

SCM-198 Prevents Endometriosis By Reversing Low Autophagy Levels Of Endometriotic Stromal Cells Via Inhibiting TNF- α -Aromatase-Estrogen-Era Pathway And Promoting PR Expression

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Abstract

Background: Endometriosis (EMS), an estrogen-dependent disease, is characterized by dysregulated inflammation and increased estrogen in ectopic lesions. However, the crosstalk and pathogenic mechanism of inflammation and estrogen has not been fully explored. SCM-198 is the synthetic form of leonurine with multiple pharmacological activities. Whether SCM-198 could inhibit the progress of EMS by regulating inflammation and estrogen signaling remains unknown.

Methods: The therapeutic effects and potential mechanisms of SCM-198 on EMS were analyzed by establishing EMS mice models and performing RNA-seq assay. ELISA was performed to detect estrogen and TNF- α concentration in normal endometrial stromal cells (nESCs) and ectopic endometrial stromal cells (eESCs), with or without SCM-198 treatment. Western blotting, RNA silencing and plasmid overexpression were utilized to analyze the relationship among inflammation, endocrine and autophagy as well as the regulation of SCM-198 on inflammation-endocrine-autophagy axis.

Results: Increased estrogen-ER α signaling and decreased PR expression co-led to the hypo-autophagy state in eESCs, which further inhibited the apoptosis of eESCs. Highly expressed TNF- α in eESCs enhanced low-autophagy mediated anti-apoptosis effect by activating aromatase-estrogen-ER α signaling. SCM-198 inhibited the growth of ectopic lesions in EMS mouse model and promoted the apoptosis of eESCs both *in vivo* and *in vitro*. The apoptosis effect of SCM-198 on eESCs were realized by upregulating the autophagy level via inhibiting TNF- α activated aromatase-estrogen-ER α signaling and increasing PR expression.

Conclusion: Inflammation facilitated the progress of EMS by disturbing estrogen regulatory axis. SCM-198 restrained the growth of EMS by regulating inflammation-endocrine-autophagy axis.

Background

Endometriosis (EMS) is defined as the presence of endometrial-like tissue outside the uterine cavity, with periodic bleeding of the ectopic lesions under the influence of ovarian hormones[1]. EMS is an estrogen-dependent and inflammation disordered disease that affects 10% women in reproductive age[2], and is associated with pelvic pain and infertility[3-6]. None of the proposed pathogenic theories can (retrograde menstruation, coelomic metaplasia and immune disorder theory) fully explain the origin and progression of EMS[7, 8]. Recent studies have pointed out that the aetiology of endometriosis is more related to the interaction between inflammation and endocrine[9, 10].

The treatment of EMS is tricky for its high recurrent rate (~50%)[11]. The surgical treatment is traumatic [12, 13] and medical therapy (e.g., progesterone, gonadotropin-releasing hormone (GnRH) agonists) is inefficient reducing the locally produced estrogen by ectopic lesions[11]. The expenditure of EMS is identified with the cost of diabetes, while the curative effect fails to meet the expectations. Therefore, it is urgent to comprehensively understand the pathogenesis of EMS and to find appropriate treatment.

Enhanced survivability leads to continuous development of EMS[14]. Elevated estrogen level and resisted progesterone effect co-enhanced the survival of ectopic endometrium[15, 16]. Accumulated pro-inflammatory factors TNF- α enhanced the proliferation of ectopic endometrial stromal cells (eESCs)[17, 18]. Moreover, meta-analysis showed that TNF- α was associated with susceptibility to EMS and anti-TNF- α therapy can relieve the pelvic pain associated with EMS[19, 20]. However, the specific crosstalk between endocrine and inflammation and its role in the occurrence and development of EMS needs to be further investigated.

Recently, the role of autophagy has been emphasized on the progress of EMS[21]. The autophagy level of eESCs is downregulated, which further promotes the survival and inhibits the apoptosis of eESCs[22-24]. Abnormal upregulated estrogen signaling has been found to inhibit autophagy in EMS[10, 25]. Inflammation and autophagy can negatively regulate each other. Autophagy activation ameliorates inflammation[26, 27] and enhancing inflammation inhibits autophagy[28, 29]. Whether inflammation, endocrine and autophagy have regulatory networks to jointly mediate the process of EMS needs to be further explored.

SCM-198 is a synthetic form of leonurine[30, 31]. Studies have shown that SCM-198 has therapeutic effects on cardiovascular[32, 33] and cerebrovascular disease[34]. Importantly, SCM-198 has been demonstrated to alleviate hyperalgesia in adenomyosis mice[35]. However, no study was reported to explore the effect of SCM-198 on EMS.

In the present study, we focused on the therapeutic effects of SCM-198 on EMS and explored the regulatory roles of SCM-198 in inflammation, endocrine and autophagy network of EMS. We found that SCM-198 obviously reversed the downregulated expression of PR and alleviated the higher production of TNF- α in eESCs. The activation of aromatase-estrogen-ER α signaling induced by augmented TNF- α was dramatically inhibited by SCM-198. Further, SCM-198 promoted the apoptosis of eESCs via reversing the inhibited autophagy level facilitated by activated TNF- α -aromatase-estrogen-ER α signaling and weakened PR expression. This study explained the complicated mechanisms involving immune-endocrine dysregulation of EMS and supplied a promising therapy for the refractory disease.

Methods

Reagents and mice EMS model

SCM-198 was kindly gifted by Dr Zhu Yizhun's lab. Female C57BL/6 mice (6-8 weeks old) were purchased from Shanghai JieSiJie Laboratory Animal Co. Ltd (shanghai., China). After two weeks of adaptation, mice were randomly selected as the donors of EMS model. Donor mice were intraperitoneally injected with 17 β estradiol (E2) (#E2758, Sigma, St. Louis, USA) (0.2ug/g bodyweight) three times for one week. Vaginal smears were utilized to select estrus mice as the recipients of EMS mice model. Mixture of the donor mouse uterine fragments and PBS were intraperitoneally injected to recipient mouse. Recipient mice were allowed to rest for one week.

To investigate the effects of SCM-198 on EMS, recipient mice were randomly divided into three groups: EMS group, EMS + low-dose SCM-198 group (EMS+SCM-198 L, 7.5mg/kg) and EMS + high-dose SCM-198 group (EMS+SCM-198 H, 15mg/kg). Intraperitoneally inject 200 μ l of SCM-198 to the recipient mice according to the corresponding dose (once a day for a total one week). EMS group were given the same dose and frequency of PBS. One week later, the mice were sacrificed. Collect endometriotic tissue, uterus and peritoneal fluid for subsequent treatment.

