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MATERNAL-FETAL MEDICINE

Retinoic acid regulates endometriotic stromal cell growth through upregulation of Beclin1

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Abstract
Purpose To elucidate the role of retinoic acid (RA) in autophagy-mediated endometriosis.
Methods The mRNA and protein expressions of autophagy markers were examined in Ishikawa cells and endometriotic stromal cells (ESCs) after RA treatment. Beclin1 expression was specifically analyzed in clinical samples of endometriosis. The effect of Beclin1 knockdown on ESC growth was assessed, and the effect of autophagy inhibition on the sensitivity of endometriotic cells to RA was analyzed.
Results RA treatment enhanced the autophagy in ESCs, and Beclin1 expression showed a negative correlation with the clinical stage of endometriosis. Beclin1 knockdown enhanced ESC growth, whereas RA treatment reversed this effect. Furthermore, inhibition of autophagy by chloroquine (CQ) and Beclin1 knockdown did not show any positive effect on the sensitivity of endometriotic cells to RA.
Conclusions RA treatment induces autophagy and Beclin1 may play an important role in endometriosis progression.

Keywords Retinoic acid · Autophagy · Endometriosis · Beclin1

Introduction

Endometriosis refers to the condition that endometrial tissues (glands and stroma) grow outside of uterine cavity. It is a common disease typically associated with chronic pain. Clinical features include irregular menstruation [1] and infertility, as high as 40% of infertility rate [2]. Its exact pathogenesis remains unclear, and so far, the most accepted hypothesis about endometrial implantation supports Sampson's hypothesis [3] that the exfoliated endometrial tissue from uterus is shed through the fallopian tubes and reaches the peritoneal cavity during menstruation [4–6]. The shed endometrial cells can survive, implant, grow and invade at ectopic locations and eventually develop into endometriotic lesions [5]. In addition, the ectopic endometrial cells display a high invasive capability.

Autophagy is a process by which the cytoplasmic components of the cell are degraded and recycled for energy generation. It is also described as self-eating and is an important process in the development of multiple diseases. To date, approximately 30 yeast genes and 16 human genes have been identified as autophagy-related genes (ATGs). Among these, Beclin1, a mammal homologous gene of yeast autophagy-related genes Atg6/Vps30, has been shown to play a key role in the mammalian autophagy process [7, 8] as an important protein for autophagosome formation [9] and to be expressed in endometriotic cells. Another protein, p62, autophagy degradation-related protein [10], can also be used as an autophagy marker protein [11] and accumulates in cells when autophagy occurs improperly. Similarly, ATG3, as a key ubiquitin-conjugated enzyme, is involved in the function of the ATG12–ATG5 complex in ubiquitin–proteasome system during autophagosome formation [12] and plays an important role in LC3 (microtubule-associated protein 1 light chain 3) protein maturation [13].

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Retinoic acid (RA) is a metabolite of natural vitamin A and has been shown to be involved in the regulation of cell proliferative disorders and to affect vascular endothelial growth factor (VEGF) expression in malignant tumors. A recent study indicated that RA is involved in hormonal regulation in endometrial cells as well as gene expression in mesenchymal glandular epithelial cells [14]. However, the exact mechanism and functions of RA in endometriosis remain incompletely understood, and it is not known whether it can act as a potential therapeutic target.

To the best of our knowledge, the roles of autophagy especially Beclin1 in the induction of endometriosis have not yet been investigated, and its correlation with clinicopathological characteristics remains to be identified. Furthermore, the role of RA in endometriosis and its relationship to Beclin1 are unclear. Thus, the overall goals of our study were to: (a) clarify the role of autophagy in RA-mediated endometriosis, (b) explore the relationship between Beclin1 expression and RA in endometriotic cells, and (c) investigate whether autophagy regulation can enhance RA sensitivity.

