

Original Research

Paeonol alleviates migration and invasion of endometrial stromal cells by reducing HIF-1 α -regulated autophagy in endometriosis

Conghui Pang¹, Zhijuan Wu², Xiaoyan Xu¹, Wenxiu Yang¹, Xiaoxuan Wang¹, Yinghua Qi^{1,*}

¹Center of Reproduction and Genetics Integrated Traditional Chinese and Western Medicine, The Hospital Affiliated to Shandong University of Traditional Chinese Medicine, 250014 Jinan, Shandong, China, ²Department of Gynecology of Traditional Chinese Medicine, Shandong University of Traditional Chinese Medicine, 250014 Jinan, Shandong, China

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Materials and methods
4. Results
5. Discussion
6. Conclusions
7. Author contributions
8. Ethics approval and consent to participate
9. Acknowledgment
10. Funding
11. Conflict of interest
12. Availability of data and materials
13. References

1. Abstract

Background: Dysregulated migration and invasion of endometrial stromal cells is implicated in the pathogenesis of endometriosis. Hypoxia functions as critical microenvironmental factor that results in promotion of endometrial stromal cells migration and invasion through up-regulation of autophagy. Paeonol functioned as a tumor suppressor in human ovarian cancer and promoted cytoprotective autophagy. However, the role of paeonol in hypoxia-induced autophagy in endometriosis remains unknown. **Methods:** Stromal cells were isolated from endometriotic patients by enzymatic digestion of ectopic endometrial tissues, and then characterized by immunohistochemical analysis of cytoskeleton 19 (CK19) and vimentin. Cellular morphology was evaluated under microscope. Cell viability, proliferation and apoptosis of stromal cells were assessed by Cell Counting Kit-8, EdU labeling and flow cytometry, respectively. Wound healing and transwell assays were performed to detect metastasis of the stromal cells. Hypoxia-induced autophagy was evaluated through immunohistochemistry and western blot. **Results:** Paeonol treatment dosage dependently decreased

cell proliferation and metastasis of the ectopic endometrial stromal cells (ecESCs), while promoted the cell apoptosis. Hypoxia-induced autophagy in the ecESCs was repressed by paeonol through down-regulation of LC3-II/LC3-I and Beclin-1, while up-regulation of p62. Hypoxia-inducible factor-1 α (HIF-1 α) was reduced post paeonol treatment, and paeonol-induced increase of p62 and decrease of LC3-II/LC3-I and Beclin-1 were reversed by over-expression of HIF-1 α . Over-expression of HIF-1 α also attenuated the suppressive effect of paeonol on cell growth of ecESCs. **Conclusions:** Paeonol attenuated HIF-1 α -induced promotion of ecESCs migration and invasion through reducing autophagy, and reduced HIF-1 α -induced endometriotic lesion in rats, providing potential therapeutic strategy for the treatment of endometriosis.

2. Introduction

Endometriosis is an estrogen-dependent benign chronic disease [1], and affects 2–10% of premenopausal women as a major cause of infertility and chronic pelvic pain [2]. Endometriosis is generally characterized by the

presence of endometrial glands and extracellular matrix, and defined as extra-growth of the endometrial tissues [3]. The pathogenesis of endometriosis is still unclear, and the most widely accepted theory is the implantation theory in which endometrial tissue is implanted outside the uterus through an open fallopian tube during the menstrual cycle [4]. Meanwhile, the enhanced proliferation, adhesion, migration and invasion of endometrial cells, including stromal and epithelial cells, facilitated the ectopic implantation of the endometrial tissues and development of endometriotic lesions [5]. The suppression of endometrial stromal cells metastasis contributed to amelioration of endometriosis [5].

Autophagy is an evolutionary conservative mechanism that eliminates and circulates non-essential cytoplasmic components to maintain homeostasis [6]. Dysregulated autophagy in the eutopic or ectopic endometrium retarded cell apoptosis and promoted hyperplasia of stromal cells and endometriotic tissues, thus contributing to the pathogenesis of endometriosis [7]. The level of autophagy is relatively high in the ectopic endometrium of patients with ovarian endometriosis [8], and autophagy could promote the migration and invasion of endometrial stromal cells under the condition of hypoxia [9]. Inhibition of autophagy inhibited the angiogenesis of endometriosis and attenuated the ectopic endometriotic lesions [10]. Therefore, regulation of autophagy might be helpful for the suppression of endometrial stromal cells metastasis.

Paeonol (4-methoxy-2-hydroxyacetophenone), an active compound extracted from traditional Chinese medicine, demonstrated antioxidant, anti-tumor, anti-inflammatory and immunomodulatory effects [11]. For example, paeonol promoted colorectal cancer cell apoptosis [12], and retarded non-small-cell lung cancer motility [13]. Moreover, paeonol decreased vascular endothelial growth factor to enhance radiotherapy-induced apoptosis of ovarian cancer [14]. However, whether paeonol has an effect on endometriosis has not been studied yet. Paeonol activated autophagy flux in ovarian cancer [15], and promoted autophagy to demonstrate anti-atherosclerotic in vascular smooth muscle cell [16]. Additionally, oxidized low-density lipoprotein-induced vascular endothelial cell autophagy was suppressed by paeonol [17]. The detailed role of paeonol on autophagy in endometriosis needs further research.

This study aimed to investigate roles of paeonol on migration and invasion of isolated ectopic endometrial stromal cells (ecESCs), as well as endometriotic lesion in rats. Specifically, whether autophagy was implicated in paeonol-mediated progression of endometriosis was also determined.

3. Materials and methods

3.1 Isolation and culture of ecESCs

Patients (N = 7; age of 18 to 50) with ovarian endometriosis were recruited at The Hospital Affiliated to Shandong University of Traditional Chinese Medicine. The presence of endometriosis was confirmed by pathological examination and laparoscopy. The pathological examination was performed by an experienced fertility specialist based on the criteria of Noyes. The study was approved by the Center of Reproduction and Genetics, The Hospital Affiliated to Shandong University of Traditional Chinese Medicine and the patients signed written informed consents. Laparoscopy was performed to isolate the ectopic endometrial tissues, and the normal endometrial tissues were also isolated from regularly cycling women without endometriosis. The tissues were digested with collagenase A and DNase (Sigma Aldrich, St. Louis, MO, USA) for 2 hours. The cells were then cultured in DMEM medium (Lonza, Basel, Switzerland) containing 10% fetal bovine serum (Lonza) at 37 °C incubator. The adherent cells were grown to 80% confluence before the evaluation under microscope (Olympus Inc., Tokyo, Japan) and immunohistochemical analysis of CK19 and vimentin.

3.2 Immunohistochemistry

The isolated ecESCs were formalin-fixed and soaked in 0.3% Triton X-100. Following incubation with 3% H₂O₂, the cells were incubated with specific antibody against CK19 (1:200, Abcam, Cambridge, MA, USA) and vimentin (1:200, Abcam, Cambridge, MA, USA). The cells were incubated with diaminobenzidine and hematoxylin after incubation with corresponding secondary antibody. Slides were observed under microscope (Olympus, Tokyo, Japan).

