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ORIGINAL ARTICLE

IGF-I stimulates ERβ and aromatase expression via IGF1R/PI3K/AKT-mediated transcriptional activation in endometriosis

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Abstract
Estrogen receptor beta (ERβ, encoded by *ESR2* gene) and cytochrome P450 aromatase (encoded by *CYP19A1* gene) play critical roles in endometriosis, and the levels of insulin-like growth factor-I (IGF-I) in the peritoneal fluid are significantly higher in patients with endometriosis compared with those in normal women. However, the effects and mechanisms of IGF-I on ERβ and aromatase expression remain to be fully elucidated. In this study, human endometriotic stromal cells (ESCs) and endometrial cells (EMs) derived from ovarian endometriomas and eutopic endometrial tissues. ESCs were cultured with IGF-I, signal pathway inhibitors, and siRNAs. ERβ and aromatase expression were measured by real-time PCR and Western, respectively. The binding of c-Jun and CREB to the *ESR2* and *CYP19A1* promoters was assessed by chromatin immunoprecipitation assay. Animal experiments were performed in a xenograft mouse model. Levels of *IGF-I* mRNA in ESCs were markedly higher than those in EMs. IGF-I upregulated ERβ and aromatase expression in ESCs after stimulation of the IGF1R/PI3K/AKT pathway. Following IGF-I treatment, a marked increase in c-Jun and CREB phosphorylation occurred, enhancing binding to the *ESR2* and *CYP19A1* promoters. An IGF1R inhibitor in vivo reduced IGF-I-induced endometriosis graft growth and ERβ and aromatase expression. In conclusion, this is the first report to describe a mechanistic analysis of ERβ and aromatase expression regulated by IGF-I in ESCs. Moreover, an IGF1R inhibitor impeded ectopic lesion growth in nude mice. These findings suggest that an inhibitor of IGF1R might have therapeutic potential as an antiendometriotic drug.

Key messages
- Level of *IGF-I* mRNA in ESCs is markedly higher than that in EMs.
- IGF-I up-regulates ERβ and aromatase expression via IGF1R/PI3K/AKT pathway.
- C-Jun and CREB are recruited to *ESR2* or *CYP19A1* promoter by IGF-I stimulation.
- IGF-1R inhibitors in vivo impede the growth of ectopic lesions in nude mice.

Keywords IGF-I · IGF1R inhibitor · ERβ · Aromatase · IGF1R/PI3K/AKT pathway · Endometriosis

Introduction

Endometriosis is an estrogen-dependent disease that affects one in ten women of reproductive age [1, 2]. Only ~50 % of all women with a previous diagnosis of endometriosis achieve pain relief upon receipt of hormone therapy or conservative surgery [1]. Therefore, there is a clear need to understand the underlying mechanisms to develop novel and effective therapies for the treatment of endometriosis.

Local estrogen production in endometriotic lesions is essential for the growth and persistence of endometriosis [3–5]. The actions of estrogen are primarily mediated via its nuclear estrogen receptor (ER) [6], which are encoded by separate genes (*ESR1* and *ESR2*). Although ERα and ERβ are expressed in the endometrium, ERα appears to be the primary mediator of estrogenic actions in this tissue [7]. However,

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endometriosis seems to be associated with a unique complement of ER subtypes compared with those of the eutopic endometrium. It has been reported that markedly higher levels of ERβ and lower levels of ERα are found in human endometriotic tissues and primary stromal cells compared with those in eutopic endometrial tissues and cells [8]. ERβ is thought to play a key role in endometriosis growth regulation, and ERβ-selective estradiol antagonists might represent novel therapeutics for endometriosis in the future [9]. Additionally, a large body of evidence demonstrates that high levels of steroidogenic acute regulatory protein (StAR) and aromatase (encoded by *CYP19A1*) are expressed in endometriotic stromal cells (ESCs), which give rise to local estrogen production [5, 10]. In de novo estrogen biosynthesis [11], StAR facilitates the entry of cholesterol into the mitochondrion, while aromatase catalyzes the final step of estrogen production by converting C19 steroids to estrogens. These are both key enzymes for estrogen biosynthesis [12]. Additionally, ERβ expression is partially regulated by a promoter region that contains multiple consensus sites for several transcription factors, such as the activator protein 1 (AP-1) family members c-Fos and c-Jun [13]. Transcriptional regulation of *CYP19A1* can be mediated by several transcription factors, which are located in promoter II (P II), including the cAMP response element (CRE)-binding protein (CREB)/CRE modulator, c-Fos, and c-Jun [3, 14, 15].

Insulin-like growth factor (IGF) is known to be a factor that prevents apoptosis and acts as a mitogen on endometrial stromal cells cultured in vitro [16, 17]. A recent study demonstrates the importance of the IGF system in younger endometriosis patients from an epidemiological perspective [18]. Indeed, it has been previously reported that levels of IGF-I in the peritoneal fluid are significantly higher in patients with endometriosis compared with those in normal women [19]. Binding of IGF-I to the type 1 IGF receptor (IGF1R) and the subsequent intracellular modifications that occur results in the activation of PI3K/AKT and MEK/ERK, two signaling pathways that are involved in the regulation of various cellular and nuclear proteins, including transcription factors such as c-Jun and CREB [20–22]. Additionally, the pro-proliferative and anti-apoptotic PI3K/AKT and MEK/ERK signaling pathways have been shown to be hyperactive in endometriosis [23, 24]. Crosstalk exists between the estrogen and IGF-1 molecular axes in mammary gland tumorigenesis and lung cancer [25, 26]. In human granulosa cells, IGF-I can induce the expression of aromatase and other estrogen biosynthetic enzymes [25, 27]. Currently, however, there is little article about the effect of IGF-I on ERβ expression, and the role of IGF-I in aromatase expression during endometriosis remains poorly understood.

Based on these previous findings, we investigated whether the local overproduction of IGF-I could be one of the mechanisms that determines ERβ and aromatase overexpression in ESCs through the activation of specific transcription factors, c-Jun and CREB, via the MEK/ERK and PI3K/AKT signaling pathways. Additionally, we demonstrated that treatment of endometriosis with an IGF1R inhibitor abrogated IGF-I-induced growth of endometriosis xenografts.

Materials and methods

Subjects and primary cell culture

Ectopic endometrial tissues from the cyst walls of ovarian endometriomas and eutopic endometrial tissues were obtained from 12 subjects (age range, 23–40 years) immediately after they underwent surgery, composing 12 self-control pairs. All the patients had regular menstrual cycles, and none received any preoperative hormonal therapy. The experimental procedures were approved by the institutional review board of the First Hospital of Peking University (No. 2014[789] and No. 2014[790]), and signed informed consents for use of the samples were obtained from each patient. Human ESC and endometrial cells (EMs) were isolated from tissue samples using the protocol previously described by Ryan et al. [28] with minor modifications [11].

