YAP1 inhibits ovarian endometriosis stromal cell invasion through ESR2

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Abstract

Endometriosis is an estrogen-dependent disease, and estrogen receptor 2 (*ESR2*) plays a critical role in the pathogenesis of ovarian endometriosis by promoting cell invasion. Yes-associated protein 1 (*YAP1*) plays suppressive roles in several types of tumors. However, the relationship between *YAP1* and *ESR2* is not fully understood. The aim of this study was to investigate the regulatory mechanism of *YAP1* in terms of *ESR2* and *YAP1* regulation of endometriotic stromal cell (ECSC) invasion in ovarian endometriosis. Our results demonstrated that *YAP1* mRNA and protein levels in eutopic endometrium (EU) tissues were higher than those in paired ectopic endometrium (EC) tissues. ECSCs transfected with *siYAP1* exhibited a significant increase in both *ESR2* mRNA levels and protein expression. Simultaneously, *YAP1* overexpression in ECSCs yielded the opposite results. Co-IP assays demonstrated *YAP1*-NuRD complex formation by *YAP1*, CHD4 and MTA1 in ECSCs. *YAP1* bound to two sites, (-539, -533) and (-158, -152), upstream of the *ESR2* transcription initiation site. *YAP1* binding to the two sites of the *ESR2* promoter in ECSCs was significantly lower than that in eutopic endometrial stromal cells (EUSCs) from EU tissues. ECSCs transfected with *siYAP1* exhibited increased invasion activity, while ECSCs transfected with *siESR2* showed inhibition of invasion. However, transfection with *siYAP1* and *siESR2* together decreased the number of invading cells compared with transfection with *siYAP1* alone. Therefore, we conclude that decreased levels of *YAP1* in ovarian endometriomas enhance *ESR2* expression via formation of a *YAP1*-NuRD complex, which further binds to the *ESR2* promoters. Furthermore, *YAP1* inhibits ECSCs invasion.

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Introduction

Endometriosis is an estrogen-dependent disease (Bulun 2009) affecting up to 10% of women of reproductive age (Ryan & Taylor 1997). Hormonal treatment or conservative surgery achieves significant pain relief in only approximately 50% of women diagnosed with endometriosis, and the symptom recurrence rate is high. Thus, novel and effective therapies for endometriosis are urgently needed (Vercellini *et al.* 2014, Greene *et al.* 2016, Liu *et al.* 2017). Estradiol is a key hormone for the growth and persistence of endometriotic tissue (Maia *et al.* 2012). Ovarian endometriotic lesions show high estradiol biosynthesis compared with the normal endometrium (Jarzabek *et al.* 2013). However, the effects of estrogen are mediated primarily via nuclear estrogen receptors, including ER α and ER β , which are encoded by *ESR1* and *ESR2*, respectively (Jarzabek *et al.* 2013). Previous studies have suggested that estrogen receptor 2 (*ESR2*)-selective agonists might be therapeutic in a rodent endometriosis model (Xiu-Li *et al.* 2009) and that *ESR2* interacts with the inflammasome complex and cytoplasmic apoptotic machinery to enhance the proliferative and adhesive

activities of endometriotic tissues and prevent tumor necrosis factor α (TNF- α) induced cell death (Han *et al.* 2015), indicating a key role for *ESR2* in endometrial and endometriosis growth regulation.

Yes-associated protein 1 (*YAP1*) is a critical component of the size-controlling Hippo signaling pathway (Pan 2010). Through a kinase cascade, this pathway targets *YAP1* for phosphorylation, preventing its translocation to the nucleus, where it functions as a transcriptional coactivator. As a transcriptional coactivator, *YAP1* can bind to oncogenes or tumor suppressor gene transcription factors, the most important of which are the TEAD transcription factors. A *YAP1*/TAZ-TEAD complex recruits the nucleosome remodeling and histone deacetylase (NuRD) complex to deacetylate histones and alters nucleosome occupancy at target genes (Kim *et al.* 2015). The current dogma suggests that restriction of *YAP1* transcriptional activity is the principal mechanism of growth and tumor suppression by the Hippo pathway (Mo *et al.* 2014). Indeed, nuclear *YAP1* is a powerful mediator of organ growth, cancer cell proliferation and invasion, and tumor growth (Camargo *et al.* 2007, Dong *et al.* 2007). The role of *YAP1* in cancer

development remains controversial (Zanconato *et al.* 2016). *YAP1* was originally found to promote cell proliferation and transformation. *YAP1* overexpression has been linked to tumor progression and worse survival in the context of certain malignancies. However, *YAP1* has been recently recognized as a tumor suppressor gene because it also induces apoptosis. Therefore, the expression and function of *YAP1* in specific diseases require further investigation.