Collection of human samples and isolation of endometrial stromal cells

Human ectopic endometrial tissues were obtained from 46 women with EMS (aged 22–45 years) via laparoscopy and human normal endometrial samples were collected from 10 healthy women (aged 23–46 years) by uterine curettage at the Obstetrics and Gynecology Hospital of Fudan University. The endometrial tissues were minced (2–3-mm pieces) and digested in DMEM/F-12 containing collagenase type IV (0.1%, Sigma, USA) for 30min at 37°C. The dispersed cells were then filtered through a 400-mesh wire sieve to remove the undigested tissue pieces containing the glandular epithelium. After gentle centrifugation, the supernatant was discarded, and the cells were resuspended in DMEM/F-12 containing 10% fetal bovine serum (Gemini, Calabasas, USA), 100 IU/ml penicillin (Sigma, USA), 100 μ g/ml streptomycin (Sigma, USA), and 1 μ g/ml amphotericin B (Sangon, Shanghai, China) at 37°C in 5% CO₂.

Immunohistochemical

Placing the immunohistochemical sections at 60°C for 2h. Using xylene and gradient alcohol to Dewax and rehydrate the sections. It was incubated with 3% hydrogen peroxide and 5% bovine serum albumin successively to block endogenous peroxidase. Tissue sections were incubated with anti-mouse ER α (#ab32063, Abcam, Cambridge, UK), PR (#ab101688, Abcam, UK), overnight in a humid chamber at 4°C. Wash the sections 3 times with PBS for 5 minutes each time. Cover the section with peroxidase-conjugated goat anti-rabbit or mouse IgG (#GK500710, Gene Teck, San Francisco, USA) and incubate the secondary antibody for 30 minutes. Then it was reacted with 3,3-diaminobenzidine (DAB) and stained the nucleus with hematoxylin. Finally, the slices are dehydrated in gradient alcohol and xylene, and then the slices are mounted.

Western blotting analysis

The total proteins of endometrial stromal cells (ESCs), mouse uterine and ectopic lesions were extracted using radioimmunoprecipitation (RIPA) assay buffer (Beyotime, Shanghai, China) supplemented with protease and phosphatase inhibitors (Sigma, USA). The protein concentration was measured using a BCA protein assay kit (Beyotime, China). After denaturation, equal amounts of protein were separated via SDS-polyacrylamide gel electrophoresis (PAGE) before wet transfer onto polyvinylidene difluoride membranes. Nonspecific binding sites were blocked by incubating the membranes with 5% skim milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 1h. Then, the membranes were incubated overnight at 4°C with primary antibodies (1:1000) against aromatase(#14528, CST, Boston, USA), ER α (#ab32063, Abcam, UK), PR(#ab32085, Abcam, UK), LC3B(#3868, CST, USA), BECN1(#ab207612, Abcam, UK), Bcl-2(#2870, CST,

USA), Bax(#12105, CST, USA), FN1(#ab2413, Abcam, UK), vimentin(#5741, CST, USA), α -tubulin(#ab7291, Abcam, UK) and GAPDH (#10112, Arigo, Taiwan, China). Subsequently, membranes were incubated with appropriate horseradish peroxidase-conjugated anti-rabbit (#65351, Arigo, China) or anti-mouse (#65350, Arigo, China) IgG secondary antibodies for 1h at room temperature. After three washes with TBS-T, immunopositive bands on the blots were visualized using chemiluminescent HRP substrate (#WBKLS0100, Millipore, Boston, USA) on the enhanced chemiluminescence detection system (Merck Millipore, USA).

RNA-Seq Data Analysis

Differential expression was computed with DESeq2, and the corrected gene or transcriptome expression value, the corrected fold-change, and the p-value and FDR value of the significance of the difference can be obtained. Set p-value<0.05 and Fold change greater than 1.2 times or less than 0.83333 times as difference, then we get 1616 differential genes. Based on the differentially expressed genes between the different groups, the GO database and the KEGG database were used for functional enrichment and pathway enrichment, respectively. Statistical analysis was performed in the R version 4.0.4 according to p-value<0.05. Bubble charts and volcano plot were performed by using ggplot and cluster profiler packages.

Quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, California, USA) and then reverse-transcribed into first-strand complementary DNA (cDNA) (Takara, Kyoto, Japan) according to the manufacturer's instructions. The synthesized cDNA was amplified with specific primers and SYBR Green (Takara, Japan) using an ABI PRISM 7900 Sequence Detection System (Applied Biosystems, California, USA). Triplicate samples were examined for each condition. A comparative threshold cycle value was normalized for each sample using the $2^{-\Delta\Delta Ct}$ method.

ELISA

The supernatant of normal endometrial stromal cells (nESCs) and differently treated eESCs was harvested and assayed by ELISA according to the manufacturer's instructions (estrogen, #CSB-E07286h, CUSABIO, Shanghai, China; TNF- α , #BDEL-0049, Biodragon, Beijing, China), to detect the levels of secreted estrogen and TNF- α .

Plasmid Overexpression and siRNA transfection

Aromatase overexpression (Aromatase^{over}) plasmid and negative control plasmid were purchased from Shanghai Genechem Co., LTD. (shanghai, China). Aromatase siRNA (siAromatase) and control siRNA were purchased from Shanghai Genepharma Co., LTD. (shanghai, China). Aromatase overexpressed plasmid and negative control plasmid (Ctrl) were transfected into eESCs cells by liposome transient transfection when the fusion degree reached about 70-80% in 6-well plate. Transfected cells were

incubated for 24 h at 37°C and then collected for further study. The transfection process of siAromatase were similar to the overexpressed aromatase transfection.

Statistical analysis

Prism 8 software (GraphPad) was used for data analysis. Statistical significance was determined using Student's t-test for 2-group or one-way ANOVA for multiple group comparisons. The data are presented as mean±SD. Statistical significance was attained when $P < 0.05$.

Results

SCM-198 suppresses the endometriotic growth both *in vivo* and *in vitro*

We first explored whether SCM-198 could alleviate the development of EMS by using mice EMS model. Fig. 1A showed the general process of establishing EMS mice model. As shown, SCM-198 not only decreased the weight and size of mice ectopic lesions (Fig. 1B, C), but also reduced the wall thickness of EMS lesions (Fig. 1D). In addition, masson staining showed that SCM-198 treatment significantly reduced collagen accumulation in ectopic lesions (Fig. 1E). Western blotting results revealed that SCM-198 inhibited the expression of anti-apoptosis protein Bcl-2 and promoted the expression of pro-apoptosis protein Bax in ectopic lesions at both low and high administration (Fig. 1F). Consistent with *in vivo* results, *in vitro* analysis showed that SCM-198 inhibited Bcl-2 and promoted Bax expression in eESCs (Fig. 1G). Meanwhile, fibrosis related molecules fibronectin 1 (FN1) and vimentin were also decreased in SCM-198 dose-dependently treated eESCs (Fig. 1H). These results suggest that SCM-198 has the ability to accelerate the apoptosis and attenuate the growth and fibrosis of EMS both *in vivo* and *in vitro*.

