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RESEARCH ARTICLE

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Sunitinib induces primary ectopic endometrial cell apoptosis through up-regulation of STAT1 in vitro

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Abstract

Background: Endometriosis (EMS) is a prevalent gynecological condition characterized by the growth of endometrial tissue outside the uterine cavity. This study aimed to clarify the targeted therapeutic effect of sunitinib in an endometriosis in vitro experiment.

Methods: Primary culture of ectopic endometrial cells and normal endometrial cells. Six tumor targeting drugs were selected to screen. MTT was used to determine the IC50, flow cytometry, and DAPI staining of the targeted drugs, in order to determine the apoptosis. The differential proteins after seeding were analyzed by protein spectrum, the correlation between the specific protein and cell apoptosis was determined by small molecule interference, and the expression of each related protein was detected by Western blot. Immunohistochemistry and ELISA were used to detect the expression of p-PDGFR and p-STAT1 in clinical samples, and the correlation between p-STAT1 expression and ectopic focal size was analyzed by SPSS 19.

Results: Through the drug screening, it was found that sunitinib has a significant inhibitory effect on ectopic endometrial cells. It was determined that the IC50 of sunitinib on ectopic stromal endometrial cells was 3.32 μM, while the IC50 on normal endometrium was 7.9 μM. Meanwhile, the flow cytometry and DAPI nuclear dye that took out sunitinib had an inhibition effect on the ectopic endometrium at a concentration of 4 μM. Protein spectrum analysis was conducted on ectopic intimal cells after sunitinib treatment, and it was found that STAT1 is specifically expressed in ectopic endometrial cells. In vitro, and through fludarabine interference, it was revealed that sunitinib specifically inhibited the phosphorylation site Tyr751 of PDGFR, while the expression of STAT1, p-STAT1, and caspase-3 was significantly upregulated, and the expression of STAT1 and p-STAT1 was positively correlated with the expression of caspase-3. Finally, the expression of p-PDGFR and p-STAT1 in ectopic foal tissues was both higher than that in normal endometrium, and p-STAT1 expression was positively with ectopic focal size.

Conclusion: The in vitro experiments revealed that sunitinib could upregulate the expression of STAT1 by inhibiting the phosphorylation site Tyr751 of PDGFR, thereby specifically inducing the apoptosis of the primary heterotopic mesenchymal endometrium.

Jiajie Li and Maidinaimu Abudula are contributed equally to this work.

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KEYWORDS
apoptosis, endometriosis, PDGFR, STAT1, sunitinib

1 | INTRODUCTION

Endometriosis (EMS) is a prevalent gynecological condition characterized by the growth of endometrial tissue outside the uterine cavity. It has significant implications for a patient's health and well-being, particularly when it comes to pelvic pain and infertility. EMS accounts for approximately 10%-15% of women of childbearing age, and in recent years, this has exhibited a significant upward trend, which seriously endangers a women's health.[1,2] At present, surgery and hormone drugs are the major clinical treatments, such as compound oral contraceptives, gonadotropin-releasing hormone agonist (GnRHa),[3] and progesterone.[4] However, the postoperative recurrence rate of EMS within 5 years can reach as high as 50%.[5] Although most patients of reproductive age are more inclined to conservative treatment, the vast majority of EMS drug treatments cannot achieve radical cure. Therefore, the top priority is to find drugs that are safer, more effective, and more compliant.

In recent years, with the EMS in-depth study of molecular biology and pathophysiology, a number of scientists have gradually found that non-hormonal medications have great potential for the treatment of EMS. Anti-angiogenesis drugs, which is one of the non-hormonal drugs, can inhibit EMS migration, invasion, and recurrence.[6] Therefore, anti-angiogenesis agents, such as sunitinib, have also increasingly become a focus in the treatment of EMS. Sunitinib, which was approved by the Food and Drug Administration (FDA) in 2006, is a multi-target tyrosine kinase inhibitor against angiogenesis.[7] In the mouse model of human tumors, sunitinib was found to reduce the permeability of blood vessels and inhibit the angiogenesis and destruction of existing blood vessels.[8]

As it is known, EMS has a "tumor-like condition" characteristic. Hence, some researchers have evaluated the efficacy of anti-vascular drugs on EMS in animal models.[9-12] However, the efficacy and possible adverse reactions of sunitinib on human EMS remain unclear, and the specific molecular mechanism has not been clarified. Therefore, the present research group intended to investigate the molecular mechanism of sunitinib based on human ovarian ectopic endometrial cells, in order to provide a more powerful laboratory basis for clinical trials.

