DMEM)/F12 (1:1; HyClone, Logan, Utah) supplemented with 10% fetal bovine serum (FBS; GIBCO/BRL, Grand Island, New York) and 100 U/mL penicillin (Lonza, Basel, Switzerland), 100 U/mL streptomycin (Lonza), and 250 ng/mL amphotericin B (Lonza) at 37°C in a humidified atmosphere containing 5% CO_2.

RNA Extraction and Quantitative Analysis by Real-Time Quantitative Polymerase Chain Reaction

Total RNA was extracted from ESCs and EMs using TRIzol reagent (Invitrogen, Carlsbad, California) according to the manufacturer's protocol. A 2-μg sample of total RNA was converted into complementary DNA (cDNA) using an ABI High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, California). Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using an ABI 7500 sequence detection system and an ABI Power SYBR Green gene expression system (Applied Biosystems) to quantify *18S, GAPDH*, *NR1H4, CYP19A1*, *and ESR2* mRNA expression levels. Relative quantities of all transcripts were analyzed using the comparative threshold cycle method as previously described.[4] GAPDH or 18S mRNA was used as a control for normalization. The primers were as follows: *NR1H4*, forward 5′-TGCTCTGCTTACAGCAATTGTTATC-3′, reverse 5′-CCTGAAGCTTCTCTACTGCCTCTCT-3′; *CYP19A1*, forward 5′-CACATCCTCAATACCAGGTCC-3′, reverse 5′-CAGAGATCCAGACTCGCATG-3; *ESR2*, forward 5′-ATGATCAGCTGGGCCAAGAA-3′, reverse 5′-CCACATC AGCCCCATCATTAA-3′; *GAPDH*, forward 5′-GAAGGTG AAGGTCGGAGTC-3′, reverse 5′-GAAGATGGTGATGGG ATTTC-3′.; and *18S*, forward 5′-AGGAATTCCCAGTAAGT GCG-3′, reverse 5′-GCCTCACTAAACCATCCAA-3′.

Western Blotting

Endometriotic stromal cells and EMs were washed and lysed in RIPA buffer (KeyGen Biotech, Nanjing, China) supplemented with a protease inhibitor cocktail (Amresco, Solon, Ohio) and phosphatase inhibitor (KeyGen Biotech). A micro-BCA protein assay kit (KeyGen) was used to determine protein concentrations. Briefly, equal amounts of protein were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membranes, and incubated with the following antibodies: anti-FXR antibody (1:500 dilution; Santa Cruz, Dallas, Texas), anti-aromatase antibody (1:1000 dilution; Abcam, Cambridge, United Kingdom), and anti-ERβ antibody (1:1000 dilution; Merck Millipore). Anti-GAPDH antibody (1:1000 dilution; ZSGB-BIO, Beijing, China) was used as a loading control. Protein bands

were visualized by enhanced chemiluminescence solution (Syngene, Cambridge, United Kingdom), and the intensities of the Western blot bands were analyzed by AlphaEaseFC software and normalized with respect to the loading control.

Drug Treatments

After starvation overnight, the ESCs were incubated in serum-free medium containing GW4064 (Sigma) with different concentrations (0, 0.5, 1, 2 μM) for 24 hours. After starvation overnight, the ESCs were preincubated with the Stat3 inhibitor AG490 (AG, 10 μM; Sigma), AMPK inhibitor Compound C (CC, 5 μM; Millipore), P38 inhibitor SB202190 (SB, 5 μM; Sigma), and ERK1/2 inhibitor PD98059 (PD, 25 μM; Sigma) for 1 hour at the indicated concentrations and then treated with or without GW4064 for 24 hours.

Enzyme-Linked Immunosorbent Assay

Endometriotic stromal cells were cultured in DMEM/F12 media (HyClone) and treated with or without GW4064 and inhibitors for the indicated times. Conditioned cell culture medium derived from treated and untreated cells was harvested. After centrifugation, the concentrations of estradiol in the supernatants were measured using standard Enzyme-Linked Immunosorbent Assay (ELISA) kits (Cayman Chemical, Ann Arbor, Michigan) according to the manufacturer's instructions. Estradiol levels were normalized to the cell number.

Small Interfering RNA Knockdown

Endometriotic stromal cells were transfected with either a non-specific negative control small interfering RNA (siRNA; Invitrogen) or siRNAs against human signaling pathway proteins (Invitrogen) at 100 nmol/L using Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM reduced-serum medium (Invitrogen) after culturing to approximately 70% to -80% confluence. Twenty-four hours after siRNA transfection, ESCs were serum-starved for 16 hours and treated with 2 μM GW4064 for 24 hours. Subsequently, the cells were processed for RT-PCR and Western blot analysis.

Coimmunoprecipitation Assay

Endometriotic stromal cells were washed and lysed in a non-denaturing lysis buffer (Applygen Technologies, Beijing, China) supplemented with 1% protease inhibitors. Cell lysates were then harvested and centrifuged. The supernatants were stored at −80°C or used immediately for immunoprecipitation. For immunoprecipitation, equal amounts of protein (500-1000 μg) were first immunoprecipitated with anti-CREB and anti-FXR antibodies (Santa Cruz) at 4°C for 6 hours. Protein A agarose (Roche, Basel, Switzerland) was then added and incubated at 4°C overnight. The immunoprecipitants were collected, washed 3 times, and eluted with SDS-PAGE sample buffer. The immunoprecipitated were then analyzed by Western blotting with anti-FXR and anti-CREB antibodies as described above.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) was performed using a ChIP assay kit (Pierce, Rockford, Illinois) according to the protocol provided by the manufacturer. Briefly, the cells were cross-linked, harvested, and subjected to ChIP using an anti-FXR antibody (Santa Cruz), anti-CREB antibody (Santa Cruz), anti-Stat3 antibody (Santa Cruz) or control antibody (IgG; Santa Cruz) at 4°C overnight with rotation. Protein/DNA complexes were then eluted from the beads. The purified DNA was analyzed by real-time qPCR using the following primers: *ESR2* promoter, forward 5′-CATTAAGCTGGGGGAACTGG-3′, reverse 5′-ACCAGAGAGGCTTTGGGTTT-3′; and *CYP19A1* promoter, forward 5′-ATTGAAGTCACTAGAGATGGCCT-3′, reverse 5′-CTTATCATCTTGCCCTTGAGTGG-3′. The method used to analyze real-time ChIP results was previously described.[4]