SCM-198 promotes the autophagy level and inhibits estrogen signaling of EMS

To investigate the under mechanism of SCM-198 in restraining EMS, we performed RNA-seq in SCM-198 treated and untreated ectopic lesions of EMS mice model. We observed a total of 1616 differentially expressed genes, with 701 genes being upregulated and 915 genes being downregulated in SCM-198 treated ectopic lesions compared with that in EMS groups (Fig. 2A). GO enrichment and KEGG pathway analysis revealed that SCM-198 downregulated the autophagy inhibitor molecules and estrogen receptor pathway of ectopic lesions (Fig. 2B, C).

To confirm the results from bioinformatics analysis, we first analyzed the autophagy related gene expression in ectopic lesions. The results in Fig. 2D showed that SCM-198 could extensively promote the mRNA expression of autophagy positively-related proteins in ectopic lesions, such as Map1lc3b, Becn1Ulk1, Atg3, Atg4b, Atg5, Atg7, Gabarap, Atg9a and Atg10. Further, western blotting results confirmed that SCM-198 could promote the autophagy level by upregulating the ratio of autophagy-positively related proteins LC3B-II/□ and BECN1 expression (Fig. 2E). Meanwhile, SCM-198 reversed the imbalanced expression of ERα and PR in EMS ectopic lesions by up-regulating PR and down-regulating ERα

expressions (Fig. 2F, G). These results suggest that SCM-198 could promote the autophagy level and inhibit estrogen signaling of EMS.

The ER α /PR imbalance contributes to the hypo-autophagy state of eESCs.

We then assessed estrogen, hormone receptor, and autophagy level of eESCs in EMS patients. Higher production of estrogen (Fig. 3A) and upregulated ER α (Fig. 3B) were observed in eESCs. Compared with nESCs, LC3B-II/III and BECN1 were downregulated in eESCs, suggesting a lower autophagy level in eESCs (Fig. 3C). To explore the relationship between estrogen signaling and autophagy, eESCs were treated with E2. The results showed that E2 treatment dose dependently increased ER α expression and inhibited autophagy level by reducing LC3B-II/III and BECN1 expression (Fig. 3D). Previous study has demonstrated that ER α inhibited autophagy in eESCs[10]. Thus, locally produced high level of estrogen led to increased ER α , which further inhibited autophagy. Meanwhile, PR expression was decreased in eESCs (Fig. 3E). Progesterone increased PR expression and promoted autophagy in a dose-dependent manner (Fig. 3F). Further, PR silencing downregulated the autophagy of eESCs by decreasing LC3B-II/III and BECN1 protein expression, implying that decreased PR contributed to the hypo-autophagy state in eESCs (Fig. 3G). Together, these results suggest that local high estrogen level leads to increased ER α level and the imbalanced of ER α /PR expression in ectopic lesions promotes the hypo-autophagy in eESC.

SCM-198 promotes autophagy mediated apoptosis of eESC by inhibiting estrogen-ER α pathway and promoting PR expression

To investigate whether SCM-198 promotes autophagy level by inhibiting estrogen signaling, eESCs were treated with SCM-198. The results showed that SCM-198 dose dependently downregulated estrogen level, ER α expression (Fig. 4A, B) and enhanced autophagy by upregulating LC3B-II/III and BECN1 of eESCs (Fig. 4B). Importantly, augmented ER α expression and inhibited autophagy induced by E2 were reversed by SCM-198 administration in eESCs (Fig. 4C). Meanwhile, SCM-198 dose-dependently upregulated PR expression (Fig. 4D) and reversed the inhibited autophagy mediated by PR silencing (Fig. 4E). Further, by using autophagy inhibitor 3-MA, we demonstrated that low autophagy level was conducive to cell anti-apoptosis (Fig. 4F). And SCM-198 exerted pro-apoptosis effects on eESCs via promoting autophagy (Fig. 4F).

Together, these results imply that SCM-198 could promote the apoptosis of eESCs by enhancing autophagy via inhibiting estrogen pathway and promoting PR expression.

TNF- α positively regulates aromatase-estrogen-ER α pathway in eESCs

Locally disordered inflammatory factors and increased estrogen both promoted the growth and proliferation of EMS ectopic lesions. To investigate the crosstalk between inflammation and estrogen signaling pathway in EMS, we first detected the level of pro-inflammation cytokine TNF- α and the expression of aromatase (key enzyme of estrogen production) in eESCs. As shown, the mRNA expression and concentration of TNF- α was significantly increased (Fig. 5A) and the expression of aromatase was

also upregulated in eESCs (Fig. 5B). Next, eESCs were treated with TNF- α or R-7050, a tumor necrosis factor receptor (TNFR) antagonist. TNF- α obviously promoted estrogen signaling pathway by increasing the expressions of aromatase and ER α (Fig. 5C), and elevating estrogen concentration (Fig. 5D). Aromatase silencing decreased estrogen level and ER α expression (Fig. 5E, F). Aromatase overexpression increased estrogen level and ER α expression (Fig. 5G, H), indicating that aromatase-estrogen signaling positively regulate ER α . Further, to demonstrate TNF- α positively regulate estrogen-ER α signaling through aromatase, aromatase was silenced under the treatment of TNF- α . As shown in Fig. 5I and J, aromatase silencing abolished the promotive effect of TNF- α on estrogen production and ER α expression. These data indicate that TNF- α can positively regulate the aromatase-estrogen-ER α pathway and inflammatory disorders can promote estrogen production to accelerate the development of EMS.

The pro-apoptotic effects of SCM-198 are realized by down-regulating aromatase-estrogen pathway via inhibiting TNF- α

To explore whether the promotive effects of SCM-198 on autophagy are realized by inhibiting TNF- α -aromatase-estrogen-ER α pathway, we treated eESCs with different concentrations of SCM-198. SCM-198 dose dependently decreased the concentrations of TNF- α (Fig. 6A) and estrogen (Fig. 4A), and suppressed the expression of aromatase and ER α in eESCs (Fig. 6B). Furthermore, the anti-apoptosis effect by TNF- α -aromatase-estrogen-ER α signaling mediated low autophagy could be reversed by SCM-198 administration (Fig. 6C, D). These results suggest that inflammation suppresses autophagy via estrogen signaling, thereby inhibiting the apoptosis of eESCs. SCM-198 could downregulate TNF- α -aromatase-estrogen-ER α pathway, eventually promoting autophagy and ultimately accelerating the apoptosis of eESCs (Fig. 7).