Currently, few studies have considered the function of *YAP1* in endometriosis, and the relationship between *YAP1* and *ESR2* in ovarian endometriosis is still not well understood. Because *ESR2* has been shown to regulate the invasion of ectopic endometrial cells in ovarian endometriosis, whether *YAP1* suppresses cell invasion activity by regulating *ESR2* is worth exploring. Thus, the aim of this study was to investigate the regulatory mechanism of *YAP1* in terms of *ESR2* and *YAP1* regulation of endometriotic stromal cell invasion in ovarian endometriosis. We propose that *YAP1* inhibits *ESR2* expression by participating in NuRD transcriptional repression complexes and that *YAP1* inhibits *MMP2* and *MMP9* expression by inhibiting *ESR2* expression.

Materials and methods

Participants and primary cell culture

Ectopic endometrium (EC) tissues from the cyst walls of ovarian endometriomas and eutopic endometrium (EU) tissues were obtained from 25 women with endometriosis immediately after surgery, resulting in 25 self-controlled pairs. All patients (age range: 23–40 years) had regular menstrual cycles and none received hormonal therapy for at least 3 months before surgery. The diagnoses for all samples were histologically confirmed. The experimental protocol was approved by the Institutional Review Board of Peking University (No. 2014 (789)), and an informed consent form was signed by each patient before the samples were used. Human endometriotic or ectopic stromal cells (ECSCs) from ovarian endometriomas and eutopic endometrial stromal cells (EUSCs) were isolated from 17 tissue samples using the protocol previously described by Ryan *et al.* with minor modifications (Ryan *et al.* 1994). Briefly, endometriotic or endometrial tissues were digested with collagenase (1 mg/mL; Sigma-Aldrich) and deoxyribonuclease I (0.1 mg/mL; Sigma-Aldrich). Epithelial cells were removed by filtration of the cell suspension through a 75- μ m sieve. Stromal cells were then harvested and cultured to confluence in DMEM/F-12 containing 10% fetal bovine serum (Gibco, Thermo Fisher Scientific).

RNA extraction and real-time polymerase chain reaction

Total RNA was extracted from cultured primary stromal cells using TRIzol reagent (Life Technologies, Thermo Fisher Scientific) and quantified with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Two micrograms of total RNA was used to synthesize cDNA using High-Capacity

cDNA RT kits (4368814, Life Technologies, Thermo Fisher Scientific) according to the manufacturer's instructions. Real-time (RT) quantitative PCR (qPCR) was performed using an ABI 7500 Sequence Detection system and an ABI Power SYBR-Green gene expression system (4367660, Applied Biosystems, Thermo Fisher Scientific) to quantify *YAP1*, *ESR2*, *MMP2* and *MMP9* mRNA levels. The following primers were used: *YAP1*: (F) 5'-ACCCCAGATGACTTCCTGAACA-3', (R) 5'-GCAGGGTGCTTTGGTTGATAG-3'; *ESR2*: (F) 5'-ATGATCAGCTGGGCCAAGAA-3', (R) 5'-CCACATCAGCCC-CATCAT-TAA-3'; *MMP2*: (F) 5'-CCCACTGCGGTTTCTCGAAT-3', (R) 5'-CAA-AGGGGTATCCATCGCCAT-3'; *MMP9*: (F) 5'-AGA-CCT-GGGCAGATTCCAAAC-3', (R) 5'-CGGCAAGTCTCCGAGTAGT-3'; and 18S rRNA: (F) 5'-AGGAATCCCAAGTAAGTGC-3', (R) 5'-GCCTCACTAAACCATCCAA-3'. Human 18S rRNA was used as an internal control. The relative quantification of all transcripts was analyzed by the comparative threshold cycle method.

Western blot analysis

ECSCs were washed in ice-cold PBS and lysed in mammalian protein extraction reagent (KeyGen Biotech, China) containing a protease inhibitor cocktail (Amresco, USA). Lysates were centrifuged at 17,949 g for 20 min at 4°C. Protein concentrations were determined using a micro-BCA protein assay kit (KeyGen, Biotech). Equal amounts of total protein were resolved on 8 or 10% polyacrylamide gels using sodium dodecyl sulfate-PAGE and transferred onto nitrocellulose membranes. Membranes were incubated for 1 h at room temperature with Tris-buffered saline (2 mM Tris-HCl (pH 8.0) and 15 mM NaCl (pH 7.6)) with 0.1% Tween 20 containing 5% nonfat dry milk powder to saturate nonspecific binding sites. Membranes were incubated with primary antibodies at the following dilutions: anti-human *YAP1* antibody at 1:1000 (14074, Cell Signaling Technology), anti-human *ESR2* antibody at 1:1000 (04-824, Merck-Millipore), anti-human *MMP2* antibody at 1:1000 (40994, Cell Signaling Technology), anti-human *MMP9* antibody at 1:1000 (ab38898, abcam) and anti- β -actin antibody at 1:1000 (TA-09 and TA-08; ZSGB-BIO, China). All protein bands were quantified by densitometry using ImageJ software (National Institutes of Health, USA).