Hormone treatments

The cells were incubated in serum-free medium containing IGF-I (PeproTech, Rocky Hill, NJ) with different concentrations (0, 1, 10, 100, and 1000 ng/mL) or different durations (0, 6, 24, and 48 h). When IGF1R inhibitor AG1024 (AG) (Millipore, Boston, MA), PI3K inhibitor LY294002 (LY) (Millipore), and MEK inhibitor PD98059 (PD) (Sigma) were applied respectively, the cells were preincubated for 1 h with 1 μM AG, 10 μM LY, or 25 μM PD.

RNA extraction and quantitative analysis by real-time semi-quantitative PCR

RNA extraction and real-time semi-quantitative PCR were performed as previously described [5]. Relative quantification for all transcripts was analyzed by the comparative threshold cycle method described previously [29]. The primers were listed in Table 1.

Small interfering RNA knockdown

The method has been descried previously [5]. Thirty-six hours after small interfering RNA (siRNA) transfection, ESCs were serum-starved for 12 h, treated with 100 ng/mL IGF-I for 48 h. After this, the cells were processed for real-time PCR and Western blot analysis or aromatase activity assay.

Table 1 Primers

Primer list	
Human:	
GAPDH-forward primer	GAAGGTGAAGGTCGGAGTC
GAPDH-reverse primer	GAAGATGGTGATGGGATTTC
IGF-I-forward primer	GCTCTTCAGTTCGTGTGTGG
IGF-I-reverse primer	TGACTTGGCAGGCTTGAGG
IGF1R-forward primer	ACCCGGAGTACTTCAGCGC
IGF1R-reverse primer	CACAGAAGCTTCGTTGAGAA
ESR2-forward primer	ATGATCAGCTGGGCCAAGAA
ESR2-reverse primer	CCACATCAGCCCCATCATTAA
CYP19A1-forward primer	CACATCCTCAATACCAGGTCC
CYP19A1-reverse primer	CAGAGATCCAGACTCGCATG
CHIP-*ESR2*-forward primer	ACTGGCTCCTTAGAATCAGACAT
CHIP-*ESR2*- reverse primer	ATTTAAGAGGTCTGGAGTAGGGC
CHIP-*CYP19A1*-forward primer	ATTGAAGTCACTAGAGATGGCCT
CHIP-*CYP19A1*- reverse primer	CTTATCATCTTGCCCTTGAGTGG
Mice:	
GAPDH-forward primer	ACCACAGTCCATGCCATCAC
GAPDH-reverse primer	TCCACCACCCTGTTGCTGTA
ESR2-forward primer	GTGTGTGAAGGCCATGATTC
ESR2-reverse primer	CCATGCCCTTGTTACTGATG
CYP19A1-forward primer	CCTGACGAAAGAGAACGTGA
CYP19A1-reverse primer	CCCACAACAGTGTGGATCTC

Western blot analysis

Western blot was performed as previously described [5]. A densitometric analysis (with imageJ software) of the related experiments was used to quantify the results.

Aromatase activity assay

The aromatase activity of cultured ESCs was measured by a [^{3}H]H_2O release assay as previously described [30]. After 42 h of the hormone therapy, a mixture of labeled and cold androstenedione was added to each well, and the cells were incubated for another 6 h.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was conducted as previously described [5]. The purified DNA was then analyzed by semi-quantitative PCR and real-time PCR using primers of *ESR2* promoter.

Tissue culture for grafting

Human endometrial biopsies were obtained in ice-cold sterile PBS on day 1 and dissected into 1-mm^3 fragments. Tissue fragments were cultured in DMEM/F12 (1:1) (HyClone) with 2 % stripped FBS (GIBCO/BRL), 10 nM E2 (Sigma), and 100 U/mL penicillin (Lonza) with 100 U/mL streptomycin (Lonza) at 37 °C in a humidified atmosphere with 5 % CO_2 for 24 h before injection into mice.

Xenograft mouse model

A xenograft mouse model was adapted from the model previously described by Bruner–Tran et al. [31–33]. The First Hospital of Peking University Animal Care Committee approved the use of mice for this study (No. J201403). The 6-week-old nude mice (nu/nu, $n = 30$) were obtained from the Animal Research Laboratory of Peking University First Hospital and surgically ovariectomized. On day 1, E2 pellets (1.7 mg/60 days sustained release) were implanted sc in all mice. On day 2, ten fragments of fresh human endometrium in 200-uL sterile PBS human endometrial tissue fragments were injected sc into nude ovariectomized mice. Each mouse received two injections of tissue, one in each flank. After 3 days, mice were randomly divided into three groups: blank control, IGF-I, and IGF-I + AG groups. Subsequently, IGF-I (0.025 μg/g) (PeproTech) by graft local injection and AG (0.2 nM/g) (Millipore) by oral gavage were performed every 3 days for 15 days. Then, the mice were sacrificed, and tissue volume was calculated with the following formula: volume = 0.5 * length * width^2. The tissues were used for RNA and protein extraction to determine the aromatase or ERβ levels. The primers were listed in Table 1.

Immunohistochemistry

Serial paired endometriotic and endometrial samples from ten subjects were fixed in 4 % paraformaldehyde for 24 h and embedded in paraffin. Embedded tissues were cut into 4-μM sections and then mounted on glass slides. Immunohistochemical staining for IGF-I (1:200 dilution; Abcam, Cambridge, MA) and IGF1R (1:200 dilution; Cell Signaling) was processed. Normal rabbit IgG was used instead of the primary antibodies for negative controls.

Statistical analyses

All experiments were carried out at least three times. Statistical analyses were performed by two-tailed Student's *t* test for comparison of the two treatment groups. One-way ANOVA followed by Tukey's multiple-comparison test was used for comparison of more than two groups. A p value of <0.05 was considered statistically significant. All the values are given as the mean, with the bars showing the standard error of mean (SEM).

Results

IGF-I and IGF1R expression in endometriosis

Real-time PCR was used to quantify mRNA expression levels of *IGF-I* and *IGF1R* in cultured ESCs and EMs from paired endometriotic and endometrial samples. Levels of *IGF-I* in primary ESCs were markedly higher (55-fold) than those in primary EMs, whereas the expression levels of *IGF1R* mRNA were not different between these two cell types (Fig. 1a). Immunohistochemical analysis revealed that in human ovarian endometriomas and eutopic endometrial tissues, IGF-1 and IGF-1R were expressed both in the stromal cell and in the glandular epithelium (Fig. 1b).