3.3 Cell treatment and transfection

pcDNA-mediated over-expression of HIF-1 α (forward Primer: 5'-GAACGTCGAAAAGAAAAGTCTCG-3' and reverse Primer: 5'-CCTTATCAAGATGCGAACTCACA-3') was constructed by GenePharma (Suzhou, China). Isolated ecESCs were transfected with pcDNA-HIF-1 α or the empty vector by Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The ecESCs with or without transfection were cultured under hypoxic condition (1% O₂, 5% CO₂, 94% N₂) for 24 hours, and then treated with different concentrations of paeonol (0, 10, 30, 50, 100 μ m; Sigma Aldrich, St. Louis, MO, USA) for another 24 hours before functional assays. Normal endometrial stromal cells and normal ovarian epithelial IOSE80 cells were also treated with different concentrations of paeonol (0, 10, 30, 50, 100 μ m; Sigma Aldrich, St. Louis, MO, USA) for 24 hours before functional assays.

3.4 Cell Counting Kit-8 and EdU staining

The ecESCs with indicated transfection and treatment, as well as normal endometrial stromal cells and IOSE80 cells, were plated and incubated with Cell Counting Kit-8 solution (Dojindo, Tokyo, Japan) for 2 hours. Absorbance at 450 nm was determined by Microplate Autoreader (Thermo Fisher Scientific). For EdU staining, cells were incubated with diluted EdU solution (1:1000, Sigma Aldrich, St. Louis, MO, USA) for 2 hours, and then fixed in 4% paraformaldehyde. Cells were incubated with 100 μ L 1 \times Apollo® dyeing reaction solution (Sigma Aldrich, St. Louis, MO, USA) and DAPI solution (Sigma Aldrich, St. Louis, MO, USA) for 1 hour. The fluorescence was measured under fluorescence microscope (Olympus, Tokyo, Japan).

3.5 Flow cytometry

The ecESCs with indicated transfection and treatment were stained using an Annexin V-fluorescein isothiocyanate apoptosis detection kit (Beyotime, Beijing China). The proportion of apoptotic cells was determined via flow cytometer (Attune, Life Technologies, Darmstadt, Germany).

3.6 Wound healing and transwell assays

The ecESCs with indicated transfection and treatment were plated and scratched by a plastic tips. Forty-eight hours later, the wound was calculated under microscope (Olympus). For transwell assay, cells in serum-free medium were seeded in the upper Matrigel-coated Transwell chamber, and medium with 20% fetal bovine serum was added into the bottom chamber. Forty-eight hours later, the invasive cells in the bottom chamber were fixed with paraformaldehyde and stained with 0.1% crystal violet before measurement under microscope (Olympus).

3.7 Immunofluorescence

The ecESCs with indicated transfection and treatment were fixed with 4% paraformaldehyde before incubation with 5% bovine serum albumin for 1 hour. Specific antibody against LC3B (1:300; Abcam, Cambridge, MA, USA) was applied to incubated the cells overnight. Following incubation with FITC-conjugated secondary antibody (1:100; Abcam, Cambridge, MA, USA), DAPI was used to stain cell nucleus before measurement under fluorescence microscope (Olympus).

3.8 qRT-PCR

Total RNAs extracted from ecESCs by TRIzol Reagent (Sigma Aldrich) were reversed into cDNAs under cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). SYBR Green Master (Roche, Mannheim, Germany) was applied to determined mRNA expression of HIF-1 α and GAPDH by $2^{-\Delta\Delta C_t}$ method. GAPDH was used as internal reference. The primers were listed below: HIF-1 α : F 5'-AACATAAAGTCTGCAACATGGAAG-

3' and R 5'-AACATAAAGTCTGCAACATGGAAG-3'; GAPDH: F 5'-GGATTGTCTGGCAGTAGCC-3' and R 5'-ATTGTGAAAGGCAGGGAG-3'.

3.9 Animal model

Experiments were performed according to National Institutes of Health Laboratory Animal Care and Use Guidelines. and approved by The Hospital Affiliated to Shandong University of Traditional Chinese Medicine. Female SD rats (6–8 weeks old, 200–220 g) were purchased from Envigo (Indianapolis, IN, USA), and housed in cages with 12:12 h light and dark cycles and controlled environment (24 ± 2 °C). Rats were divided into sham and endometriosis groups. To establish endometriotic rats, rats were anaesthetized by halothane in oxygen, and the bowels were exposed by mid-ventral incision. The right uterine horn was removed, opened longitudinally via scissors in sterile PBS, and cut into four square pieces (4 mm²). The pieces were sutured to the intestine mesentery with single stitch. The abdominal wall was closed, and the rats were kept for endometriosis induction for 4 weeks. Laparotomy was performed to confirm the endometriotic lesions. Rats in the sham group (N = 6) were conducted with surgery without transplantation of uterine tissue towards intestine mesentery. Rats in the endometriosis group were randomly divided into 3 groups: endometriosis (EMs) group (N = 6), EMs with 20 mg/kg paeonol group (N = 6) and EMs with 50 mg/kg paeonol group (N = 6). Rats were orally treated with paeonol for 21 days before the functional analyses.

3.10 ELISA

Rats were sacrificed by carbon dioxide inhalation, the serum samples were collected, and then performed with commercial ELISA kits (Krishgen Biosystem, Mumbai, India) to determine levels of IL-1 β , IL-6 and TNF- α .

3.11 Histological examination

The endometriotic lesions were surgically removed from the rats, and fixed in formalin. The paraffin-embedded tissues were cut into 5 μ m thick sections. The sections were stained with hematoxylin and eosin (Sigma-Aldrich), and examined under microscope.

3.12 Western blot

Proteins extracted from ecESCs or endometriotic lesions were separated by SDS-PAGE, and then transferred to PVDF membranes (Sigma Aldrich). The membrane was blocked in 5% bovine serum albumin before incubation with primary antibodies against HIF-1 α (1:2000, Abcam, Cambridge, MA, USA), LC3B (1:2500, Abcam, Cambridge, MA, USA), Beclin-1 and p62 (1:3000, Abcam, Cambridge, MA, USA) and β -actin (1:3500, Abcam, Cambridge, MA, USA). HRP-conjugated secondary antibody (1:5000, Abcam, Cambridge, MA, USA) incubation with the membranes were performed before analysis with chemiluminescence system (Tanon, Shanghai, China).

3.13 Statistical analysis

Data with at least three times repeat were presented as mean \pm standard deviations. Student's *t* test or one-way analysis of variance were used to perform statistical comparison under Graphpad Prism® statistical analysis software. A *p* values < 0.05 was considered statistically significant.

4. Results

4.1 Paeonol repressed cell proliferation of ecESCs

To explore the effects of paeonol on endometriosis, ecESCs were isolated and characterized under microscope. The cells showed spindle shape (Fig. 1A). Moreover, immunohistochemical analysis demonstrated negative staining of CK19 and positive staining of vimentin (Fig. 1A), indicating that endometrial stromal cells were successful isolated. The ecESCs under hypoxia treatment were applied to paeonol condition. Results showed that paeonol condition dosage dependently reduced cell viability of ecESCs (Fig. 1B), while dosage dependently promoted cell apoptosis of ecESCs (Fig. 1C). However, paeonol 100 μ M had no significant effect on the cell viability of normal endometrial stromal cells and IOSE80 cells (Supplementary Fig. 1A). Lower EdU staining of ecESCs under paeonol condition (Fig. 1D) further demonstrated the anti-proliferative role of paeonol on ecESCs.