Materials and methods

Cell culture

Endometriotic stromal cells (ESCs) were obtained from endometriosis patients aged 25–40 years via hysterectomy during the proliferative phase. Normal endometrium tissues were collected from infertility patients with a regular menstrual cycle via diagnostic curettage. None of the patients received any hormonal medication for at least 3 months prior to ESC collection. The Ishikawa cell line (human endometrial cancer cells) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RPMI-1640 medium (HyClone Laboratories, Inc., Logan, UT, USA) containing 10% newborn calf serum (Lanzhou Minhai Bioengineering Co., Ltd., Gansu, China) in an incubator at 37 °C with 5% CO_2. Cells in the exponential growth phase were used for all experiments.

Quantitative RT-PCR

Total RNA was extracted from 100 mg of tissue using TRIzol reagent (Invitrogen Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instruction. The cDNA was synthesized using AMV Reverse Transcriptase (Promega Biotech, Beijing, China) from 1 μg total RNA. The thermal cycling conditions involved an initial step of denaturation at 95 °C for 30 s and then 40 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, and elongation at 72 °C for 1 min, on a PCR machine (Mastercycler Realplex, Eppendorf, Hamburg, Germany). Subsequently, the RT-qPCR results were analyzed using the $2^{-\Delta\Delta Ct}$ method. The following primer pairs were used for amplification of different genes.

GAPDH:	5′-GGTCTCCTCTGACTTCAACA-3′
	5′-AGCC AAATTCGTTGTCATAC-3′
LC3- II:	5′-AGTCCAATGCTCCAGACTTGG-3′
	5′-TAGCTGAAGTGGCATGTCTATC-3′
Beclin-1:	5′-GCTCCATGCTTTGGCCAATAAC-3′
	5′-ATGGTCAAACTTGTTGTCCCAG-3′
P62:	5′-TCAGTCTCTGACAGAGCAAATG-3′
	5′-CGATTCTGGCATCTGTAGAGAC-3′
ATG3:	5′-AAGGGAAAGGCACTGGAAGT-3′
	5′-GTGATCTCCAGCTGCCACAA-3′

Western blotting

The proteins (30 μg) isolated from the cells were separated by electrophoresis on a 10% sodium dodecyl sulfate (SDS) gel and then transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked for 3 h in Tris-buffered saline containing 0.1% Tween-20 (TBST; 50 mM Tris-Cl, pH 7.5, and 150 mM NaCl) and 3% bovine serum albumin (BSA), and subsequently incubated overnight at 4 °C with a primary antibody against Beclin-1 (sc-11427, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at a dilution of 1:500. The membrane was then washed three times with TBST and incubated with an appropriate horseradish peroxidase-conjugated secondary antibody (anti-mouse IgG, 1:5000; Cell Signaling Technology, Danvers, MA, USA). Finally, the protein bands were visualized using an enhanced chemiluminescence (ECL) reagent (Thermo Fisher Scientific, Waltham, MA, USA).

siRNA knockdown

The small interfering (si) RNAs against Beclin1 and the control siRNA were purchased from Cell Signaling Technology. Cells plated in 96-well plates were transfected with siRNAs (20 nM) using Lipofectamine 2000 reagent. The cells were collected after 48 h of transfection for Western blotting to detect Beclin1 protein to verify the knockdown efficiency.

Cell proliferation

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cell growth assay (Sigma-Aldrich) was used to assess cell proliferation after transfection with Beclin1 or control siRNAs, according to the manufacturer's instructions. The cytotoxicity of RA was also measured using a MTT colorimetric assay. Data are presented

as mean ± standard deviation (SD) of triplicate experiments and expressed as a percentage of the DMSO control. RA was purchased from Enzo Life Sciences (Farmingdale, NY, USA), and chloroquine disphosphate was purchased from Sigma-Aldrich.

Statistical analysis

All statistical comparisons were performed using *t* test with SPSS version 21.0 software (IBM Corp., Armonk, NY, USA). $p < 0.05$ represented a statistically significant difference.