Tissue Culture for Grafting

Endometrial tissues were collected from patients (age range, 23-40 years) who had regular menstrual cycles and had no history of endometriosis, endometrial polyps or endometrial carcinoma. The tissue samples were in the proliferative phase with an endometrial thickness ≥9 mm. No patients had received hormonal therapy within the previous 3 months. Informed consent forms were signed by each patient before samples were used. Fresh endometrial tissue biopsies were washed in ice-cold sterile PBS to remove residual blood. Subsequently, biopsies were dissected into 1-mm^3 fragments and cultured in DMEM/Ham F-12 medium (HyClone) supplemented with 2% stripped FBS (GIBCO), 10 nM E2 (Sigma), 100 U/mL penicillin (Lonza), and 100 U/mL streptomycin (Lonza) at 37°C in a humidified chamber with 5% CO_2 for 24 hours prior to injection into mice.

Xenograft Mouse Model

The xenograft mouse model of endometriosis was prepared as previously described by Bruner-Tran et al.[20] Animal studies were approved by the First Hospital of Peking University Animal Care Committee (No. J201803). Fourteen 5-week-old ovariectomized female nude mice (nu/nu) were obtained from the Animal Research Laboratory of Peking University First Hospital. On day 1, a pellet of 17β-estradiol (1.7 mg/60-day release; Innovative Research of America) was implanted subcutaneously into the neck region of all nude mice. On day 2, each mouse received 2 subcutaneous injections of fresh endometrial tissue fragments in 200 μL of sterile PBS—one in each flank. Three days after tissue implantation, GW4064 (25 mg/kg; Sigma) was administered by intraperitoneal (ip) injection every 2 days for 15 days. Mice were then killed at 18 days postinoculation, and tissue volume was calculated as