2 | MATERIAL AND METHODS

2.1 | Cell culture

Ex vivo human endometrial and endometriotic cells in culture were investigated. Stromal cells were extracted from endometrial and endometriotic biopsies obtained from patients with and without EMS. The fresh tissue samples were cut up and digested in the basic medium which included type IV collagenase (final concentration of 1 mg/mL) and Penicillin-Streptomycin in 37°C water bath. After 1 hour, cell suspension liquid was centrifuged with 400 *g* for 10 minutes. The supernatant was abandoned and resuspend the cells with complete medium DMEM/F12 containing 10% FBS. All of mixture cells were filtrated through 100 mesh and 200 mesh of cell strainers. Epithelial cells could be intercepted by 200 mesh of strainer, while almost of stromal cells were in the filter liquor. Stroma cells were used in this experiment and identified with vimentin antibody by immunofluorescence. These cells were maintained in DMEM/F12 (Thermo Fisher Scientific), which was supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) and 500 mg/mL of penicillin/streptomycin (Solarbio) at 37°C in a humidified atmosphere with 5% CO_2. P2-P3 primary cells were used in all experiments.

2.2 | Cell cytotoxicity assay

Cells were seeded in a 96-well plate at a density of 5×10^3 cells for each well, and incubated in DMEM/F12 medium overnight. After seeding for 24 hours, certain drugs were added at a specified concentration gradient for treatment, and these cells were continually cultured for 48 hours. On the next day, 15 ul of MTT reagent was added into each well, and these cells were incubated at 37°C for four hours. Afterward, 150 uL of DMSO was added, followed by four hours of cell incubation. The optical density (OD) values were assessed, according to manufacturer instructions, using a MK3 microplate reader (Thermo Fisher Scientific).

2.3 | Flow cytometry analysis (apoptosis)

Cells were seeded in 6-well plates at a concentration of 1×10^3 cells for each well and exposed to the drug at the indicated concentration gradient for 48 hours at 37°C. Then, these cells were washed by pre-cold phosphate-buffered saline (PBS) twice. Afterward, these cells were re-suspended in $1 \times$ BD buffer (300 μL); fluorescein isothiocyanate (FITC) and propidium iodide (PI) dye were added into the suspension lipid; and these incubated in the dark at room temperature (BD Biosciences). The flow cytometry analysis for apoptosis was performed according to manufacturer's protocol. The data were analyzed by C6 flow cytometry (BD Biosciences).

2.4 | Western blot

Cells with sunitinib treatment at a certain concentration ingredient for 12, 24, and 48 hours were harvested and lysed with RIPA buffer supplemented with proteinase inhibitors (Beyotime Biotechnology, China). Then, the protein concentrations were

determined using a BCA protein assay kit, according to manufacturer's protocols. Afterward, the proteins were resolved using 10%-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene difluoride (PVDF) membranes. Next, these membranes were blocked with 5% skim milk for one hour at room temperature and incubated with primary antibodies at 4°C overnight. The antibodies were as follows: anti-PDGFR (1:1,000; CST), anti-p-PDGFR Tyr751 (1:1,000; CST), anti-p-PDGFR Tyr740 (1:1,000; CST), anti-STAT1 (1:500; Bios), anti-pSTAT1 (1:500; Bios), anti-caspase3 (1:1,000; Bios), and anti-GAPDH (1:3,000; Bios). After incubation with the appropriate horseradish peroxidase-conjugated secondary antibody, the immune-reactive protein bands were visualized using chemiluminescence reagents (CST), followed by imaging on an Electrophoresis Gel Imaging Analysis System (D-Digital).

2.5 | Nano-LC-MS/MS and data processing

The desalted peptides were re-solubilized in 10 μL of 0.1%(vol/vol) trifluoroacetic acid. The sample was loaded onto a peptide trap column, then separated by a C18 capillary column (ChromXP, Eksigent Technologies) at 300 nL/min delivered by an Eksigent nanoLC pump (Silicon valley). A tripleTPF 5600+ mass spectrometer coupled with a nanospray source was used to analyze peptides elute from capillary C18 chromatography. MS data analysis software ProteinPilot5.0 was used for protein database searching against SwissProt database.

2.6 | Nuclear fluorescent staining

Cells were seeded in a 6-well plate that contained coverslips and exposed to sunitinib at different concentrations for 48 hours at room temperature. Then, these coverslips were taken from the plate, and the cell nucleus was counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen), and visualized using a fluorescence microscope (Leica).

2.7 | Fludarabine inhibited p-STAT1 in ectopic endometrial cells

Cells were seeded in 6-cm dishes, treated with 4 μM of sunitinib and 4 μM of sunitinib + 8 μg/mL of the p-STAT1 inhibitor fludarabine for 48 hours, respectively. Western blot was performed to determine the expression of STAT1, p-STAT1, and caspase-3. Flow cytometry was performed to analyze the apoptosis of ectopic endometrial cells.