Small-interfering RNA knockdown

Primary ECSCs were cultured in growth medium as described previously to achieve approximately 70 to 80% confluence at the time of transfection. Transfection was performed using a small-interfering RNA (siRNA) (Life Technologies, Thermo Fisher Scientific) against *YAP1*: (F) 5'-CAGCAGAAUAGAUGAACUCGGCUU-3'; (R) 5'-AAGCCGAGUUAUCAUAUUCUGCUG-3', *ESR2*: (F) 5'-CCUUUA-GUGGUCCAUCGCCAGUUAU-3'; (R) 5'-AUAACUGGCGAU-GGACCACUAAAGG-3' or a nontargeting negative control siRNA (low GC content; Life Technologies, Thermo Fisher Scientific) at a final concentration of 100 nM using Lipofectamine RNAiMAX (Life Technologies, Thermo Fisher Scientific). The control group included non-treated cells. Cells were collected 48 h after transfection for RT-qPCR and Western blot analysis.

Plasmid overexpression

ECSCs were cultured to approximately 80–90% confluence at the time of transfection. Lipofectamine 3000 (Life Technologies, Thermo Fisher Scientific) was used to transfect either empty pENTER plasmid or pENTER-YAP1 plasmid (CH862329, Vigene Biosciences, China) in Opti-MEM reduced-serum medium (Life Technologies, Thermo Fisher Scientific). The control group included non-treated cells. At 48 h post transfection, the cells were harvested and processed for RT-qPCR and Western blotting to verify overexpression efficiency and detect gene expression.

Coimmunoprecipitation assay

ECSCs were lysed with nondenaturing lysis buffer (Appligen Technologies, China) with 1% protease inhibitors and incubated on ice for 20 min. The cell lysates were then centrifuged in a refrigerated microcentrifuge at 13,000g for 20 min at 4°C. The supernatants were used either directly for immunoprecipitation or stored at 80°C. For immunoprecipitation, equal amounts of protein (500 g) were first immunoprecipitated with anti-YAP1 (14074, Cell Signaling Technology), anti-CHD4 (ab72418, abcam), or anti-MTA1 (ab71153, abcam) primary antibody at 4°C for 4 to 6 h, followed by the addition of protein A agarose (11719408001, Roche) and incubation at 4°C overnight. The immunoprecipitates were collected by centrifugation, washed three times with PBS containing 0.5% protease inhibitors, and eluted with SDS-PAGE sample buffer. Immunoprecipitates were then analyzed by Western blotting as described previously.

Chromatin immunoprecipitation and real-time qPCR

Chromatin immunoprecipitation (ChIP) was performed using a ChIP assay kit (Pierce Chromatin Prep Module; Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, cells were crosslinked with 1% formaldehyde for 10 min at room temperature and collected in PBS containing 1% protease inhibitors. The crosslinked cells were then lysed and enzymatically digested with micrococcal nuclease to shear genomic DNA. The following antibodies were used for immunoprecipitation: anti-YAP1 and polyclonal rabbit IgG. Protein/DNA complexes were eluted from the beads and treated with proteinase K solution at 65°C for 2.5 h. RT-qPCR was performed on the purified DNA in the presence of SYBR Master Green Mix (Life Technologies, Thermo Fisher Scientific) using the *ESR2* primers (F) 5'-aaatgtgagtttcagtcctca-3'; (R) 5'-TCCAGGGGTAAGATGGATTG-3'; and 5'-ttgagcaatcac-acctcagc-3'; (R) 5'-ctcacgcctgtaatccaat-3' for amplification.

The $\Delta\Delta C_t$ method was used to analyze real-time ChIP results. Briefly, RT-qPCR was performed with a primer set targeting a positive locus and a primer set targeting a negative locus. Each ChIP DNA fraction's C_t value was normalized to the input DNA fraction C_t value for the same qPCR assay to account for differences in chromatin sample preparation: $C_t(\text{normalized ChIP}) = C_t(\text{ChIP}) - (C_t(\text{input}) - \log_2(\text{input dilution factor}))$. The difference between the ChIP fraction C_t values ($\Delta\Delta C_t$) of the normalized experimental sample (S2) and the control sample (S1) was then determined: $\Delta\Delta C_t(S2 - S1) = \Delta C_t(S2:\text{normalized ChIP}) - \Delta C_t(S1:\text{normalized ChIP})$. Finally, the differential occupancy fold change (linear conversion of the second $\Delta\Delta C_t$

to yield a fold change in site occupancy) was calculated: fold change in occupancy = $2^{(-\Delta\Delta C_t(S2-S1))}$.

