TGF-β1 in Seminal Plasma Promotes Endometrial Mesenchymal Stem Cell Growth via p42/44 and Akt Pathway in Endometriosis

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Research

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Abstract

**Background:** The cause of endometriosis, which is characterized by the existence of functional endometrial tissue outside the uterine cavity, is poorly understood. Seminal plasma (SP) is rich in multiple cytokines may promote endometrial tissue survival.

**Methods:** We evaluated the effect of SP on growth of endometrial mesenchymal stem cells (MSCs) from women with endometriosis (E-MSCs), and women without endometriosis (NE-MSCs). Additionally, effects of SP on endometriotic implants were analyzed using a xenograft model of endometriosis in immunodeficient nude mice.

**Results:** Proliferation, cell foci formation, cell cycle progression, and growth marker expression of E- and NE-MSCs were promoted by SP. SP significantly stimulated phosphorylation of p42/44 and Akt and increased expression of the proliferation marker in E- and NE-MSCs, which was attenuated by transforming growth factor beta 1 (TGF-β1) receptor inhibitor SB431542, Akt inhibitor LY294002, or p42/44 MAPK inhibitor PD98059. SP enhanced CDK2 and CDK6 expression and accelerated cell cycle progression in E-MSCs. Xenografts exposed to SP exhibited a three-fold increase in volume and four-fold increase in weight after 14 days.

**Conclusions:** Our findings demonstrate that TGF-β1 in SP may promote endometrial tissue survival. These effects may be mediated through activation of TGF-β1, Akt, and p42/44 signaling, which enhances CDK2 and CDK6 expression and accelerates cell cycle progression.

**Background**

Endometriosis, a major cause of infertility, is a prevalent gynecologic disease characterized by the existence of functional endometrial tissue outside the uterine cavity, which causes pain and affects fertility in almost 10% of reproductive women. The precise mechanisms that cause endometriosis remain unknown. Sampson's theory posits that endometriotic implants arise from retrograde menstruation of endometrial tissue through the fallopian tubes into the peritoneal cavity. The human endometrium is a unique tissue exhibiting regular cyclical periods of growth, denudation, and renewal. Mesenchymal stem cells (MSCs) from endometrium have been characterized as multipotent cells of mesenchymal origin that can differentiate into a number of different cell types, and were implicated in cyclic endometrial regeneration. Endometrial MSCs have been detected in the basalis layer of the endometrium and endometriotic implants, and may be responsible for the development of endometriosis. However, factors capable of triggering the proliferation of endometrial MSCs require further study.

Endometriosis is relatively common in women of childbearing age. Sexual activity in females allows contact with semen, which contains sperm and seminal plasma (SP) rich in numerous cytokines. SP can induce epithelial-mesenchymal transdifferentiation and the expression of myofibroblastic metaplasia.
markers in endometriotic and endometrial cells. In the absence of a barrier contraceptive, the growth of endometrium can be further modulated by mediators present in SP, which is derived from sexual activity. Previous research showed that SP can promote endometriotic development and induce global transcriptomic changes that increase the risk for endometriosis. Moreover, patients with endometriosis may be oversensitive to stimulation from SP. In vitro experiments showed that endometrial epithelial and stromal cells had an enhanced proliferative response to SP compared with cells from women without endometriosis. In vivo experiments showed that SP enhanced endometrial survival and facilitated endometriotic lesion development. Although a few studies have focused on the role of SP in promoting endometriotic development, downstream signaling mechanisms are not fully understood. SP may also activate the development of endometrial MSCs, which are sensitive to extracellular environments that promote endometriotic lesion survival.

As the acellular fraction of seminal fluid, SP is rich in cytokines, chemokines, growth-factors, and steroid hormones. In particular, SP contains high concentrations of TGF-β1, TGF-β2, and TGF-β3. TGF-β1 is a multifunctional regulatory cytokine involved in several cellular functions, including proliferation, migration, invasion, and differentiation. TGF-β1 expression is increased in peritoneal fluid and lesions of women with endometriosis. Ibrahim et al. described novel evidence that even when semen is washed twice, SP is still detectable. After sexual intercourse, SP components were found to enter into the endometrial bed or peritoneal bed as a result of hematogenous dissemination or direct tissue perfusion through the anterior or posterior vaginal fornix. Therefore, it is possible that TGF-β1 in SP, which comes into contact with the endometrial bed after unprotected sexual intercourse, can induce biological changes in those tissues and cells. Here, we explored whether SP could activate MSCs in endometrium through TGF-β1.

Mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt pathways play important roles in stimulating proliferation of endometriotic cells, production of inflammatory factors, and neovascularization. Honda et al. reported activation of PI3K/Akt and MAPK signaling pathways in endometriosis. MAPKs are activated by multiple extracellular signals that promote cell proliferation, whereas Akt phosphorylation is mediated by PI3K in response to numerous extracellular signals. Both MAPK and PI3K/Akt pathways are crucial for the regulation of cell survival and proliferation. The molecular mechanisms of TGF-β1-regulated MSC proliferation have been extensively studied. Here, we determined whether MAPK and Akt pathway activation is involved in the stimulation of endometrial MSC proliferation. In addition, the TGF-β receptor inhibitor SB431542, Akt inhibitor LY294002, and p42/44 MAPK inhibitor PD98058 were examined for their ability to reverse the effect of SP on endometrial MSC proliferation.

Understanding whether SP can activate the proliferation of MSCs is of great importance because unprotected sexual intercourse is common. In the present study, we found that TGF-β1 in SP promotes the growth of endometrial MSCs via p42/44 and Akt pathways, indicating a potential prevention and treatment strategy for endometriosis.
Methods

Patients

This study was approved by the ethics committee of Sir Run Run Shaw Hospital (Hangzhou, China). The patient group consisted of women with endometriosis (stage III–IV) undergoing laparoscopy for pain or infertility (n = 6). The control group consisted of women with no endometriosis undergoing laparoscopy for tubal disease (n = 6). Human SP was obtained from male partners [(n = 15), aged 25 to 39 years] of women undergoing treatment for female-factor infertility at Sir Run Run Shaw Hospital in China and healthy fertile men. None of the donors had received hormonal therapy for at least 6 months prior to surgery. Participants had regular menstrual cycles (27–32 days). Additional clinical characteristics of patients are listed in Supplementary Tab. 1. Prior to enrolling in the study, informed written consent (procedure approved by the local Ethics Committee) was obtained from each patient. All female patients exhibited stage III or IV endometriosis, as classified by the American Society for Reproductive Medicine. Sperm analyses of male patients were conducted according to recommendations of the World Health Organization (WHO). Samples were included if all parameters were consistent with normal values in the WHO Laboratory Manual for the Examination and Processing of Human Semen.

Isolation of MSCs

To isolate and generate MSC lines from the endometrium of women with endometriosis (E-MSCs) and without endometriosis (NE-MSCs), endometrial tissue biopsies were obtained just prior to surgery using an endometrial suction catheter (Lilycleaner, Ningbo, China). Cells were isolated from biopsy specimens of the endometrium and seeded in triplicate at low density (approximately 200 cells per 100-mm dish) in Dulbecco’s Modified Eagle’s Medium with F12 Supplement (DMEM/F12; Gino167 Biological, Hangzhou, China). Large colonies were isolated and trypsinized into single-cell suspensions following incubation for 21 days. The resulting cell suspensions were diluted and seeded into 96-well plates at a density of approximately one cell per well. After 14 days in culture, clonally derived proliferating colonies were trypsinized and individually cultured in individual 100-mm dishes. Cells were allowed to grow in DMEM/F12 with 10% fetal bovine serum (FBS), and adherent cells were cultured until they reached 80%–90% confluence. Cells were trypsinized, subcultured, and used for experiments during passages two to four.