2.8 | Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPF) mouse ectopic endometrial tissue sections were deparaffinized in xylene, rehydrated through graded ethanol, and boiled for 10 minutes in citrate buffer (10 mM, pH 6.0) for antigen retrieval. Endogenous peroxidase activity was suppressed by exposure to 3% hydrogen peroxide for 10 minutes. Tissue slides were then blocked with 5% BSA (bovine serum albumin; Boster Bioengineering) and incubated overnight at 4°C with following primary antibodies: anti-p-PDGFR(CST) and anti-p-STAT1(CST), followed by incubation with corresponding secondary antibody for 20 minutes. Slides were visualized adding DAB (3,3'-diaminobenzidine) substrate, counterstained with hematoxylin, and mounted for observation under microscope.

2.9 | The expression of p-STAT1 was detected in clinic tissue proteins by ELISA assay

All clinical samples were homogenized and cracked. Enzyme-linked immunosorbent assay (ELISA) was used to quantify p-STAT (JiangLai Bio) concentrations. The preparation of all reagents, the working standards, and the protocol was followed according to the manufacturer's instructions. The absorbance was read at 450nm using a microplate spectrophotometer (MK3, Thermo Inc).

2.10 | Statistical analysis

The statistical analysis was performed using the SPSS software (version 20.0; IBM Corp., Armonk, NY, USA) and GraphPad Prism version 6.0 (GraphPad Software). Comparisons between pairs were performed using Student's *t* test, and multiple comparisons between the groups were analyzed using *multi-t* test. Correlation analysis used SPSS20 software. All experiments were performed in triplicate, and the results were presented as mean ± standard deviation. $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Sunitinib induced the proliferation inhibition in ectopic endometrial cells

Targeted drugs have been widely used in clinic treatment for patients with cancer or other medical conditions. Since EMS has been regarded as a tumor-like condition, in order to determine whether there is an underlying targeted drug that can be used for endometriosis treatment, six kinds of tumor targeted drugs were screened, which included erlotinib, imatinib, sorafenib, icotinib, gefitib, and sunitinib. Then, the cell viability of treatment with these different drugs was tested. Ectopic endometrial stromal cells (n = 3), who identified the expression of vimentin by immunofluorescence, were incubated with the targeted drugs at gradient concentrations (2, 4, 8, and 16 μM) for 48 hours, respectively. Then, the MTT assay was performed to assess the cell viability. The results indicate that cells treated with sunitinib were observed to have a more obvious

proliferation inhibitory effect, when compared to the other groups (Figure 1).

3.2 | Effect of sunitinib on normal and ectopic endometrial cell proliferation and apoptosis

In order to determine the impact of sunitinib on ectopic endometrial cells, ectopic and normal endometrial cells were treated with sunitinib at appointed concentrations of 0, 1, 2, 4, 8, and 16 uM for 48 hours. The MTT assay revealed that the half maximal inhibitory concentration (IC_{50}) of normal endometrial cells to sunitinib (IC_{50} = 7.9 μM) was significantly higher, when compared to ectopic endometrial cells (IC_{50} = 3.32 μM), suggesting that sunitinib has no effect on the normal endometrium within the therapeutic concentration range, since it is on the ectopic endometrium in vitro (Figure 2A). Then, it was measured that sunitinib reduced the cell apoptosis by nuclear-fluorescence staining. These results show that the number of apoptotic cells in the ectopic endometrial group (100x) increased with the increase in sunitinib concentration (Figure 2B). Furthermore, in order to confirm the results above, the cell apoptosis was determined by flow cytometry. The cell apoptosis rate (FITC + plus FITC+/PI+) in ectopic endometrial cells was 51.9%±8.3% and 78.8% ± 3.2% at a sunitinib concentration of 4 μM and 8 μM, respectively, and both were significantly higher than that in normal endometrial cells, with 0.2% ± 1.2% (vs ectopic endometrial cells, $P < .0001$, $t = 26.89$) and 68.1% ± 2.1% (vs ectopic endometrial cells, $P = .025$, $t = 3.49$), respectively (Figure 2C). Taken together, these results demonstrate that sunitinib can affect the cell proliferation and apoptosis of ectopic endometrial cells in a dose-dependent manner.