Discussion

EMS, a common obstetrics and gynecology diseases, is characterized by the growth of endometrial tissue appears outside the uterine cavity. Owing to the severe complications (pelvic pain and infertility) and high prevalence (~10%), multiple therapies have been proposed to deal with the disease[36, 37]. However, still up to 50% recurrence rate has been observed in EMS patients, because neither medication nor surgical treatment is effective in eradicating the growth of ectopic lesions[11]. Finding and identifying effective medication for the treatment of EMS is beneficial for improving the quality of women life and relieving substantial economic burden. SCM-198, a synthetic form of leonurine, proved to have pain relief pharmacological effects on adenomyosis[35]. In the present study, we first identified the therapeutic effects of SCM-198 on EMS, as evidenced by decreased weight and size, reduced collagen accumulation and augmented Bax/Bcl-2 ratio of mice ectopic lesions.

Aberrant autophagy has stepped into the spotlight on the stage of EMS pathogenesis[21, 38]. Cumulative studies have uncovered that the autophagy level of ectopic endometrium is decreased[39, 40]. More important, low-autophagy level contributes to the survival of ectopic endometrium, as corroborated by enhanced apoptosis and decreased proliferation of eESCs under the induction of autophagy[24, 41].

Then, we inquired whether autophagy is involved in the mechanisms of SCM-198 on treating EMS. RNA-Seq results based on ectopic foci suggested that SCM-198 treatment significantly promoted autophagy of eESCs. The upregulated mRNA levels of autophagy related genes in ectopic lesions and increased LC3B and BECN1 expression both *in vitro* and *in vivo* under SCM-198 treatment further confirmed the promotive effects of SCM-198 on autophagy level of eESCs.

The imbalanced endocrine microenvironment of ectopic endometrium, manifested with high estrogen signaling and progesterone resistance, have inescapable responsibility for the growth of ectopic endometrium. Increased estrogen production supported by elevated aromatase expression potentiates the proliferation of ectopic lesion[42-44], which could be reinforced by resisted progesterone signaling mediated by decreased PR expression[45, 46]. Multiple studies have emphasized that inhibiting autophagy is an important pathway for estrogen to restrain the apoptosis and facilitate growth of ectopic lesions[10, 25]. Whether the promotive effects of SCM-198 on autophagy are realized by repairing the damaged hormonal endocrine networks. We verified that increased estrogen signaling and impaired progesterone signaling co-drive the decline of autophagy in eESCs. In consistent with the hypothesis, the upregulation effects of SCM-198 on autophagy are mediated by increasing PR expression and decreasing aromatase-estrogen-ER α signaling in eESCs.

The current consensus is that dysregulated pelvic inflammatory process plays a crucial role in the initiation and progression of EMS[47]. Abnormally upregulated pro-inflammatory cytokine TNF- α is closely related to the pelvic pain and infertility associated with EMS[20, 48]. Our data confirmed that there was an obviously upregulation in both mRNA expression and concentration of TNF- α . The upraised production of TNF- α could be suppressed the treatment of SCM-198, which has been identified as an anti-inflammation drug. More important, we demonstrated that elevated TNF- α augmented the activation of estrogen-ER α signaling by increasing the expression of aromatase. Further, TNF- α inhibited the autophagy of eESCs by promoting estrogen signaling, suggesting inflammation-endocrine-autophagy axis plays an indispensable role in ectopic endometrial survival. Notably, we found that SCM-198 abated TNF- α induced estrogen signaling activation and autophagy inhibition, ultimately restrained the apoptosis of eESCs.

Conclusions

In summary, the disturbed inflammation, endocrine and autophagy made EMS to be complicated and difficult to cure. Elevated levels of inflammation, imbalanced estrogen and progesterone and inactivated autophagy co-contribute to the progression of EMS. SCM-198 focused on rectifying the aberrant inflammation-endocrine-autophagy axis. SCM-198 reversed the low autophagy level of eESCs via inhibiting TNF- α -aromatase-estrogen-ER α pathway and promoting PR expression, and ultimately alleviated the development of EMS. To sum up, this study provided a theoretical basis for the potential application of SCM-198 on the treatment of EMS.

Abbreviations

EMS: endometriosis; eESCs: endometrial stromal cells; nESCs: normal endometrial stromal cells; eESCs: ectopic endometrial stromal cells; GnRH: gonadotropin-releasing hormone; E2: 17 β -estradiol; RIPA: radioimmunoprecipitation; cDNA: complementary DNA; DAB: diaminobenzidine; FN1: fibrosis related molecules fibronectin 1; TNFR: tumor necrosis factor receptor.

Declarations

Ethics approval and consent to participate

Our research was approved by the Human Research Ethics Committee of the Obstetrics and Gynecology Hospital of Fudan University and all experiments were performed in accordance with relevant guidelines and regulations (No. Kyy2016-4) (Shanghai, China).

Consent for publication

All authors have read the manuscript and approved the final version.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests.

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Authors' contributions

Yikong Lin and Yunyun Li designed and performed the experiments, and drafted the manuscript; Yue Li performed literature searches, data analysis and revised the manuscript; Li Wang, Dajin Li, Xiaolin Wang performed data interpretation and revised the manuscript. Meirong Du Jiajing Cheng and Yizhun Zhu conceived the project, analyzed the data and revised the manuscript. All authors reviewed the manuscript, and approved the final manuscript.