4.2 Paeonol repressed cell migration and invasion of ecESCs

The role of paeonol on metastasis of endometriosis was then investigated. Data from wound healing (Fig. 2A) and transwell (Fig. 2B) assays demonstrated that cell migration and invasion of ecESCs were dosage dependently repressed by paeonol treatment, suggesting the anti-invasive role of paeonol on ecESCs.

4.3 Paeonol repressed autophagy of ecESCs

To explore role of paeonol in autophagy of ecESCs, indicators of autophagy, including LC3B, Beclin and p62, were examined. As shown in Fig. 3A, paeonol treatment dosage dependently induced decrease in LC3-II and Beclin, and increase in p62, suggesting that paeonol repressed autophagy of ecESCs. Moreover, there was a gradual decrease in the immunofluorescence of LC3 in ecESCs (Fig. 3B), further confirming that paeonol could induce suppression of autophagic flux in ecESCs.

4.4 Paeonol repressed HIF-1 α in ecESCs

Hypoxia-induced HIF-1 α was shown to be positively correlated with autophagy in ectopic endometrium. Expression level of HIF-1 α was determined in ecESCs post paeonol treatment. There was a gradual decrease in the mRNA (Fig. 4A) and protein (Fig. 4B) expression of HIF-

1 α in ecESCs post paeonol treatment, further confirming the suppressive role of paeonol on HIF-1 α expression in ecESCs.

4.5 Paeonol repressed autophagy of ecESCs through down-regulation of HIF-1 α

The ecESCs transfected with pcDNA-mediated HIF-1 α over-expression were treated with 50 μ M paeonol to examine the effect of HIF-1 α on paeonol-mediated autophagy in ecESCs. The transfection efficiency of pcDNA-mediated HIF-1 α over-expression was shown in **Supplementary Fig. 1B**. Over-expression of HIF-1 α attenuated paeonol-induced decrease in LC3-II and Beclin-1, and the increase in p62 (Fig. 5A), suggesting that paeonol repressed autophagy of ecESCs through down-regulation of HIF-1 α . Moreover, the decrease in the immunofluorescence of LC3 was reversed by in pcDNA-mediated HIF-1 α over-expression in ecESCs (Fig. 5B), further confirming that paeonol could induce suppression of autophagic flux in ecESCs through down-regulation of HIF-1 α .

4.6 Paeonol repressed migration and invasion of ecESCs through suppression of autophagy

The ecESCs transfected with pcDNA-mediated HIF-1 α over-expression were treated with 50 μ M paeonol to examine the effect of HIF-1 α on paeonol-mediated migration and invasion of ecESCs. Over-expression of HIF-1 α counteracted with the suppressive effect of paeonol on cell viability of ecESCs (Fig. 6A). The promoted cell apoptosis of ecESCs by paeonol was also reversed by pcDNA-mediated HIF-1 α over-expression (Fig. 6B). Moreover, over-expression of HIF-1 α also counteracted with the suppressive effect of paeonol on cell migration (Fig. 6C) and invasion (Fig. 6D) of ecESCs. These results showed that paeonol repressed migration and invasion of ecESCs through suppression of autophagy.

4.7 Paeonol ameliorated endometriotic lesions of rats in a HIF-1 α dependent manner

To investigate role of paeonol in *in vivo* endometriosis, endometriotic rats were established through transplantation of uterine tissue towards intestine mesentery. Histopathological analysis by H&E staining showed hallmarks of endometriosis in the endometriotic rats, as demonstrated by perforation of stroma, increased number of glands and thickening walls of endometrial glands compared with the sham group (Fig. 7A). In addition, the number and thickened walls of glands were reduced by paeonol (Fig. 7A). Serum levels of IL-1 β , IL-6 and TNF- α in endometriotic rats were down-regulated by paeonol (Fig. 7B). Paeonol attenuated endometriosis-induced increase in HIF-1 α , Beclin-1 and p62 in the rats (Fig. 7C), suggesting that paeonol ameliorated endometriotic lesions of rats in a HIF-1 α dependent manner.

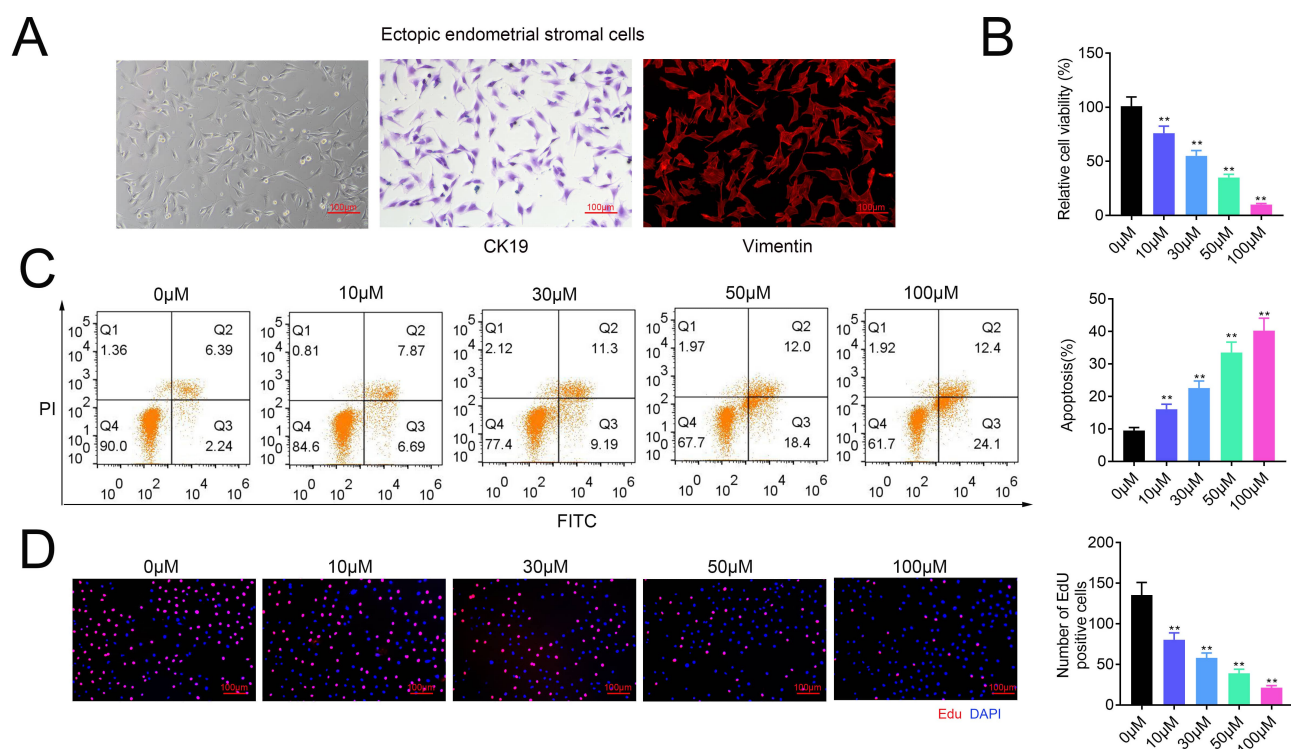


Fig. 1. Paeonol repressed cell proliferation of ecESCs.

(A) Isolated ecESCs showed spindle shape, negative staining of CK19 and positive staining of vimentin. Scale bar: 100 μ m.