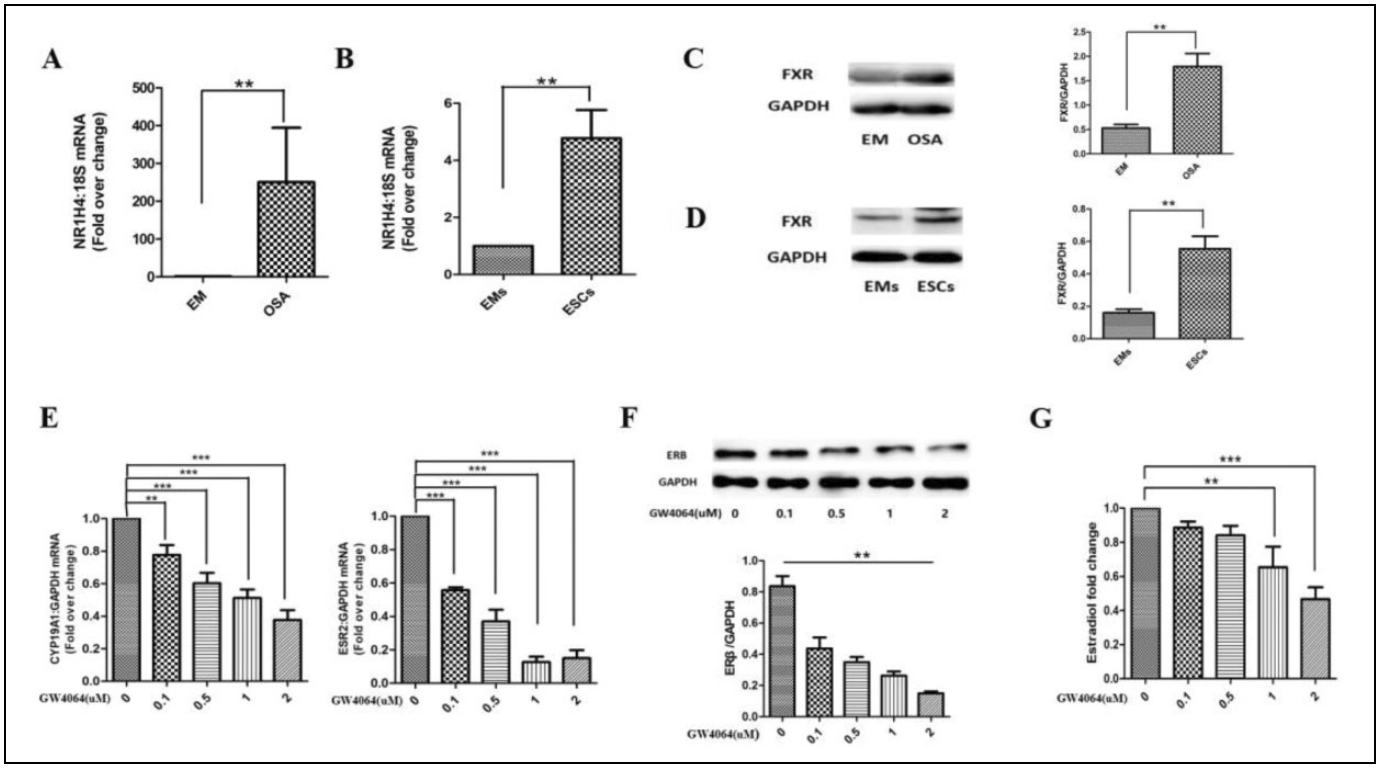

Figure 1. Expression of FXR in ESCs and EMs and the regulation of aromatase and ERβ expression by GW4064 in ESCs. A, Total RNA was extracted from paired endometriotic tissues and endometrial tissues. *NR1H4* mRNA levels were measured by RT-PCR (n = 6; **, $P < .01$, *t* test). B, Human primary EMs and ESCs were isolated, and *NRIH4* mRNA levels were measured by RT-PCR (n = 7; **, $P < .01$, *t* test). C, Western blotting was performed to compare the protein levels of FXR between endometriotic and paired endometrial tissues (n = 6; **, $P < .01$, *t* test). D, Western blotting was performed to compare the protein levels of FXR between EMs and ESCs (n = 6; **, $P < .01$, *t* test). Densitometric analysis (with AlphaEaseFC software) was used to quantify FXR protein expression. E, After starvation overnight, ESCs were cultured with GW4064 (0.1, 0.5, 1, and 2 μM) for 24 hours. GW4064 inhibited *CYP19A1* and *ESR2* mRNA expression (n = 4; **, $P < .01$, ***, $P < .001$; ANOVA). F, Western blotting was performed, and densitometric analysis (with AlphaEaseFC software) was used to quantify ERβ protein expression. GW4064 reduced ERβ protein expression, with a maximal effect at 2 μM (n = 3; **, $P < .01$; ANOVA). G, Effects of GW4064 on estradiol production (n = 3; **, $P < .01$, ***, $P < .001$; ANOVA). ANOVA indicates 1-way analysis of variance; EM, endometrial stromal cells; ERβ, estrogen receptor β; ESCs, endometriotic stromal cell; FXR, farnesoid X receptor; PCR, polymerase chain reaction.

follows: volume = 0.5 × length × width^2. The implanted endometrial lesions were harvested, and RNA and protein were extracted for quantification. The primers were as follows: *GAPDH*, forward 5′-ACCACAGTCCATGCCATCAC-3′, reverse 5′-TCCACCACCCTGTTGCTGTA-3′; *CYP19A1*, forward 5′-CCTGACGAAAGAGAACGTGA-3′, reverse 5′-CCCACAACAGTGTGGATCTC-3′; and *ESR2*, forward 5′-GTGTGTGAAGGCCATGATTC-3′, reverse 5′-CCATGCCCTTGTTACTGATG-3′.

Statistical Analyses

All experiments were performed at least 3 times using samples from different women. Comparisons of 2 groups were conducted using 2-tailed Student *t* test. Comparisons of more than 2 groups were conducted using 1-way analysis of variance. All values are shown as the mean ± standard error of the mean, and a *P* value <.05 was considered statistically significant.

Results

Expression of FXR and Regulation of Aromatase and ERβ Expression by GW4064 in ESCs

Real-time reverse transcription PCR and immunoblotting analyses were performed to quantify the expression levels of FXR in endometriotic and endometrial samples. We found that the expression of FXR in endometriotic tissues was higher than that in paired endometrial tissues both on the mRNA level (251-fold; Figure 1A) and protein level (3.41-fold; Figure 1C). Moreover, human primary EMs and ESCs were isolated from the collected endometrial and endometriotic tissues. Consistently, the expression of FXR was more enriched in ESCs than in EMs on both the mRNA (5.2-fold; Figure 1B) and protein levels (3.76-fold; Figure 1D).

GW4064 is a synthetic agonist of FXR. Treatment of ESCs with GW4064 at different concentrations reduced the levels of *CYP19A1* and *ESR2* mRNA in a dose-dependent manner. GW4064 inhibited *CYP19A1* and *ESR2* mRNA