3.3 | Identification of apoptosis-related protein (STAT1) by proteomic analysis

It has been presumed that there may be some underlying proteins that specifically express in ectopic endometrial cells treated with sunitinib, which make an effort to induce cell apoptosis. Therefore, proteomic analysis was performed in ectopic endometrial cells with and without sunitinib treatment. The protein samples were prepared after exposing ectopic endometrial cells to sunitinib at a concentration of 4 μM for 48 hours, and 0 μM was taken as the control group. Then, the protein sample was analyzed to identify the differentially expressed cellular proteins through proteomic approaches. As is known to all, sunitinib is a multi-target anticancer drug, the alternative targets include VEGFR1-3, PDGFRβ, c-Kit, FLT-3, and RET. Previous results showed that sunitinib cause apoptosis of ectopic endometrium. According to the results, we integrated all of target proteins of sunitinib, caspase family proteins which were related with apoptosis and all differential proteins from protein mass spectrometry to predictive protein signal pathway through SRTING website (sting.db.org). It was only predicted pathway showed up: PDGFRβ-STAT1-Caspase3. Cellular proteins that were specifically detected in samples obtained from ectopic endometrial cells treated with sunitinib were selected, and these are listed in Table S1.

3.4 | Fludarabine inhibits the sunitinib-induced STAT1 and caspase-3 activation

In order to deeply investigate the biological function of sunitinib, the expression of platelet-derived growth factor receptor (PDGFR) and

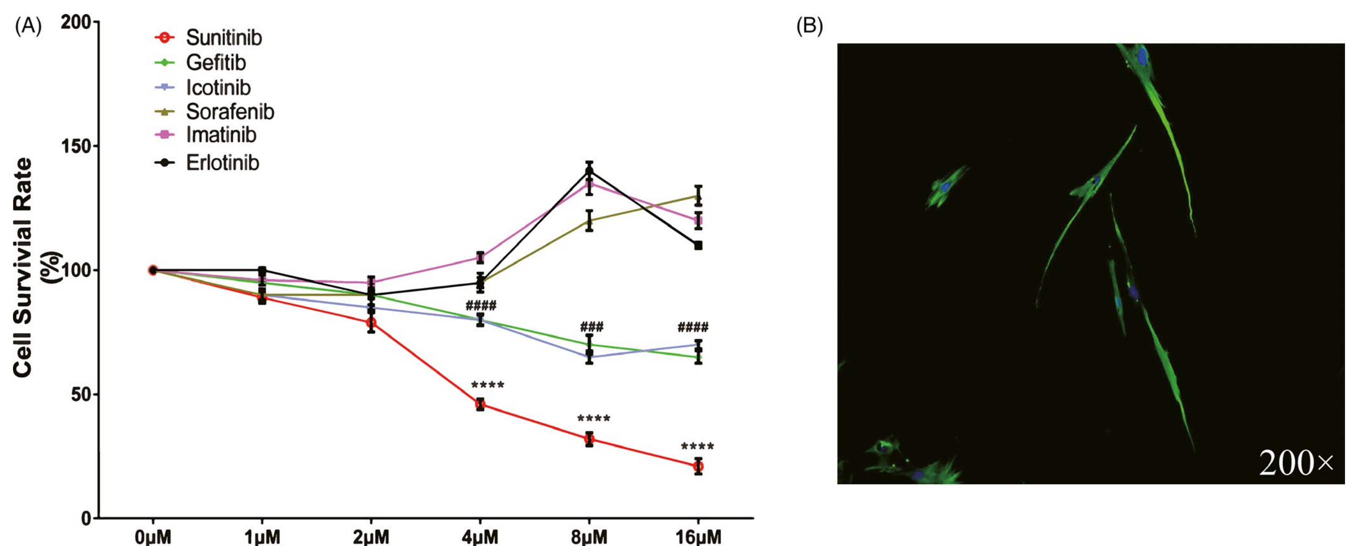

FIGURE 1 A, The cell proliferation assay of ectopic endometrial cells (n = 3) after treatment with six kinds of targeted drugs (erlotinib, imatinib, sorafinib, icotinib, gefetinib, and sunitinib), *, Icotinib vs. Sunitinib, ****<0.0001; #, Gefetinib vs. Sunitinib, ###<0.001, ####<0.0001. B, The expression of vimentin in the endometrial stromal cell (200×)

