Endometrial cell-derived exosomes facilitate the development of adenomyosis via the IL-6/JAK2/STAT3 pathway

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Abstract. Interleukin (IL)-6 upregulation is involved in the pathogenesis of adenomyosis, but the underlying mechanism remains to be elucidated. Exosomes mediate intercellular communication, therefore the present study investigated whether endometrial cell-derived exosomes mediated the crosstalk between the endometrium and the myometrium via IL-6 signaling. Primary adenomyotic myometrial (AM) cells and eutopic endometrial cells were isolated from patients with adenomyosis. Exosomes were obtained from endometrial cells and incubated with AM cells in the presence or absence of tocilizumab (an IL-6 inhibitor). MTT, flow cytometry and wound-healing assays were performed to examine AM cell proliferation, apoptosis, cell cycle distribution and migration. Western blotting and reverse transcription-quantitative PCR were conducted to determine the expression of the IL-6/Janus kinase 2 (JAK2)/STAT3 pathway proteins. Incubation with endometrial cell exosomes suppressed cell apoptosis of AM cells compared with controls, accompanied by increases in IL-6 production and JAK2/STAT3 phosphorylation. Endometrial cell exosomes promoted cell proliferation, increased the percentage of S-phase cells and enhanced the migration of AM cells. These effects were completely reversed by tocilizumab, along with substantial decreases in IL-6 production and JAK2/STAT3 phosphorylation. Endometrial cell-derived exosomes promote cell proliferation, migration and cell cycle transition of AM cells through IL-6/JAK2/STAT3 activation, facilitating the development of adenomyosis by mediating the crosstalk between the endometrium and the myometrium, and IL-6 targeted therapy could be a complementary approach against adenomyosis.

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Introduction

Adenomyosis is a benign gynecological disorder defined by the ectopic presence of endometrial tissue in the myometrium. It is clinically characterized by diffuse enlargement of the uterus, secondary dysmenorrhea and irregular vaginal bleeding (1). The prevalence of adenomyosis worldwide ranges widely from 8.8-61.5%, and the gold standard for diagnosis is the histopathologic examination of the uterus after hysterectomy (2). In recent years, patients with adenomyosis tend to be younger and the incidence of the disease has increased annually (3). Thus, it is of great importance to unveil the etiology and pathogenesis of adenomyosis. It is commonly accepted that adenomyosis results from the increased invasive properties of endometrial cells, including increased proliferation and migration and decreased apoptosis. These changes facilitate the migration of endometrial cells across the boundary between the endometrium and the myometrium, along with an excessive proliferation of ectopic endometrial cells in the myometrium (4,5). Therefore, targeting the proliferation, migration and apoptosis of endometrial cells might be a promising therapeutic strategy in adenomyosis treatment (6,7).

The proinflammatory cytokine interleukin (IL)-6 is elevated in eutopic and ectopic endometrium in women with adenomyosis compared with control endometrium, probably playing an important role in the formation of ectopic endometrial implants in adenomyosis (8-10). IL-6 acts as a growth regulator of human endometrial stromal cells through its receptor IL-6R (11). Once bound to IL-6R, IL-6 can activate Janus kinase 2 (JAK2) and trigger the phosphorylation and nuclear localization of signal transducer and STAT3 (12-14). The IL-6/JAK2/STAT3 pathway plays a key role in the growth and development of human types of cancer, including endometrial carcinoma (15,16). The IL-6/JAK2/STAT3 signaling pathway is hyperactivated in human endometrial cells cocultured with macrophages and promotes epithelial-mesenchymal transition in adenomyosis (10). Thus, IL-6/JAK2/STAT3 may promote the invasive behavior of endometrial cells in adenomyosis, but the mechanism underlying the activation of IL-6/JAK2/STAT3 in adenomyosis remains to be elucidated.

Exosomes are a subtype of extracellular vesicles characterized by a diameter of 30-150 nm and the presence of marker proteins such as CD63 and CD9. Exosomes can

transfer molecular cargoes, such as DNA, RNA, proteins and lipids, from the parental cells to the recipient cells, playing an important role in intercellular communication (17-19). Endometrial cell-derived exosomes contain important cargoes that are involved in the pathogenesis of endometriosis (20). Proinflammatory cytokines are increased in endothelial cells treated with exosomes from patient-derived endometriotic epithelial cells (21). Hence, these studies suggest that exosomes from endometrial cells play a role in cell behavior and immune modulation of the recipient cells. Thus, it was hypothesized that endometrial cell exosomes might mediate the communication between the endometrium and the myometrium through IL-6 signaling, contributing to the development of adenomyosis.

The present study isolated primary adenomyotic myometrial (AM) cells and eutopic endometrial cells from patients with adenomyosis and examined the effects and the underlying mechanism of endometrial cell-derived exosomes on AM cell proliferation, apoptosis, cell cycle distribution and migration. The findings suggested that endometrial cell exosomes promote the invasive properties of AM cells by activating IL-6/JAK2/STAT3 signaling, providing new information about the etiology of adenomyosis.