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Figures

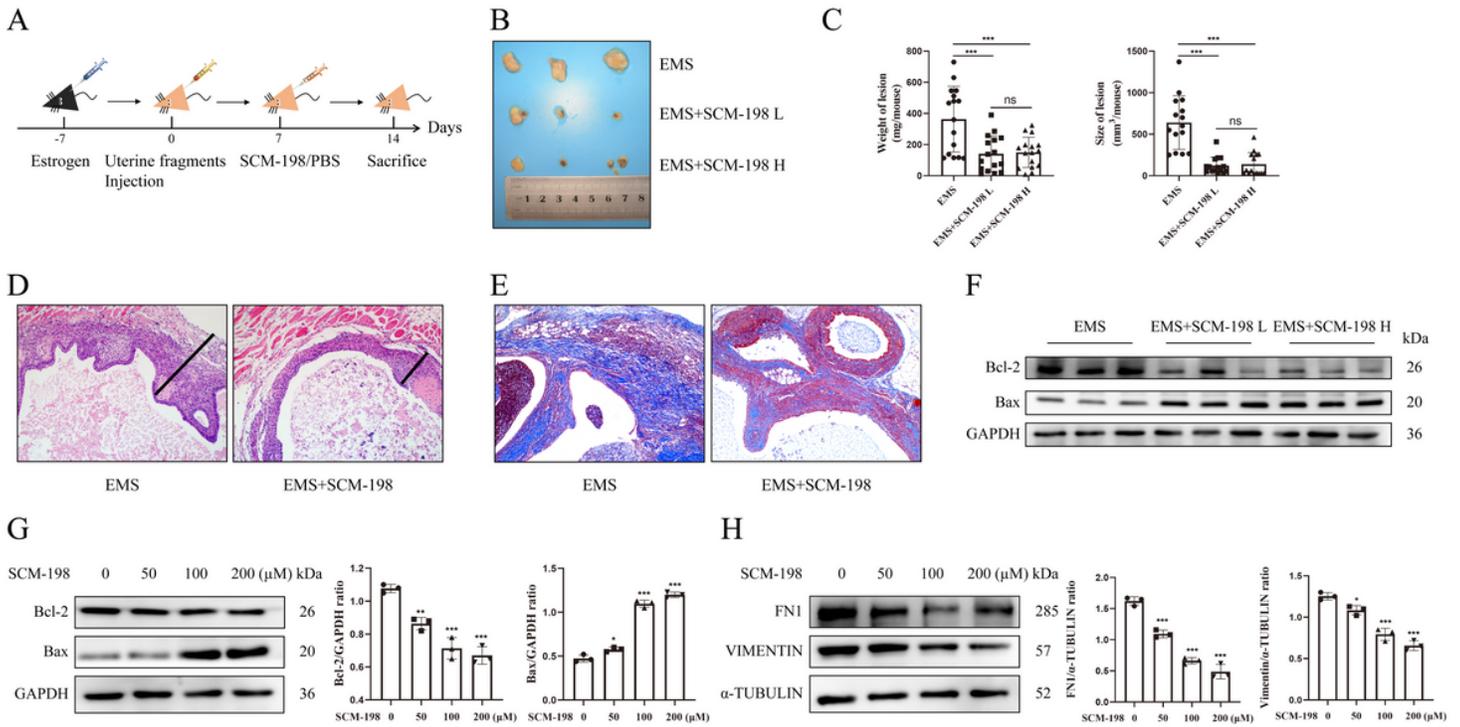


Figure 1

SCM-198 inhibits the endometriotic growth both *in vivo* and *in vitro*.

EMS mice were treated with or without SCM-198 at low (EMS+SCM-198 L, 7.5mg/kg) or high (EMS+SCM-198 H, 15mg/kg) dose once a day for one week. **(A)** The flow chart of establishing mice EMS model. **(B)** Representative images of the ectopic lesions from EMS mice. **(C)** Quantitative results for the weight (n=16) and size (n=15) of ectopic lesions from EMS mice. **(D)** Thickness of ectopic cyst wall from EMS mice. **(E)** Masson staining was performed to detect the collagen fibers of ectopic lesions. **(F)** Western blotting was utilized to analyze the protein levels of Bcl-2 and Bax in ectopic lesions. **(G, H)** The eESCs were treated with different doses of SCM-198 for 48h, then western blotting was utilized to analyze the protein levels of Bcl-2, Bax, FN1, vimentin (n=3). Data are the presented as the mean ± SD (**P* < 0.05, ***P* < 0.01, and ****P* < 0.001; ns, not significant).

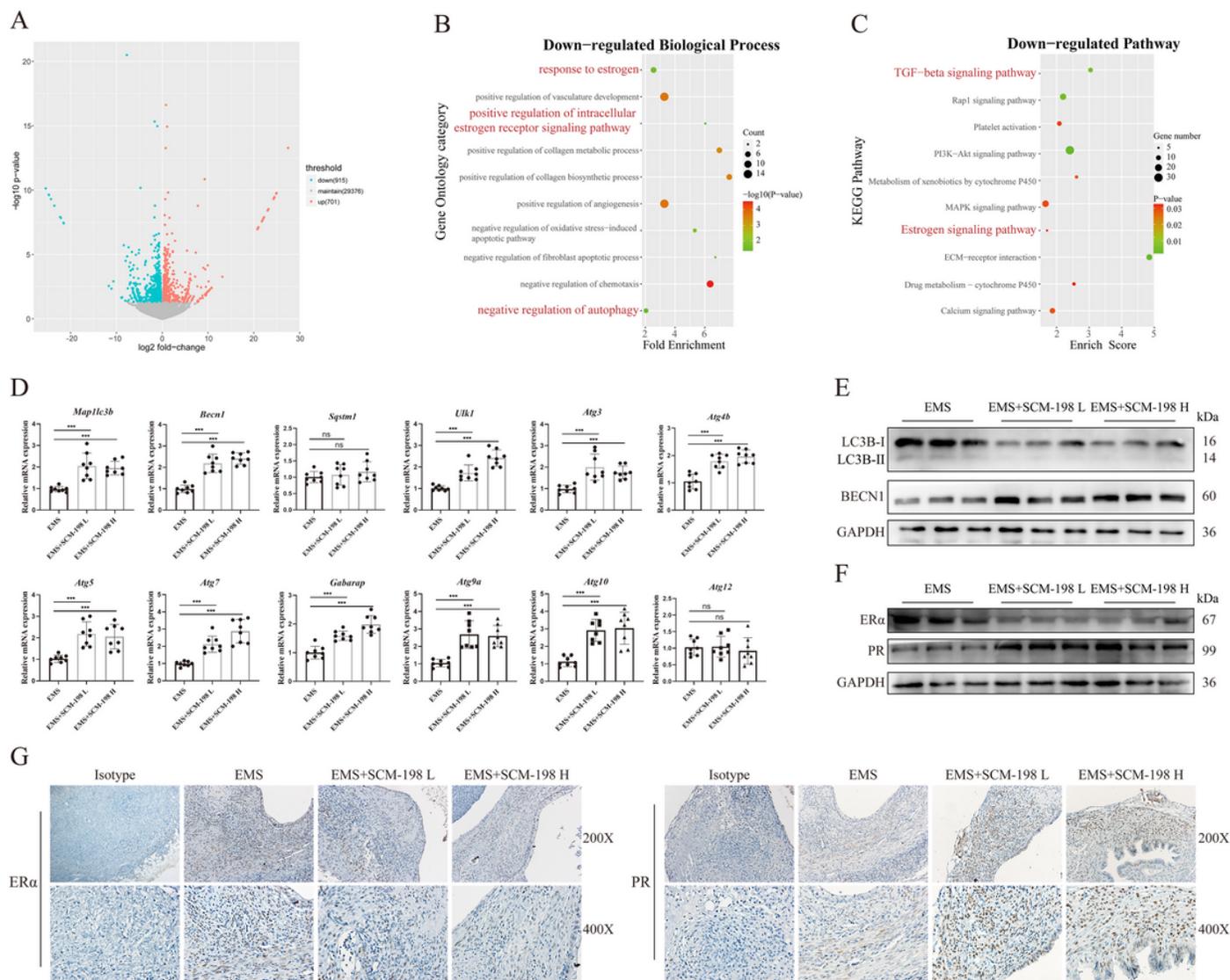


Figure 2

SCM-198 promotes the autophagy and inhibits estrogen signaling in EMS mouse model.