(B) Paeonol dosage dependently reduced cell viability of hypoxia-induced ecESCs. N = 3.

(C) Paeonol dosage dependently promoted cell apoptosis of hypoxia-induced ecESCs. N = 3.

(D) Paeonol dosage dependently reduced cell proliferation of hypoxia-induced ecESCs. Scale bar: 100 μ m. N = 3. ** $p < 0.01$.

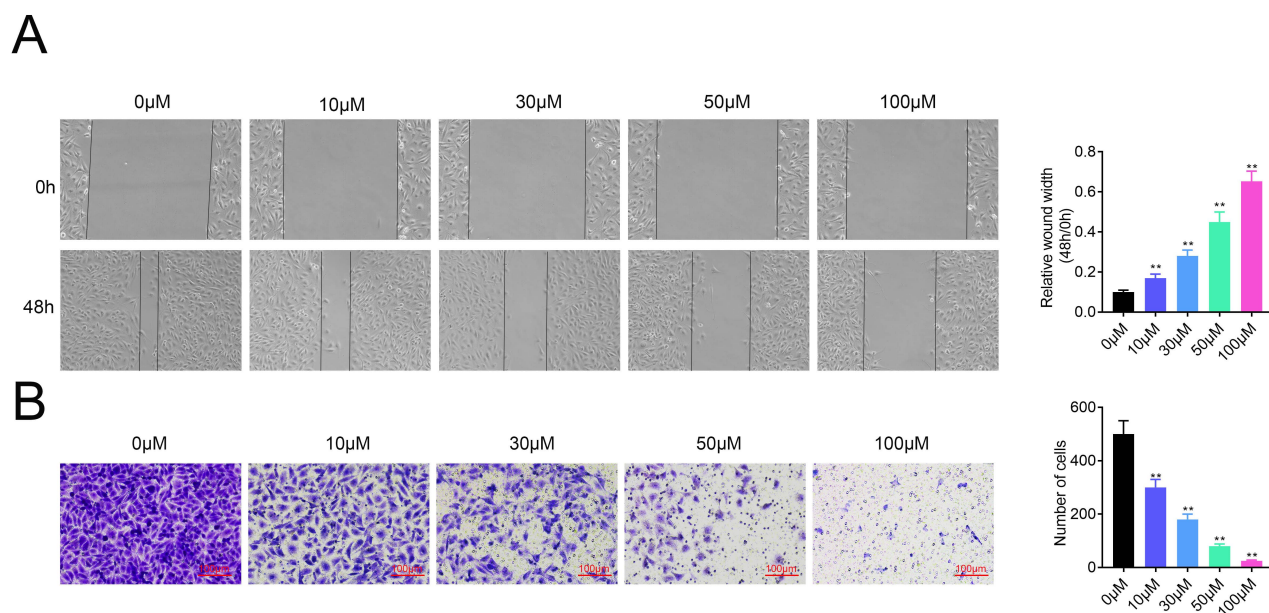


Fig. 2. Paeonol repressed cell migration and invasion of ecESCs.

(A) Paeonol dosage dependently reduced cell migration of hypoxia-induced ecESCs. N = 3. Scale bar: 100 μ m.

(B) Paeonol dosage dependently reduced cell invasion of hypoxia-induced ecESCs. Scale bar: 100 μ m. N = 3. ** $p < 0.01$.

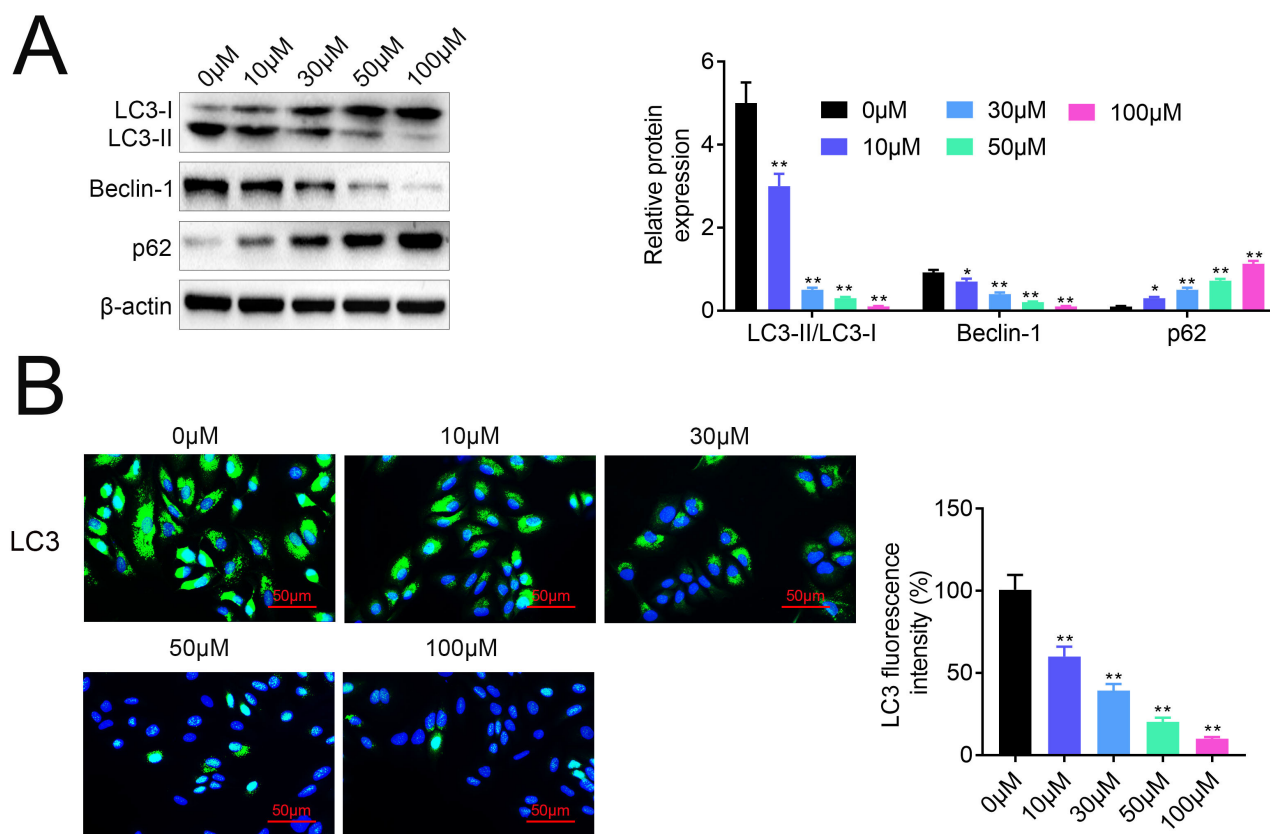


Fig. 3. Paeonol repressed autophagy of ecESCs.

(A) Paeonol dosage dependently reduced LC3-II and Beclin, while increase of p62 in hypoxia-induced ecESCs. N = 3.

(B) Paeonol dosage dependently reduced LC3 through immunofluorescence analysis. N = 3. * $p < 0.05$.