Multipotent differentiation

The multipotency of endometrial MSCs for osteogenesis and adipogenesis was determined as previously described. Briefly, to induce osteogenesis, MSCs were plated at a density of $5 \times 10^3$ cells per cm$^2$ and treated with 10 mm β-glycerol phosphate, 0.1 μM dexamethasone, and 50 μg/ml ascorbic acid (all from Sigma-Aldrich, St. Louis, MO, USA) for 2 weeks. On Day 14, cells were stained with Alizarin Red and alkaline phosphatase. To induce adipogenic differentiation, MSCs were cultured in DMEM supplemented with adipogenic supplements (Stem Cell Technologies, Vancouver, Canada). On Day 14, lipid vacuoles within cells were stained with Oil Red O.
SP Collection

Semen samples obtained from 15 donors were allowed to liquefy for 2 hours. After liquefaction, each sample was centrifuged at 700 × g for 10 minutes at room temperature within 2 hours of ejaculation. The supernatant was centrifuged at 10,000 × g for 30 minutes to remove spermatozoa. The collected samples were pooled, filtered and concentrated using Centricon Plus-20 centrifugal concentration tubes (3,000 NMWL; Millipore, Cork, Ireland) (Supplementary Fig. S1A). Acid activation of cytokines in SP was achieved by treating concentrated SP with 1 N HCl, followed by incubation at room temperature for 10 minutes. SP was then neutralized with 1.2 N NaOH/0.5 M HEPES before resuspending in DMEM/F12. The treated SP was aspirated and frozen at -80 °C. Prior to use, concentrated SP was thawed on ice, resuspended, and diluted 1:100 with DMEM/F12 for in vitro studies, or 1:500 with sterile-filtered phosphate-buffered saline (PBS) for in vivo studies.

Treatment of cells

Cells were seeded into 96-well plates at a density of 2 × 10^3 cells per well for proliferation analyses, and 5 × 10^4 cells per 60-mm dish for western blotting (WB). For cell proliferation analyses, cells were cultured at 37 °C for 1 day in DMEM/F12 containing 10% FBS, followed by incubation with control (DMEM/F12 + 2% FBS) or SP containing 2% FBS for the indicated time. For foci formation assay, flow cytometry analysis, and WB, cells were cultured at 37 °C for 1 day and then incubated for 1 day in serum-free culture media. Subsequently, culture media were replaced and cells were treated with vehicle control or SP. Foci formation assay was performed after 2 days of culture. WB analysis was performed after 2 days of culture or at specified time (0, 1, 2 and 6 hours).

Flow cytometry analysis

Endometrial MSCs (1 × 10^6) were resuspended in PBS and incubated with 1 µg of fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat monoclonal antibodies for 1 hour at 4 °C in 100 ml of PBS containing 0.1% bovine serum albumin (BSA). The following antibodies were used: anti-CD44, CD90, CD176, CD29, CD117, CD 34, CD45, HLA-DR, and FITC-conjugated isotype-matched immunoglobulin G (1:50; all from Becton Dickinson, Franklin Lakes, NJ). Cells were washed twice with PBS before analysis using an Epics XL flow cytometer (Beckman Coulter, Fullerton, CA, USA).

Cell-cycle parameters were determined by flow cytometry of propidium iodide (PI)-stained endometrial MSCs. Briefly, MSCs (2 × 10^6) were fixed in 70% ethanol at 4 °C overnight. The ethanol was discarded and MSCs were resuspended in PBS. Cell pellets were stained with 50 µg/ml PI and 100 µg/ml RNase A in PBS for 30 minutes at room temperature. After washing with PBS, MSCs were analyzed by an Epics XL flow cytometer.

Flow cytometric analysis of apoptosis markers was performed. Endometrial MSCs were washed in cold PBS and incubated with FITC-conjugated Annexin and PI solution (Invitrogen, Carlsbad, CA, USA) at room temperature for 15 minutes in the dark. Stained cells were analyzed using an Epics XL flow cytometer.
Cell proliferation assay

Cell proliferation was determined using a Cell Counting Kit 8 (CCK-8) assay. Endometrial MSCs were seeded into 96-well plates at a density of $2 \times 10^3$ cells per well. After cell attachment, control (phenol red-free DMEM/F12 containing 2% FBS) or resuspended SP containing 2% FBS was added to cells. To evaluate changes in cell proliferation, cell counting was performed using a CCK-8 kit (Tojindo, Shanghai, China) according to the manufacturer's instructions.