Stimulation of ERβ and aromatase expression by IGF-I in ESCs

ESCs treated in the presence or absence of IGF-1 for different durations displayed elevated levels of *ESR2* and *CYP19A1* mRNA in a time-dependent manner. Levels of IGF-I-induced basal *ESR2* and *CYP19A1* mRNA expression peaked (3.01- and 5.03-fold, respectively) at 48 h (Fig. 1c). IGF-1 stimulated *ESR2* and *CYP19A1* mRNA expression, with a maximal effect (3.36- and 4.58-fold, respectively) observed

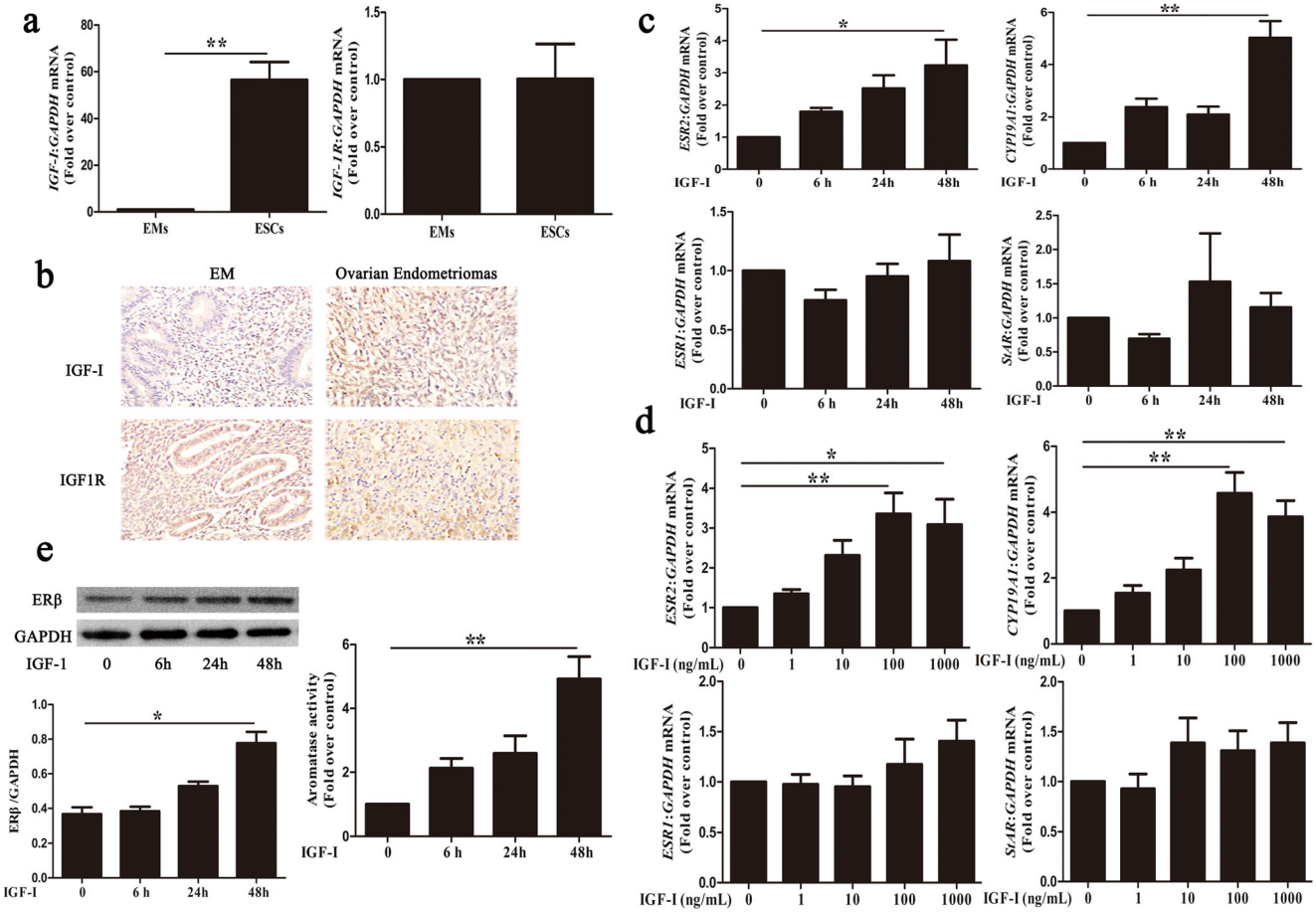

Fig. 1 Differences in IGF-I expression between ESCs and EMs and the effects of IGF-I on ERβ and aromatase expression. **a** Total RNA was extracted from EMs and ESCs. *IGF-I* ($n = 10$; $^{**}P < 0.01$, *t* test; upper) and *IGF1R* ($n = 10$; $P > 0.05$, *t* test; lower) mRNA levels were measured by real-time PCR. **b** Immunohistochemical analysis showed that IGF-1 and IGF-1R were similarly expressed in both human ovarian endometriomas and eutopic endometrial tissues ($n = 10$). Original magnification was × 400. **c** After starvation overnight, ESCs were treated in the presence or absence of IGF-I (100 ng/mL) for 6, 24, or 48 h. *ESR2* and *CYP19A1* mRNA levels were elevated by IGF-I ($n = 4$; $^{*}P < 0.05$; $^{**}P < 0.01$, ANOVA; upper), but IGF-I showed no significant effect on *ESR1* and *StAR* mRNA expression levels ($n = 4$; $P > 0.05$, ANOVA; lower). **d** ESCs were cultured with or without IGF-I (1, 10, 100, and 1000 ng/mL) for 48 h. IGF-1 stimulated *ESR2* and *CYP19A1* mRNA expression, with a maximal effect (3.36- and 4.58-fold, respectively) observed at 100 ng/mL ($n = 4$; $^{*}P < 0.05$; $^{**}P < 0.01$, ANOVA; upper), but IGF-I showed no significant effect on *ESR1* and *StAR* mRNA expression levels ($n = 4$; $P > 0.05$, ANOVA; lower). **e** Western blotting and aromatase activity assays were performed. A densitometric analysis (with imageJ software) was used to quantify ERβ protein expression. The basal aromatase activity in the control group was normalized to 1. IGF-I induced basal ERβ protein expression (left) and aromatase activity (right), with a maximal effect observed at 48 h ($n = 4$; $^{*}P < 0.05$; $^{**}P < 0.01$, ANOVA). Values are the mean ± SEM

at 100 ng/mL (Fig. 1d). However, IGF-I had no such effect on levels of *ESR1* or *StAR* mRNA expression (Fig. 1c, d). As shown in Fig. 1e, similar data were obtained for the ERβ protein expression (2.12-fold) and aromatase activity assay (4.92-fold) (Fig. 1e).