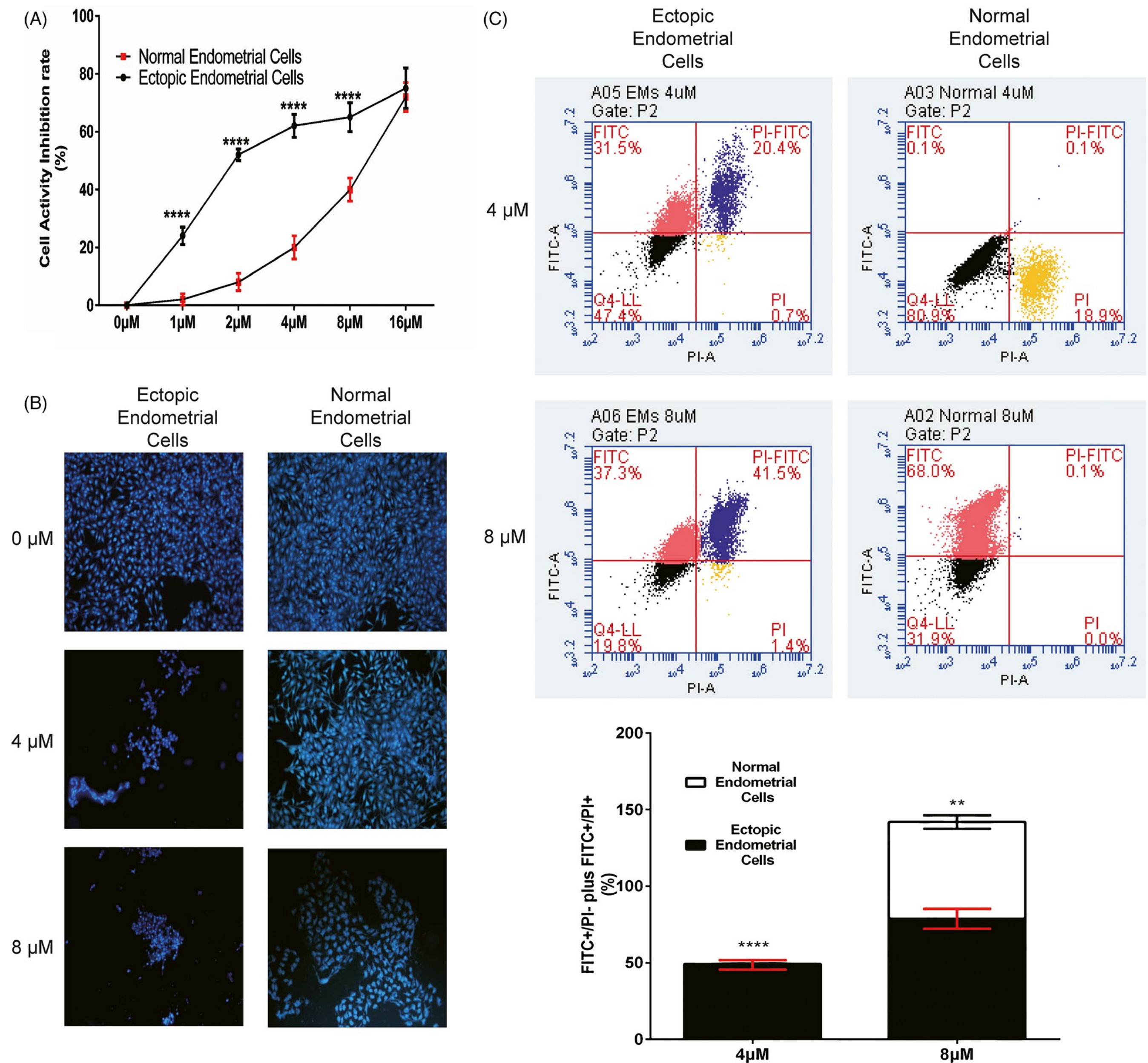

FIGURE 2 The effect of sunitinib on cell proliferation and cell apoptosis. A, The cell proliferation inhibition (%) of ectopic and normal endometrial cells after sunitinib treatment, ****<0.0001. B, The nuclear-fluorescence staining with DAPI after 0, 4, and 8 μM of sunitinib treatment. C, The cell apoptosis assay of ectopic and normal endometrial cells after 4 and 8 μM of sunitinib treatment for 48 hours (n = 3), ****<0.0001, **<0.01

its phosphorylation levels in ectopic and normal endometrial cells were examined by Western blot (Figure 3A and 3B). The results revealed that sunitinib reduced the phosphorylation level of PDGFR Tyr751 in ectopic cells, which was greater than that in normal endometrial cells, suggesting that p-PDGFR Tyr751 might be a major target of sunitinib in EMS. In order to further determine the role of STAT1 in regulating cell apoptosis, Western blot was performed to examine the variation in the STAT1 signaling pathway. As presented in Figure 3B, the ratio of P-STAT1/STAT1 increased in ectopic endometrial cells at 48 hours after sunitinib treatment. In the meantime, the regulatory role of STAT1 in the expression of caspase-3 was investigated, which is also an apoptosis-related protein. Ectopic endometrial cells were treated with sunitinib and sunitinib + fludarabine of 8 mM for 48 hours, respectively. The effect of fludarabine on the expression of STAT1 and caspase-3 was determined by Western blot (Figure 3B). As expected, fludarabine reversed the expression of P-STAT1 and caspase-3 induced by the sunitinib treatment. The ectopic endometrial cell apoptosis after sunitinib treatment with or without fludarabine was also determined by flow cytometry, and it was found that for cells treated sunitinib with fludarabine, the percentage of cell apoptosis decreased by 24% ± 1.3% (P = .0018, t = 7.383), when compared with sunitinib treatment (Figure 3C).