Colony-forming assay

MSCs were seeded into six-well culture plates at a density of $1 \times 10^2$ cells per well and incubated at 37 °C for 14 days. Subsequently, MSCs were stained with a crystal violet solution and imaged with a microscope equipped with a digital camera.

Antibodies and western blotting

Cell lysates were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the resulting proteins were transferred onto polyvinylidene fluoride membranes. Membranes were blocked in Tris-buffered saline containing Tween-20 and 5% BSA, and then probed with the following primary antibodies: proliferating cell nuclear antigen (PCNA; 1:500; Abcam, Cambridge, UK), B-cell lymphoma-2 (Bcl-2; 1:500; Abcam), β-Tubulin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:3000; Abcam), total Akt, p-Akt-Ser473, p-Akt-Thr308, p-p42/44, total p42/44, CDK2, CDK4, CDK6, cyclin A, cyclin D1, and cyclin E2 (1:1000, Cell Signaling Technology, Beverly, MA, USA). Anti-rabbit and anti-mouse antibodies (1:1000; Cell Signaling Technology) and Immobilon Western Chemiluminescent HRP Substrate (Millipore, Boston, MA) were used to visualize immune-positive bands. Protein expression levels were normalized to β-Tubulin or GAPDH. ImageJ software (National Institutes of Health, Bethesda, MD, USA; http://imagej.nih.gov/ij) was used to evaluate protein band densities.

Mouse model of endometriosis

Mature female athymic nude mice (6 weeks old) purchased from Shanghai Laboratory Animal Co. Ltd. (Shanghai, China) were housed in a pathogen-free and climate-controlled environment (23–25 °C) with regulated 12-hour light/dark cycles. One week was allowed for acclimatization prior to experimental proceedings. Three days prior to endometrial transplantation, mice received a daily intraperitoneal injection of 200 µg/kg 17β-estradiol. The nude mouse model of endometriosis was established as previously described 18,20. Proliferative-phase human endometrial fragments (1–2 mm³) from endometriosis patients were washed with sterile serum-free DMEM/F-12 culture medium. Human endometrial fragments were implanted subcutaneously into mice under general anesthesia. To achieve higher local concentrations, subcutaneous injection was employed as the administration route based on previous animal experiments 18,20,21 (Supplementary Fig. S1B). Subcutaneous injections of TGF-β1 (40 ng/g), SP, or the TGF-β receptor inhibitor SB431542 (10 ug/g) were employed. Daily intrallesional injections were started on the first day after endometrial tissue implantation and continued for 14 days.
Mice were then randomly divided into four groups: control, TGF-β1, SP, and SP+SB431542. Endometrial tissues were harvested and measured after 14 days of intralesion injections commencing from day 1 post-implantation. Treatment dosages used were determined by preliminary experiments. Endometriotic implants were collected, fixed in 10% formalin-acetic acid, and embedded in paraffin for histopathological examination. Immunohistochemical staining was performed according to well-established protocols. Anti-human leukocyte antigen (HLA) antibody (1:100; Abcam) was used to identify human cells within xenograft tissue. Tissue sections were also incubated with a polyclonal antibody for the proliferative marker Ki67 (1:100; Abcam). Staining scores and parameters were calculated using a computerized image analysis system, as previously described. Areas of positive staining were calculated. In addition, the whole area of the endometriotic implant in each section was analyzed.

Data analysis and statistics

SPSS version 16.0 (SPSS, Chicago, IL) was used to analyze complete datasets. Student’s t test, one-way analysis of variance, or Scheffe’s general linear model of repeated measures method was used to compare differences between treatment groups. Statistical significance was defined as $P < 0.05$.