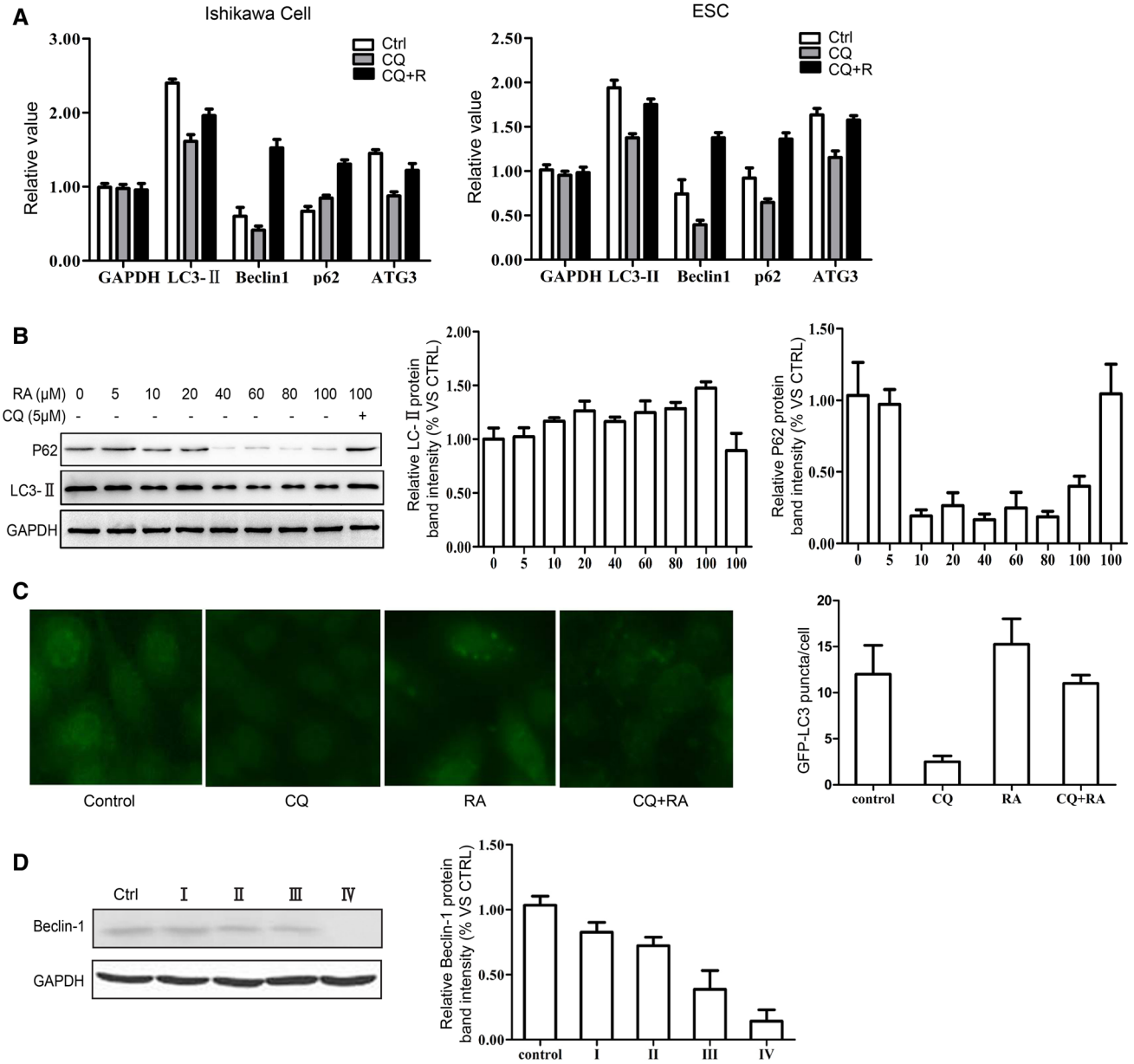

Fig. 1 Effects of RA treatment on autophagy in endometrial cells. **a** Ishikawa cells and ESCs were treated with various concentrations of CQ and RA for 24 h, and mRNA expression of LC3-II, Beclin1, p62, ATG3 and GAPDH was measured by RT-qPCR. **b** ESCs were treated with CQ and different concentrations of RA, and protein expression of p62 and LC3-II was assessed by normalization to GAPDH. **c** ESCs were transfected with a GFP-LC3 plasmid and then treated with the indicated concentrations of RA and CQ for 48 h before observation under fluorescence microscopy. GFP-LC3 puncta were counted in 50 cells per sample, and the average number of puncta per cell was plotted. The bars represent the mean ± standard deviation (SD) of triplicate experiments. **d** Tissue lysates from normal endothelium and clinical samples of different grades of endometriosis (stage I, II, III, and IV) were analyzed for Beclin1 and GAPDH expression. The data represent the normalized Beclin1 protein expression from triplicate experiments

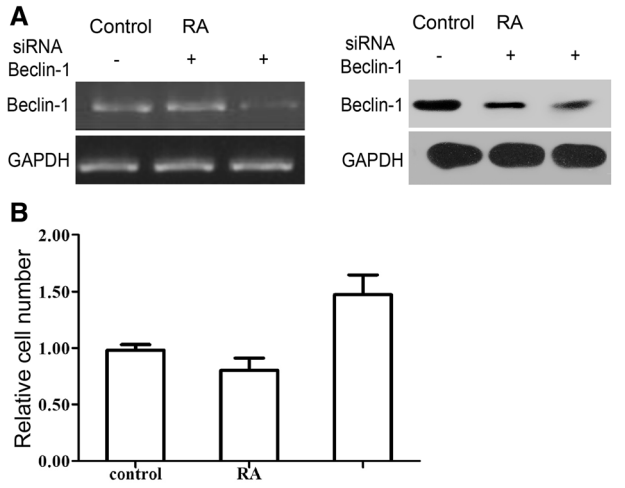

Fig. 2 Effect of Beclin1 knockdown on ESC growth. After transfection with control or Beclin1 siRNA, ESCs were treated with RA for 96 h. **a** The expression of Beclin1 and GAPDH mRNA (RT-qPCR) and protein (western blotting) was assessed. **b** The effect of Beclin1 knockdown on cell proliferation, in the presence or absence of RA, was assessed by MTT assay

Results

RA induced autophagy in endometrial cells

To determine the role of RA in the regulation of autophagy in endometriotic cells, we first treated Ishikawa cells and ESCs with different doses of RA for 24 h in the presence or absence of an autophagy inhibitor, CQ. The mRNA analysis of various autophagy markers, including LC3-II, Beclin1, and ATG3 (Fig. 1a) indicated that the CQ-mediated inhibition of their expression was rescued by RA treatment. Interestingly, the mRNA expression profile of p62 showed the opposite trend in Ishikawa cells, suggesting that RA induced autophagy in endometriotic cells. We also analyzed the expression of p62 and LC3-II at the protein levels in ESCs after treatment with various concentrations of RA and CQ. The data in Fig. 1b showed that RA treatment inhibited p62 expression in a dose-dependent manner, whereas LC3-II expression was obviously suppressed after treatment with 10–40 μM RA.