Materials and Methods

Sample selection. A total of 10 women (mean age, 46 years old) with adenomyosis were recruited from the First Affiliated Hospital of Guangdong Pharmaceutical University (Guangdong, China) between March 2020 and May 2021. None of the patients had received hormones or similar drug therapy within 6 months prior to the study. Biopsy specimens of endometrial and adenomyotic tissue were collected during surgery. Adenomyosis was confirmed by pathological examination. Endometrial tissue samples were collected from the endometrium without visible infection. The study was approved by the Research Ethics Committee of The First Affiliated Hospital of Guangdong Pharmaceutical University [approval no. 2021-(31)]. All participants provided informed consent before the study.

Isolation and characterization of primary AM cells and eutopic endometrial cells. The adenomyotic and endometrial tissue specimens were digested with collagenase type 1 (MilliporeSigma) to obtain primary AM cells and endometrial cells. Cells were cultured in high-glucose DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 1% penicillin/streptomycin and 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). The cells were passaged every 5-7 days and only the cells in passages 3-6 were used for the present study. In order to characterize the primary AM and endometrial cells, immunocytochemistry was performed to detect the expression of surface markers in the cells of passage 1. Briefly, cells were blocked with normal goat serum (OriGene Technologies, Inc.) at room temperature for 60 min and incubated with the primary antibody against keratin (cat. no. ZM-0060; OriGene Technologies, Inc.), vimentin (cat. no. ZM-0260; OriGene Technologies, Inc.), or actin (cat. no. ZM-0003; OriGene Technologies, Inc.; all diluted 1:200) overnight at 4˚C. The cells were incubated with a secondary antibody (cat. no. TA130040; OriGene Technologies, Inc.) for 30 min at 37˚C and visualized with an Olympus PM-20 optical microscope (Olympus Corporation).

Isolation and characterization of exosomes from endometrial cells. The endometrial cells were cultured in serum-free high-glucose DMEM for 24 h. The supernatant was collected for exosome isolation using a MagCapture exosome extraction kit (FUJIFILM Wako Pure Chemical Corporation) following the manufacturer's instructions. Briefly, the extraction reagent was mixed with the cell supernatant at 1:5. After centrifugation (10,000 x g, 30 min, 4˚C) overnight at 4˚C the supernatant was discarded. The exosome pellet was resuspended in phosphate-buffered saline solution and identified by transmission electron microscopy (TEM), nanoparticle tracking analysis and western blot analysis.

TEM examination. TEM was used to examine the morphology of the exosomes. The samples were prepared as previously described (22). The sample was fixed with 2.5% glutaraldehyde at 4˚C for 20 min and washed with PBS 3 times for 2 min each time. Then, 10 μl exosome resuspension was added to the copper mesh and adsorbed at room temperature for 10 min. And 10 μl of 2% phosphotungstic acid (pH 6.8) was added to the copper mesh to stain the sample at room temperature for 5 min. The exosomes were observed under a JEM-1200EX microscope (JEOL, Japan) at a magnification of x6,000.

Cell proliferation assay. AM cells were seeded in a 96-well plate at a density of $1x10^5$ cells per well and cultured overnight. After starvation for 24 h, the cells were incubated with $1x10^4$ endometrial cell-derived exosomes for 24 h. Untreated cells were used as a negative control. Then, the cells were incubated with MTT solution (Hangzhou Haotian Biotechnology, Co., Ltd.) for 4 h at 37˚C. The absorbance was measured at 490 nm using a microplate reader. Cell viability was calculated as

$$\frac{\text{the absorbance of the experimental group}}{\text{the absorbance of negative control}} x100\%.$$

Cell migration assay. Cell migration was investigated using a wound-healing assay. AM cells were seeded in a 12-well plate and grown overnight. A scratch was made using a sterile pipette tip. The cells were incubated with exosomes in the presence or absence of 20 μM tocilizumab (Roche Diagnostics) in a serum-free medium. Untreated cells were used as a negative control. Images were acquired at 0 and 24 h after incubation using a PM-20 optical microscope (Olympus Corporation). The wound area was measured using ImageJ software (version 1.53c; National Institutes of Health). The percentage of wound closure was calculated as

$$\frac{\text{The initial wound area at 0 h} - \text{the wound area at 24 h}}{\text{the initial wound area}} x100\%.$$

Flow cytometry assay. Cell apoptosis and cell cycle were examined using flow cytometry. For the apoptosis assay, AM cells were seeded in a 96-well plate at a density of $1x10^5$ cells per well and cultured overnight. After starvation for 24 h, the cells were treated with $1x10^4$ endometrial cell-derived exosomes. Untreated cells were used as a negative control. The cells were added with 5 μl Annexin V-FITC and stained with 5 μl propidium iodide (PI), avoiding light at 4˚C for 15 min by using a FITC Annexin V apoptosis detection kit (BD Biosciences) following the manufacturer's instructions.