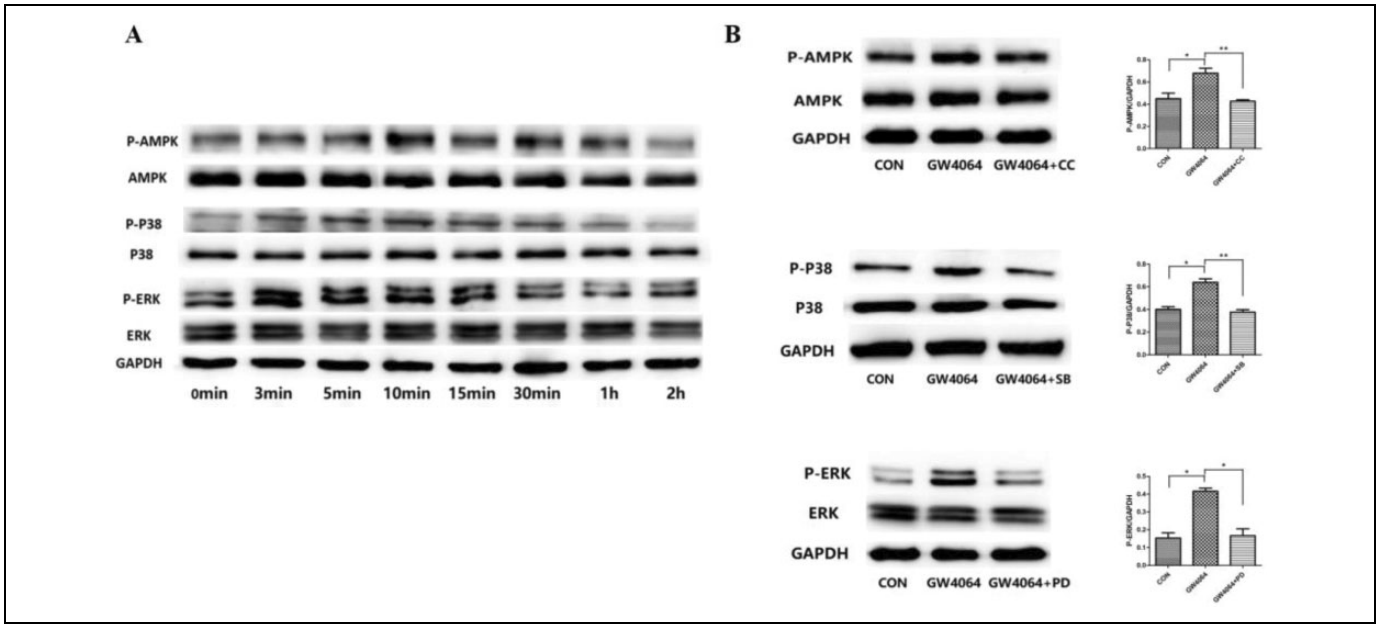

Figure 2. GW4064 treatment stimulates the ERK1/2, P38 and AMPK pathways in ESCs. A, After starvation overnight, ESCs were treated with 2 μM GW4064 for the indicated times. Whole-cell lysates were prepared, subjected to SDS-PAGE and analyzed by Western blotting with the indicated antibodies (n = 3). GW4064 stimulation induced the phosphorylation of ERK1/2, P38, and AMPK proteins. B, ESCs were pretreated with PD, SB, or CC for 1 hour and were then treated without (control) or with 2 μM GW4064 for 3, 10, and 10 min, respectively. The levels of protein phosphorylation were analyzed by Western blotting (n = 3). ESCs indicates endometriotic stromal cell; SDS-Page, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

expression, with maximal effects (63% and 87%, respectively) at doses of 2 μM and 1 μM, respectively (Figure 1E). Moreover, following GW4064 treatment, similar effects on ERβ protein levels were observed, with maximal inhibition (82%) observed at a dose of 2 μM (Figure 1F). Estradiol levels were lower in GW4064-treated ESCs than in the vehicle control (Figure 1G).

Signaling Pathways Involved in the Inhibitory Effect of GW4064 on Aromatase and ERβ Expression in ESCs

To identify the downstream signaling events involved in GW4064-induced downregulation of aromatase and ERβ, we examined the phosphorylation status of several signaling proteins, including ERK1/2, p38, and AMPK, in ESCs. Phosphorylation of ERK1/2, p38, and AMPK proteins was rapidly induced by 2 μM GW4064 and peaked at 3, 10, and 10 min, respectively (Figure 2A). Additionally, to determine whether the activation of these proteins was required in the regulation of aromatase and ERβ expression by GW4064 in ESCs, the ERK1/2 inhibitor PD, p38 inhibitor SB, and AMPK inhibitor CC were used. The inhibitors could reverse the corresponding GW4064-induced levels of protein phosphorylation (Figure 2B). We examined the mRNA levels of *CYP19A1* and *ESR2* in ESCs treated with GW4064 for 24 hours in the presence or absence of PD, SB, and CC and found that GW4064 reduced *CYP19A1* and *ESR2* mRNA expression levels. The addition of PD reversed this inhibitory effect on *CYP19A1* expression by 2.3-fold (Figure 3A). In addition, the addition of CC or SB reversed this inhibitory effect on *ESR2* expression by 1.7-fold and 3.9-fold, respectively (Figure 3D). Similar effects on aromatase (Figure 3B) and ERβ (Figure 3E) protein levels and estradiol production (Figure 3C) were also observed. Altogether, these data might indicate that activation of ERK1/2 is involved in the GW4064-mediated reduction of aromatase expression, while activation of p38 and AMPK might be necessary for the GW4064-mediated inhibition of ERβ expression.

GW4064 Enhances Stat3 Binding to the ESR2 *Promoter Region in ESCs to Repress ERβ Expression*

In addition to the signaling proteins mentioned above, the Stat3 signaling pathway has been reported to be regulated by GW4064 in hepatocellular carcinoma (HCC) cells.[21] Furthermore, Wang et al revealed that the promoter region of *ESR2* has a potential binding site (-717/-414) for Stat3 protein.[22] Thus, we attempted to clarify whether the Stat3 signaling pathway was involved in the GW4064-induced reduction of ERβ expression. We found that GW4064 treatment induced Stat3 phosphorylation in ESCs, which peaked at 10 minutes (Figure 4A). Addition of the Stat3 inhibitor AG490 reversed the reduction of both *ESR2* mRNA (2.1-fold, Figure 4B) and protein (2.73-fold, Figure 4C).

To provide further support for the roles of Stat3 in the regulation of ERβ expression by GW4064, we used siRNAs to knockdown Stat3. The efficacy of siRNA knockdown was determined by Western blotting (Figure 4D). In control siRNA-transfected ESCs, 2 μM GW4064 treatment resulted