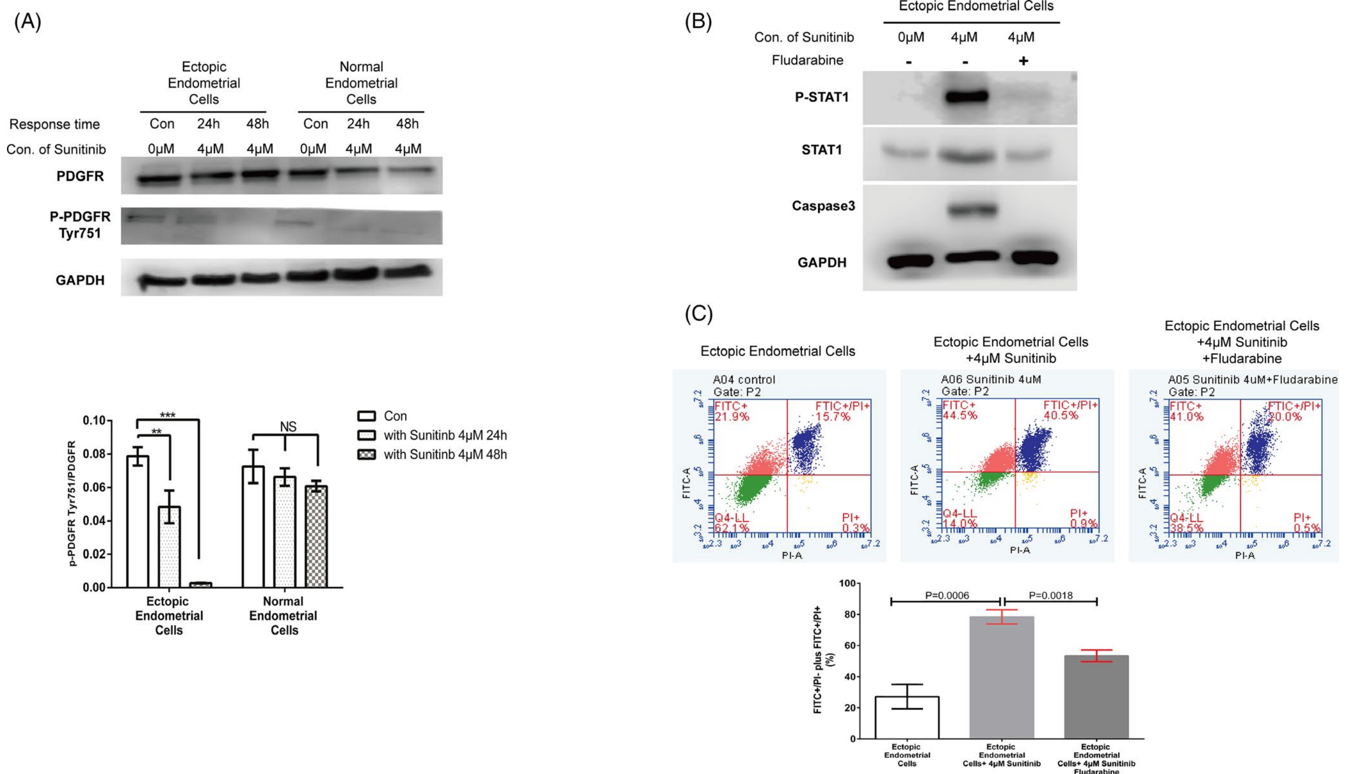

FIGURE 3 The effect of sunitinib and fludarabine on the expression level of PDGFR/P-PDGFR, STAT1, and caspase-3. A, The Western blot analysis revealed the expression of PDGFR and P-PDGFR in ectopic and normal endometrial cells after 0, 4, and 8 uM of Sunitinib treatment for 24 and 48 hours, respectively. B, The effect of sunitinib on STAT1 and caspase-3 expression with and without fludarabine treatment. C, The cell apoptosis assayed by flow cytometry (n = 3)

3.5 | The expression of p-STAT1 in the ectopic tissues of EMs patients was positively correlated with the size of the heterotopia

We collected ectopic tissue samples from 10 patients with chocolate cyst and the normal endometrium of the scraped patients (excluding EMs, the patient information is in Appendix S1). The expression of p-PDGFR and p-STAT1 in tissue samples was detected by immunohistochemical assay, showed both of two proteins in ectopic tissue of EMs patients were significantly higher than that of normal tissue (Figure 4A). Meanwhile, the concentration of p-STAT1 in the two groups was quantified by ELISA assay, the results displayed that the average expression of p-STAT1 in EMs groups was 83.93 ± 8.89 pg/mL, which was observably higher than that in the normal group, $P = .0004$, $t = 4.393$ (Figure 4B). Finally, we analyzed the correlation of between p-STAT1 expression in heterotopic tissues and the size of heterotopic foci, we found the expression of p-STAT1 and the size of heterotopic foci were positively correlated, pearson: .775, $P = .012$ (Figure 4C).