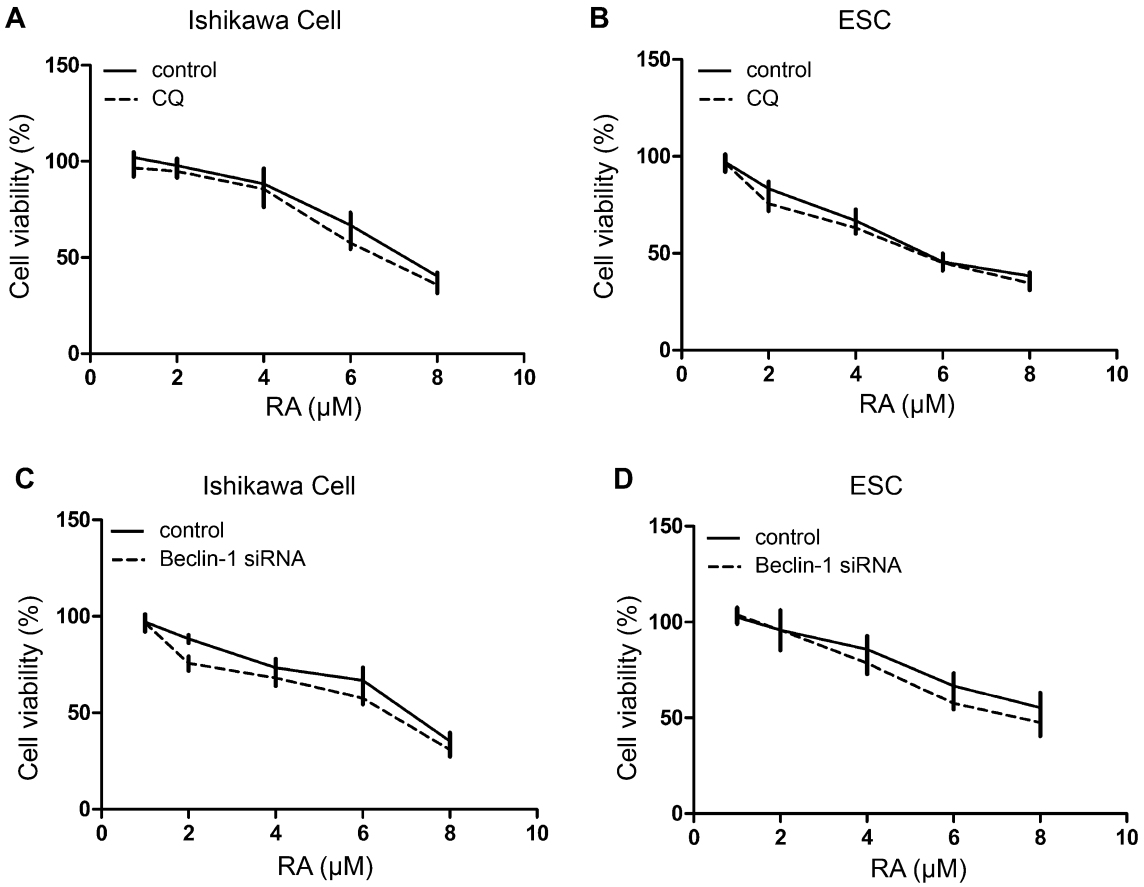

Fig. 3 Effect of autophagy inhibition on RA sensitivity in endometriosis. **a**, **b** Ishikawa cells and ESCs were treated with the indicated concentrations of RA for 48 h in the presence or absence of CQ (5 μM), and cell viability was measured using MTT assay. **c**, **d** Ishikawa cells and ESCs were treated with the indicated concentrations of RA for 48 h after control or Beclin1 siRNA treatment, and then cell viability was measured

Further, addition of CQ reversed the expression patterns of p62 and LC3-II (last lane in Fig. 1b). The stimulatory effect of RA on autophagy was further verified using a green fluorescent protein (GFP)-LC3 puncta formation assay, in which ESCs that had been transfected with a LC3 plasmid expressing GFP were treated with RA in the presence or absence of CQ. As shown in Fig. 1c, RA-treated cells showed greater numbers of puncta/cells in comparison to cells that received the control treatment. The effect of RA treatment on puncta formation was reduced by CQ treatment, whereas CQ treatment alone showed very few puncta. These data indicate that RA can increase the autophagic flux in ESCs. Moreover, we analyzed the expression of Beclin1 protein expression in clinical samples of endometriosis (Fig. 1d) and observed that it decreased with the increasing grade of clinical samples, thereby showing an inverse correlation between Beclin1 expression and higher clinical stage of endometriosis.

