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Puerarin suppresses proliferation of endometriotic stromal cells in part *via* differential recruitment of nuclear receptor coregulators to estrogen receptor-α

Mei Ji[a,c,1], Yuhuan Liu[c,1], Shengsheng Yang[b,1], Dongxia Zhai[a], Danying Zhang[a], Lingling Bai[a], Zhenzhi Wang[a], Jin Yu[a], Chaoqin Yu[a,*], Zailong Cai[b,**]

[a] Department of Traditional Chinese Medicine, Changhai Hospital, Second Military Medical University, Shanghai, China
[b] Department of Biochemistry and Molecular Biology, Second Military Medical University, Shanghai, China
[c] Department of Obstetrics and Gynecology, Changhai Hospital, Second Military Medical University, Shanghai, China

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ABSTRACT

Background and objectives: Puerarin, a phytoestrogen with a weak estrogenic effect, binds to estrogen receptors, thereby competing with 17β-estradiol and producing an anti-estrogenic effect. In our early clinical practice to treat endometriosis, a better therapeutic effect was achieved if the formula of traditional Chinese medicine included *Radix puerariae*. This study was to investigate whether puerarin could suppress the proliferation of endometriotic stromal cells (ESCs) and to further elucidate the potential mechanism.

Methods and results: The ESCs were successfully established. The effects of puerarin on the proliferation of ESCs, cell cycle and apoptosis were determined by Cell Counting Kit-8 assay and flow cytometry. The mRNA and protein levels of cyclin D1 and cdc25A were detected by real-time PCR and Western blot analysis. Coimmunoprecipitation was applied to examine the recruitment of nuclear receptor coregulators to the estrogen receptor-α. We found that puerarin can suppress estrogen-stimulated proliferation partly through down-regulating the transcription of cyclin D1 and cdc25A by promoting the recruitment of corepressors to estrogen receptor-α as well as limiting that of coactivators in ESCs.

Conclusions: Our data suggest that puerarin could suppress the proliferation of ESCs and could be a potential therapeutic agent for the treatment of endometriosis.

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1. Introduction

Endometriosis affects up to 5–10% of reproductive-age women. Its symptoms include several types of severe pain and infertility, which significantly impair the quality of life of patients. Clinical and experimental data have clearly established that endometriosis grows and regresses in an estrogen-dependent manner [1]. The effects of estrogen are mediated by estrogen receptors (ERs) and subsequent alterations in transcription. ER-α activity can be regulated through interactions with a multitude of coregulatory factors called coactivators and corepressors [2]. These factors bind to steroid receptors in a ligand-dependent fashion, and the receptor-bound cofactors then bind to the basal transcriptional machinery of the target genes, resulting in transcription. Steroid receptor cofactors are thus important molecules that intervene between receptors and target genes [3].

Phytoestrogens represent a group of natural non-steroidal compounds that exhibit estrogen-like activities because of their structural similarity to endogenous estrogens, which have two-way adjustments to endogenous estrogens. Puerarin, a phytoestrogen derived from the Chinese medicinal herb *Radix puerariae*, has been proven practical in the management of various cardiovascular disorders, alcoholism, and neurological disease [4]. Based on our early clinical practice in the treatment of endometriosis, better therapeutic effects are achieved if the traditional Chinese medicine formulation to be used includes *R. puerariae*. To further understand the molecular basis mediating such an effect by puerarin, we investigated the ability of coregulator proteins to modulate human ER-α transcriptional activity in the presence of estrogen and/or puerarin and determined the regulatory contribution of coregulator proteins to the expression of specific ER-responsive genes associated with cell proliferation in endometriotic stromal cells (ESCs).

* Corresponding author at: Department of Chinese Traditional Medicine, Changhai, Hospital, Second Military Medical University, 168 Changhai Road, Shanghai 200433, China.
** Corresponding author at: Department of Biochemistry and Molecular Biology, 800 Xiangyin Road, Second Military Medical University, Shanghai 200433, China. Tel.: +86 2181870970.
E-mail addresses: chqyu81@sina.com (C. Yu), czl8003@163.com (Z. Cai).
[1] Worked equally on this study.

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2. Materials and methods

2.1. Tissue collection and cell culture

ESCs were obtained from premenopausal patients who had undergone salpingo-oophorectomy or evisceration for ovarian endometriotic cysts. All patients had been free of any hormonal treatments before the operation. All the samples were obtained in the proliferative phase of the cycle, which was confirmed histologically. This study was approved by the human investigation committee of the Changhai Hospital, and written informed consent was obtained from all patients. The tissues were collected under sterile conditions and transported to the laboratory on ice in Dulbecco's modified Eagle's medium (DMEM) (PAA, Linz, Austria). The ESCs were isolated and cultured in flasks with DMEM containing 10% charcoal stripped fetal bovine serum (FBS) (Biological Industries, Israel), 100 IU/mL penicillin, and 100 IU/mL streptomycin, which were then incubated in 5% CO_2 at 37 °C. The purity of ESCs in isolated cells was determined as previously described [5]. The isolated cells with the purity of ESCs > 90% were used to perform the following experiments.