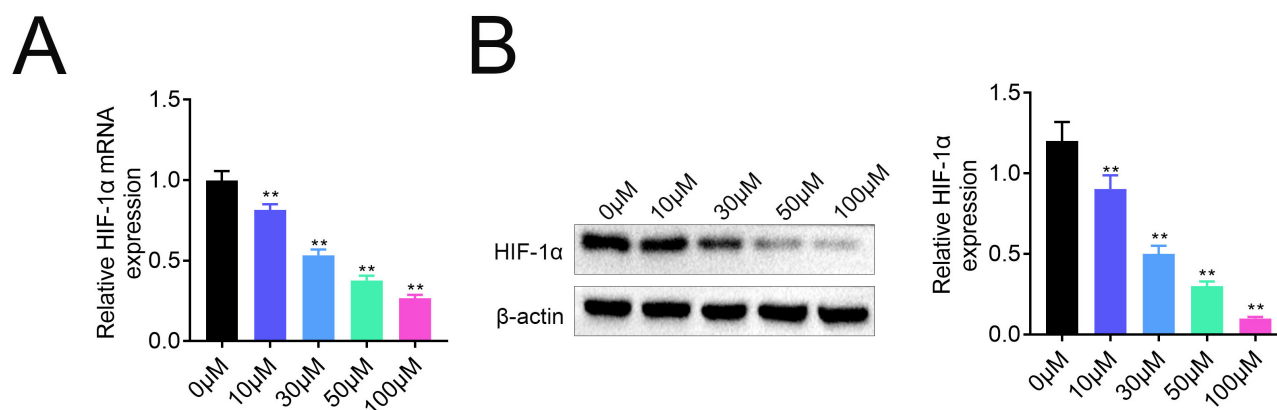


Fig. 4. Paeonol repressed HIF-1 α in ecESCs.

(A) Paeonol dosage dependently reduced mRNA expression of HIF-1 α in hypoxia-induced ecESCs. N = 3.

(B) Paeonol dosage dependently reduced protein expression of HIF-1 α in hypoxia-induced ecESCs. Scale bar: 100 μ m. N = 3. ** $p < 0.01$.

5. Discussion

Previous study has shown that the dysregulation of endometrial stromal cells leads to endometriotic lesions during the development of endometriosis [5]. Endometriosis was found to be related to increased risk of clear-cell carcinoma and other malignancies [18]. Impaired autophagy

was found to be closely associated with stage and prognosis of endometriosis, and mediated the cell proliferation, apoptosis, migration and invasion of endometrial cells [19]. Inhibition of autophagy contributed to suppression of endometrial cell migration and invasion [20]. Since paeonol promoted cytoprotective autophagy in ovarian cancer cells

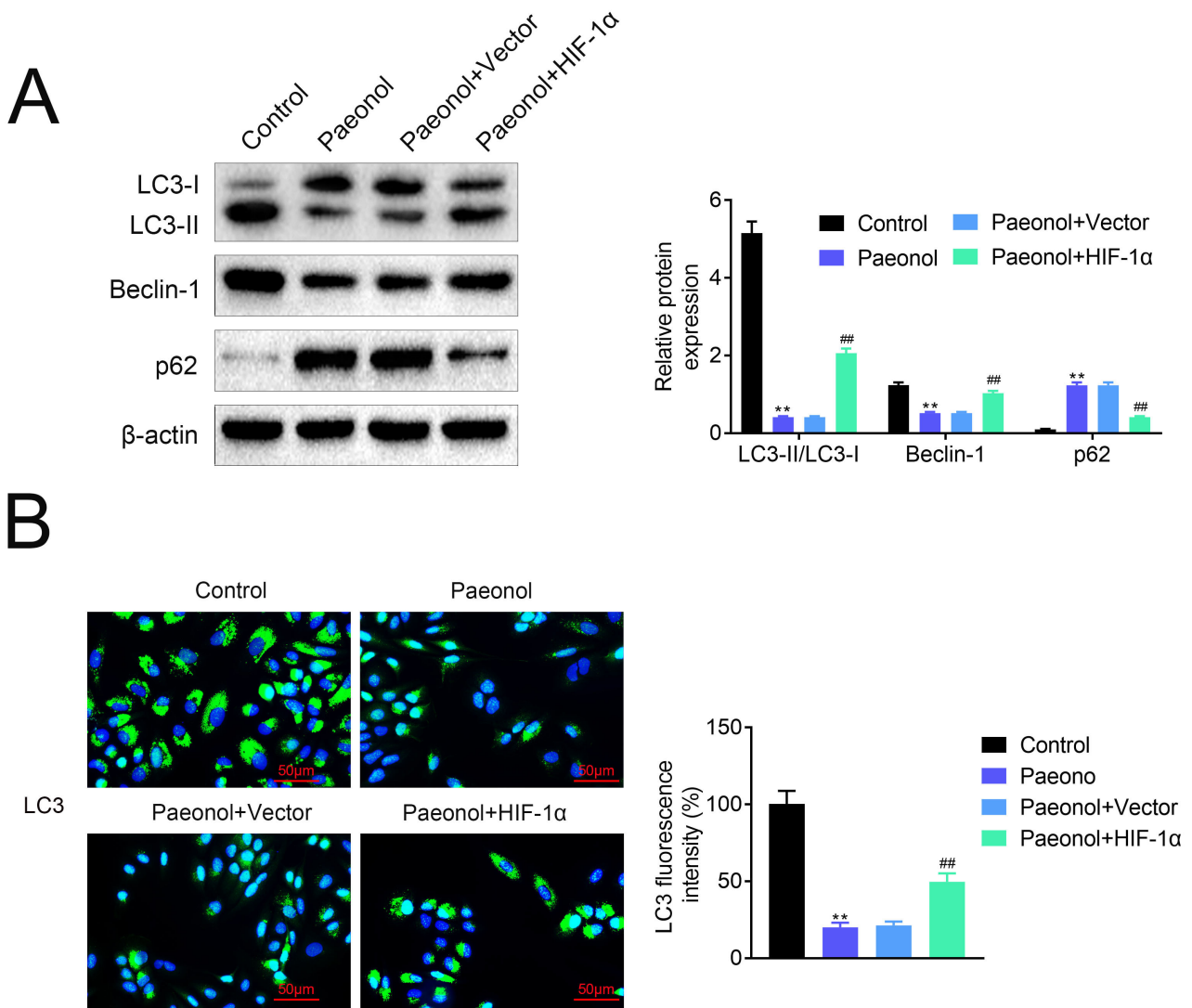


Fig. 5. Paeonol repressed autophagy of ecESCs through down-regulation of HIF-1α.

(A) Over-expression of HIF-1α attenuated paeonol-induced decrease of LC3-II and Beclin-1, and the increase of p62 in hypoxia-induced ecESCs. $N = 3$. (B) Over-expression of HIF-1α attenuated paeonol-induced decrease of LC in hypoxia-induced ecESCs through immunofluorescence analysis. Scale bar: 100 μm . $N = 3$. **, ## $p < 0.01$.

[15], and suppressed the oxidized low-density lipoprotein-induced vascular endothelial cell autophagy [17], the functional role of paeonol in autophagy, migration and invasion of endometrial stromal cells was investigated in this study.