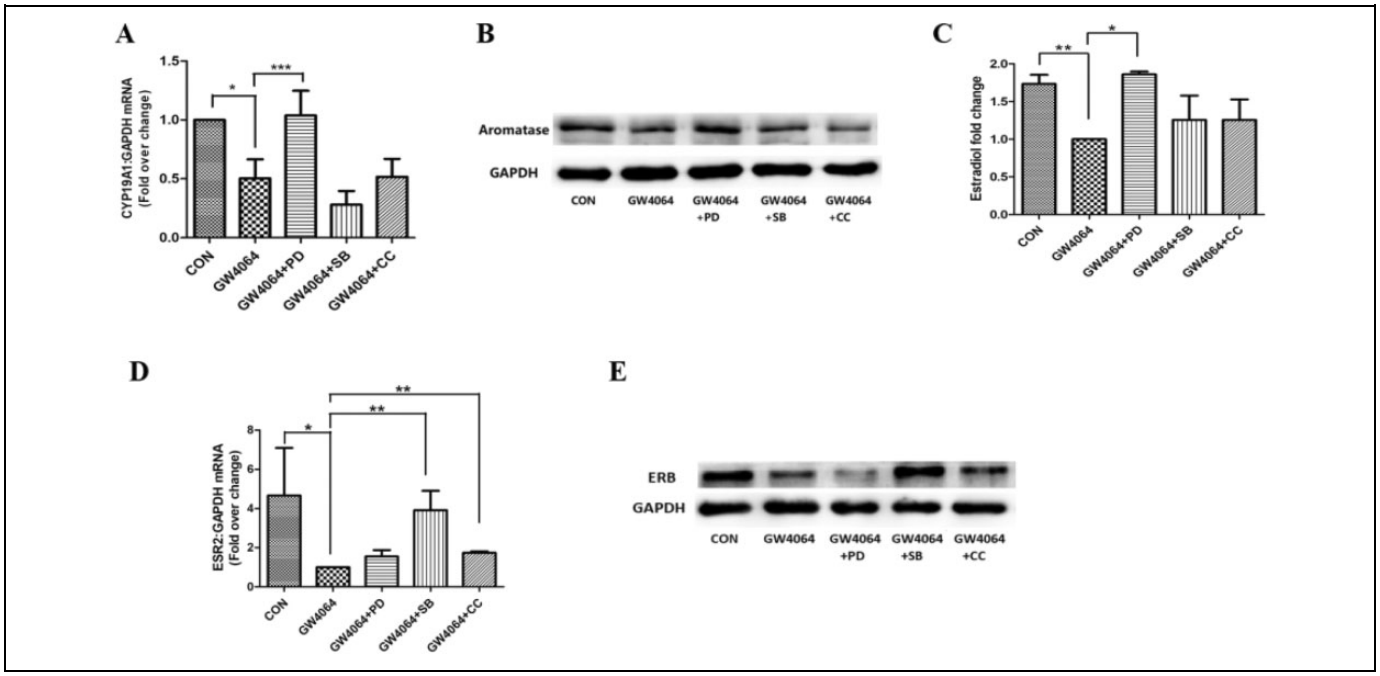

Figure 3. Signal pathways involved in GW4064 responsiveness in ESCs. A and B, ESCs were serum-starved overnight and pretreated with 25 μM PD, 5 μM CC, or 5 μM SB followed by treatment with 2 μM GW4064 for 24 hours. Cells were collected for real-time quantitative PCR and Western blotting (n = 3; *, $P < .05$, ***, $P < .001$, ANOVA). C, Conditioned media were harvested and assayed by ELISA (n = 3; *, $P < .05$, **, $P < .01$, ANOVA). D and E, Real-time quantitative PCR and Western blotting were conducted (n = 3, *, $P < .05$, **, $P < .01$; ANOVA). ANOVA indicates 1-way analysis of variance; ESCs, endometriotic stromal cell; PCR, polymerase chain reaction.

in a decrease in *ESR2* mRNA expression levels. Transfection with siStat3 following GW4064 treatment reversed this inhibitory effect on *ESR2* expression at both the mRNA level (2-fold, Figure 4E) and protein level (1.5-fold, Figure 4F). Collectively, these findings indicate that activation of Stat3 is involved in the GW4064-mediated reduction of ERβ expression.

By mapping the human *ESR2* promoter region, a potential Stat3 binding site was located at -717/-414.[22] We investigated whether the binding of Stat3 to the promoter region of *ESR2* could be influenced by GW4064 treatment. The ESCs were treated with 2 μM GW4064 for 2 hours, and ChIP assay was performed. We observed a marked increase in Stat3 occupancy (5.7-fold) of the *ESR2* promoter after GW4064 treatment (Figure 4G), which suggested that GW4064 enhances Stat3 binding to the *ESR2* promoter region in ESCs to repress ERβ expression.

Farnesoid X Receptor Competes With CREB in Binding the CYP19A1 *Promoter Region After GW4064 Treatment*

Previous studies have suggested that FXR and CREB share a common binding site in promoter regions of a large number of genes.[15] Thus, to determine whether FXR and CREB are physically associated in the regulation of *CYP19A1* in human ESCs, coimmunoprecipitation assays were conducted. We demonstrated that FXR and CREB could work as a transcription complex (Figure 5A). Additionally, the binding of CREB to the *CYP19A1* promoter region could contribute to transcriptional activation, which has been reported in endometriosis.[23] To further investigate the recruitment of these transcription factors to the *CYP19A1* promoter region by the treatment of GW4064 in ESCs, ChIP assays were performed. We found that the binding of CREB to the *CYP19A1* promoter region was decreased by 97% following GW4064 treatment (Figure 5B, left), while the binding of FXR to the *CYP19A1* promoter region was increased by 21.8-fold (Figure 5B, right). Our results suggested that FXR activation by GW4064 treatment could compete with CREB for binding to the promoter region of *CYP19A1* in ESCs.

GW4064 Inhibited the Expression of Aromatase and ERβ in Vivo

To confirm the effects of GW4064 on endometriotic tissues in vivo, human endometrium was injected subcutaneously into ovariectomized female nude mice to mimic ectopic implantation as previously described.[20] Before human tissue injections, pellets of 17β-estradiol were implanted subcutaneously in the neck region of all mice to facilitate the survival of endometriotic lesions. Fourteen mice were randomly divided into 2 groups: the PBS and PBS+GW4064 groups. GW4064 (25 mg/kg) was administered by ip injection every 2 days for 15 days. Mice were then killed at 18 days postinoculation, and grafts were harvested (Figure 6A). Real-time PCR of the tissue grafts showed that *CYP19A1*