Matrigel invasion assay

In vitro invasion assays were performed using Matrigel-coated (1:8 dilution) 24-well transwell chambers (8- μ m pore size, 6.5-mm diameter, Corning). Next, 2×10^5 primary, siRNA-transfected ECSCs were plated into the upper chambers in media without serum. The lower chambers were filled with 600 μ L of DMEM/F12 containing 20% FBS. The cells were then incubated at 37°C for 48 h. The cells on the upper surfaces of the filters together with the Matrigel were removed by wiping with a cotton swab. The inserts were then fixed in methanol for 30 min at room temperature and stained with hematoxylin. The stained cells reaching the lower surfaces of the transwells were observed and photographed using an Olympus DP71 microscope (Olympus). Five randomly selected fields were quantified for each experiment.

Statistical analyses

All experiments were performed at least three times. All data are presented as the mean and s.e.m. Comparisons of two groups were performed using the two-tailed Student's *t*-test. Comparisons of more than two groups were performed using one-way ANOVA. Statistical analyses were performed using the Statistical Package for the Social Science (SPSS) computer software version 22.0 (IBM SPSS Statistics). Figures were constructed using Prism 5 version 6.0f (GraphPad Software, Inc.). Power analyses were conducted using G*Power version 3.1.9.6 based on the assumption of $\alpha=0.05$ (two-tailed). Powers $\geq 80\%$ were considered statistically powered, and *P*-values < 0.05 were considered statistically significant.

Results

Differential expression of YAP1 in paired eutopic and ectopic endometrial tissues

RT-qPCR and Western blotting results demonstrated differential expression of YAP1 in EU and EC tissues from the same patient with endometriomas. Paired endometriotic and endometrial tissues were used to measure YAP1 expression, and the results showed that YAP1 mRNA levels were significantly increased in the EU compared with the EC (5.9-fold; $n=20$; $P<0.01$; power=0.998; Fig. 1A). Western blotting showed that YAP1 protein expression was also significantly higher in the EU ($n=20$; $P<0.05$; Fig. 1B).

YAP1 functions as a transcriptional corepressor to suppress ESR2 expression in ECSCs

As ESR2 plays a critical role in the pathological process of endometriosis, we asked whether YAP1, as a transcriptional coregulator, could regulate ESR2 expression. We used siRNAs to specifically knockdown YAP1 in ECSCs to measure the mRNA and protein levels

Figure 1 Differential expression of YAP1 in paired eutopic and ectopic endometrial tissues. Western blotting and quantification of YAP1 mRNA levels were performed as described. The values are the means $\pm$ S.E.M. (A) YAP1 mRNA levels in paired eutopic endometrial tissues were higher than those in ectopic endometrial tissues ($n=20$; **, t -test, $P<0.01$). (B) YAP1 protein expression in paired eutopic endometrial tissues was higher than that in ectopic endometrial tissues ($n=20$; *, t -test, $P<0.05$). EU, eutopic endometrial tissues; EC, ectopic endometrial tissues. Error bars indicate $\pm$ S.E.M. All experiments were repeated three times.

of *ESR2*. The control group included non-treated cells, and no significant difference was observed between the control group and the control siRNA group ($P>0.05$). The efficacy of YAP1 siRNA knockdown was determined by both RT-qPCR and Western blotting (Fig. 2A). ECSCs transfected with siYAP1 exhibited diminished YAP1 mRNA and protein levels and significant increases in *ESR2* mRNA levels (3.3-fold; $n=6$; $P<0.01$; power=0.953) and protein expression (Fig. 2B). Simultaneously, plasmid transfection was used to overexpress YAP1 in ECSCs (Fig. 2C), and no significant difference was observed between the control group and the control plasmid group ($P>0.05$). The results showed that transfection of YAP1 overexpression plasmids suppressed *ESR2* mRNA expression (35%; $n=6$; $P<0.05$; power=0.804) and protein levels (Fig. 2D), indicating that YAP1 exerts an inhibitory effect on *ESR2* expression in ECSCs.

YAP1 directly binds to the promoter region of the *ESR2* gene by recruiting the NuRD complex

Previous studies have demonstrated that YAP1 exerts an inhibitory effect on gene expression by recruiting NuRD

transcriptional repression complexes, particularly CHD4 and MTA1 (Kim et al. 2015). Thus, Co-IP assays were used to determine the interactions among YAP1, CHD4 and MTA1 in ECSCs. When YAP1 was immunoprecipitated from ECSCs, both CHD4 and MTA1 were present in the immunoprecipitate. Reciprocally, YAP1 was present in the CHD4 and MTA1 immunoprecipitates. Thus, we observed the formation of the YAP1-NuRD complex by YAP1, CHD4 and MTA1 ($n=6$; Fig. 3A), showing that YAP1 could interact with the CHD4 and MTA1 proteins to form the YAP1-NuRD complex in ECSCs.