The increase in endometrial stromal cell migration and invasion contributed to epithelial-to-mesenchymal transition in endometriosis [21]. Suppression of endometrial stromal cell migration and invasion could ameliorate endometriotic lesions [22]. Results in this study indicated that paeonol treatment decreased cell viability, proliferation, migration and invasion of ecESCs, and promoted the cell apoptosis. *In vivo* endometriosis showed that paeonol ameliorated hallmarks of endometriosis in the endometriotic rats, suggesting that paeonol might ameliorate endometriotic lesions during the development of endometriosis.

Since epithelial-to-mesenchymal transition was considered as the original establishment of endometriotic lesions [23], the effects of paeonol on epithelial-to-mesenchymal transition of ecESCs should be investigated in the further study.

Autophagy was found to be promoted in ovarian endometriosis [8], and the induction of autophagy resulted in abnormal apoptosis during development of endometriosis [24]. Expression of HIF-1α and LC3 were reduced in eutopic endometrium in women with and without endometriosis compared with ectopic endometrial lesions [25], while hypoxia induced increase in HIF-1α, LC3 and Beclin-1, and decrease in p62 [9]. Our results demonstrated that HIF-1α expression was reduced in ecESCs under paeonol treatment, and hypoxia-induced autophagy

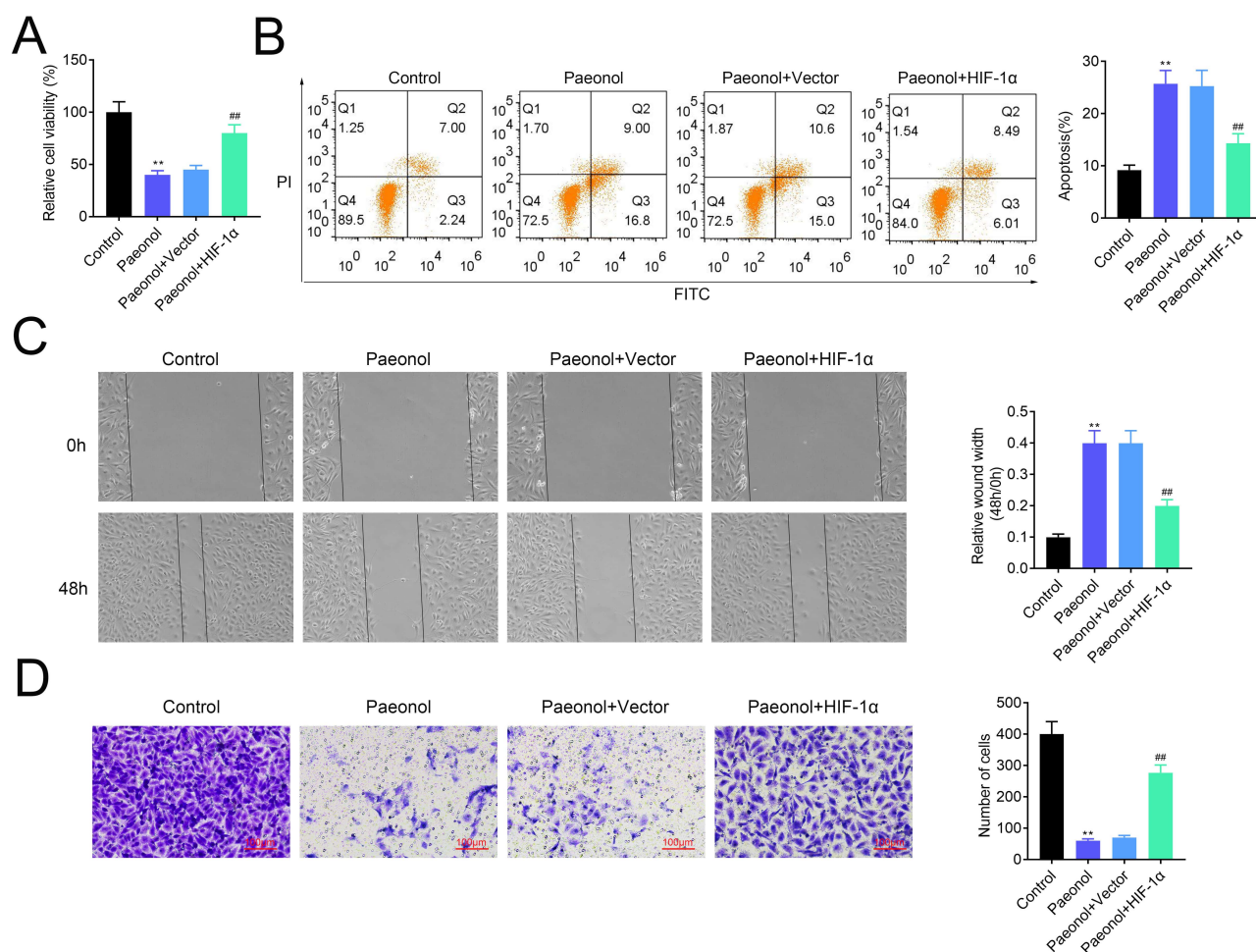


Fig. 6. Paeonol repressed migration and invasion of eCSCs through suppression of autophagy.

(A) Over-expression of HIF-1 α attenuated paeonol-induced decrease of cell viability in hypoxia-induced eCSCs through immunofluorescence analysis. N = 3.

(B) Over-expression of HIF-1 α attenuated paeonol-induced increase of cell apoptosis in hypoxia-induced eCSCs through immunofluorescence analysis. N = 3.

(C) Over-expression of HIF-1 α attenuated paeonol-induced decrease of cell migration in hypoxia-induced eCSCs through immunofluorescence analysis. N = 3. Scale bar: 100 μ m.

(D) Over-expression of HIF-1 α attenuated paeonol-induced decrease of cell invasion in hypoxia-induced eCSCs through immunofluorescence analysis. Scale bar: 100 μ m. N = 3. **, ## $p < 0.01$.

was also ameliorated by paeonol treatment through down-regulation of LC3 and Beclin-1, and up-regulation of p62, suggesting that paeonol suppressed autophagy during the development of endometriosis to ameliorate endometriotic lesions. However, previous research also showed that autophagy was reduced in eCSCs compared with the normal women [26], and the enhanced autophagy in eCSCs could retard the ectopic lesions through stimulation of cytotoxic activity of NK cells [27]. The protective effect of paeonol against autophagy in eCSCs might dependent on the immune microenvironment and individual characteristic of the patients.

HIF-1 α was reported to promote the development of endometriosis through induction of epithelial-

mesenchymal transition of endometrial epithelial cells [28]. Endometrial stromal cells migration and invasion [9], as well as autophagy and epithelial-mesenchymal transition [29], were also provoked to participate in the pathophysiology of endometriosis. Moreover, HIF-1 α promoted autophagy to facilitate for the migration and invasion of endometrial stromal cells [9], and hypoxia-induced pro-survival autophagy was suppressed by knockdown of lncRNA MALAT1 to enhance the cell apoptosis [30]. pcDNA-mediated HIF-1 α over-expression in eCSCs attenuated paeonol-induced increase in cell apoptosis, and decrease in cell viability, proliferation, migration and invasion, suggesting that paeonol repressed migration and invasion of eCSCs through suppression of autophagy.

