

Involvement of angiotensin II receptor type 1/NF- κ B signaling in the development of endometriosis

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Abstract. Endometriosis (EM) is a common disease in women; however, the signaling pathways and related genes underlying the mechanisms of EM remain unclear. The present study aimed to investigate the role of angiotensin II receptor type 1 (AGTR1) in the pathogenesis of EM. Human EM tissues were collected, and the expression levels of AGTR1 and NF- κ B in the tissues were analyzed using immunohistochemistry and western blotting, while the estrogen levels in the EM tissues were determined by ELISA. *In vitro* human endometrial stromal cells were used to investigate the expression levels of AGTR1 following exposure to estrogen; the interaction between AGTR1 and NF- κ B was determined using reverse transcription-quantitative PCR and western blotting; and the effects of AGTR1 on cell proliferation, as well as the apoptotic and migratory abilities of the cells were evaluated using WST-1 assays, wound healing assays and flow cytometry, respectively. It was observed that both the expression levels of AGTR1 and the activity of NF- κ B were increased in human EM tissues and stromal cells, and this activation of AGTR1 subsequently increased the activity of NF- κ B. Moreover, estrogen was found to regulate the expression levels of AGTR1 in stromal cells. The activation of AGTR1 was demonstrated to promote cell proliferation and migration, in addition to preventing cells from undergoing apoptosis. In conclusion, the present study suggested that the increased activity of the AGTR1-NF- κ B axis following the decreased exposure to estrogen may be

important for the pathogenesis of EM. In addition, AGTR1 may be a potential therapeutic target for the treatment of EM.

Introduction

Endometriosis (EM) is a disorder in which endometrial tissue grows outside of the uterine cavity, such as within the fallopian tubes or peritoneum (1). It has been suggested that EM affects 6-10% of women of reproductive age in the USA, whilst in the clinic, EM is diagnosed in 4% of women undergoing tubal ligation and 50% of teenagers with intractable dysmenorrhea (2). EM is generally considered as an inflammatory, estrogen-related disease that can induce numerous symptoms in patients, including pelvic pain or infertility; however, the pathogenesis of EM remains relatively unclear. The most current hypothesis is that EM occurs following retrograde menstruation, which suggests that menstrual uterine contractions stimulate the reflux of endometrial tissue into the fallopian tubes and peritoneal cavity. However, retrograde menstruation is observed in 76-90% of women during menstruation, whereas EM only affects 6-10% of women of reproductive age (3). Recent molecular cytogenetic studies have provided novel evidence suggesting that acquired alterations in specific genes or signaling pathways may induce EM (4,5). In fact, in a previous study, through reanalyzing multiple genetic datasets of EM, it was identified that numerous important genes may contribute to the pathogenesis of EM, including angiotensin II receptor type 1 (AGTR1), membrane metalloendopeptidase, myosin heavy chain 11 and 15-hydroxyprostaglandin dehydrogenase (6). These genes regulate pathways involved in the renin-angiotensin system (RAS), smooth muscle contraction, lipoxin synthesis and the NF- κ B signaling pathway (6). AGTR1 is a gene coding receptor for angiotensin II (AngII) and is considered to mediate major cellular events; for example, AGTR1 was reported to be expressed in human epithelial ovarian carcinoma (7), and it was found to be involved in the invasion, migration and tumorigenesis of endometrial carcinoma (8). Notably, in a genome-wide association study, Hsieh *et al* (9) identified polymorphisms in the AGTR1 gene in EM. In addition, AGTR1 activation has also been observed to induce inflammation through the increased expression of leukocytic and endothelial adhesion molecules and the

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increased production of pro-inflammatory mediators (10). It is widely accepted that inflammation serves an important role in the pathogenesis and symptoms of EM.

NF- κ B is a well-established pathway that participates in the inflammatory response, and previous studies have reported the activation of the NF- κ B signaling pathway in EM. For example, Wei and Shao (11) suggested that the activation of the NF- κ B signaling pathway promoted the development of EM, while Taniguchi *et al* (12) suggested that activated NF- κ B signaling may contribute to EM through inhibiting apoptosis in endothelial cells.

Therefore, in the present study, AGTR1 expression levels and the activity of NF- κ B in human EM tissues were investigated. AGTR1 expression levels were significantly increased in human EM tissues, and *in vitro*, it was demonstrated that the increased expression levels of AGTR1 were in response to the inhibition of the estrogen receptor. AGTR1 was observed to activate the NF- κ B signaling pathway, and promote cell viability and migration, whilst preventing apoptosis in EM cells, which may contribute to EM development.

Materials and methods

Patient studies. Study protocols involving human subjects were approved by the Institutional Ethics Committee of the Fourth Hospital of Shijiazhuang City, and written informed consent was obtained from all subjects. From June 2017 to December 2018, 34 women with EM were recruited to this study from the Fourth Hospital of Shijiazhuang City; however, 2 cases withdrew, 6 cases were confirmed to not have EM by laparoscopy and 9 cases were excluded due to the quality control of the samples. In the control group, 32 patients were recruited in the Fourth Hospital of Shijiazhuang City; however, 5 cases withdrew and 6 cases were excluded due to the quality control of the samples. Altogether, 17 patients (age, 22-38 years old) with EM with ovarian endometriotic cysts and 21 controls (age, 23-36 years old) were enrolled in the present study. The collected tissues were histologically confirmed by pathologists. Data concerning the patients' menstrual cycle phases were collected by ZZ, YY and LH, and were classified into the proliferative phase (days 1-14) and the secretory phase (days 15-29). The exclusion criteria of this study included patients who received hormonal treatment or any anti-inflammatory treatment for ≥ 6 months prior to surgery.