Activation of MEK/ERK and PI3K/AKT signaling by IGF-I treatment in ESCs

To identify the signaling events involved in the up-regulation of aromatase and ERβ expression by IGF-I, we investigated the phosphorylation of molecules, including IGF1R, AKT, and ERK1/2, in downstream signaling pathways. Phosphorylation of IGF1R, ERK1/2, and AKT proteins was rapidly induced by IGF-I stimulation (Fig. 2a), and peaked at 5, 5, and 10 min, respectively. Activation of p-IGF1R, p-ERK1/2, and p-AKT by IGF-I was analyzed using an IGF1R inhibitor AG, MEK inhibitor PD, and PI3K inhibitor LY, each of which severely affected the corresponding levels of protein phosphorylation (Fig. 2b). However, these inhibitors did not affect the expression levels of IGF1R, ERK1/2, or AKT protein. Therefore, changes in the levels of p-IGF1R, p-ERK1/2, and p-AKT induced by IGF-I reflected increases in protein phosphorylation rather than protein synthesis, suggesting that IGF-I could activate the MEK/ERK and PI3K/AKT signaling pathways in endometriosis.

IGF-I stimulated ERβ expression and aromatase activity via the PI3K/AKT rather than the MEK/ERK signaling pathway in ESCs

To determine whether the activation of IGF1R, ERK1/2, and AKT by IGF-I was required for IGF-I-stimulated ERβ expression and aromatase activity in ESCs, we used specific inhibitors, including PD, LY, and AG. We measured *ESR2* and *CYP19A1* mRNA expression levels in the presence or absence of inhibitors following IGF-I treatment for 48 h by qPCR. We then established that IGF-I increased *ESR2* mRNA expression levels (3.66-fold). By contrast, the addition of LY or AG, but not PD, markedly reduced this induction by 58 and 85 %, respectively. Additionally, LY, but not PD, could inhibit IGF-I-induced *CYP19A1* mRNA expression (4.47-fold) by 56 %, while AG caused 89 % inhibition (Fig. 3a). Similar

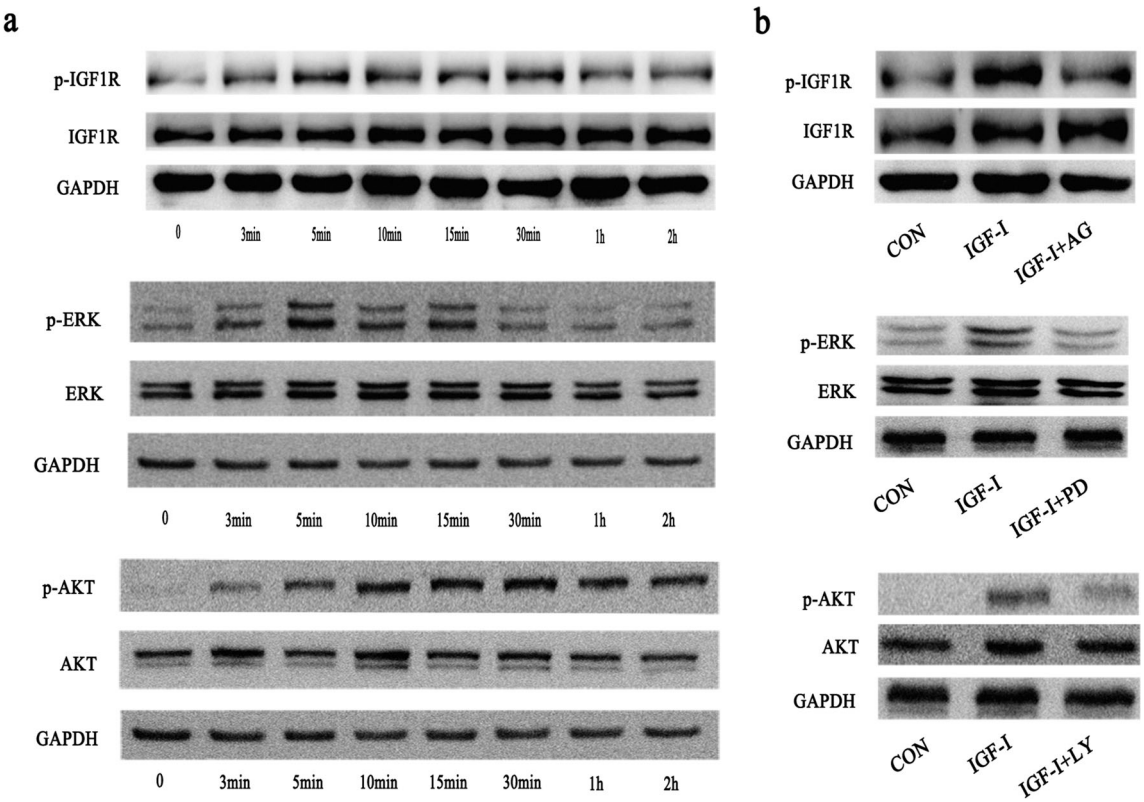

Fig. 2 The specific involvement of the IGF1R, ERK1/2, and AKT signaling pathways in IGF-I responsiveness in ESCs. **a** ESCs were serum-starved overnight and treated with IGF-I (100 ng/mL) for the indicated amounts of time. Whole-cell lysates were prepared and subjected to SDS–PAGE and analyzed by Western blotting with the indicated antibodies. The phosphorylation of IGF1R, ERK1/2, and AKT proteins was rapidly induced and peaked at 5, 5, and 10 min, respectively ($n = 4$). **b** ESCs were pretreated with an IGF1R inhibitor AG1024 (AG, 1 μM), a MEK inhibitor PD98059 (PD, 25 μM), or a PI3K inhibitor LY294002 (LY, 10 μM) for 1 h, and then were stimulated for 5, 5, and 10 min, respectively, without (control) or with 100 ng/ml IGF-I. Cellular protein levels were analyzed by Western blotting. Each of the above inhibitors severely affected the corresponding amounts of protein phosphorylation ($n = 4$)