For the cell cycle analysis, AM cells were seeded in a 24-well plate at a density of $5x10^4$ cells per well and cultured for 24 h. The cells were treated with exosomes in the presence or absence of tocilizumab for 48 h. Untreated cells were used as a negative control. The cells were harvested and fixed in pure ethanol at 4˚C overnight. The cells were resuspended in DNA staining solution and incubated in the dark for 30 min at room temperature. Flow cytometry analysis was performed using an ALTRA flow cytometer (Beckman Coulter). Data were analyzed using the EXPO32 software (version 1.1.2; Applied Cytometry). The percentage of apoptotic rate was calculated as the percentage of early apoptotic cells + the percentage of late apoptotic cells.

Western blot analysis. AM cells were cultured in a six-well plate and treated with exosomes in the presence or absence of 10 ng/ml tocilizumab for 48 h at 37˚C in a serum-free medium. Untreated cells were used as a negative control. Cells were lysed with 200 μl RIPA lysis buffer (Thermo Fisher Scientific, Inc.) and protease inhibitors were added to each plate and placed on ice for 5 min, and 5x loading buffer was added and mixed well, then the samples were denatured in a boiling water bath for 10 min. Total protein was detected by using the BCA protein quantification kit (cat. no. 23250; Thermo Fisher Scientific, Inc. USA) and subjected to 8-12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were washed, blocked at room temperature for 1 h with a 5% skim milk powder solution and incubated with antibodies to detect the expression of IL-6 (cat. no. ab214429; 1:1,000; Abcam), JAK2 (cat. no. ab32101; 1:1,000; Abcam), phosphorylated (p-)JAK2 (cat. no. ab195055; 1:2,000; Abcam), STAT3 (cat. no. ab68153; 1:2,000; Abcam), p-STAT3 (cat. no. ab76315; 1:1,000; Abcam), CD63 (cat. no. ab134045; 1:1,000; Abcam), CD9 (cat. no. ab236630; 1:1,000; Abcam) and GAPDH (cat. no. ab9485; 1:2,500; Abcam), followed by incubation with HRP-linked secondary antibody (cat. no. HS201-01; Boster Biological Technology; 1:5,000) for 1 h at room temperature. Then the protein bands were visualized using an ECL kit (cat. no. 34075; Thermo Fisher Scientific, Inc.). And the grey values of the protein bands were quantified with Quantity One 4.1 software (Bio-Rad Laboratories, Inc.) and the results were the average of three independent experiments.

Reverse transcription-quantitative (RT-q) PCR. The cells ($1x10^6$) in each group were treated with TRIzol® (Thermo Fisher Scientific, Inc.) and chloroform was added at a ratio of 200 μl of chloroform to 1 ml of TRIzol. The aqueous phase of the upper layer was extracted after centrifugation (12,000 x g, 5 min, 4˚C). Isopropanol was added in a ratio of 0.5 ml of isopropanol to 1 ml of TRIzol and then mixed and the supernatant was discarded after centrifugation (12,000 x g, 10 min, 4˚C). Ethanol was added in a ratio of 1 ml of 75% ethanol to 1 ml of TRIzol and the supernatant was discarded after centrifugation (7,500 x g, 5 min, 4˚C). The bottom layer was allowed to air-dry at room temperature for 5-10 min and mixed with 20 μl of DEPCI. After 10 min, 20 μl DEPC water was added to dissolve the precipitate. Total RNA was extracted according to manufacturer's instructions and the absorbance was measured at 260 and 280 nm for quality control and quantification. The RNA was reverse transcribed into cDNA according to the manufacturer's instruction of the TaKaRa Reverse Transcription Kit (Takara Bio, Inc.) and then diluted 10-fold, using a 10 μl system: 2 μl template cDNA, 0.4 μl each of the upper and lower primers and 5 μl amplification probe II and then make up the total amount to 10 μl with sterilized double-distilled water and then placed into a fluorescent quantitative PCR instrument for the reaction using a Super SYBR Green kit (Transgene SA). Gene amplification was carried out according to the manufacturer's instructions: 95˚C for 10 min; and 40 cycles of 95˚C for 10 s and 60˚C for 34 sec. All reactions were run in triplicate and the gene expression was quantified using the $-2^{\Delta\Delta Cq}$ method (23), with GAPDH as the internal reference. The primer sequences were GAPDH (internal reference) F 5'-GAAGGTGAAGGTCGGAGTC-3' and R 5'-GAAGATGGTGATGGGATTTC-3'; IL-6 F 5'-ACTCACCTCTTCAGAACGAATTG-3' and R 5'-CCATCTTTGGAAGGTTCAGGTTG-3'; JAK2 F 5'-GTTTGGAGCTTTGGAGTGGTT-3' and R 5'-AATCATACGCATAAATTCCGC-3'; STAT3 F 5'-CACCACCAAGCGAGGACT-3' and R 5'-CAGCCAGACCCAGAAGGA-3'.