To confirm that YAP1 could bind to the promoter region of *ESR2* and participate in the regulation of *ESR2* expression, we first examined the *ESR2* gene sequence and found two possible YAP1 binding sequences (CATTCC) near the transcription initiation site of *ESR2*. ChIP assays were used to measure differences in the binding ability of YAP1 to *ESR2* genes in paired eutopic and ectopic endometrial stromal cells. The results showed that YAP1 could bind to two sites, (-539, -533) and (-158, -152), upstream of the *ESR2* transcription initiation site ($n=4$). The binding of YAP1 to the two sites of the *ESR2* promoter in ECSCs was significantly lower than that in EUSCs (4.44- and 3.43-fold, respectively; $n=4$; $P<0.01$, $P<0.05$, respectively; power=0.999, power=0.944, respectively; Fig. 3B), suggesting that YAP1 might inhibit *ESR2* expression by directly binding to its promoter region.

YAP1 inhibits the invasiveness of ECSCs and the expression of MMP2 and MMP9 by suppressing *ESR2* expression

YAP1 has been shown to regulate cell invasion in different types of cancer cells, and a previous study confirmed that *ESR2* can affect the invasive ability of endometriotic cells by regulating the expression of Snail (Han et al. 2015). To investigate whether YAP1 influences cell invasion by regulating *ESR2* expression, we used siRNAs to knock down YAP1, *ESR2* or both and measured changes in the mRNA and protein levels of MMP2 and MMP9, which are closely related to the invasiveness of ECSCs. The control group included non-treated cells, and no significant difference was observed between the control group and the control siRNA group ($P>0.05$). The efficacy of siRNA knockdown was determined by RT-PCR and Western blotting ($n=6$; $P<0.01$; Fig. 4A). ECSCs transfected with siYAP1 exhibited 1.58-fold higher MMP2 mRNA levels than those of the controls, while ECSCs transfected with si*ESR2* showed MMP2 mRNA levels that were markedly decreased by 35%. However, MMP2 mRNA expression was reduced by 26% in cells transfected with siYAP1 and si*ESR2* together compared with that in cells transfected with siYAP1 alone ($n=6$; $P<0.01$, $P<0.01$, $P<0.01$, respectively; power=0.998; Fig. 4B left). Likewise, the MMP9 mRNA levels in the siYAP1 group were 3.72-fold

Figure 2 YAP1 functions as a transcriptional corepressor to suppress ESR2 expression in ECSCs. (A) siRNA-mediated knockdown of YAP1 blocked YAP1 expression in ECSCs. ECSCs were serum-starved overnight and transfected with control siRNA or *siYAP1*. The control group included non-treated cells, and no significant difference was observed between the control group and the control siRNA group ($P > 0.05$). Cells were harvested for SYBR® Green RT-qPCR and Western blotting 48 h after transfection for verification of siRNA knockdown efficiency. (B) siRNA-mediated knockdown of YAP1 increased ESR2 mRNA levels ($n = 6$; $**P < 0.01$) and ESR2 protein expression ($n = 6$). (C) ECSCs were transfected with the control plasmid or YAP1 plasmid for 48 h. The control group included non-treated cells, and no significant difference was observed between the control group and the control plasmid group ($P > 0.05$). Cells were then harvested for RT-PCR and Western blotting for verification of YAP1 overexpression efficiency. (D) Plasmid-mediated overexpression of YAP1 suppressed ESR2 mRNA ($n = 6$; $*P < 0.05$) and ESR2 protein levels ($n = 6$). NS, not significant. Error bars indicate $\pm$ S.E.M. All experiments were conducted in primary cultured cells and repeated three times.

higher than those in the control group, while the *siESR2* group showed a 55% decline in MMP9 mRNA levels. Compared with the *siYAP1* group, the *siYAP1* plus *siESR2* group (*siYAP1*+*siESR2*) showed a 70% reduction ($n = 6$; $P < 0.05$, $P < 0.05$, $P < 0.05$, respectively; power = 0.921; Fig. 4B right). Similar results for MMP2 and MMP9 protein expression were observed, as shown in Fig. 4C. To further validate the role of ESR2 in YAP1 regulation of cell invasion, we conducted transwell experiments to evaluate invasion activity. ECSCs transfected with *siYAP1* exhibited increased invasion activity ($n = 5$; $P < 0.05$), while ECSCs transfected with *siESR2* showed inhibited invasiveness ($n = 5$; $P < 0.05$). However, transfection with *siYAP1* and *siESR2* together decreased the number of invading cells compared with transfection with *siYAP1* alone ($n = 5$; $P < 0.05$; Fig. 4D). Taken together, these findings indicated that YAP1 could inhibit the invasiveness of ECSCs and the expression of MMP2 and MMP9 by suppressing ESR2 expression.

Discussion

To our knowledge, this study provides the first direct evidence of the function of YAP1 in the regulation of ESR2 and invasion of ovarian endometriosis. In this study, we showed that YAP1 expression was decreased in ectopic endometrium tissues from the cyst walls of ovarian endometriomas. YAP1 inhibited ESR2 expression by recruiting an NuRD transcriptional repression

complex, which further downregulated invasion activity and MMP2 and MMP9 expression.