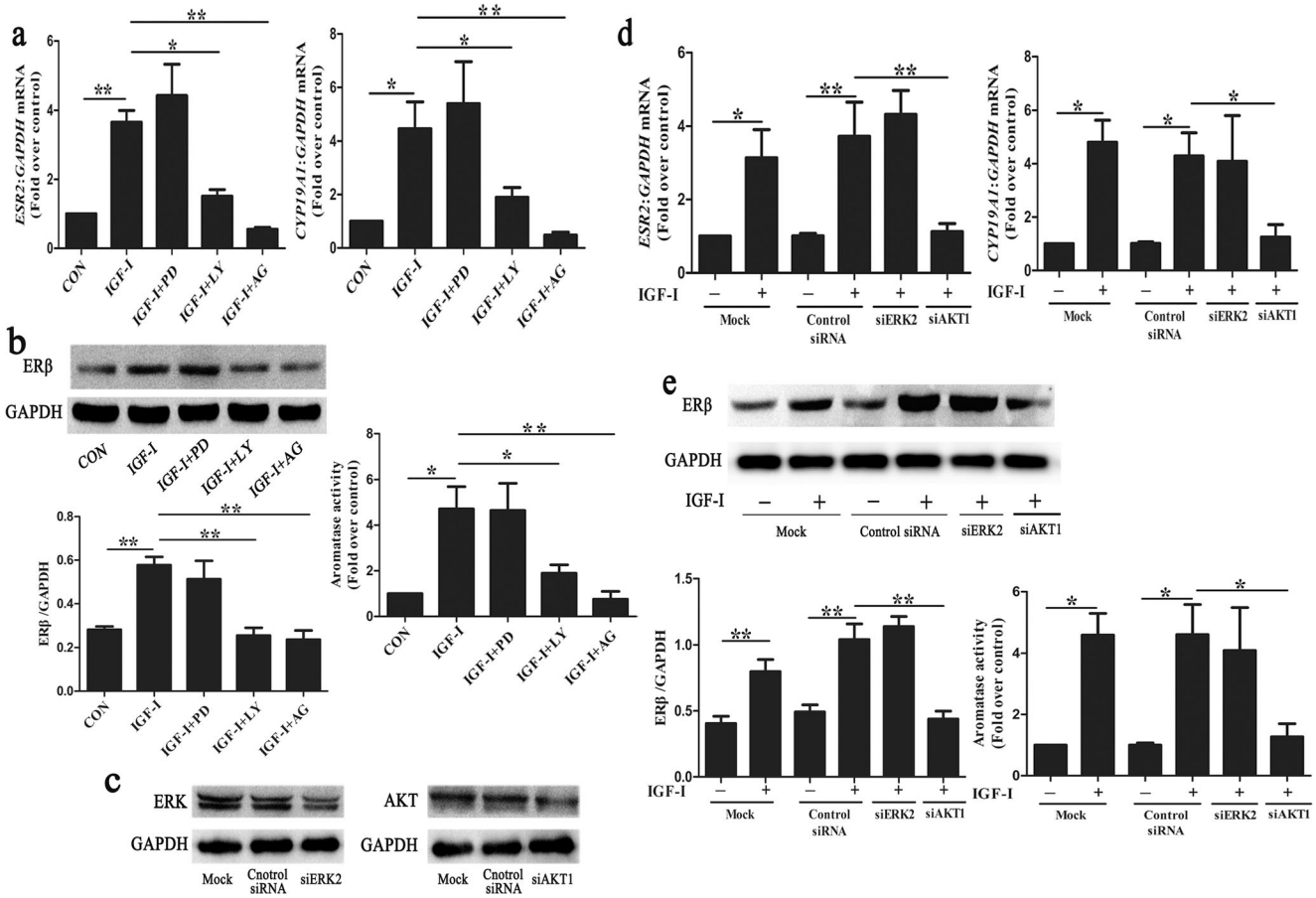

Fig. 3 IGF-I stimulates ERβ and aromatase expression via the PI3K–AKT rather than the MEK–ERK signaling pathway in ESCs. **a** Following overnight serum-starvation, ESCs were pretreated with 25 μM PD, 10 μM LY, or 1 μM AG for 1 h, then were subsequently treated with 100 ng/ml IGF-I for 48 h. Real-time quantitative PCR was used to quantify the mRNA expression levels of *ESR2* and *CYP19A1* ($n = 4$; $^*P < 0.05$; $^{**}P < 0.01$, ANOVA). **b** Western blotting and aromatase activity assays were performed ($n = 4$; $^*P < 0.05$; $^{**}P < 0.01$, ANOVA). **c** ESCs were mock-transfected or transfected with the indicated siRNAs for 48 h; then, cells were harvested for immunoblotting with anti-ERK1/2 or anti-AKT antibody for verification of the siRNA knockdown efficiency ($n = 4$). **d** ESCs were mock-transfected or transfected with the indicated siRNAs, serum-starved overnight, and then treated with or without 100 ng/ml IGF-I for 48 h. Cells were harvested for real-time PCR ($n = 4$; $^*P < 0.05$; $^{**}P < 0.01$, ANOVA). **e** Western blotting and aromatase activity assays were performed ($n = 4$; $^*P < 0.05$; $^{**}P < 0.01$, ANOVA). Values are the mean ± SEM

inhibitory patterns were also observed for ERβ protein content and aromatase activity (Fig. 3b).

To provide additional evidence for the roles of ERK1/2 and AKT in the regulation of ERβ expression and aromatase activity by IGF-I, we used siRNAs to knockdown *ERK2* and *AKT1*. The efficacy of siRNA knockdown was determined by Western blotting (Fig. 3c). In mock or control siRNA-transfected ESCs, IGF-I treatment resulted in a marked increase in *ESR2* and *CYP19A* mRNA expression levels. Transfection with siAKT1 suppressed this induction by 68 and 73 %, respectively. However, the function of siERK2 was very weak and not significantly different (Fig. 3d). Moreover, transfection of ESCs with siRNAs had equivalent effects on ERβ protein levels and aromatase activity (Fig. 3e). Together, these findings indicated that activation of PI3K/AKT, but not MEK/ERK, was necessary for IGF-I-mediated induction of ERβ expression and aromatase activity.

C-Jun and CREB phosphorylation and relevance to ESR2 and CYP19A1 gene transcription induced by IGF-I

Based on previous findings that showed the ability of IGF-I to activate c-Jun and CREB in rat tumor Leydig cells [21, 22], we investigated the role of these two factors in the regulation of ERβ and aromatase in ESCs. Cells treated with 100 ng/ml IGF-I showed in an increase in the phosphorylation of c-Jun (p-c-Jun) and CREB (p-CREB) in a time-dependent manner, peaking at 30 min. No changes were observed in the levels of c-Jun or CREB protein during this 120-min period (Fig. 4a). To establish the influence of these factors in the IGF-I signal transduction pathway in modulating p-c-Jun and p-CREB, we used specific inhibitors of IGF-IR (AG) and PI3K (LY). Both inhibitors severely inhibited levels of phosphorylated c-Jun and CREB (Fig. 4b). To obtain additional insights into these mechanisms, we performed a ChIP assay to investigate how