Results

Isolation and characterization of NE- and E-MSCs

Isolated NE-MSCs and E-MSCs were kept in culture until passage 20, indicating a self-renewal ability typically associated with MSCs. To verify NE- and E-MSC phenotypes, we evaluated the expression of positive mesenchymal markers CD44, CD90, CD73, and CD29, as well as negative MSC markers CD117, CD34, CD45, and HLA-DR. Mesenchymal markers were highly expressed in more than 90% of NE- and E-MSCs, while mesenchymal-negative markers were observed in less than 10% of NE- and E-MSCs (Supplementary Fig. S2A). An osteogenic differentiation assay confirmed that the majority of NE- and E-MSCs had the capacity to undergo osteogenesis. Mineralized calcium was deposited after osteogenic induction, as confirmed by positive Alizarin Red S and alkaline phosphatase staining. Following adipogenic induction conditions, Oil Red O staining demonstrated the accumulation of lipids, indicating MSCs had differentiated into adipogenic lineages (Supplementary Fig. S2B). Collectively, these results indicated the successful isolation of MSCs.

SP promoted NE- and E-MSC proliferation, cell foci formation, cell cycle progression, and growth marker expression

To investigate the effect of SP on MSC proliferation, NE- and E-MSCs were cultured with SP in vitro, and CCK-8 assays were performed. SP treatment significantly increased the proliferation of MSCs compared with controls (Fig. 1A). In addition, SP could significantly increase the ability of MSCs to form colonies, as demonstrated by the foci formation assay (Fig. 1B). Flow cytometry was used to determine the influence of SP on the cell cycle progression of NE- and E-MSCs. Compared with the control group, the number of cells in G0/G1 of the SP-treated group was decreased, and the number of S-phase cells was elevated by
SP treatment; however, the distribution of G2/M phase cells did not change significantly (Fig. 1C). SP promoted MSC entrance into S phase from G0/G1, which was considered indicative of cell growth. Apoptosis of MSCs was determined by flow cytometric analysis of annexin V FITC/PI staining. There was no significant reduction or increase in the percentage of live cells in the SP-treated group compared with the control group. After treatment with SP or vehicle, a small fraction of apoptotic cells was observed. The percentage of apoptotic cells was similar in SP-treated and control groups (Fig. 1D). We also evaluated the effect of SP on expression of proliferating cell nuclear antigen (PCNA), an essential component of DNA polymerase δ\(^\text{23}\), and the antiapoptotic protein Bcl-2. WB assay results demonstrated that SP elevated the protein expression of PCNA (Fig. 1C), while Bcl-2 protein expression was unchanged (Fig. 1E). These results indicate that SP activation promoted the proliferation of MSCs and accelerated their cell cycle.

**TGF-β1 in SP promoted MSC proliferation via phosphorylation of p42/44 and Akt**

We next examined the role of p42/44 and Akt pathways, which control cell proliferation, differentiation, and survival\(^\text{24}\). Expression of p-p42/44, T-p42/44, p-Akt-S, p-Akt-T, T-AKT, and β-tubulin was assessed by WB. We found that SP stimulated phosphorylation of p42/44 and Akt both at serine 473 (Ser473) and threonine 308 (Thr308) at various time points (1, 2, and 6 hours) after incubation with SP, indicating activation of p42/44 and Akt pathways (Fig. 2A). Next, the involvement of p42/44 and/or Akt signaling in SP-enhanced proliferation of MSCs was examined. SP contains high concentrations of TGF-β1, which is involved in the activation of p42/44 and/or Akt signaling pathways\(^\text{25}\). TGF-β1 (2 ng/ml), SP, SP with the TGF-β receptor inhibitor SB431542, SP with the AKT inhibitor LY294002, or SP with the p42/44 MAPK inhibitor PD98059 were used to stimulate NE- and E-MSCs, and determine whether MSC-secreted TGF-β1 contributes to cell proliferation through p42/44 or Akt pathways. Pretreatment with inhibitors was conducted prior to stimulation with SP. TGF-β1, SP significantly increased the expression of the proliferation marker PCNA. However, addition of the TGF-β receptor inhibitor SB431542, Akt inhibitor LY294002, or p42/44 MAPK inhibitor PD98059 markedly attenuated PCNA protein expression in SP-treated MSCs (Fig. 2B). These results indicate that p42/44 and Akt signaling are involved in the enhanced MSC proliferation caused by SP activation.