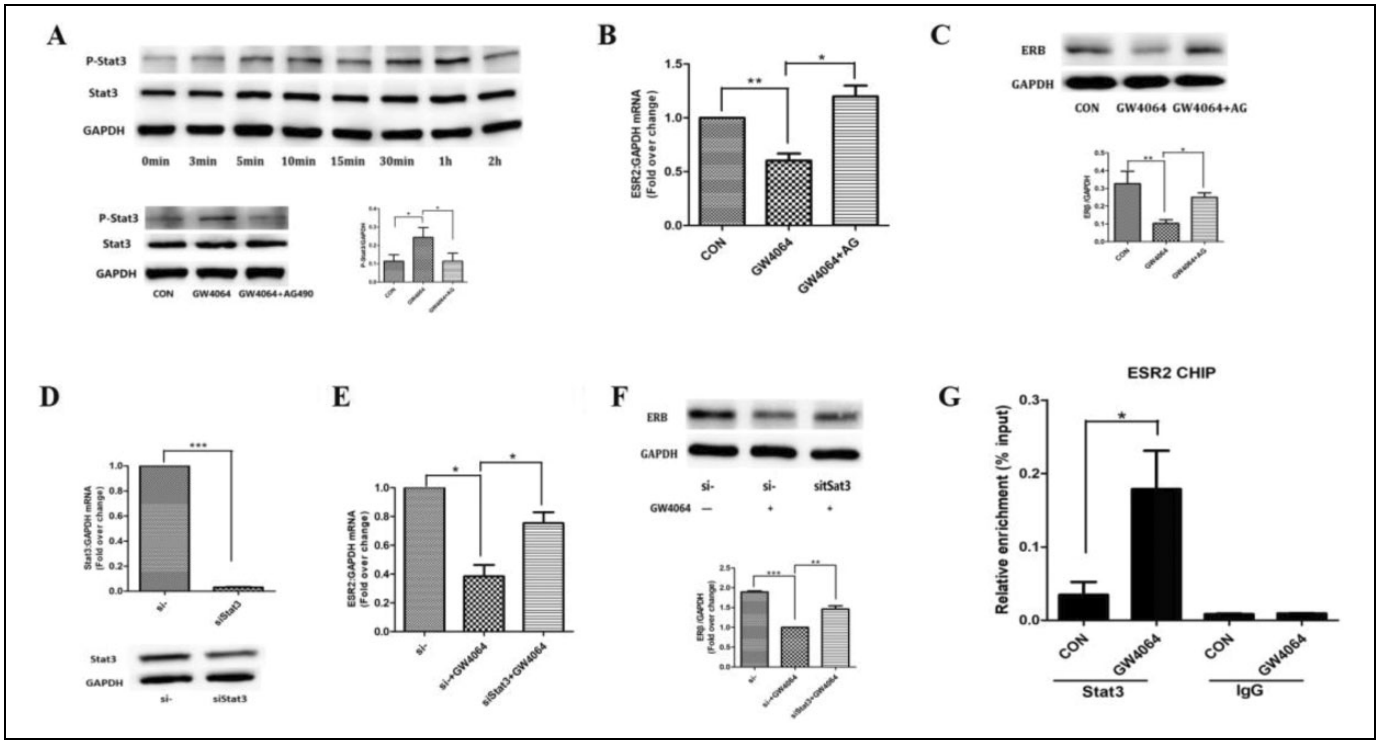

Figure 4. GW4064 inhibits ERβ expression via the Stat3 signaling pathway in ESCs. A, ESCs were serum-starved overnight and treated with 2 μM GW4064 for the indicated times. Western blotting was performed to detect the phosphorylation status of Stat3 (n = 3, upper). Endometriotic stromal cell were pretreated with AG490 for 1 hour and then were treated without (control) or with 2 μM GW4064 for 10 min. The protein phosphorylation levels were analyzed by Western blotting (n = 3, lower). B and C, ESCs were serum-starved overnight and pretreated with 10 μM AG490 followed by treatment with 2 μM GW4064 for 24 hours. Real-time quantitative PCR was used to quantify the *ESR2* mRNA expression levels (n = 3; *, $P < .05$, **, $P < .01$, ANOVA; left). Western blotting was also performed (n = 3; *, $P < .05$, **, $P < .01$, ANOVA; right). D, ESCs were transfected with the indicated siRNAs for 48 hours, and cells were harvested for real-time quantitative PCR or immunoblotting with anti-Stat3 antibody to verify the siRNA knockdown efficiency (n = 3; ***, $P < .001$, *t* test). E and F, ESCs were mock-transfected or transfected with siStat3, serum-starved overnight, and then treated with or without 2 μM GW4064 for 24 hours. Real-time PCR and Western blotting (n = 3; *, $P < .05$; **, $P < .01$, ***, $P < .001$; ANOVA) were conducted. G, After starvation overnight, cells were treated with or without GW4064 for 2 hours. The cells were harvested and subjected to chromatin immunoprecipitation (ChIP) with anti-Stat3 antibody; ChIP products were also measured by real-time PCR (n = 3, *, $P < .05$, *t* test). ANOVA indicates 1-way analysis of variance; ERβ, estrogen receptor β; ESCs, endometriotic stromal cell; PCR, polymerase chain reaction; siRNA, small interfering RNA.

and *ESR2* mRNA levels were 62% and 64% lower in the GW4064 group than in the control group (Figure 6B). The protein levels of ERβ exhibited the same trend as the mRNA levels of ERβ (Figure 6C). A reduced endometriotic lesion volume was observed in the GW4064 group compared to that in the PBS group (Figure 6D). Thus, these data suggest that GW4064 suppresses aromatase and ERβ expression in vivo and might be a promising therapeutic agent.

Discussion

Farnesoid X receptor is highly expressed in the enterohepatic system and is related to bile acid, lipid, glucose metabolism.[24,25] Fiorucci et al reported that FXR activation could protect against E2-induced cholestasis.[26] Estrogen dependence is well known as a feature of testicular tumors and breast cancer, while excessive estrogen production plays a significant role in sustaining tumor growth and progression. Farnesoid X receptor, which is expressed in R2C Leydig tumor cells, suppresses estrogen-dependent tumor cell proliferation by reducing aromatase expression.[12] Similarly, FXR activation has been demonstrated to negatively regulate aromatase activity and inhibit breast cancer cell proliferation.[11] The above evidence supports that FXR may be an important regulator of estrogen biosynthesis. Endometriosis is well known as an estrogen-dependent disease; however, few studies have reported the role of FXR in endometriosis. In this study, we first detected the expression of FXR in endometriotic and endometrial tissues and stromal cells and then demonstrated that treatment with the FXR agonist GW4064 repressed aromatase and ERβ expression by increasing the recruitment of FXR and Stat3 to the promoter regions of *CYP19A1* and *ESR2* in ESCs. Moreover, GW4064 administration resulted in decreased expression of aromatase and ERβ in endometriosis xenografts of mouse models.