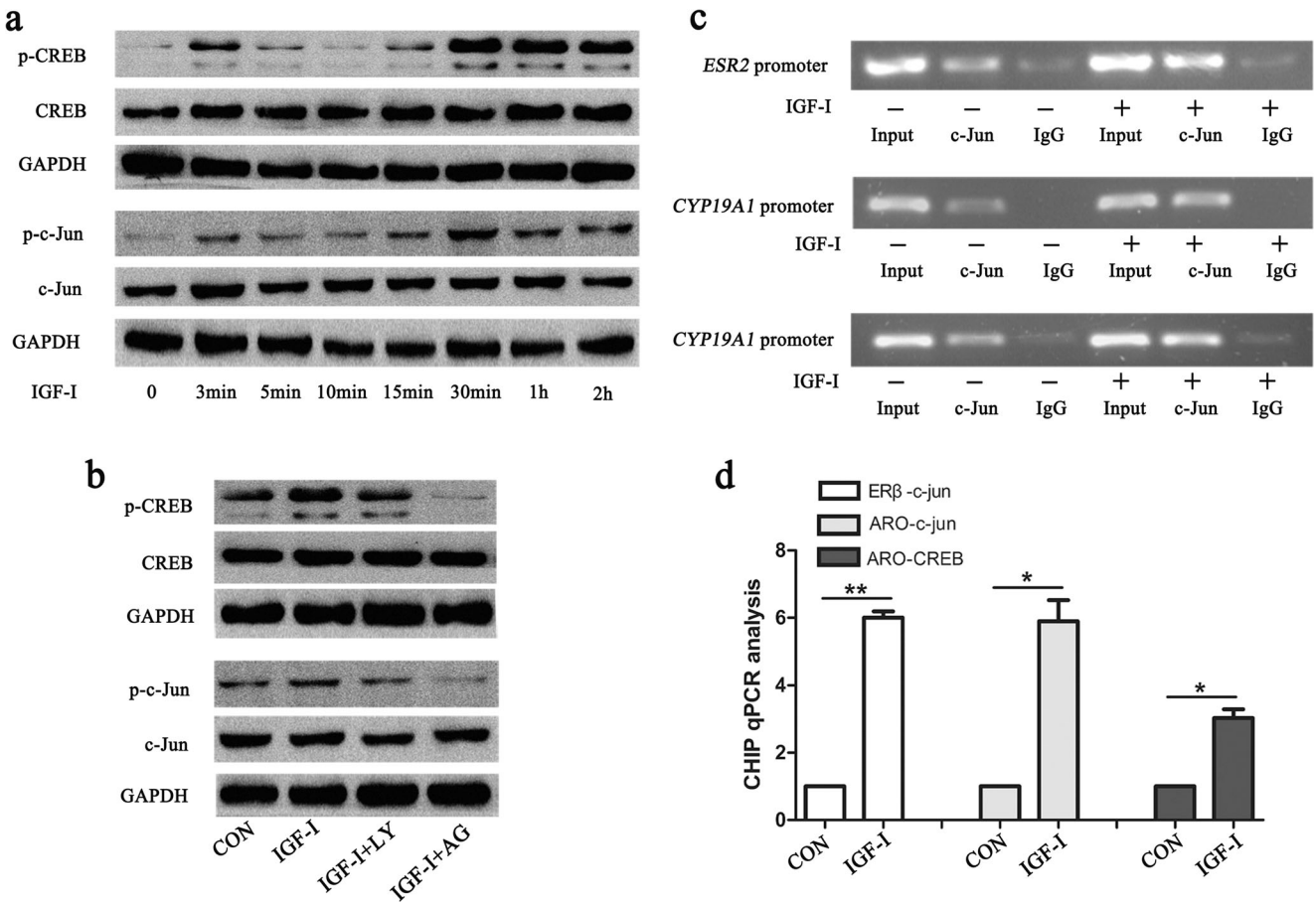

Fig. 4 Levels of c-Jun and CREB phosphorylation and their link to *ESR2* and *CYP19A1* transcription induced by IGF-I. **a** ESCs were treated with IGF-I (100 ng/mL) after starvation for the indicated amounts of time. Cell lysates were prepared and analyzed by Western blot with anti-c-Jun or anti-CREB antibody. Protein phosphorylation of c-Jun and CREB both rapidly peaked at 30 min ($n=4$). **b** ESCs were preincubated with LY (10 μM) or AG (1 μM) for 1 h, and then were stimulated for 30 min with or without 100 ng/ml IGF-I. Cellular proteins were analyzed by Western blotting with anti-c-Jun or anti-CREB antibody. Both of these inhibitors attenuated c-Jun and CREB protein phosphorylation ($n=4$). **c** After starvation overnight, cells were treated with or without IGF-I for 1 h. The treated and untreated cells were harvested and subjected to chromatin immunoprecipitation (ChIP) with anti-c-Jun, anti-CREB antibody, or control IgG followed by semi-quantitative PCR ($n=3$). **d** ChIP products were also measured by real-time PCR ($n=3$; $^{*}P<0.05$; $^{**}P<0.01$, ANOVA). Values are the mean ± SEM

IGF-I influenced the binding of transcription factors to the promoters of the *ESR2* and *CYP19A1* genes. By mapping the human *ESR2* promoter region, it has been found to contain multiple recognition motifs for sequence-specific transcription factors, including c-Jun but not CREB [13, 26]. Therefore, we treated ESCs with IGF-I for 1 h and then used ChIP with anti-c-Jun antibodies followed by *ESR2* promoter PCR (Fig. 4c). We observed a marked increase in c-Jun (6.01-fold) binding after IGF-I treatment (Fig. 4d). Similarly, we conducted the ChIP experiment with anti-c-Jun and anti-CREB antibodies to confirm the enhanced recruitment of c-Jun and CREB to *CYP19A1* PII by 5.90- and 3.03-fold, respectively (Fig. 4c, d).

An IGF1R inhibitor reduced IGF-I-induced endometriosis graft growth in vivo

To confirm the effect of the IGF1R inhibitor AG on human endometriotic tissues in vivo, tissue fragments from human endometrium fragments were injected subcutaneously into nude ovariectomized mice, as described by Bruner–Tran et al. [31–33]. E2 was included in all the treatment arms to establish endometriotic lesions. The 30 mice were randomly assigned to one of three treatment arms: control, IGF-I, or IGF-1 + AG (Fig. 5a). Then, two weeks later, mice were sacrificed, grafts were harvested, and standard pathological analyses were carried out to confirm the distinctive histological characteristics of endometriosis (Fig. 5b). Compared with the control group, the endometriotic lesions volume increased in the IGF-I treatment group (Fig. 5c; 15.26 vs. 22.59 mm^3). A trend towards a reduced endometriotic lesion volume was noted in the IGF-1 + AG group compared with that in the control group (Fig. 5c; 22.59 vs. 14.73 mm^3). Real-time qPCR analysis of the tissue grafts showed that the *ESR2* and *CYP19A1* mRNA expression levels were significantly higher in the IGF-1 group than those in the control group, 7.48- and 9.56-fold, respectively (Fig. 5d). By contrast, treatment with the IGF1R

inhibitor AG reduced the levels of *ESR2* and *CYP19A1* mRNA by 70 and 78 %, respectively (Fig. 5d). Similar inhibitory patterns could also be observed for ERβ protein (Fig. 5e).