**SP induced modulation of cell cycle-related protein CDK2 and CDK6 expression via p42/44 and Akt signaling**

The cell cycle is driven, in part, by cyclin dependent kinases (CDKs) and their activation by cyclins\(^\text{26}\). Cyclin D/CDK4/CDK6 activity occurs in mid to late G1 phase, upstream of CDK2/cyclin E activity. The complexes formed by cyclins and CDKs can regulate kinase activity and progression through G1/S phase. Cyclin A may be required for both S phase and M phase\(^\text{27}\). In the present study, CDK2 and CDK6 protein levels were higher in SP-treated E-MSCs whereas, CDK4, cyclin A2, cyclin D1, and cyclin E2 protein levels remained largely unchanged (Fig. 3A). SP activation may accelerate the cell cycle by increasing expression of CDK2 and CDK6, resulting in enhanced E-MSC proliferation. We also examined effects of the AKT inhibitor LY294002 and p42/44 MAP kinase inhibitor PD98059 on SP-induced cell cycle-related
protein expression. SP treatment increased CDK2 protein levels, which, conversely, were markedly attenuated by LY294002. CDK6 protein levels were also markedly attenuated by LY294002 and PD98059 (Fig. 3B). These results indicate that SP-induced increases in p42/44 and Akt signaling may result in upregulation of CDK2 and CDK6 expression, consequently accelerating the cell cycle.

Exposure to SP promoted endometriosis-like lesion development in mice

To confirm our hypothesis, an in vivo SCID mouse model was used. All mice developed endometriosis-like lesions showing typical characteristics of endometriosis with glandular structures and stroma. Endometriosis-like lesions harvested from mice treated with TGF-β1 and SP were found to be significantly larger and heavier than lesions treated with vehicle alone (Fig. 4A–C). Xenografts exposed to SP showed a three-fold increase in volume and four-fold increase in weight after 14 days. Endometriosis-like lesions may contain both donor human and host mouse tissue. Part of the xenograft tissue stained positively for HLA, indicating its human origin. There was a significantly greater proportion of donor tissue in endometriosis-like lesions from TGF-β1 and SP groups (Fig. 4D, F). Moreover, cells positive for Ki67, a marker for proliferation, were significantly more numerous in endometriosis-like lesions after TGF-β1 and SP treatment compared with controls (Fig. 2E, F). However, the growth-promoting effect of SP was attenuated by the TGF-β receptor inhibitor SB431542 (Fig. 4A–F). These results indicate that SP directly supports the proliferation of cells of endometrial origin via TGF-β1.

Discussion

Endometriosis is one of the most common gynecological disorders. Its pathogenesis remains unknown despite extensive study. Studying the role of stem cells in the etiology of endometriosis may illuminate some of the factors that initiate this disease. Many studies have demonstrated the presence of MSCs in endometrium and menstrual blood. Recent studies showed that menstrual blood-derived MSCs from women with endometriosis showed a higher proliferative capacity compared with MSCs from women without endometriosis; they also revealed different phenotypic and functional characteristics.

Aggressive overgrowth of MSCs may be a cause of endometriosis. SP may influence endometriotic lesion development in human endometrial explant cultures. The endometrium has unique regenerative properties arising from the existence of MSCs. Once in the abdominal cavity, endometrial MSCs can proliferate, invade, and differentiate into endometrial cells, finally generating ectopic implants. SP contains high concentrations of cytokines, such as TGF-β1, a multifunctional regulatory cytokine. Indeed, the susceptibility of MSCs to SP may be a cause of endometriosis.