Several studies support that GW4064 regulates gene expression in a classic small heterodimer partner (SHP)-dependent

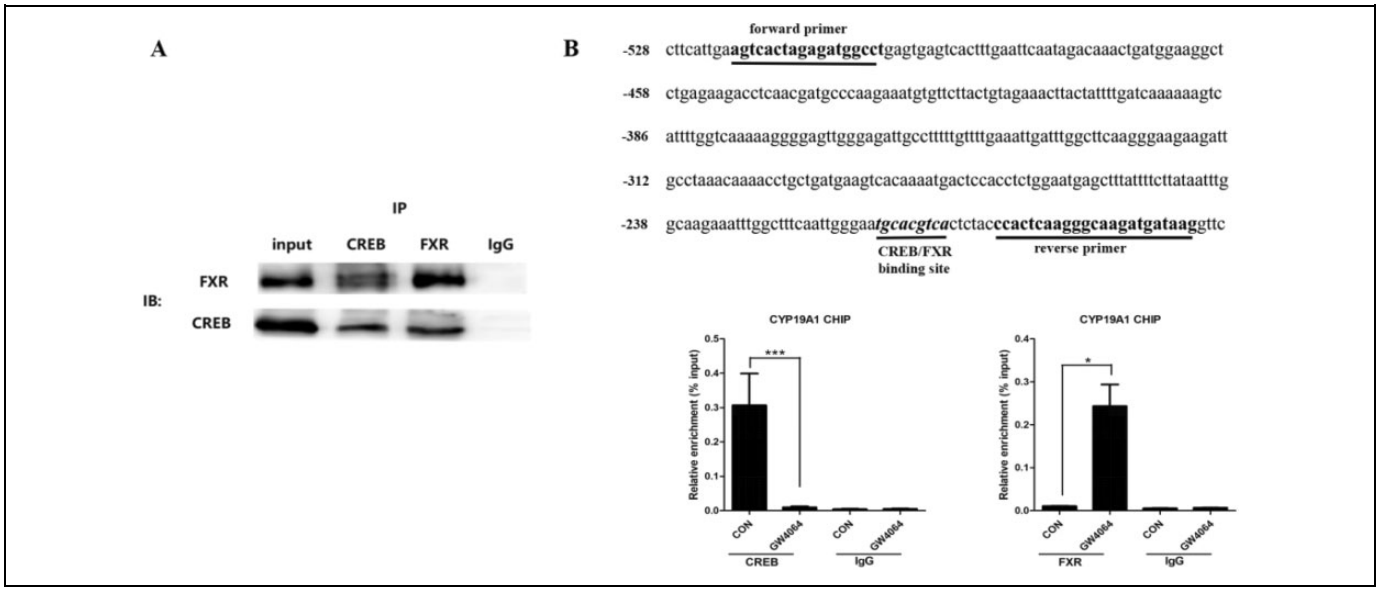

Figure 5. GW4064 interrupts the binding of the FXR-CREB complex to the *CYP19A1* promoter in ESCs. A, ESC lysates were collected, immunoprecipitated using an anti-FXR or Anti-CREB antibody, and analyzed by Western blotting with an anti-CREB or anti-FXR antibody (n = 3; IB, immunoblot; IP, immunoprecipitation). B, The putative binding site for FXR/CREB is italicized and underlined. The location and sequence of primers were indicated. After starvation overnight, cells were treated with or without GW4064 for 2 hours. The cells were harvested and subjected to chromatin immunoprecipitation (ChIP) with anti-FXR or anti-CREB antibody; CHIP products were also measured by real-time pCR (n = 3; *, $P < .05$, ***, $P < .001$, *t* test). ESCs indicates endometriotic stromal cell; FXR, Farnesoid X receptor.