Beclin1 knockdown enhanced cell growth but did not affect migration or invasion of ESCs

Since we observed that Beclin1 expression was negatively associated with the clinical stage of endometriosis, we speculated that it might have a tumor suppressor role. To further analyze Beclin1 function, we ablated its expression in ESCs and examined cell growth. As shown in Fig. 2a, Beclin1 siRNA knockdown indeed resulted in significant reduction in its mRNA and protein expression. However, RA treatment restored Beclin1 expression to some extent. Cell growth analysis showed that Beclin1 knockdown promoted ESCs growth (Fig. 2b), but addition of RA to Beclin1-knockdown cells suppressed cell growth. However, Beclin1 knockdown did not show obvious effects on ESC migration or invasion (data not shown).

Autophagy inhibition did not enhance RA sensitivity of ESCs

To determine whether autophagy inhibition can enhance the sensitivity of ESCs to RA, we next analyzed the cytotoxicity of RA in ESCs and Ishikawa cells treated with various concentrations of RA for 48 h, in the presence or absence of 5 μM of the autophagy inhibitor CQ. As shown in Fig. 3a, b, no obvious differences in cell viability were observed between two conditions. Moreover, we tested RA sensitivity in both Ishikawa and ESCs after Beclin1 knockdown (Fig. 3c, d) and observed no significant difference in cell viability between the control and Beclin1 knockdown groups of both cell types.

Discussion

The detached endometrial fragments (glandular epithelial and stromal cells) have the potential to move to and grow on other tissues besides the uterine cavity. Endometriosis appears not to develop without an active process of angiogenesis via an intricate network of interactions with local pelvic immune cells and host tissue [15]. Therefore, shed endometriotic cells have been observed to behave somewhat like tumor cells, sufficient vascular supply is necessary for the development of endometriotic lesions [16]. High level of VEGF was detected in the peritoneal fluid of endometriotic patients, and its level appeared to correlate with the stage of disease [17]. Takehara et al. [18] observed increased VEGF mRNA expression in endometriotic tissue (early stage) compared with the eutopic endometrium.

Among various signaling pathways that regulate tumor progression, autophagy has been shown to play an important role. Its importance in tumor development and treatment has been well established and widely accepted. During tumor progression, the body is unable to clear away abnormally proliferated malignant cells through autophagy [19, 20]. However, the exact mechanism of autophagy in endometriosis development is not clear. Importantly, recent studies have highlighted the role of RA in endometrial cells through hormonal regulation. Thus, in the present study, we investigated whether autophagy is involved in RA-mediated endometriosis and the underlying molecular mechanisms.

Our data showed that RA treatment did induce autophagy in ESCs. We observed the increase in LC3-II, Beclin1, and ATG3 mRNA expression, and a decrease in p62 expression. Similar profiles were observed at the protein level for p62 and LC3-II expression. In addition, with a more relevant autophagy assay based on puncta formation, we observed an increase in GFP-puncta after RA treatment. As a control, treatment with CQ, an inhibitor of autophagy, led to very little puncta formation. Next, among the multiple autophagy markers, Beclin1 expression was analyzed in endometriotic clinical samples of different grades. Zhang et al. [21] showed that the expression of Beclin-1 mRNA and protein was lower in both eutopic and ectopic endometrium of patients with endometriosis than that in the control group. Interestingly, Beclin1 showed an inverse correlation with higher clinical grade, showing very low expression in higher clinical grade samples. This finding highlights the probable importance of Beclin1 in endometriosis progression. Consistent with our observation, Ren et al. [22] also reported the importance of Beclin1 in endometriosis. By RT-PCR analysis, they showed that Beclin1 mRNA expression was significantly lower in adenomyosis eutopic endometrium compared with normal endometrium and concluded that autophagy was involved to certain degree in endometrial invasion and metastasis.