Statistical analysis. Data were expressed as the mean ± standard deviation and analyzed using SPSS 19.0 (IBM Corp.). Statistical analysis was conducted using one-way ANOVA. The differences between groups were analyzed using the Bonferroni method when homogeneity of variance was observed and Tamhane's T2 test when heterogeneity of variance was observed. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Characterization of primary cells and exosomes. AM and endometrial cells from patients were characterized by surface markers. Immunocytochemical staining showed that AM cells expressed keratin, vimentin and actin (Fig. 1Aa-c), whereas endometrial cells expressed keratin and vimentin (Fig. 1Ad-e). Under TEM, endometrial cell-derived exosomes appeared as spheres with clear and holonomic membranes. Western blot analysis showed that the exosomes expressed the typical exosomal markers CD63 and CD9 but not the endoplasmic reticulum protein calnexin. The diameters of the exosomes ranged from 45 to 120 nm, peaking at 73 nm (Fig. 1B). These results suggest that the primary AM and endometrial cells and endometrial cell exosomes were successfully obtained.

Endometrial cell exosomes inhibit apoptosis of AM cells. AM cells were treated with endometrial cell exosomes to examine cell apoptosis. As shown in Fig. 2A, exosome treatment significantly reduced the apoptotic rate of AM cells compared with the control (5.28±1.87% vs. 18.64±3.97%, $P<0.05$), suggesting that endometrial cell exosomes inhibit apoptosis of adenomyotic cells.

Endometrial cell exosomes enhance IL-6 expression and JAK2/STAT3 activation of AM cells. IL-6 upregulation is closely involved in the pathogenesis of adenomyosis (9). Thus, IL-6 expression and JAK2 and STAT3 phosphorylation were detected in AM cells exposed to exosomes. Western blotting