Discussion

This study, for the first time, attempted to demonstrate of the effects of IGF-I on ERβ expression both in vitro and in vivo, and also the underlying mechanisms for these functions. We demonstrated that IGF-I stimulated ERβ and aromatase expression in human ESCs by enhancing binding of the transcription factor c-Jun and CREB to the *ESR2* and *CYP19A1* promoter regions. This involved stimulation of the IGF1R/PI3K/AKT signaling pathways. Furthermore, inhibition of IGF1R could impede IGF-I-induced growth in endometriosis. This is especially relevant for our understanding of the mechanism of action for IGF-I in women with endometriosis.

IGF-1 can be synthesized by various cell types, including endometrial cells, and is involved in cell proliferation, differentiation, and apoptosis [16, 17, 34]. The reciprocal crosstalk that exists between estrogen and the IGF-I molecular axis can regulate the progression of estrogen-dependent diseases [35]. Indeed, it has been previously reported that endometriotic cysts contain significantly lower levels of IGF-I expression, both at the mRNA and protein levels [36, 37]. In our study, IGF-I and IGF-1R were expressed both in paired eutopic endometrium and ovarian endometrioma tissues using an immunohistochemical approach. It may be due that immunohistochemical results are mainly used for localization and semiquantitation with some inaccuracy and highly affected

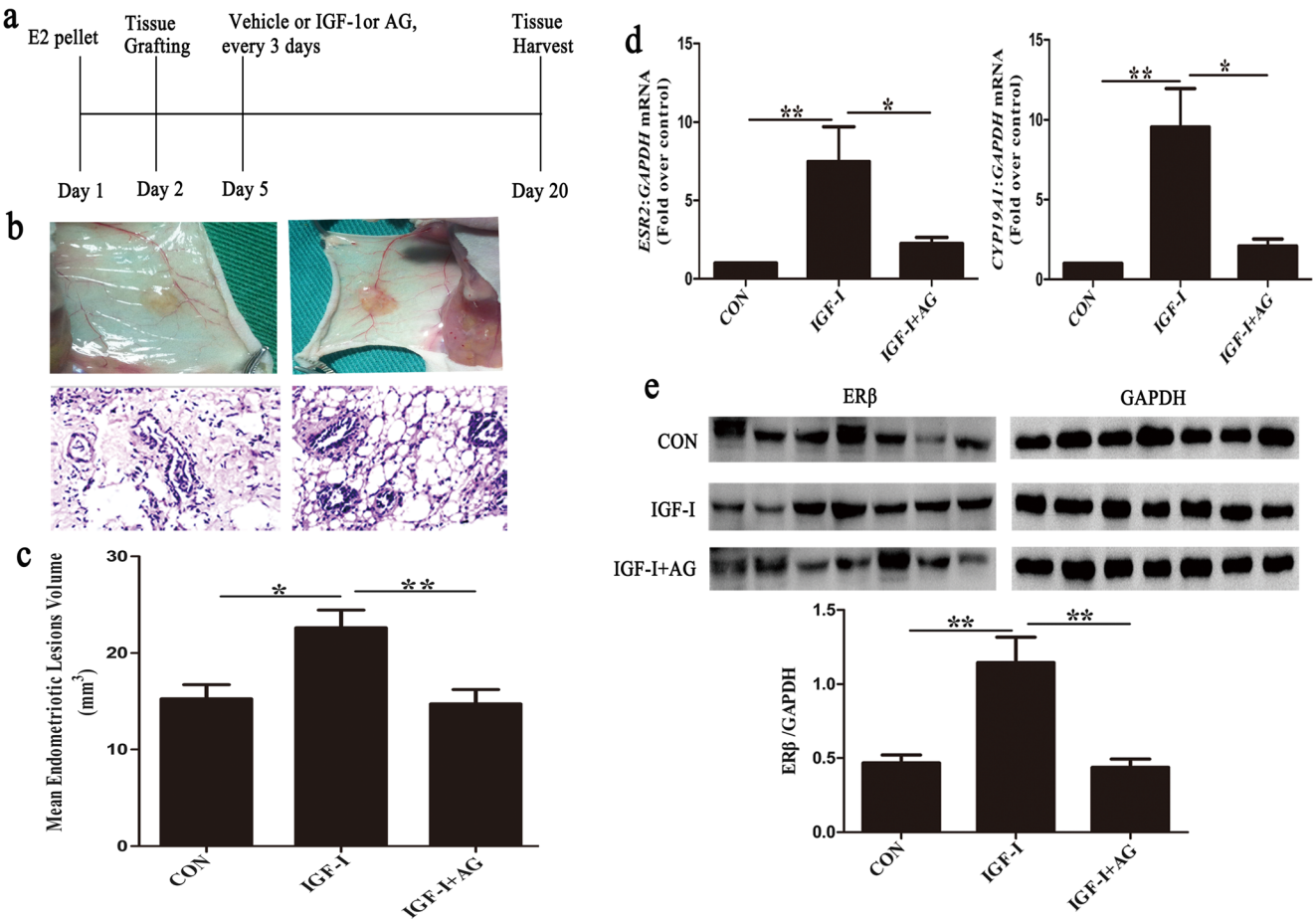

Fig. 5 The IGF1R inhibitor AG reduces IGF-I-induced ERβ and aromatase expression and the growth of endometriosis grafts in vivo. **a** Estradiol hormone pellets were implanted subcutaneously in nude mice. The next day, endometrium tissue fragments were injected subcutaneously into both flanks of mice. Three days later, administration of IGF-I (0.025 μg/g) by local graft injection and AG (0.2 nM/g) by oral gavage was performed every 3 days for 15 days. **b** Mice were sacrificed and grafts were obtained. H&E staining was carried out to confirm the distinctive histological characteristics of endometriosis ($n = 10$; original magnification, ×400). **c** Graft volumes were measured on the last day, and data were expressed as means ± SEM from 60 lesions in 30 mice ($n = 20$ per group; $^{*}P < 0.05$; $^{**}P < 0.01$, ANOVA). **d** Total RNA was extracted from 30 lesions in the three groups, and SYBR® Green-based RT-PCR quantification of ESR2 and CYP19A1 mRNA levels was performed ($n = 10$ per group; $^{*}P < 0.05$; $^{**}P < 0.01$, ANOVA). **e** Cellular proteins were extracted from 21 lesions in the three groups, which were analyzed by Western blotting with anti-ERβ antibody ($n = 7$ per group; upper). Levels of ERβ expression normalized to the amount of GAPDH were standardized based on the vehicle control ($n = 7$ per group; $^{**}P < 0.01$, ANOVA; lower). Values are the mean ± SEM