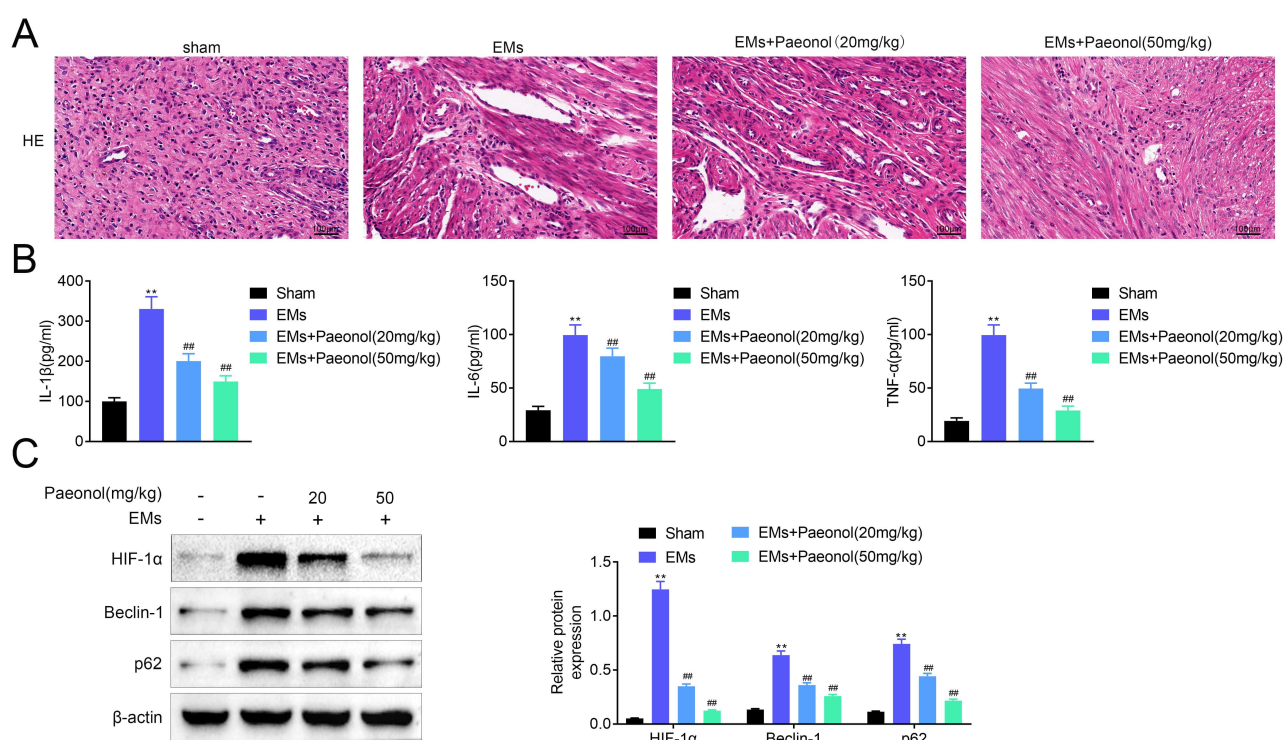


Fig. 7. Paeonol ameliorated endometriotic lesions of rats in a HIF-1 α dependent manner.

(A) H&E staining showed that paeonol administration decreased number of glands and reduced thickened walls of glands in endometriotic rats. Scale bar: 100 μ m. N = 3.

(B) Paeonol administration attenuated endometriosis-induced increase in serum levels of IL-1 β , IL-6 and TNF- α . N = 3.

(C) Paeonol administration attenuated endometriosis-induced increase in HIF-1 α , Beclin-1 and p62 in the rats. N = 3. **, ## $p < 0.01$.

Paeonol attenuated endometriosis-induced increase in HIF-1 α , Beclin-1 and p62 in the rats, suggesting that paeonol ameliorated endometriotic lesions of rats in a HIF-1 α -dependent manner. In addition, inflammatory response was reported to participate in the progression of endometriosis through migration and invasion of endometrial cells in the ectopic regions [31], and paeonol could attenuate the inflammation [32]. Serum levels of IL-1 β , IL-6 and TNF- α in endometriotic rats were down-regulated by paeonol in this study, suggesting that paeonol might exert anti-inflammatory effect against endometriosis.

6. Conclusions

In general, our results showed that paeonol repressed migration and invasion of eECs through attenuation of hypoxia-triggered autophagy, and ameliorated histopathological changes and inflammatory response of endometriotic rats in a HIF-1 α dependent manner, suggesting that paeonol might serve as a novel therapeutic strategy for the treatment of endometriosis. Moreover, according previous study [33], paeonol might be intraperitoneally introduced into the patients with endometriosis.

7. Author contributions

CP and ZW designed the study, supervised the data collection, XX analyzed the data, interpreted the data, WY, XW and YQ prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

8. Ethics approval and consent to participate

Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article. All animal care and treatments were done as approved by Animal Care Committee, the Hospital Affiliated to Shandong University of Traditional Chinese Medicine.

9. Acknowledgment

Thanks to all the peer reviewers for their opinions and suggestions.

10. Funding

This work was supported by the Project of Shandong Traditional Chinese Medicine Administration (Grant No. 2019-0116) and National Natural Science Foundation of China (Grant No. 81774355).

11. Conflict of interest

The authors declare no conflict of interest.

12. Availability of data and materials

All data generated or analyzed during this study are included in this published article.