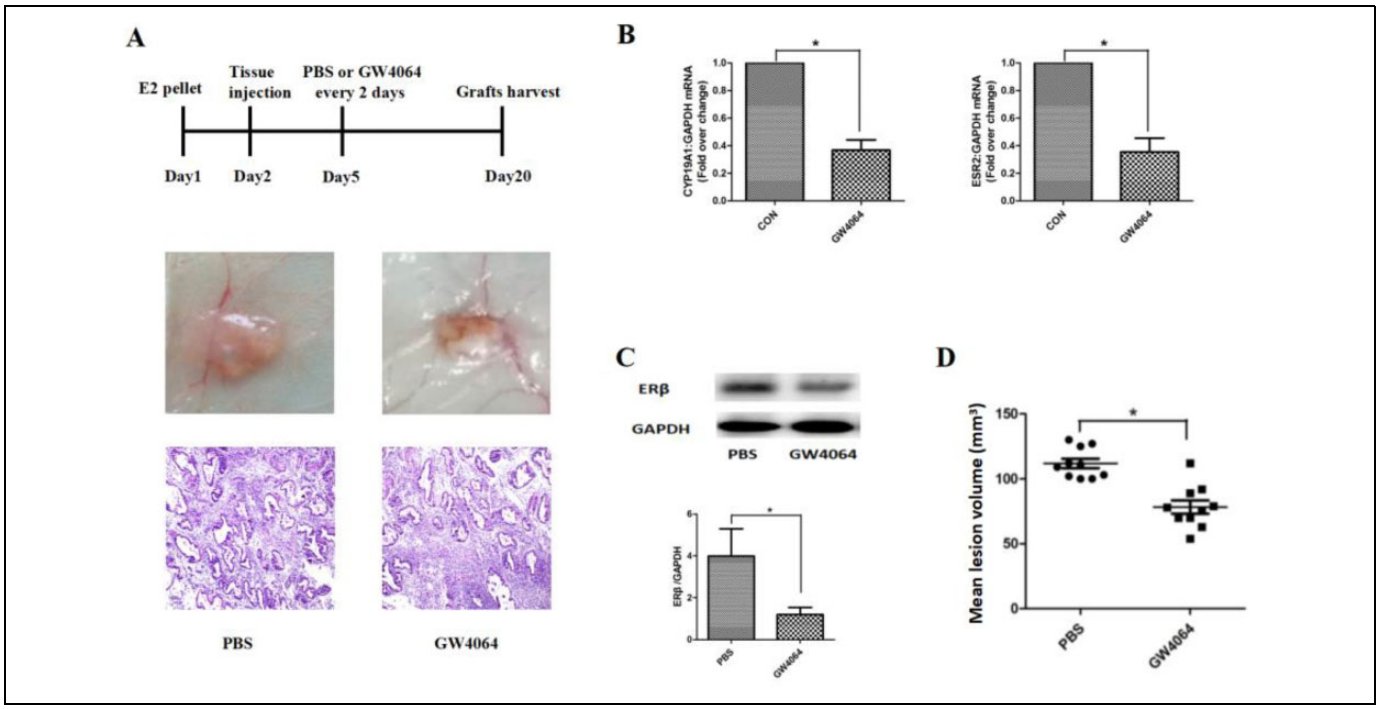

Figure 6. GW4064 reduces aromatase and ERβ expression in vivo. A, Estradiol pellets were subcutaneously implanted in nude mice. One day later, endometrium fragments were injected subcutaneously into both flanks of the mice. Three days later, GW4064 was administered (25 mg/kg) by ip injection every 2 days for 15 days. The mice were killed, and the grafts were harvested. Immunohistochemical staining was performed to confirm the histological characteristics of endometriosis. B, Total RNA was extracted from 14 lesions in the 2 groups, and SYBR green-based RT-PCR quantifications of *CYP19A1* and *ESR2* were carried out (n = 7 Per Group; *, $P < .05$; *t* test). C, Proteins were extracted from 14 lesions in the 2 groups, and Western blotting was performed using anti-ERβ antibody. ERβ expression levels were normalized to the levels of GAPDH (n = 7 per Group; *, $P < .05$, *t* test). D, Xenograft volumes were measured from 20 lesions (n = 10 per group; *, $P < .05$; *t* test). ERβ indicates estrogen receptor β; ip, intraperitoneal; PCR, polymerase chain reaction.

manner. Small heterodimer partner, a representative target gene of FXR, contributes to transcriptional repression.[27] However, in this study, we demonstrated that GW4064 downregulated the expression of aromatase and ERβ in endometriotic tissues in an SHP-independent manner both in vitro and in vivo.

Various functional motifs have been identified in the promoter region of *CYP19A1*, including CRE and SF-1 binding sites.[6,28] In Leydig tumor cell lines, FXR was demonstrated to compete with SF-1 for binding to the aromatase promoter region to repress aromatase expression, which suggested that FXR activation might combat estrogen-dependent disease by inhibiting estrogen production. Furthermore, FXR and CREB shared binding sites, mostly in the promoter regions, in a considerable number of genes, such as Tfeb and Atg3.[15,29] Farnesoid X receptor activation may disrupt the complex formed by CREB and its coactivator CRTC2, suppressing the transcriptional activity of CREB in mouse models.[15] However, here, we found that FXR could form a complex with CREB in human ESCs.

Additionally, our data showed that GW4064 attenuated ERβ expression with a maximal inhibitory effect of 83% in human ESCs. However, the underlying molecular mechanism involved in this regulatory event is not yet well characterized. In lung adenocarcinoma cells, Wang et al illustrated using a luciferase assay that the -717/-414 region of the *ESR2* promoter was a critical element modulating ERβ expression. Interleukin-6 induces Stat3 phosphorylation, which then enhances the binding of Stat3 to the *ESR2* promoter, thereby upregulating ERβ expression in lung adenocarcinoma cells.[22] In the HCC tumor xenograft model, phosphorylation of Stat3 was reduced after GW4064 administration.[21] Combining all the above data, we attempted to uncover whether Stat3 mediated the downregulation of *ESR2* in human ESCs in response to GW4064. Our results demonstrated that Stat3 inhibition by a specific Stat3 inhibitor or siStat3 rescued the suppressive effect of GW4064 on ERβ expression. Additionally, our ChIP assay identified increased occupancy of the ERβ promoter region by Stat3, which resulted in transcriptional repression. In contrast, a previous report showed that Stat3 activation and binding resulted in transcriptional activation of ERβ expression.[22] Indeed, the results of whole-transcriptome profiling revealed that the transcription factor Stat3 could serve as both a transcriptional activator and suppressor, with a comparable number of up- and downregulated genes.[5,30,31] Therefore, we proposed that this discrepancy in reports may be attributed to cell specificity.

Previous studies have shown the therapeutic effects of FXR agonists in atherothrombotic disease, breast cancer, biliary tract cancer, HCC, diabetes, cholesterol gallstones, and other diseases.[21,27,32-34] Herein, we found that GW4064 could reduce aromatase and ERβ expression both in ESCs and in the endometriosis mouse model. Our findings may thus expand the therapeutic indications of FXR agonists.

In summary, our study demonstrated that activation of FXR, which was highly expressed in endometriotic tissues, reduced aromatase and ERβ expression. Farnesoid X receptor was able to compete with CREB for binding to the promoter region of *CYP19A1* after GW4064 treatment. Meanwhile, GW4064 treatment repressed the expression of ERβ through increased Stat3 binding to the promoter region of *ESR2* via the Stat3 signaling pathway. GW4064 treatment could also suppress aromatase and ERβ expression in our endometriosis mouse model. Our results therefore suggest that FXR might be a novel molecular target for the treatment of endometriosis.

Acknowledgments

Authors thank Professors Yu Qi and Ding-Fang Bu for their generous advice regarding this study.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the National Natural Science Foundation of China [grant number 81671427].

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