The role of YAP1 in cancer development remains controversial. Accumulative evidence suggests that the different roles of YAP1 in oncogenesis might be tissue- and cell context-specific. YAP1 overexpression in a wide variety of solid tumors, such as esophageal cancer, cervical cancer, hepatoma, and gastric cancer, and its association with a more aggressive tumor biology and worse survival strongly suggest that YAP1 plays oncogenic and anti-apoptotic roles in these tumor types (Song *et al.* 2015, Xiao *et al.* 2015, Cheng *et al.* 2016). However, YAP1 is defined as a tumor suppressor that induces apoptosis (Yuan *et al.* 2008, Bertini *et al.* 2009). Decreased or absent expression of YAP1 is highly correlated with tumor progression and worse survival for other tumors, such as breast cancer, colorectal cancer (CRC) and head and neck cancers (HNSCCs) (Wang *et al.* 2014). Inhibition of YAP1 in breast cancer cell lines suppresses breast cancer cell anoikis and increases cell migration and invasion (Tufail *et al.* 2012). Additionally, significantly decreased YAP1 expression has been noted in invasive carcinoma compared to normal breast tissues, suggesting that YAP1 acts as a tumor suppressor in invasive breast carcinomas (Cao *et al.* 2017). Furthermore, knockdown of YAP1 enhances cell proliferation, survival, migration, and resistance to cisplatin in HNSCC cell lines (Ehsanian *et al.* 2010). Moreover, YAP1 has also been reported to act as a tumor

Figure 3 YAP1 binds to the ESR2 promoter region and inhibits its expression by recruiting the NuRD complex. (A) ECSC lysates were collected, immunoprecipitated using an anti-YAP1, anti-CHD4, anti-MTA1 or anti-IgG antibody, and analyzed by Western blotting with an anti-YAP1, anti-CHD4 or anti-MTA1 antibody. Formation of the YAP1-NuRD complex by YAP1, CHD4 and MTA1 was observed ($n=6$). (B) YAP1 inhibits ESR2 expression by directly binding to its promoter region. Paired eutopic and ectopic endometrial stromal cells were harvested and subjected to ChIP using an anti-YAP1 or control IgG antibody, followed by SYBR® Green RT-qPCR. The binding of YAP1 to the two sites (-539, -533) and (-158, -152) upstream of the ESR2 promoter in ECSCs was significantly lower than that in EUSCs (4.44- and 3.43-fold, respectively; $n=4$; $*P<0.05$, $**P<0.01$). IB, immunoblot; IP, immunoprecipitation; EUSCs, eutopic endometrial stromal cells; ECSCs, ectopic endometrial stromal cells. All experiments were conducted in primary cultured cells and repeated three times.

suppressor in human CRC and restrict the growth of CRC xenografts (Barry *et al.* 2013). Consistent with the results of the previously mentioned studies, our study showed that YAP1 inhibits the expression of ESR2 and further suppresses cell invasion in ectopic stromal cells from ovarian endometriomas, thereby acting as a gene suppressor. The mechanism of YAP1's inhibitory effects on different cells is still not clear, and the specific mechanism of YAP1's action in cell suppression requires further exploration.

The correlation between YAP1 and the estradiol synthesis pathway has been studied in breast and ovarian granulosa cell carcinomas. Wang *et al.* discovered that YAP1 knockdown reduces FSH-induced aromatase protein expression and estrogen production in KGN cells, demonstrating that YAP1 plays an important role in regulating GCT cell proliferation, migration and steroidogenesis (Fu *et al.* 2014). Guan *et al.* proved that verteporfin, which disrupts the interaction between YAP1/TAZ and TEADs, inhibits breast cancer cell proliferation and migration and tumor growth (Zhou *et al.* 2015). Endometriosis is an estrogen-dependent disease, and ectopic lesions grow in the presence of estrogen (Bulun

2009, Xiong *et al.* 2015). Previous researchers and our team have found that aromatase, the key rate-limiting enzyme that catalyzes the conversion of androgens to estrogens, is highly expressed in the ECSCs of ovarian endometriomas (Bulun *et al.* 2005, Zeng *et al.* 2015). The important role of ESR2 in ovarian endometriosis has also been confirmed. Previous studies have shown that epigenetic ESR2 promoter regulation can account for high ESR2 levels and that ESR2 is also responsible for low ESR1 expression in ECSCs (Xue *et al.* 2007). Increased expression of ESR2 inhibits the transcriptional activity of ESR1 in endometriosis, resulting in a decrease in the expression level of the progesterone receptor (PGR) (Bulun *et al.* 2010). Additionally, a positive role for ESR2 in endometriotic stromal cell cycle progression and proliferation has been proposed (Trukhacheva *et al.* 2009). Recently, O'Malley *et al.* (Han *et al.* 2015) proposed that the SRC-1 isoform/ESR2 complex can be a next-generation endometriosis therapeutic target. Thus far, the relationship between YAP1 and ESR2 has not been studied. Our study is the first to show that YAP1 regulates ESR2 transcription and ESR2 translation. SiRNA knockdown of YAP1 increases ESR2 mRNA expression and protein levels in ECSCs of ovarian endometriomas and that plasmid overexpression of YAP1 significantly decreases ESR2 expression. Our study proposes that YAP1 might be a protective factor in ovarian endometriosis and provides new evidence for the role of YAP1 in suppressing gene expression.