(A) Volcano plot of differentially expressed genes of ectopic lesions in SCM-198-treated EMS mice compared with that from the untreated EMS mice. The x-axis showed Log2 (Fold Change) in expression, and the y-axis showed the -Log10 (p-value) of gene being expressed differentially (blue: downregulated genes; red: upregulated genes). (B) GO enrichment of biological process for the downregulated genes. (C) KEGG pathway analysis for the downregulated genes. (D) The mRNA expressions of Map1lc3b, Becn1, Sqstm1, Ulk1, Atg3, Atg4b, Atg5, Atg7, Gabarap, Atg9a, Atg10 and Atg12 of ectopic lesion (n=8) treated with or without SCM-198. (E, F) The protein expressions of LC3B-II, BECN1, ERα and PR in ectopic lesions were measured via Western blotting. (G) The expressions of ERα and PR in ectopic lesion were analyzed by IHC. Data are presented as the mean ± SD (***) $P < 0.001$, compared with EMS group; ns, not significant).

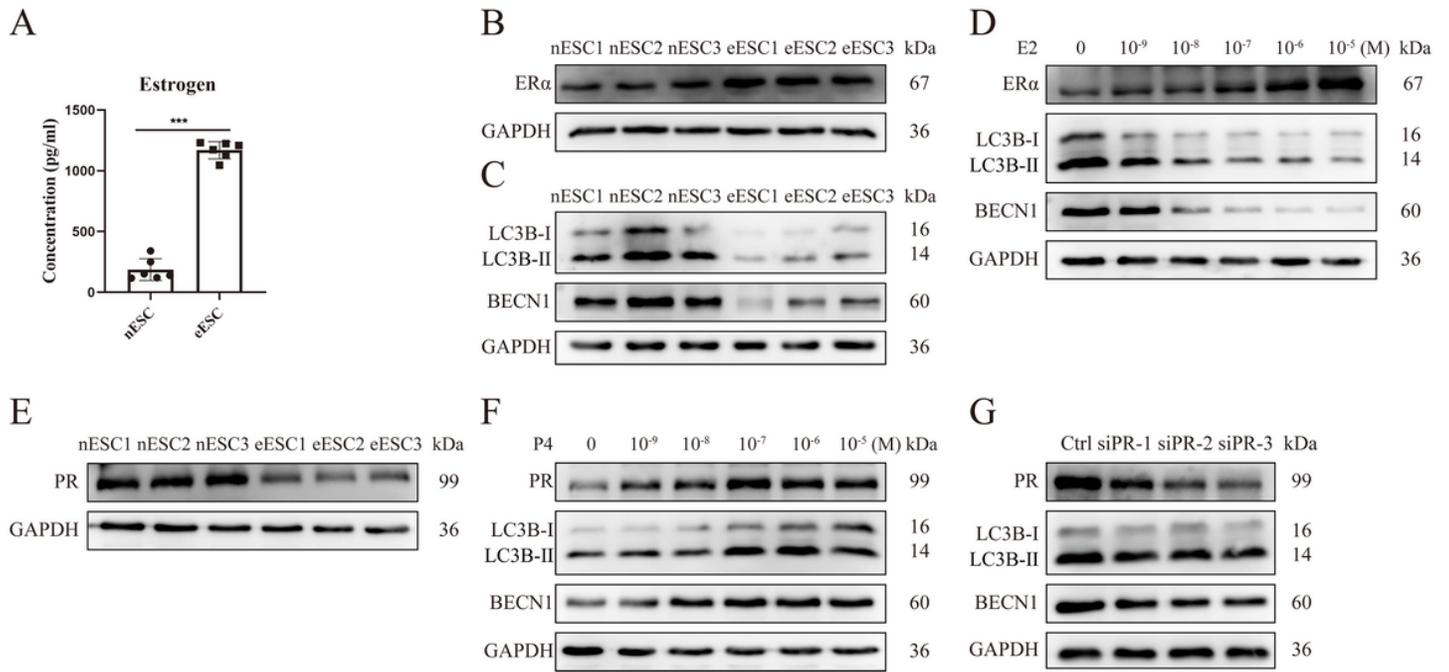


Figure 3

The ER α /PR imbalance contributes to the hypo-autophagy state of eESCs.

(A) ELISA was utilized to detect the estrogen concentrations of eESCs and nESCs (n=6), both of which were cultured in culture flask for 24h. (B, C) The protein expressions of ER α , LC3B-II/□ and BECN1 of eESCs and nESCs were analyzed by western blotting. (D) After treated with different concentrations of E2 for 48h, the expression levels of ER α , LC3B-II/□ and BECN1 in eESCs were analyzed by western blotting. (E) The protein expressions of PR in eESCs and nESCs were detected by western blotting. (F) After treated with different concentrations of progesterone for 48h, the protein levels of PR, LC3B-II/□ and BECN1 in eESCs were analyzed by western blotting. (G) The protein levels of PR, LC3B-II/□ and BECN1 in eESCs after PR silencing were analyzed by western blotting. Data are the presented as the mean \pm SD (***) $P < 0.001$).