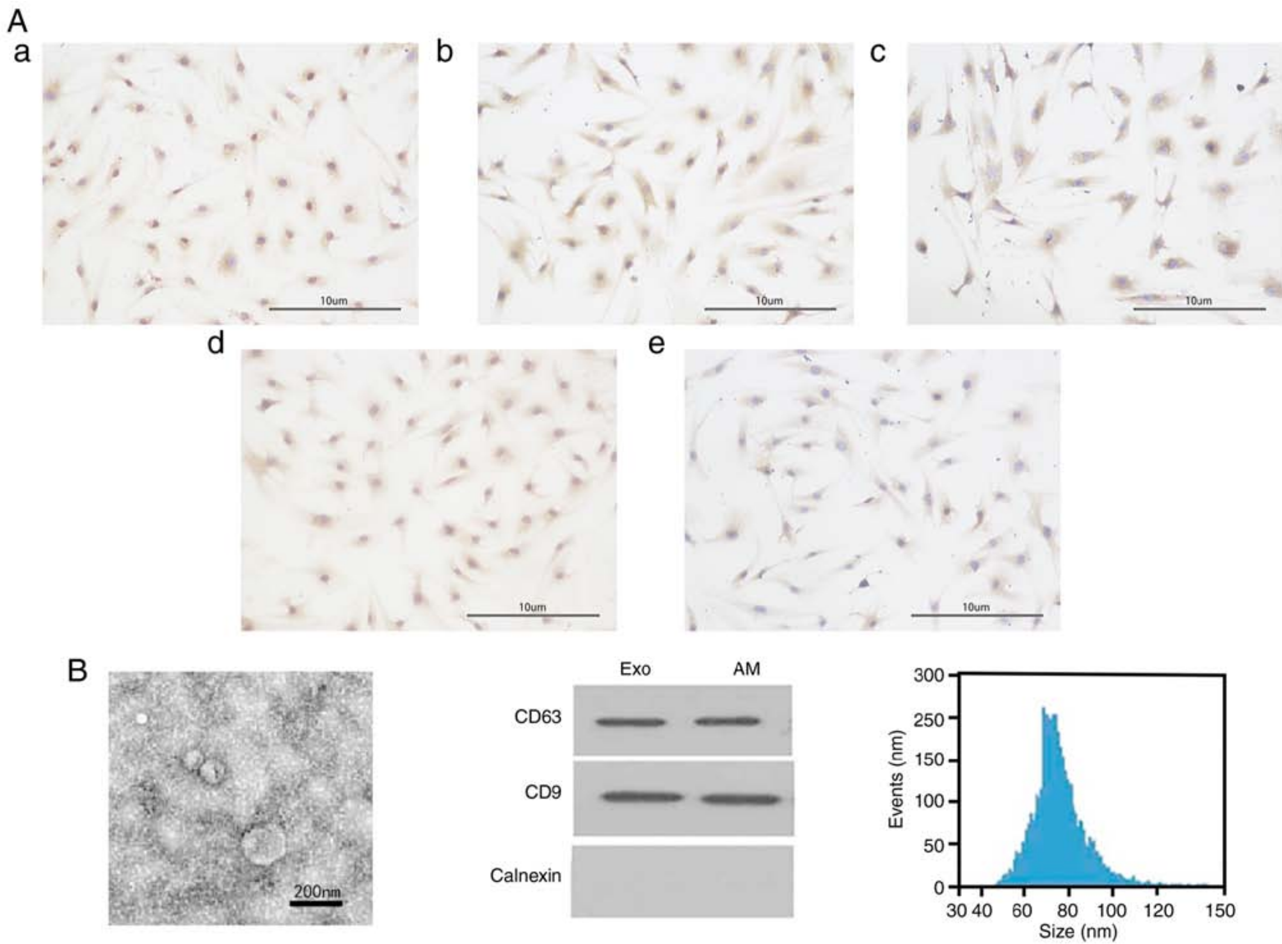

Figure 1. Identification of primary AM cells and endometrial cells and endometrial cell-derived exosomes. (A) Immunocytochemistry was performed to identify AM and endometrial cells. AM cells were positive for keratin (a), vimentin (b) and actin (c) expression. Endometrial cells were positive for keratin (d) and vimentin (e) expression. Magnification x100, scale bar=100 μm. (B) Endometrial cell exosomes were observed under transmission electron microscopy. Nanoparticle tracking analysis was performed to measure the exosome sizes (scale bar=200 nm). Western blot analysis was conducted to determine the expression of surface markers CD63 and CD9. AM, adenomyotic myometrial.

revealed that endometrial cell exosomes significantly enhanced IL-6, JAK2, p-JAK2, STAT3 and p-STAT3 protein expression (Fig. 2B), suggesting that the activation of IL6/JAK2/STAT3 signaling contributes to the effect of endometrial cell exosomes on AM cells.

IL-6 inhibition abolishes endometrial cell exosome-induced proliferation, cell cycle progression and migration of AM cells. In order to investigate whether IL-6 mediates the effects of endometrial cell exosomes on AM cells, AM cells were treated with exosomes in the presence or absence of IL-6 inhibitor tocilizumab. As shown in Fig. 3A, AM cells exposed to exosomes and tocilizumab exhibited apoptotic features, including decreases in size, cytoplasmic and nuclear condensation, massive accumulation of vacuoles in mitochondria, massive aggregation and chromatin fragmentation. These phenomena were not observed in untreated cells or cells exposed to exosomes alone. Furthermore, the MTT assay showed that the cell viability of exosome-treated cells was markedly increased in the absence of tocilizumab compared with untreated cells ($P<0.05$) but substantially decreased in the presence of tocilizumab ($P<0.05$; Fig. 3B). In addition, the percentage of exosome-treated cells in S-phase was significantly decreased in the presence of tocilizumab compared with untreated cells (17.67±1.37% vs. 28.17±2.76%, $P<0.05$). The presence of tocilizumab blocked the cells in the G1/S phase (78.57±3.92% vs. 51.59±4.85%, $P<0.05$; Fig. 3C). Moreover, the wound-healing assay showed that endometrial cell exosomes promoted the migration of AM cells compared with untreated cells and it was abolished by tocilizumab (Fig. 3D). Western blot analysis demonstrated that tocilizumab reversed the activation of IL-6/JAK2/STAT3 signaling in AM cells induced by endometrial cell exosomes (Fig. 3E). As shown in Fig. 3F, IL-6, JAK2 and STAT3 mRNA expression was higher in the exosome group than in the blank group. The expression of the IL-6, JAK2 and STAT3 mRNA was significantly reduced by the addition of the IL-6 inhibitor and the difference was statistically significant compared with the exosome group ($P<0.05$). Taken together, these data suggested that endometrial cell exosomes promoted cell proliferation, cell cycle progression and migration of AM cells and that IL-6 signaling was involved.

Discussion

The etiology of adenomyosis remains elusive and the IL-6/JAK2/STAT3 pathway is involved in the pathogenesis