As a transcriptional coactivator, YAP1 can bind to oncogenes or tumor suppressor gene transcription factors, the most important of which are the TEAD transcription factors. Knockdown of TEADs or disruption of the YAP1-TEAD complex abolishes YAP1-dependent gene transcription and diminishes YAP1-induced cell proliferation, oncogenic transformation, and the epithelial-to-mesenchymal transition (EMT). Lamar *et al.* found that YAP1 promotes the metastasis of breast cancer and melanoma cells and that the TEAD-interacting domain of YAP1 is essential for YAP1-mediated tumor growth and metastasis (Lamar *et al.* 2012). However, studies have shown that YAP1 not only promotes gene transcription but also regulates gene expression. Mechanistically, the YAP1/TAZ-TEAD complex recruits the NuRD complex to deacetylate histones and alters nucleosome occupancy at target genes. NuRD is a complex comprising many subunits, including CHD4, MTA1 and GATAD2A. The NuRD complex has been proposed to mediate the repressor activity of the YAP1/TAZ-TEAD-Smad3 complex in human ES cells (Beyer *et al.* 2013). Lim *et al.* generalized the role of the YAP1/TAZ-TEAD-NuRD complex in the regulation of cell growth and survival, which might be the major function of YAP1/TAZ (Kim *et al.* 2015). Furthermore, the results of our Co-IP analysis showed that YAP1 interacts with both CHD4 and MTA1, while YAP1 knockdown or overexpression affects the binding of CHD4 and MTA1

Figure 4 YAP1 inhibits ECSC invasion and MMP2 and MMP9 expression by suppressing the expression of ESR2. (A) ECSCs were control-transfected or transfected with the indicated siRNAs and serum-starved overnight. The control group included non-treated cells, and no significant difference was observed between the control group and the control siRNA group ($P > 0.05$). Cells were harvested for RT-PCR and Western blotting with anti-YAP1 and anti-ESR2 antibodies for verification of siRNA knockout efficiency ($n = 6$; $**P < 0.01$). (B) ECSCs transfected with *siYAP1* alone exhibited increased *MMP2* and *MMP9* mRNA levels compared to the controls (1.58-fold, 3.72-fold, respectively; $n = 6$; $*P < 0.05$, $**P < 0.01$), while ECSCs transfected with *siESR2* alone showed decreased *MMP2* and *MMP9* mRNA levels (35%, 55%, respectively; $n = 6$; $*P < 0.05$, $**P < 0.01$). *MMP2* and *MMP9* mRNA levels were reduced in cells transfected with *siYAP1* and *siESR2* together compared with those in cell transfected with *siYAP1* alone (26%, 70%, respectively; $n = 6$; $*P < 0.05$, $**P < 0.01$). Cells were harvested for SYBR® Green RT-qPCR. (C) Similar results for *MMP2* and *MMP9* protein expression were observed. Likewise, ECSCs transfected with *siYAP1* alone exhibited increased *MMP2* and *MMP9* protein expression compared with the controls, while ECSCs transfected with *siESR2* alone showed decreased *MMP2* and *MMP9* protein expression. *MMP2* and *MMP9* protein expression levels were reduced in cells transfected with *siYAP1* and *siESR2* together compared with those in cells transfected with *siYAP1* alone ($n = 6$; $P < 0.05$). Cells were harvested for Western blotting. (D) To further validate the role of ESR2 in YAP1 regulation of cell invasion, a transwell assay was conducted to evaluate invasion activity. ECSCs transfected with *siYAP1* alone exhibited increased invasion activity, while ECSCs transfected with *siESR2* alone showed inhibited invasiveness. Meanwhile, the invasiveness of ECSCs was reduced in cells transfected with *siYAP1* and *siESR2* together compared with that of cells transfected with *siYAP1* alone ($n = 5$; $*P < 0.05$). Cells were harvested for SYBR® Green RT-qPCR and Western blotting. NS, not significant. Error bars indicate $\pm$ S.E.M. All experiments were conducted in primary cultured cells and repeated three times.

to target genes for cell growth and survival. Consistent with previous studies, our study demonstrated that YAP1 first interacted with the CHD4 and MTA1 proteins and then formed a YAP1-NuRD complex to regulate ESR2 expression. Whether this complex can regulate key enzymes involved in estrogen synthesis and related pathways in endometriosis warrants further study.