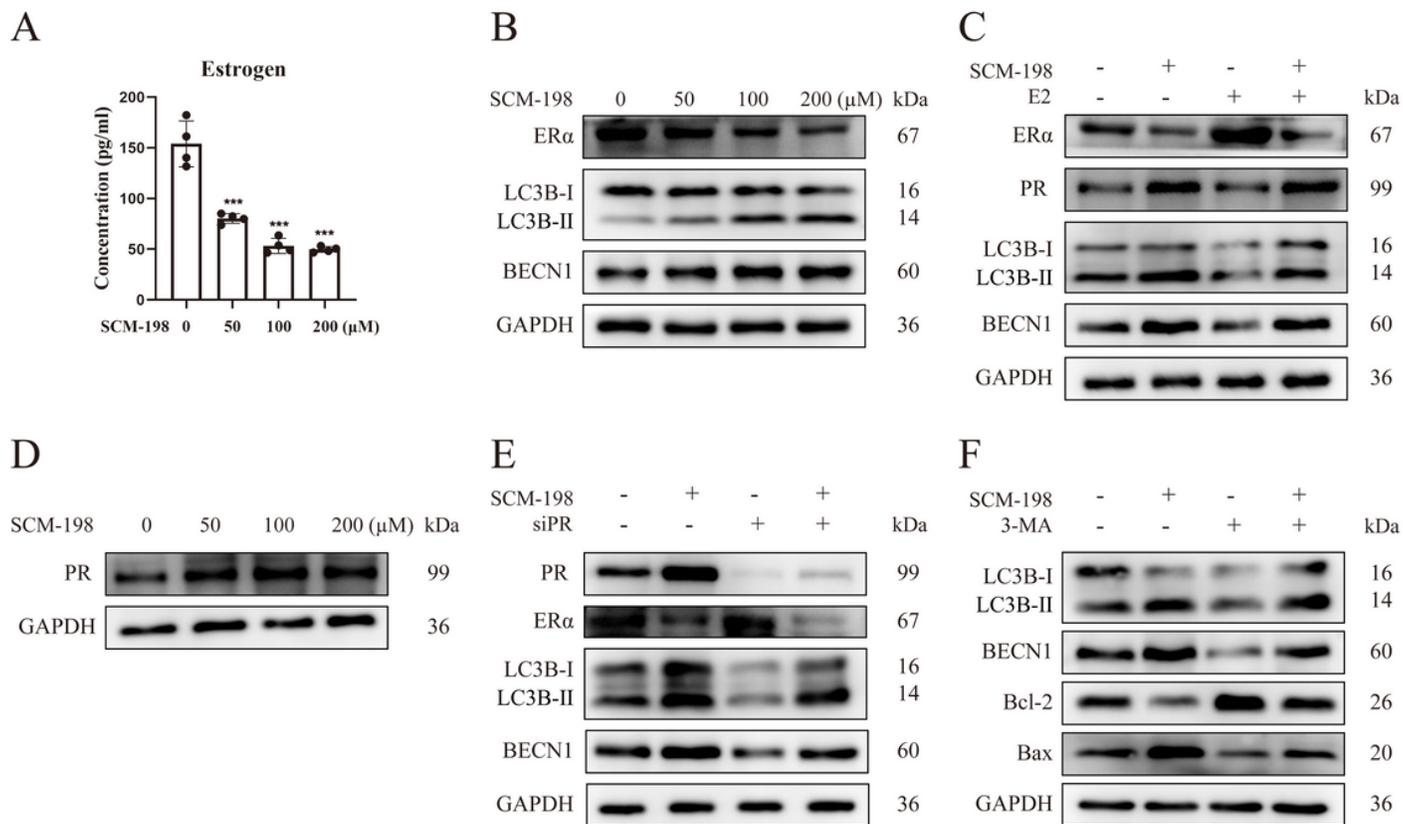


Figure 4

SCM-198 promotes autophagy mediated apoptosis of eESC by inhibiting estrogen-ER α pathway and promoting PR expression.

(A) After treated with different concentrations of SCM-198 for 48h, estrogen concentration of eESCs was analyzed by ELISA (n=4). (B) The eESCs were treated with different concentrations of SCM-198 for 48h, then protein expressions of ER α , LC3B-I/II and BECN1 were analyzed by western blotting. (C) The protein expressions of ER α , PR, LC3B-I/II and BECN1 were detected by western blotting in eESCs treated with SCM-198 (200 μ M), E2 (100nM) or SCM-198+E2 (200 μ M, 100nM) for 48h. (D) The eESCs were treated with different concentrations of SCM-198 for 48h, then protein expression of PR was analyzed by western blotting. (E) After treated with SCM-198 (200 μ M), silenced PR or SCM-198+ silenced PR for 48h, the protein expressions of PR, ER α , LC3B-I/II and BECN1 in eESCs were detected by western blotting. (F) After treated with SCM-198 (200 μ M), 3-MA (5mM) or SCM-198+3-MA (200 μ M, 5mM) for 48h, the expressions of LC3B-I/II, BECN1, Bcl-2 and Bax in eESCs were analyzed by western blotting. Data are the presented as the mean \pm SD (***) P < 0.001; compared with eESCs that were not treated with SCM-198).

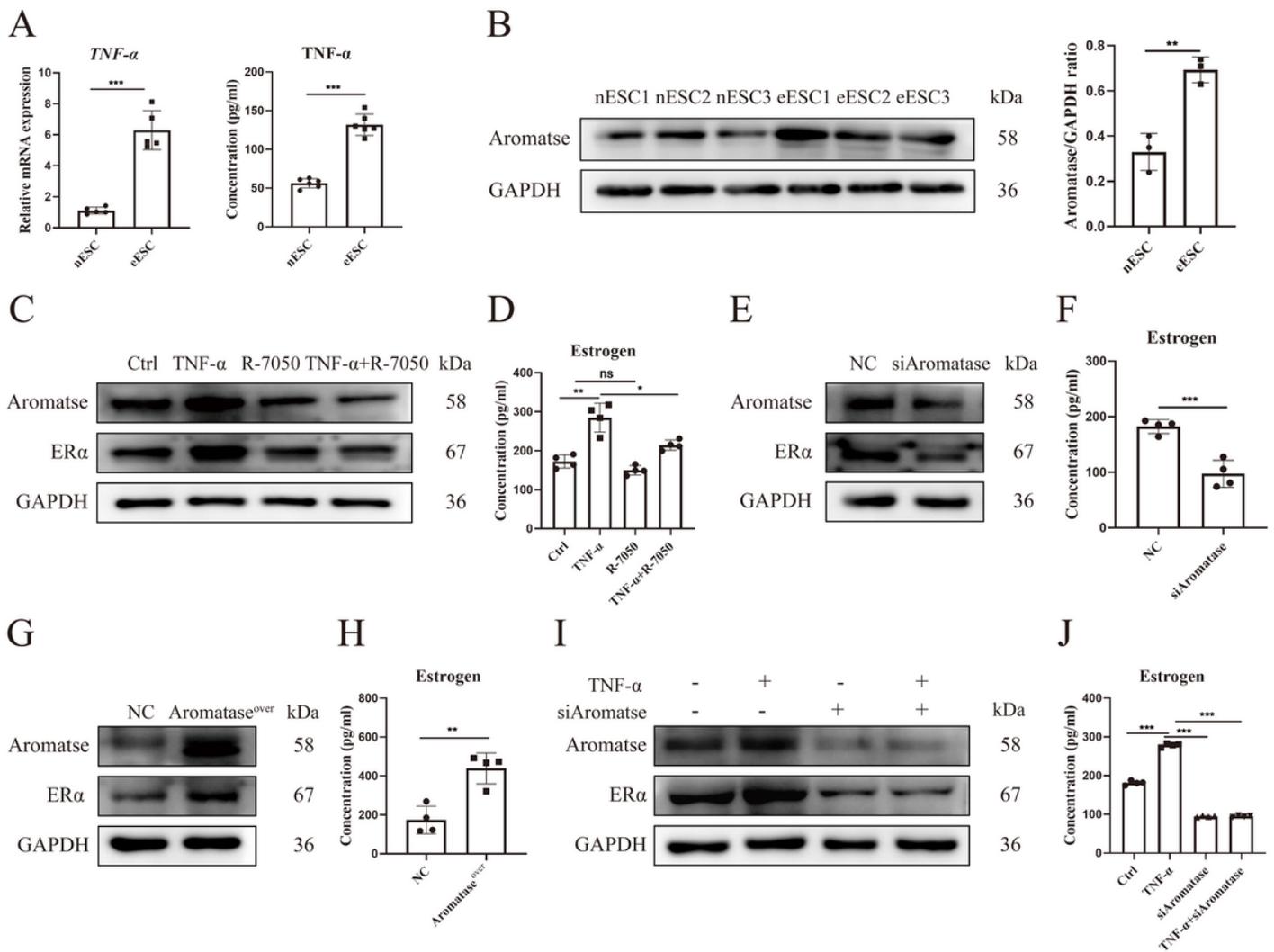


Figure 5

TNF- α upregulates aromatase-estrogen-ER α pathway in eESCs.