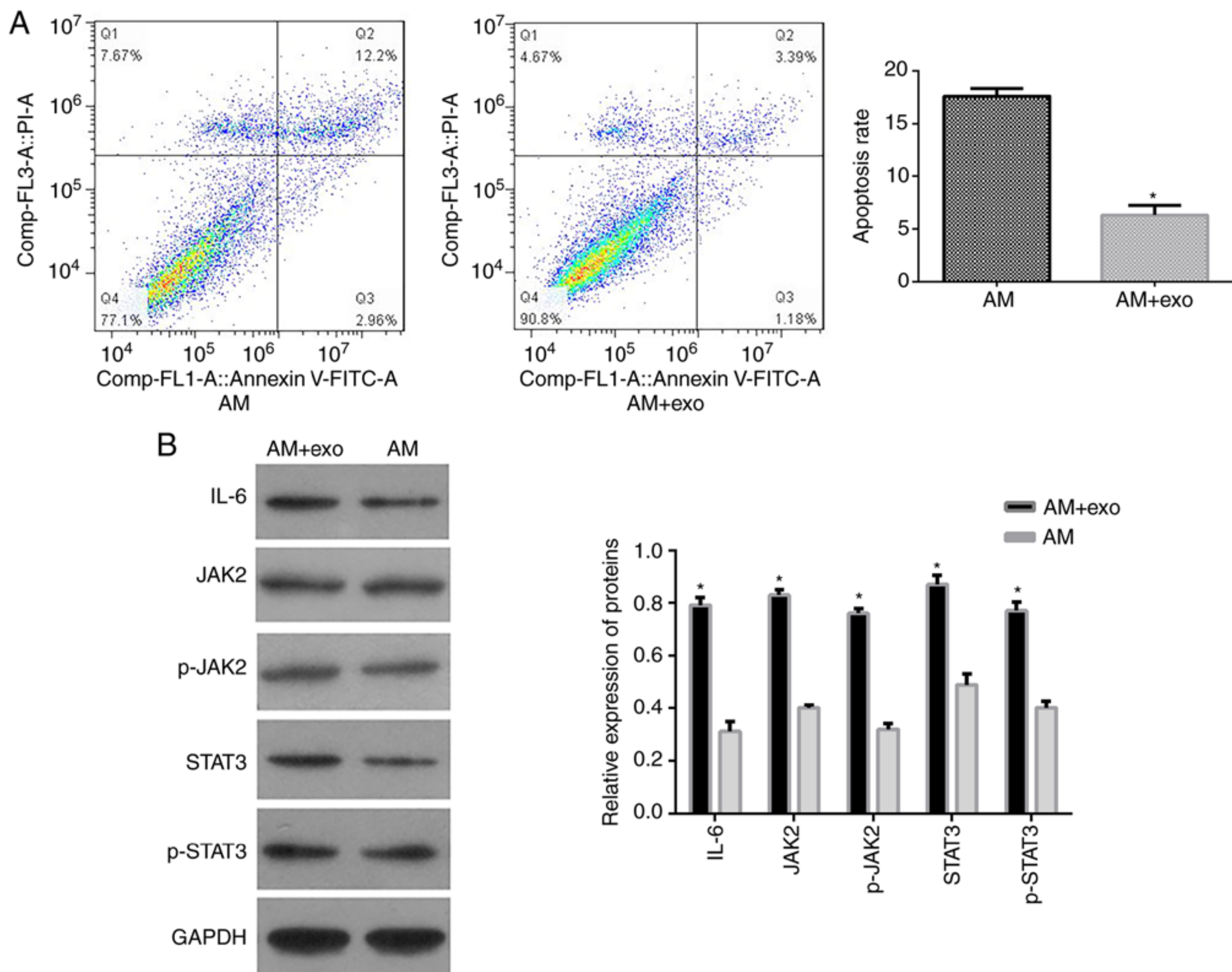

Figure 2. Endometrial cell exosomes suppressed apoptosis while enhancing IL-6 production and JAK2/STAT3 activation of AM cells. AM cells were incubated with endometrial cell exosomes for 24 h. (A) Flow cytometry analysis was performed to examine cell apoptosis. (B) Western blot analysis was conducted to measure the protein expression of IL-6, JAK2, p-JAK2, STAT3 and p-STAT3. GAPDH was used as an internal reference. Data are expressed as the mean ± standard deviation. *P<0.05 vs. AM group; n=3. AM, adenomyotic myometrial cells; exo, exosomes; p-, phosphorylated; JAK2, Janus kinase 2.

of adenomyosis (10). The present study investigated whether endometrial cell exosomes contributed to the invasive behavior of AM cells through the IL-6/JAK2/STAT3 pathway. It was demonstrated that incubation with endometrial cell exosomes significantly promoted cell proliferation, migration and cell cycle progression of AM cells while suppressing AM cell apoptosis, along with enhancement in IL-6 production and JAK2/STAT3 phosphorylation. The IL-6 inhibitor tocilizumab effectively reversed the effects of endometrial cell exosomes on AM cell proliferation and migration and blocked the cells in the G_1/S phase, accompanied by a substantial attenuation of IL-6/JAK2/STAT3 signaling. Thus, endometrial cell exosomes may contribute to the development of adenomyosis by promoting cell proliferation, migration and cell cycle progression of AM cells through the IL-6/JAK2/STAT3 pathway.