13. References

- [1] Chantalat E, Valera M-C, Vaysse C, Noirrit E, Rusidze M, Weyl A, *et al.* Estrogen Receptors and Endometriosis. *International Journal of Molecular Sciences*. 2020; 21: 2815.
- [2] Vercellini P, Viganò P, Somigliana E, Fedele L. Endometriosis: pathogenesis and treatment. *Nature Reviews Endocrinology*. 2014; 10: 261–275.
- [3] Hirsch M, Begum MR, Paniz É, Barker C, Davis CJ, Duffy JMN. Diagnosis and management of endometriosis: a systematic review of international and national guidelines. *BJOG: An International Journal of Obstetrics & Gynaecology*. 2018; 125: 556–564.
- [4] Kajiyama H, Suzuki S, Yoshihara M, Tamauchi S, Yoshikawa N, Niimi K, *et al.* Endometriosis and cancer. *Free Radical Biology and Medicine*. 2019; 133: 186–192.
- [5] Zhang M, Zhang Y, Li L, Ma L, Zhou C. Dysregulation of miR-202-3p Affects Migration and Invasion of Endometrial Stromal Cells in Endometriosis via Targeting ROCK1. *Reproductive Sciences*. 2020; 27: 731–742.
- [6] Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. *Nature*. 2008; 451: 1069–1075.
- [7] Zhan L, Li J, Wei B. Autophagy in endometriosis: Friend or foe? *Biochemical and Biophysical Research Communications*. 2018; 495: 60–63.
- [8] Allavena G, Carrarelli P, Del Bello B, Luisi S, Petraglia F, Maelaro E. Autophagy is upregulated in ovarian endometriosis: a possible interplay with p53 and heme oxygenase-1. *Fertility and Sterility*. 2015; 103: 1244–1251.e1241.
- [9] Liu H, Zhang Z, Xiong W, Zhang L, Xiong Y, Li N, *et al.* Hypoxia-inducible factor-1 α promotes endometrial stromal cells migration and invasion by upregulating autophagy in endometriosis. *Reproduction*. 2017; 153: 809–820.
- [10] Sajdak S, Markowska A, Krygowska-Zielińska J, Szarszewska M, Witek A, Olejek A, *et al.* Effect of metformin on clinical-pathological variables in women with endometrial cancer. A multicenter study. *European Journal of Gynaecological Oncology*. 2019; 40: 775–780.
- [11] Zhang L, Li DC, Liu LF. Paeonol: pharmacological effects and mechanisms of action. *International Immunopharmacology*. 2019; 72: 413–421.
- [12] Liu LH, Shi RJ, Chen ZC. Paeonol exerts anti-tumor activity against colorectal cancer cells by inducing G0/G1 phase arrest and cell apoptosis via inhibiting the Wnt/ β -catenin signaling pathway. *International Journal of Molecular Medicine*. 2020; 46: 675–684.
- [13] Zhang J, Cai L, Pang K, Dong Y, Zhang Z, Li B, *et al.* Paeonol inhibits proliferation and induces cell apoptosis of human T24 and 5637 bladder cancer cells *in vitro* and *in vivo*. *Clinical and Translational Oncology*. 2021; 23: 601–611.
- [14] Zhou H-M, Sun Q-X, Cheng Y. Paeonol enhances the sensitivity of human ovarian cancer cells to radiotherapy-induced apoptosis due to downregulation of the phosphatidylinositol-3-kinase/Akt/phosphatase and tensin homolog pathway and inhibition of vascular endothelial growth factor. *Experimental and Therapeutic Medicine*. 2017; 14: 3213–3220.
- [15] Gao L, Wang Z, Lu D, Huang J, Liu J, Hong L. Paeonol induces cytoprotective autophagy via blocking the Akt/mTOR pathway in ovarian cancer cells. *Cell Death and Disease*. 2019; 10: 609–609.
- [16] Wu H, Song A, Hu W, Dai M. The Anti-atherosclerotic Effect of Paeonol against Vascular Smooth Muscle Cell Proliferation by Up-regulation of Autophagy via the AMPK/mTOR Signaling Pathway. *Frontiers in Pharmacology*. 2018; 8: 948.
- [17] Li C, Yang L, Wu H, Dai M. Paeonol Inhibits Oxidized Low-Density Lipoprotein-Induced Vascular Endothelial Cells Autophagy by Upregulating the Expression of miRNA-30a. *Frontiers in Pharmacology*. 2018; 9: 95.
- [18] Santoro A, Angelico G, Inzani F, Spadola S, Arciuolo D, Valente M, *et al.* The Many Faces of Endometriosis-Related Neoplasms in the Same Patient: A Brief Report. *Gynecologic and Obstetric Investigation*. 2020; 85: 371–376.
- [19] Erekat NS. Autophagy precedes apoptosis among at risk cerebellar Purkinje cells in the shaker mutant rat: an ultrastructural study. *Ultrastructural Pathology*. 2018; 42: 162–169.
- [20] Li Y, Wang X, Wang X, Wan L, Liu Y, Shi Y, *et al.* PDCD4 suppresses proliferation, migration, and invasion of endometrial cells by inhibiting autophagy and NF- κ B/MMP2/MMP9 signal pathway†. *Biology of Reproduction*. 2018; 99: 360–372.
- [21] Li J, Ma J, Fei X, Zhang T, Zhou J, Lin J. Roles of cell migration and invasion mediated by Twist in endometriosis. *Journal of Obstetrics and Gynaecology Research*. 2019; 45: 1488–1496.
- [22] Zhong H, Hao L, Li X, Wang C, Wu X. Anti-inflammatory Role of Trilobatin on Lipopolysaccharide-induced Acute Lung Injury through Activation of AMPK/GSK3 β -Nrf2 Pathway. *Signa Vitae*. 2020; 16: 160–166.
- [23] Konrad L, Dietze R, Riaz MA, Scheiner-Bobis G, Behnke J, Horné F, *et al.* Epithelial-Mesenchymal Transition in Endometriosis-When Does It Happen? *Journal of Clinical Medicine*. 2020; 9: 1915.
- [24] Choi J, Jo M, Lee E, Kim HJ, Choi D. Differential induction of autophagy by mTOR is associated with abnormal apoptosis in ovarian endometriotic cysts. *Molecular Human Reproduction*. 2014; 20: 309–317.
- [25] Liu H, Du Y, Zhang Z, Lv L, Xiong W, Zhang L, *et al.* Autophagy contributes to hypoxia-induced epithelial to mesenchymal transition of endometrial epithelial cells in endometriosis. *Biology of Reproduction*. 2018; 99: 968–981.
- [26] Rhee JS, Saben JL, Mayer AL, Schulte MB, Asghar Z, Stephens C, *et al.* Diet-induced obesity impairs endometrial stromal cell decidualization: a potential role for impaired autophagy. *Human Reproduction*. 2016; 31: 1315–1326.
- [27] Mei J, Zhou W-J, Zhu X-Y, Lu H, Wu K, Yang H-L, *et al.* Suppression of autophagy and HCK signaling promotes PTGS2(high) FCGR3(-) NK cell differentiation triggered by ectopic endometrial stromal cells. *Autophagy*. 2018; 14: 1376–1397.
- [28] Xiong Y, Liu Y, Xiong W, Zhang L, Liu H, Du Y, *et al.* Hypoxia-inducible factor 1 α -induced epithelial–mesenchymal transition of endometrial epithelial cells may contribute to the development of endometriosis. *Human Reproduction*. 2016; 31: 1327–1338.
- [29] Foster WG. Hypoxia-induced autophagy, epithelial to mesenchymal transition, and invasion in the pathophysiology of endometriosis: a perspective. *Biology of Reproduction*. 2018; 99: 905–906.

- [30] Liu H, Zhang Z, Xiong W, Zhang L, Du Y, Liu Y, *et al.* Long non-coding RNA MALAT1 mediates hypoxia-induced pro-survival autophagy of endometrial stromal cells in endometriosis. *Journal of Cellular and Molecular Medicine*. 2019; 23: 439–452.
- [31] Wu M-H, Hsiao K-Y, Tsai S-J. Endometriosis and possible inflammation markers. *Gynecology and Minimally Invasive Therapy*. 2015; 4: 61–67.
- [32] Mei L, He M, Zhang C, Miao J, Wen Q, Liu X, *et al.* Paeonol attenuates inflammation by targeting HMGB1 through upregulating miR-339-5p. *Scientific Reports*. 2019; 9: 19370.
- [33] Zhang H, Geng X, Li Z, Li Y, Xu K, Wu H, *et al.* Paeonol at certain doses alleviates aggressive and anxiety-like behaviours in two premenstrual dysphoric disorder rat models. *Frontiers in Psychiatry*. 2020; 11: 295.

Supplementary material: Supplementary material associated with this article can be found, in the online version, at <https://www.fbscience.com/Landmark/articles/10.52586/4961>.

Abbreviations: CK19, cytoskeleton 19; ecESCs, ectopic endometrial stromal cells; HIF-1 α , Hypoxia-inducible factor-1 α .

Keywords: Paeonol; Metastasis; Endometrial stromal cells; HIF-1 α ; Autophagy; Endometriosis

Send correspondence to: Yinghua Qi, Center of Reproduction and Genetics Integrated Traditional Chinese and Western Medicine, The Hospital Affiliated to Shandong University of Traditional Chinese Medicine, 250014 Jinan, Shandong, China, E-mail: qiyinghuaok@126.com