2.2. Cell proliferation evaluation

Cell count was determined in 96-well plates using Cell Counting Kit-8 (Dojindo, Japan) according to the manufacturer's protocols. ESCs were grown to 80% confluence and then cultured in serum-free medium for 24 h. For dose response course, the medium was then changed to DMEM containing 10% charcoal-stripped FBS with 1×10^{-8} mol/L E2 or combination of 1×10^{-7} mol/L fulvestrant (ICI 182780), 1×10^{-13}, 1×10^{-11}, 1×10^{-9}, 1×10^{-7} mol/L puerarin respectively for 3 days. E2, ICI and puerarin are from Sigma, dissolved in dimethyl sulfoxide (DMSO) solution; for time response course, ESCs were incubated with 1×10^{-8} mol/L E2 and/or 1×10^{-9} mol/L puerarin for 1–4 days. The vehicle DMSO was used as control. For cytotoxicity assay, ESCs were incubated with 1×10^{-6}, 5×10^{-6}, 1×10^{-5}, 5×10^{-5}, 1×10^{-4}, 5×10^{-4} mol/L puerarin or DMSO solution for 3 days, the cell growth inhibit rations were calculated as follows: cell inhibitory ratio (%) = (ODv − ODp)/ODv × 100%. ODp and ODv indicated the optical density of the cells incubated with puerarin and vehicle control (DMSO solution), respectively. The half maximal inhibitory concentration (IC_{50} value) was calculated using SPSS 17.0 software.

2.3. Cell cycle and apoptosis assay

ESCs were incubated with DMSO solution, 1×10^{-8} mol/L E2 and/or 1×10^{-9} mol/L puerarin for 48 h. Cells were then trypsinized, collected after being washed twice with phosphate-buffered saline. For cell cycle assay, the cells were fixed in 75% cold ethanol, and incubated overnight at 4 °C in the dark with propidium iodide. Cell cycle was performed by FACS (Miltenyi, BergischGladbach, Germany). For apoptosis assay, 3×10^5 treated cells were collected, resuspended in 500 μl of 1× Binding Buffer, 5 μl of Annexin V-FITC and 5 μl of propidium iodide (PI) were added and incubated at room temperature for 5 min in the dark according to Annexin V-FITC Assay Protocol (BioVision). Annexin V-FITC and PI binding was analyzed by FACS.

2.4. Quantitative real-time RT-PCR and Western blot

The mRNA and protein levels of cyclin D1 and cdc25A were detected by real-time PCR and Western blot analysis [6–8]. Total RNA and cDNA were prepared according to Invitrogen's instructions (Carlsbad, USA), and quantitative PCR was performed on a real-time PCR machine (ABI7300, USA) using a standard SYBR Green PCR kit (Toyobo, Japan). The reactions were run for 40 cycles (90 °C 15 s, 60 °C 60 s). All samples were examined in duplicate. The relative expression of each target gene compared to β-actin was calculated using the $2^{-\Delta\Delta Ct}$ method. For westernblot, cells were washed by pre-cooled PBS and lysed RIPA buffer (50 mmol/L Tris–Cl pH 7.4, 150 mmol/L NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate) supplemented with 1 mmol/L PMSF. Protein concentration was determined using the BCA™ protein assay kit (Pierce, IL). 30 μg protein samples were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, MA). Primers and primary antibodies are described in supplementary material and all antibodies were diluted to 1 μg/ml in western blot. A supersignal kit (Pierce, IL) was used to visualize the bands according to the manufacturer's instructions. 1×10^{-8} mol/L E2 or/and 1×10^{-9} mol/L puerarin in the incubation experiments was used for qPCR and protein analysis.

2.5. Coimmunoprecipitation analysis

Two coactivators, SRC-1 and SRC-3, and two corepressors, nuclear receptor corepressor (NCoR) and silencing mediator for retinoid and thyroid hormone receptors (SMRT), were detected to analyze the recruitment of nuclear receptor coregulators to ER-α by CO-IP as previously described [9] with some modifications. Briefly, cells were washed three times with phosphate-buffered saline (PBS) and lysed with lysis buffer at 4 °C for 10 min. Whole lysates were centrifuged at 16,000 × *g* for 10 min at 4 °C to remove cell debris, and the protein content in the supernatants was determined by the BCA Protein Assay Kit (Thermo, USA). For coimmunoprecipitation, 500 μg samples of lysates were immunoprecipitated using 5 μg of anti-ERα antibody (Millipore, USA) per reaction. Control immunoprecipitations were carried out using 5 μg of rabbit IgG (Sigma-Aldrich, UK). Subsequently, the mixture was incubated with Protein G PLUS-Agarose beads (Santa Cruz Biotechnology). After washing with RIPA buffer, precipitates were analyzed by SDS-PAGE and Western blot with anti-ERα, anti-SRC-1(Millipore, USA), anti-SRC-3(Millipore, USA), anti-NCoR (Thermo, USA) and anti-SMRT (Millipore, USA) antibodies, and then incubated with corresponding horseradish peroxidase-conjugated secondary antibodies. The immunoblots were visualized using an enhanced chemiluminescence.

2.6. Data and statistical analysis

Data were expressed as mean ± SD from at least three independent experiments. Statistical comparisons between groups were performed using one-way ANOVA followed by *post hoc* multiple comparisons where appropriate. Results were considered statistically significant at $p < 0.05$ or highly significant at $p < 0.01$.

3. Results

3.1. Puerarin suppresses proliferation of E2-stimulated ESCs

Cell count assay revealed that E2 significantly stimulated the proliferation of ESCs compared with DMSO control ($p < 0.01$) and that E2 + ICI (1×10^{-7} mol/L) treatment inhibited such proliferation, demonstrated by the decline in cell number. Interestingly, E2 + puerarin (1×10^{-13}, 1×10^{-11}, 1×10^{-9}, or 1×10^{-7} mol/L) treatment had similar biological inhibition effect and 10^{-9} mol/L was the most effective puerarin concentration (Fig. 1A) which was confirmed as observed up to 4 days ($p < 0.05$) (Fig. 1B). *In vitro* cytotoxicity of puerarin was examined on ESCs; the results showed that relative high concentration of puerarin can non-specifically

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