Exosomes produced by pathological tissues have detrimental effects on the surrounding tissues and contribute to the development and progression of several diseases (24-27). Exosomes from pathological lesions can also exert toxic effects on nearby cells (24,25,28). Endometrial cell-derived exosomes and their cargoes contribute to the pathophysiology of endometriosis through multiple signaling pathways involved in cell proliferation, migration, apoptosis, inflammation and angiogenesis (20,29). Exosomes are also involved in developing endometrial lesions and types of cancer (30). However, few studies have assessed the role of endometrial cell exosomes in adenomyosis. Adenomyosis is closely related to endometriosis and occasionally coexists with endometriosis (31). Adenomyosis and endometriosis have been considered different phenotypes of a single disease (32). Thus, endometrial cell exosomes may also contribute to the etiology of adenomyosis. As expected, the results of the present study showed that endometrial cell exosomes significantly inhibited apoptosis of AM cells compared with control cells. Inhibition of apoptosis plays an important role in the pathogenesis of endometriosis and adenomyosis. Therapeutic agents that accelerate apoptosis have shown efficacy against adenomyosis (33,34). Therefore, endometrial cell exosomes may exacerbate adenomyosis by suppressing the apoptosis of AM cells.

IL-6 plays a central role in the growth of endometrial cells. Dysregulation of IL-6 signaling may cause disorders of the endometrium, such as endometriosis and endometrial