(A) The mRNA expression (n=5) and concentration (n=6) of TNF- α in eESCs and nESCs were detected by RT-PCR and ELISA. (B) The aromatase expression of eESCs and nESCs were detected by western blotting (n=3). (C, D) After treated with TNF- α (10ng/ml), R-7050 (5 μ M) or TNF- α +R-7050 (10ng/ml, 5 μ M), the expressions of aromatase and ER α in eESCs were analyzed by western blotting (C) and the concentration of estrogen was detected by ELISA (n=4) (D). (E-H) The aromatase was silenced or overexpressed in eESCs for 48h. Then, the expressions of aromatase and ER α were detected by western blotting (E, G) and the level of estrogen was assayed by ELISA (n=4) (F, H). (I, J) The protein expressions of aromatase and ER α were analyzed by western blotting (I) and the concentration of estrogen was detected by ELISA (n=4) (J). Data are the presented as the mean \pm SD (* P < 0.05, ** P < 0.01, and *** P < 0.001; ns, not significant).

Figure 6

The pro-apoptotic effects of SCM-198 are realized by downregulating aromatase-estrogen pathway via inhibiting TNF- α .

(A) ELISA was utilized to detect TNF- α concentration of eESCs treated with different dose of SCM-198 (n=4). (B) The protein expressions of aromatase and ER α in eESCs treated with different dose of SCM-198 were measured by western blotting. (C, D) After treated with TNF- α (10ng/ml), SCM-198 (200 μ M), or TNF- α +SCM-198 (10ng/ml, 200 μ M), the expressions of aromatase, ER α , LC3B-II/I, BECN1, Bcl-2 and Bax were analyzed by western blotting (C) and the concentration of estrogen concentration was detected by ELISA (n=4) (D). Data are presented as the mean \pm SD (** P < 0.01, and *** P < 0.001).

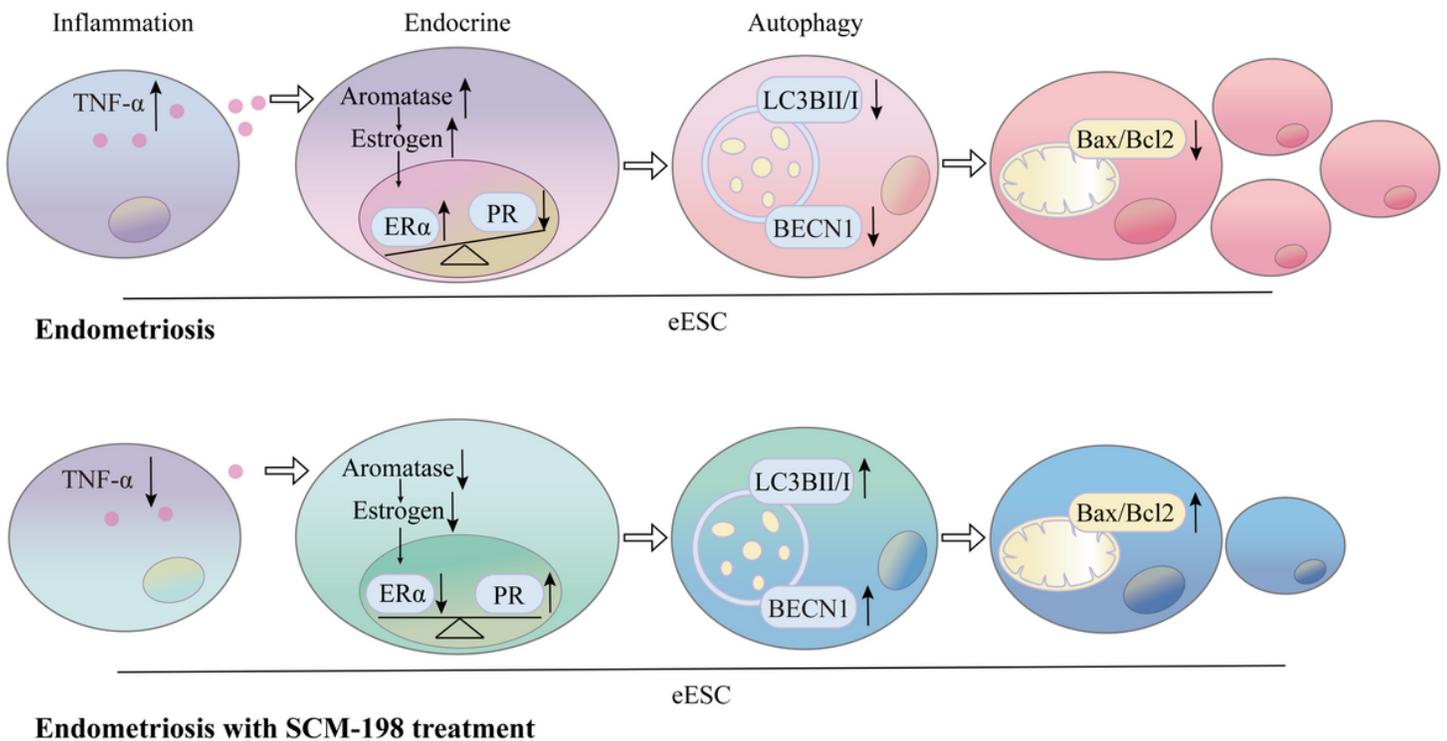


Figure 7

Schematic diagram showing therapeutic mechanism of SCM-198 on EMS.

Compared with nESCs, the production of TNF- α was higher in eESCs. Elevated TNF- α augmented the activation of aromatase-estrogen-ER α signaling. The upregulated estrogen signaling and downregulated progesterone signaling co-suppressed the autophagy level, which further conducted to the growth of eESCs. SCM-198 inhibited TNF- α -aromatase-estrogen-ER α signaling and increased PR expression. Consequently, SCM-198 promoted autophagy-mediated apoptosis of eESCs by reconstructing the balance of estrogen and progesterone signals.