Our findings demonstrate that SP can induce the proliferation of NE-MSCs and E-MSCs. SP-treated MSCs exhibited accelerated growth, cell foci formation, and cell cycle progression. In addition, SP treatment significantly increased protein levels of the proliferation marker PCNA. Thus, our results show that SP could enhance cell proliferation, which confirms the work of others.
To determine how SP enhances cell proliferation, we evaluated TGF-β1, p42/44, and Akt signaling pathways. P42/44, a part of the MAPK cascade, can be activated by extracellular or intracellular factors. Activated p42/44 is transported into the nucleus, whereby it increases proliferation-related gene expression. Activation of Akt signaling is a vital regulator of cell survival, while both p42/44 and Akt signaling are critical to cell proliferation. We found that phosphorylated p42/44 phosphorylates Akt both at Thr308 and Ser473, which were both highly expressed in SP-treated MSCs compared with controls, indicating activation of p42/44 and Akt signaling. To further explore the role of TGF-β1, p42/44, and Akt signaling pathways on SP-driven cell proliferation, signaling was inhibited by the TGF-β receptor inhibitor SB431542, Akt inhibitor LY294002, or p42/44 MAPK inhibitor PD98059. Inhibition of this signaling markedly attenuated SP-mediated proliferation, indicating that TGF-β1 can modulate Akt and p42/44 signaling pathways in cell growth and proliferation.

Cell cycle progression is dependent, in part, on tightly regulated activity of cell cycle-related proteins. By assessing the expression of such proteins, we demonstrated that increased protein expression of CDK2 and CDK6 was attenuated by the Akt inhibitor LY294002 and p42/44 MAPK inhibitor PD98059. Complexes with CDK2/CDK6 and cyclins influence progression through G1/S phase. The activation of p42/44 signal can increase the expression of CDKs and accelerate the cell cycle, which is in accordance with our results indicating that Akt and p42/44 signaling pathways have a cooperative effect on SP-enhanced proliferation of MSCs by increasing the expression of proteins regulating G1/S progression.

In vivo experiments suggest that treatment with the TGF-β receptor inhibitor SB431542 may retard the progression of endometriosis, which is conversely accelerated by TGF-β1 and SP. This result confirms a previous study conducted in a mouse model of adenocarcinoma tumor, whereby SP enhanced lesion proliferation and growth. These findings and previous in vitro results indicate a significant role for SP in the progression of endometriosis.

SP contains numerous types of inflammatory agents, such as TGF-β, prostaglandins, and glycoprotein signaling molecules including growth factors and cytokines. These molecules bind to their target cell to modulate gene expression and cell function. One limitation of our research is that we have not identified all of the signaling molecules in SP responsible for orchestrating the effects we observed in vivo and in vitro. However, these effects may result from the integration of all signaling in SP though TGF-β, making it potentially one of the most important factors.

**Conclusion**

We showed that SP promotes NE- and E-MSC proliferation, cell foci formation, cell cycle progression, and growth marker expression. In vitro experiments showed that this effect may be mediated through activation of TGF-β1, Akt and p42/44 signaling, which enhances the expression of CDK2 and CDK6, thus accelerating cell cycle progression. This study also provides in vivo evidence that TGF-β1, which is
present in SP, could accelerate endometriosis. Thus, TGF-β1 in SP may promote growth of MSCs via p42/44 and Akt pathways.

**Abbreviations**

SP: Seminal plasma; MSCs: Endometrial mesenchymal stem cells; E-MSCs: MSCs from women with endometriosis; NE-MSCs: MSCs from women without endometriosis; TGF-β1: Transforming growth factor beta 1; MAPK: Mitogen-activated protein kinase; PI3K: phosphatidylinositol 3-kinase; FBS: Fetal bovine serum; PBS: Phosphate-buffered saline; WB: Western blotting; FITC: Fluorescein isothiocyanate; BSA: Bovine serum albumin; PI: Propidium iodide; CCK-8: Cell Counting Kit 8; PCNA: Proliferating cell nuclear antigen; Bcl-2: B-cell lymphoma-2; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; HLA: Human leukocyte antigen; CDKs: Cyclin dependent kinases.

**Declarations**

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**Authors’ contributions**

JL and YD were involved in sample collection, experiments, acquisition, analysis and interpretation of data. CHL, HYZ and XYJ were involved in concept, design and drafting the article. XL and YLZ participated in the revising of the manuscript and gave conceptual advice. All authors read and approved the final version of the paper.

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**Availability of data and materials**

The dataset supporting the conclusions of this article is included within the article.

**Ethics approval and consent to participate**

This study was approved by the ethics committee of Sir Run Run Shaw Hospital Hangzhou, China. Written informed consent was obtained, and the experimental protocol was established according to the associated national guidelines from the Ministry of Science and Technology of China.
Consen for publication

All co-authors have seen and approved the final version of the paper and have agreed to its submission for publication. All patients signed informed written consent forms.