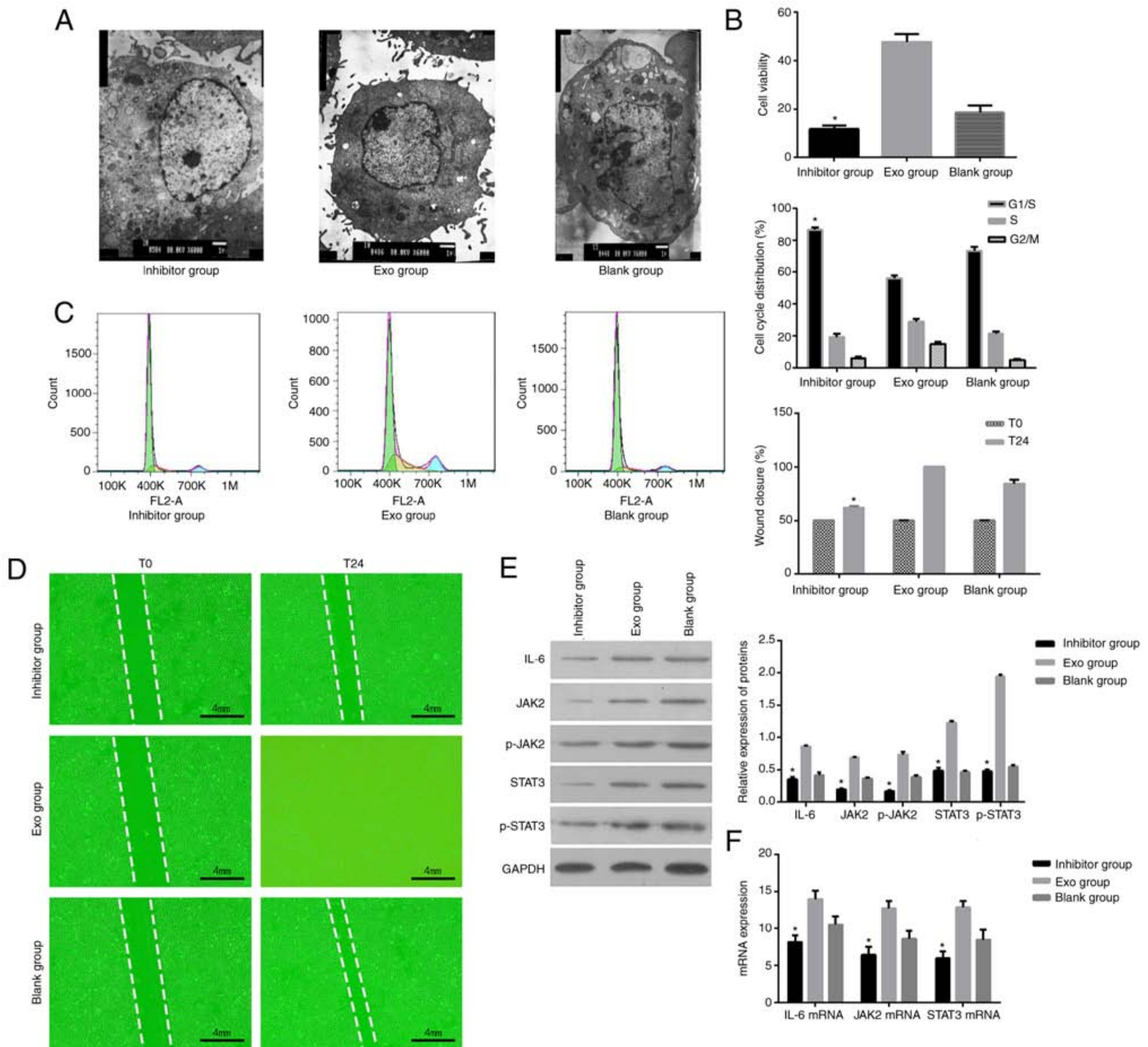

Figure 3. IL-6 inhibition reversed the effects of endometrial cell exosomes on apoptosis, cell cycle progression and migration of AM cells. AM cells were incubated with endometrial cell exosomes in the absence or presence of IL-6 inhibitor Tocilizumab (20 μM) for 24 h. (A) The ultrastructure of AM cells was observed under transmission electron microscopy. Images were acquired at a magnification x 6,000. Scale bar, 1 μm. (B) MTT assay was conducted to measure cell viability. (C) Flow cytometry analysis was performed to examine cell cycle distribution. (D) A wound-healing assay was performed to examine cell migration (scale bar, 4 mm). (E) Western blot analysis was performed to determine the protein expression of IL-6/JAK2/STAT3 signaling. (F) Reverse transcription-quantitative PCR analysis was performed to determine the mRNA expression of IL-6/JAK2/STAT3 signaling. Data are expressed as the mean ± standard deviation. *P<0.05 vs. exo group. AM, adenomyotic myometrial cells; exo, exosome; T0, 0 h; T24, 24 h.

cancer (35,36). In women with adenomyosis, IL-6 upregulation has been frequently seen in endometrial stromal cells, macrophages, endometrial biopsies, adenomyosis-derived mesenchymal stem cells and the peritoneal fluid (37-41). Incubation with exosomes from different sources can induce IL-6 production by endometrial cells (42,43). It was therefore hypothesized that IL-6 was involved in the effect of endometrial cell exosomes on the growth of AM cells. The results showed that AM cells incubated with endometrial cell exosomes had markedly enhanced IL-6 expression compared with untreated cells, consistent with the original hypothesis.

Although adenomyosis is a benign condition, AM cells share some characteristics with cancer cells. Women with adenomyosis are at a higher risk of endometrial cancer and adenomyosis is considered a precursor of endometrial cancer (44,45). Since the IL-6/JAK/STAT3 pathway is hyperactivated in endometrial cancer (16,46,47), AM cells were treated with exosomes in the presence or absence of an IL-6 inhibitor to investigate whether IL-6 is required for the effects of endometrial cell exosomes on AM cell behavior. The results revealed that IL-6 inhibition completely reversed endometrial cell exosome-induced proliferation, cell cycle progression and migration of AM cells, suggesting that endometrial cell exosomes promote the invasive properties of AM cells through the activation of IL-6 signaling.

Age is probably a major contributor in the development of adenomyosis since most patients are diagnosed in their

40s and 50s when symptoms appear (48,49), but the true incidence is unknown (48) and it is possible that younger women can have asymptomatic adenomyosis. Of note, the reported prevalence varies widely from 1-70% (48-50). Whether symptomatic adenomyosis results from slowly progressing lesions that take years to develop symptoms or whether aging plays a role in the development of adenomyosis remains unknown and the present study was not designed to answer that question. Nevertheless, inflammation and aging are two sides of the same medal (51-54). Since the present study reported a strong role of IL-6, a proinflammatory cytokine (12-14), in the development of adenomyosis and since aging is associated with a low-grade basal inflammatory state (51-54), it is possible that aging contributes to the development of adenomyosis. That hypothesis will have to be tested in future studies.

The present study has some limitations that need to be addressed in the future. First, the exosomal cargo that promotes IL-6 production by AM cells remains unknown. Second, the application of a JAK2/STAT3 inhibitor is required to verify whether JAK2/STAT3 is essential to mediate the effects of endometrial cell exosomes on AM cell growth. Third, the tissue samples were not subjected to immunohistochemistry and had to be used to isolate AMs. Indeed, the sample was limited to ~1 cm^3 to not interfere with the postoperative pathological examination and the study of adenomyosis tissues will be further supplemented and improved in future animal experiments. Fourth, a key limitation is the lack of the immune system context. Indeed, tocilizumab has direct effects on IL-6 but also has effects on immune cells (55). The immune context will have to be examined *in vivo*.

In conclusion, endometrial cell-derived exosomes promoted cell proliferation, migration and cell cycle progression of AM cells through IL-6/JAK2/STAT3 activation, suggesting that endometrial cells exosomes mediated the crosstalk between the endometrium and the myometrium via IL-6 signaling and thus facilitated the development of adenomyosis.

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Availability of data and materials

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

Authors' contributions

XJ and XC performed the experiments, participated in collecting data and drafted the manuscript. XJ and XC performed the statistical analysis and participated in its design. XJ participated in acquiring, analyzing, or interpreting data and drafted the manuscript. Both authors read and approved the final manuscript. XJ and XC confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of The First Affiliated Hospital of Guangdong Pharmaceutical University [approval no. 2021-(31)]. All participants provided informed consent before the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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