Hamacher-Brady et al. [23] also indicated that patient endometrial tissues had lower Beclin1 mRNA and protein expression than normal endometrium and suggested that autophagy might play a role in the pathogenesis of endometriosis. Similarly, Li et al. [24] observed that differences in the expression of LC3-II and Beclin1 in different clinical stages of ectopic endometrial tissues. This study showed that as endometriosis was aggravated, the expression levels of these markers changed accordingly, and they also observed a negative correlation between their expression and the clinical stage of endometriosis. Both LC3-II and Beclin1 are important autophagy markers [25, 26], and detection of the LC3-II level can clearly indicate whether autophagy has been induced or inhibited. Likewise, Beclin1expression can reflect the strength of autophagy. Other markers like p62 and ATG3 also showed consistent patterns. Thus, our results indicate that endometriosis occurrence is followed by autophagy appearance, and this disease can certainly be classified as an autophagy-related disease.

Because Beclin1 expression was negatively correlated with the clinical stage of endometriosis in our study, we further analyzed its contribution to ESC growth. Beclin1 inhibition by siRNA enhanced ESC growth. More importantly, we observed that RA treatment could reverse the loss of Beclin1 expression to some extent in Beclin1 knockdown cells and blocked the increased cell growth induced by Beclin1 siRNA. Consistent with our results, an in vitro study by Katagiri et al. [27] showed that silencing of Beclin1 using siRNA enhanced cell growth in an ovarian clear cell carcinoma (OCCC) cell line. This suggested that loss of the pro-survival role of autophagy, caused by the defect in Beclin1 expression, likely contributes to tumor progression by promoting genome damage and instability in OCCC cell development [27]. However, in contrast to our observation, Deng et al. [28] inhibited autophagy either by CQ, 3-MA, or Beclin1 siRNA and observed increased cancer cell death under starvation conditions, in comparison to normal conditions. Importantly, as an autophagy regulator, Beclin1 is mono-allelically deleted in human ovarian, breast, and prostate cancers [29]. Reduced expression of Beclin1 could prevent induction of autophagy and result in promotion and development of tumor. Some studies also pointed out that autophagy induction occurred when VEGF was expressed in aortic endotheliocytes, and alteration of Beclin1 expression could significantly enhance VEGF and matrix metalloproteinase (MMP-9) expression [30–32]. These findings imply that the autophagy response can be other target to enhance the effects of angiogenesis inhibitors.

We further analyzed whether autophagy inhibition could alter the RA sensitivity of endometrial cells. Surprisingly, both CQ treatment and Beclin1 knockdown did not change the sensitivity to RA of Ishikawa and ESCs. The mechanism for the effect of RA remains unknown. Tee et al. [33] found that RA was effective in human promyelocytic leukemia cells (HL-60) that are enriched in VEGF in the inner membrane, but had no effect on VEGF in ectopic focus. RA inhibited the generation of vascular endothelial cells by regulating the interaction of vascular endothelial cells and vascular smooth muscle cells. These effects may be associated with the Tie2 signaling pathway [34]. The VEGF-VEGFR angiopoietin-Tie systems regulate different kinds of blood and lymphatic vessel growth [35]. Song et al. [36] observed that RA obviously decreased endometriosis focus volume and angiogenesis in mice through reduced expression of VEGF. Consistent with the literature [37], RA significantly reduced implantation of ectopic focus. Moreover, a study reported that RA can inhibit the role of hyperplasia in damaged blood vessels and the proliferation and adhesion of tumor and endometrium cells, but promote cell differentiation [38]. Another recent study showed that RA is involved in hormonal regulation in endometrium and plays an important role in gene expression in mesenchymal glandular epithelial cells [39]. All these studies provide strong rationale for continued research of the effects of RA.

In summary, our study demonstrated that RA treatment induced autophagy in an endometrial cell line and stromal cells. We also observed an inverse correlation between Beclin1 expression and higher clinical grade of endometriosis. Beclin1 knockdown enhanced ESC growth, and this effect was suppressed by RA treatment. However, more details are needed to understand how Beclin l and RA are related to each other. There are some limitations in our study, we have to make advance and address the mechanism that autophagy function regulates endometriosis progression is warranted.

Author contribution HL: Project development, data collection, manuscript writing. SL: Data collection, animal model. QW: Manuscript writing.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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