4 | DISCUSSION

For EMS patients of reproductive age, the urgent problem is finding a reliable conservative treatment. In recent years, various drugs have been used in clinic due to the rapid development of molecular biology and pathology of endometriosis, which include gonadotrophin releasing hormone antagonist (GnRHA),[13] selective progesterone receptor modulators (SPRM),[14] selective estrogen receptor modulators (SERMs),[15] aromatase inhibitors (AIs), immune modulators,[16] epigenetic inhibitors,[17] and anti-angiogenic agents.[6]

Sunitinib is an anti-angiogenesis target tyrosine kinase inhibitor, which can be competitively combined to various combinations of ATP receptor tyrosine kinase domains by non-covalent binding.[18] Meanwhile, numerous studies have shown that sunitinib is also a potential multi-target inhibitor, which includes VEGFR-1, −2, and −3, PDGFR-α and -β, fibroblast growth factor receptor (FGFR-1), c-KIT, FMS tyrosine kinase receptor 3 (FLTs), ret proto-oncogene, transfected rearrangement gene (RET), and colony stimulating factor 1 receptor (CSF1R).[19] Xin et al reported that sunitinib promotes the apoptosis of tumor cells through the STAT-3 pathway,[20] which means that the mechanism of sunitinib is to switch from anti-vascular endothelial to anti-tumor. Several animal experiments have revealed that anti-angiogenic drugs, such as sunitinib, are able to reduce the vascular endothelial growth factor (VEGF) scores in EMS rats. Furthermore, 13 new studies evaluated the anti-VEGF and its receptors (VEGFR) drugs for the EMS system, in order to evaluate the efficacy of animal models, according to the results obtained from the anti-VEGF/VEGFR drugs group, when compared with the