Knockdown of *YAP1* enhances the proliferation, migration, and invasion of cancer cells, and restoration of *YAP1* expression in these knockdown cells reduces the tumorigenic properties of the cells (Wang *et al.* 2014). MMPs are important proteases that degrade the extracellular matrix and thus promote tumor metastasis and invasion. *YAP1* is highly expressed in human lung

adenocarcinomas (LACs) compared to normal lung tissue (NLT), and knockdown of *YAP1* may inhibit the proliferation and invasion of adenocarcinoma cells through downregulation of *MMP9* (Cui *et al.* 2012). The *YAP1* pathway is required for LATS1-induced inhibition of cell growth and invasion, and LATS1 restrains the nuclear transfer of *YAP1* and downregulates *YAP1*, *MMP2* and *MMP9* expression (Cheng *et al.* 2016). Furthermore, O'Malley *et al.* (Han *et al.* 2015) discovered that *ESR2* enhances invasion activity in human endometriotic epithelial cells (iHEECs). However, whether *YAP1* can influence cell invasion by regulating *ESR2* expression has not been discussed. In our study, we found that knockdown of *ESR2* reduced invasion activity by ECSCs and decreased the expression of *MMP2* and *MMP9*, further confirming the regulation of endometriosis invasion by *ESR2*. Moreover, ECSCs transfected with *siYAP1* exhibit increased *MMP2* and *MMP9* levels and enhanced invasion activity. However, invasion activity and *MMP2* and *MMP9* expression were reduced in ECSCs transfected with *siYAP1* and *siESR2* together compared to that in cells transfected with *siYAP1* alone. These results demonstrate that *YAP1* can inhibit invasion activity and MMPs expression by inhibiting *ESR2* expression in ECSCs of ovarian endometriomas, representing a new potential therapeutic target for the treatment of endometriosis.

Recently, several studies highlighted the essential role of *YAP1* in the pathogenesis of endometriosis involving different endometriotic lesions. Pei and colleagues investigated the relationship between the *YAP1* pathway and cell autophagy in endometriosis (Pei *et al.* 2019) and found that the mRNA expression of *YAP1* was increased in the EUSCs of endometriosis patients compared with healthy controls with no statistically significant difference and that the protein levels were significantly increased in the EUSCs. In this study, our results demonstrated that decreased expression of *YAP1* in ovarian endometriotic lesions is correlated with high *ESR2* expression and cell invasion, which is consistent with studies in breast cancer cell lines (Tufail *et al.* 2012). However, Lin *et al.* (2017) showed that *YAP1* expression in the EC tissues of patients with endometriosis was higher than that in the EU of normal control patients, suggesting a progressive role for *YAP1* in the development of endometriosis. Different tissue origins and cell- or tissue-specific activities should be considered for the opposite results. EC tissues from patients with endometriosis and normal EU tissues from normal participants were used as study models in their study, while the differences in *YAP1* expression in our study were detected using paired EC tissues from ovarian endometriomas and EU tissues from the same patient. Moreover, we BLAST searched *YAP1* primers from both papers, and the primer that we used included all eight transcripts formed by the *YAP1* gene (Gaffney *et al.* 2012) and was tested by other studies (Konsavage *et al.* 2012, Hayashi *et al.* 2015), but the previously mentioned

study detected five of eight transcripts of *YAP1*. Different transcripts may act as transcriptional repressors and activators, which may lead to dual functions for *YAP1* in promoting and inhibiting cell growth in human cancers. Therefore, *YAP1* expression and function in endometriosis still require further exploration. A major limitation of this study is the lack of experiments *in vivo* with an endometriosis mouse model, which may further validate the therapeutic effect of *YAP1* in the pathogenesis of endometriosis. Whether *YAP1* regulates the expression of other receptors, such as *ESR1* and *PCR*, warrants further exploration. Additionally, the regulation of *YAP1* alongside other important pathogenic factors of endometriosis, such as PGE2 and VEGF, is worth investigating.

Conclusion

In summary, our study demonstrates that decreased levels of *YAP1* in ovarian endometriotic tissues enhance *ESR2* expression via formation of a *YAP1*-NuRD complex, which further binds to the *ESR2* promoters. Furthermore, *YAP1* inhibits ovarian ESC invasion and *MMP2* and *MMP9* expression. Alterations in this *YAP1/ESR2*-mediated regulation of ovarian endometriosis cell invasion may represent a potential mechanism for women with endometriosis. Further research is needed to improve our understanding of this regulation and to identify new molecular targets for interventions and treatments for endometriosis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

C Z, Z-T D and Q X conceived the study and wrote the paper. C Z, P-L W and Z-T D performed the experiments and analyzed the data. L X and Y-F Z collected samples.

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