Competing interests

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figures
Figure 1

SP promoted cell proliferation, cell foci formation, cell cycle progression, and growth marker expression in NE- and E-MSCs. SP did not affect cell apoptosis. (A) Effects of low-serum medium, and SP with low-serum medium on the growth of NE-MSCs (n = 6) and E-MSCs (n = 6) were compared by CCK-8 assay. (B) Foci formation assay in SP-induced NE-MSCs (n = 3) and E-MSCs (n = 3). (C) Flow cytometry of cell cycle markers in SP-induced NE-MSCs (n = 3) and E-MSCs (n = 3). (D) Apoptosis of NE-MSCs (n = 3) and E-MSCs (n = 3) induced by SP was detected using flow cytometry. (E) Protein expression levels of the growth marker PCNA and antiapoptotic Bcl-2 in SP-induced NE-MSCs (n = 3) and E-MSCs (n = 3) were assessed by WB. WB bands (left) and histograms of their related expression as gray intensity values.
Figure 2

TGF-β1 in SP promoted proliferation of MSCs via phosphorylation of p42/44 and Akt. (A) Phosphorylation of p42/44 and Akt at both Ser473 (p-Akt-S) and Thr308 (p-Akt-T) were increased by transient SP treatment. Levels of total Akt (T-Akt) and total p42/44 (T-p42/44) did not change. WB bands (upper) and histograms of their related expression as gray intensity values (lower). (B) WB analyses of PCNA (proliferation marker) expression in NE-MSCs (n = 3) and E-MSCs (n = 3) treated with low-serum medium, TGF-β1 (2 ng/ml), SP, SP with the TGF-β receptor inhibitor SB431542, SP with the Akt inhibitor LY294002, or SP with the p42/44 MAP kinase inhibitor PD98059. Compared with the SP-treated MSCs group, expression of PCNA in SP groups with inhibitors was downregulated. Blocking p42/44 and Akt pathways markedly attenuated PCNA protein expression in SP-treated MSCs. WB bands (left) and histograms of their related expression as gray intensity values (right). *: P < 0.05 SP-treated MSCs versus control-treated MSCs; a: P < 0.05 control-treated NE-MSCs versus control-treated E-MSCs.
control-treated MSCs at the indicated time points. a: $P < 0.05$ versus control-treated MSCs. c: $P < 0.05$ versus SP-treated MSCs.

Figure 3

SP induced modulated expression of cycle-related proteins CDK2 and CDK6 via p42/44 and Akt signaling in E-MSCs. (A) WB analysis of CDK and cyclin protein levels in control and SP-treated E-MSCs. (B) WB analysis showed an SP-induced increase in CDK2 protein levels that was markedly attenuated by the Akt inhibitor LY294002. The SP-mediated increase in CDK6 protein levels was markedly attenuated by the Akt inhibitor LY294002 and p42/44 MAPK inhibitor PD98059. WB bands (upper) and histograms of their related expression as gray intensity values (lower). *: $P < 0.05$ SP-treated E-MSCs versus control-treated E-MSCs. a: $P < 0.05$ versus control-treated E-MSCs. b: $P < 0.05$ versus SP-treated E-MSCs.
Figure 4

SP-driven endometriosis-like lesion development in mice. The volume (A) and weight (B) of endometriosis-like lesions harvested from mice treated with vehicle, TGF-β1, SP, or SP with the TGF-β receptor inhibitor SB431542. Xenografts exposed to SP showed a three-fold increase in volume and four-fold increase in weight after 14 days. (C) Images of endometriosis-like lesions harvested from the sacrificed animal. (D) Staining scores of HLA (indicating human origin) and Ki67 (marker for...
proliferation) immunohistochemistry in an endometriosis-like lesion. a: P < 0.05 versus control-treated mice. c: P < 0.05 versus SP-treated mice. V: vehicle-treated mice; (n = 6). Scale bars = 100 μm.

**Supplementary Files**

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