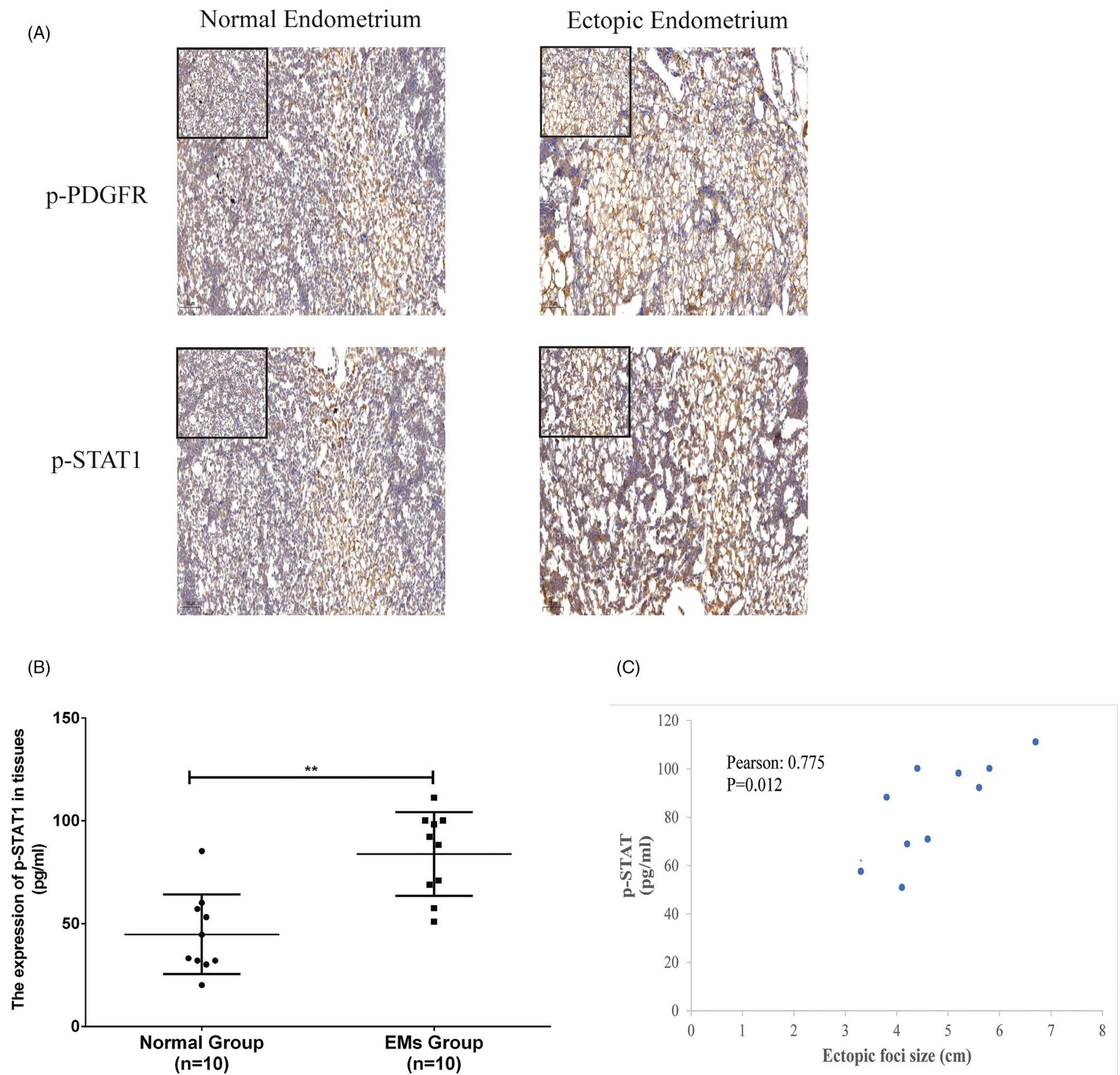

FIGURE 4 A, Immunohistochemical assay to detect the expression of p-PDGFR and p-STAT1 in ectopic tissue and normal endometrium. B, ELISA assay to detect the concentration of p-STAT1 protein in ectopic tissue and normal endometrium, **, $P < .001$. C, Correlation analysis. The data were normally distributed

control group, in which the uterus endometriosis lesions were significantly reduced, lowering the EMS evaluation.[21] However, almost all these studies were only limited to the treatment performance in animal models.[22] Furthermore, the efficacy of human EMS and the adverse reactions of anti-angiogenesis drugs remained unclear, and the molecular mechanism of anti-angiogenic drugs in ectopic diseases was still not clarified.

In the present in vitro study, six different kinds of anti-tumor drugs were tested, and it was found that sunitinib has the most significant inhibitory effect on ectopic mesenchymal intima. Meanwhile, the drug concentration of 50% was defined as the inhibition rate (IC50) of sunitinib for ectopic intima. However, sunitinib has no killing effect on normal intima at this drug concentration. Next, the differential protein expression in ectopic intimal cells was analyzed using the protein spectrum test after the sunitinib treatment. The results revealed that the specific expression of STAT1, which is a pathway molecule correlated to target protein PDGFR and caspase-3, could be detected after drug action. Next, a small molecule interference was added into the cell model, and the Western blot analysis revealed that sunitinib inhibited the

phosphorylation site Tyr751 of PDGFR, which induced the up-regulation of the expression of STAT1 and p-STAT1, and this finally activated the expression of apoptotic protein-3 to induce ectopic intima cell apoptosis. Moreover, we also confirmed in clinical samples that P-STAT1 was highly expressed in ectopic tissues and was associated with ectopic focal size. In some experiments, it has been demonstrated that STAT1 is a key regulator of caspase-3, suggesting their involvement in apoptosis.[23,24] This is the same as the present results obtained from the present experiment. When inflammatory cytokines act as stimulators, the phosphorylation of the PDGF signaling pathway regulates the transcription factor family STAT, especially STAT1, thereby activating the downstream JAK/STAT pathway.[25] STAT1 is a member of the STAT family, which regulates cell growth, differentiation and apoptosis, embryonic development, immune response, and a variety of basic biological processes.[26] Interferon(IFN) is an important activator of STAT1, others like interleukins, growth factors and cytokines, also play the role of stimulus. Phosphorylated STAT1 causes specific biological phenomena through different signaling pathways.[27] Among them, STAT1 can induce the synthesis of apoptotic protein precursors under the action of in vivo or in vitro stimulation. In mouse lymphocytes, STAT1 irritated the expression of caspase 1, 3, 11 precursors to promote cell apoptosis.[23] However, in the present in vitro experiment, sunitinib, as a targeted drug for PDGFR, inhibited the phosphorylation at this targeted site, but increased the expression of STAT1 and induced cell apoptosis. The specific molecular interactions involved in this would be the focus of future studies. At the same time, it would be noteworthy to determine whether sunitinib can have a similar therapeutic effect under the interference of hormone in future studies.

5 | CONCLUSION

In the in vitro experiments, it was found that sunitinib could upregulate the expression of STAT1 by inhibiting the phosphorylation site Tyr751 of PDGFR, thereby specifically inducing the apoptosis of the primary heterotopic mesenchymal endometrium. Combined with the results of other in vivo experiments, it was determined that sunitinib might be a potential drug for the targeted treatment of EMS.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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