by samples. Additionally, for the first time, we established that mRNA levels of *IGF-I*, but not *IGF1R*, in primary ESCs were markedly higher than those in primary EMs. The possible reason for this discrepancy with previous findings is that we used paired primary stromal cells, while *IGF-I* mRNA transcripts are expressed in the stromal cells rather than the glandular cells of the eutopic and ectopic endometrium [37]. It has been previously reported that IGF-I levels in the peritoneal fluid were higher in patients with endometriosis than those in control patients [19]. Much less data is available about IGF-1-mediated regulation of ERβ. No effect of IGF-1 on ERβ expression in the MDA-MB-231 (ERβ+) breast cancer cell line has been reported [38], although knockdown of IGF-1R in the MCF-7 breast cancer cell line using siRNA was found to increase ERβ expression and subsequently alter the ERβ–to-ERα ratio [39]. In human ESCs, our study was the first to show that IGF-I could significantly elevate ERβ, but not ERα, expression levels, both at the mRNA and protein levels. This stimulation was time-dependent and peaked at 48 h. Previous studies showed that high ERβ levels in endometriosis might be responsible for low ERα expression [8, 9], which could have influenced our results described above. Moreover, our data showed that increased *CYP19A1* mRNA expression levels in human ESCs could be induced by IGF-I, which is similar to other studies that used different cells [25, 27, 40]. More importantly, changes in *CYP19A1* mRNA expression levels could translate into significantly increased aromatase activity.

In our present study, binding of IGF-I to its receptor (IGF1R) caused receptor autophosphorylation and activation of an intrinsic tyrosine kinase that acts on various substrates, leading to the activation of two signaling pathways, the PI3K/AKT and MEK/ERK cascades, in ESCs. Activation of AKT or ERK1/2 phosphorylation in response to IGF-I was reduced by a PI3K or MEK inhibitor without affecting AKT or ERK1/2 protein synthesis. The MEK/ERK signaling cascade has been shown to regulate *CYP19A1* gene expression and steroidogenesis; however, conflicting findings have been reported regarding the mechanism in different steroidogenic cells. For example, inhibition of MEK activity with PD or U0 has been reported to be associated with stimulation [21, 41], inhibition [22, 33], or no effect [5, 42] on the steroidogenic response. These discordant findings might indicate a complex role for the MEK/ERK cascade in regulating the steroidogenic response, which appears to be dependent on receptor-effector coupling, the cell type used, and the specific stimulus. Thus, our data show that stimulation of ERβ and aromatase expression by IGF-I was independent of the MEK/ERK pathway. Activation of the MEK/ERK pathway might also play roles in other aspects of IGF-I function. Although the knockdown efficiency of siRNAs in our study is not too efficient at the protein level, the inhibitor experiments complement this work in the same direction.

To attempt to explain the molecular mechanism underlying aromatase and ERβ overexpression induced by IGF-I in human ESCs, we studied the transcription factors known to be regulators activated by IGF-I in other cell types, c-Jun and CREB [20–22, 43]. Although there was no difference between CREB protein expression levels in EMs and ESCs, it has been established that CREB can be activated by prostaglandin E2 in endometriosis [5]. Some studies have identified increased c-Jun expression in endometriotic tissues compared with that in normal endometrial tissues [44–46]. Our data showed that IGF-I could increase phosphorylation of the transcription factors c-Jun and CREB in ESCs. This posttranslational modification of c-Jun or CREB was markedly reduced by inhibition of the IGF1R/PI3K/AKT pathway. Notably, treatment with AG blocked c-Jun or CREB phosphorylation more efficiently than did treatment with LY, suggesting that the IGF1R/PI3K/AKT pathway could synergize in up-regulating c-Jun or CREB activity. Furthermore, we observed that IGF-I not only stimulated c-Jun binding to the *ESR2* promoter but also promoted the binding of c-Jun and CREB to *CYP19A1* PII, indicating the central role of the two transcription factors in regulating *ESR2* and *CYP19A1* gene transcription in human ESCs. This is the first report of a direct link between c-Jun or CREB transcription and the IGF-I signaling pathway in the regulation of ERβ and aromatase expression in endometriosis.

Many lines of evidence suggest that blockade of the IGF-I/IGF1R signaling pathway inhibits growth and metastasis in multiple cancer types, such as breast cancer and lung cancer, both in vitro and in vivo [47–49]. In endometriosis, previous studies showed that PI3K/AKT and MAPK signaling pathway inhibitors could suppress the development of endometriosis by down-regulating the expression of proinflammatory cytokines and proteolytic factors [33, 50]. However, the role of IGF1R inhibitors in vivo is not yet well characterized in endometriosis. In our present study, we found that IGF-I could promote the development of endometriosis xenografts, which was blocked by an IGF1R inhibitor (AG1024). Levels of both protein and mRNA ERβ expression were significantly higher than those in the control group, as were *CYP19A1* mRNA expression levels. In mice with lung adenocarcinoma, IGF-I alone had no effect on ERβ expression levels [48]. This finding suggested that ERβ and aromatase promoted the occurrence and development of endometriosis in mice, which is consistent with the findings of studies that used clinical specimens and cells. Additionally, an IGF-1R inhibitor could reduce IGF-I-stimulated aromatase and ERβ expression in xenografts. Thus, IGF-1R inhibitors could represent a new class of drug targets for ablation of ERβ and aromatase expression in endometriosis.

In summary, our results indicated that IGF-I activated the IGF1R/PI3K/AKT signaling pathway and then stimulated binding of c-Jun or CREB to the *ESR2* or *CYP19A1* promoter region, which resulted in the up-regulation of ERβ and

aromatase expression in endometriosis. Moreover, inhibition of IGF1R in vivo could impede the growth of ectopic lesions in nude mice. Given the current absence of an effective therapy for endometriosis, our findings suggest that further study of the potential use of IGF1R inhibitors for the treatment of endometriosis is warranted.

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Compliance to ethical standards The cell experimental procedures were approved by the institutional review board of the First Hospital of Peking University (No. 2014[789] and No. 2014[790]), and signed informed consents for use of the samples were obtained from each patient. The First Hospital of Peking University Animal Care Committee approved the use of mice for this study (No. J201403).

Conflict of interest The authors declare that they have no competing interests.

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