Cell culture and reagents. Human endometrial stromal cells (ESCs) (cat. no. CRL-4003) were purchased from the ATCC. ESCs were maintained in DMEM (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) treated with dextran-coated charcoal (final concentration, 0.25%; Sigma-Aldrich; Merck KGaA), to remove any hormonal effects from the contents of FBS; 100 U/ml penicillin (Invitrogen; Thermo Fisher Scientific, Inc.); and 10 mg/ml streptomycin (Invitrogen; Thermo Fisher Scientific Inc.), and maintained at 37°C and 5% CO₂.

Losartan potassium (AGTR1 antagonist) dissolved in DMSO, AngII (AGTR1 activator) (13), estrogen (17 β -estradiol) and tamoxifen (the estrogen receptor modulator) in DMSO (14,15) were purchased from Sigma-Aldrich. Tamoxifen is an estrogen receptor modulator (16). Cells were

treated with tamoxifen to investigate the effect of estrogen on cells when its receptor was regulated by tamoxifen. Cells were also treated with 17.9 nM TPCA-1 in DMSO (Selleck Chemicals) (17), which is a selective inhibitor of IKK β (I κ B kinase β). ESCs were divided into the various treatment groups as follows: i) 10 μ M AngII; ii) 10 μ M AngII+10 μ M losartan potassium; iii) 10 μ M losartan potassium; iv) 10 nM 17 β -estradiol; v) 10 nM 17 β -estradiol+10 μ M tamoxifen; vi) 10 μ M tamoxifen; vii) 10 μ M AngII+17.9 nM TPCA-1; viii) 17.9 nM TPCA-1; ix) DMSO (controls).

Immunohistochemistry (IHC). Endothelium tissue was fixed with 10% formalin for 24 h at room temperature, and embedded in paraffin. Paraffin-embedded tissue samples were cut into 5- μ m thick sections. The tissue sections were subsequently deparaffinized with xylene at 55°C and rehydrated with descending alcohol series, and then subjected to antigen retrieval. Deparaffinized sections were blocked with 5% goat serum (Thermo Fisher Scientific, Inc.) at room temperature for 1 h. Tissue sections were incubated with a primary rabbit monoclonal antibody targeting AGTR1 (1:200; Abcam; cat. no. ab124734) overnight at 4°C. Following the primary incubation, sections were incubated with an anti-rabbit horse-radish peroxidase-conjugated secondary antibody (1:8,000; Abcam, cat. no. ab99702) at room temperature for 1 h. The slides were subsequently stained with 3,3'-diaminobenzidine, counterstained with hematoxylin (0.5%) and visualized using a light microscope (Olympus Corporation). Expression levels were semi-quantified according to the regular IHC staining grade system (18).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from ESCs or tissues using a RNeasy kit (Qiagen, Inc.) following the manufacturer's instructions. Total RNA (500 ng) was reverse transcribed into cDNA using SuperScript™ III Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.). qPCR (SYBR™ Green; Thermo Fisher Scientific, Inc.; cat. no. 4309155) was subsequently performed using an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Cycling conditions for the reaction were as follows: An initial hold for 10 min at 95°C; then 40 cycles of 15 sec at 95°C denaturation, 30 sec at an annealing temperature of 60°C and a 30 sec extension at 72°C. The following primer pairs were used for the qPCR: β -actin forward, 5'-CATGTACGTTGCTATCCAGGC-3' and reverse, 5'-CTCCTTAATGTCACGCACGAT-3'; and AGTR1 forward, 5'-ATTTAGCACTGGCTGACTTATGC-3' and reverse, 5'-CAGCGGTATTCATAGCTGTG-3'. Gene expression levels were quantified using the 2^{- $\Delta\Delta$ C_q} method (19) and normalized to the internal reference gene β -actin. Each experiment was performed in triplicate.

Western blotting. Total protein was extracted from tissues or ESCs using RIPA lysis buffer (Beyotime Institute of Biotechnology; cat. no. P0013B) containing proteinase inhibitor (Beyotime Institute of Biotechnology; cat. no. P1006). Total protein was quantified using BCA assays, and 20 μ g protein/lane was separated by 8-10% SDS-PAGE. The separated proteins were subsequently electrotransferred onto

PVDF membranes (EMD Millipore) and blocked with 5% bovine serum albumin for 1 h at room temperature. (Beyotime Institute of Biotechnology; cat. no. ST023-50 g). The membranes were incubated with the following primary antibodies (all 1:3,000) at 4°C overnight: Anti-AGTR1 (Abcam; cat. no. ab124734), anti-p65 (Santa Cruz Biotechnology, Inc.; cat. no. sc-109), anti-phosphorylated (pho)-p65 (Ser536; Santa Cruz Biotechnology, Inc.; cat. no. sc-101752) and anti- β -actin (Santa Cruz Biotechnology, Inc.; cat. no. sc-7210). Following the primary antibody incubation, membranes were incubated with anti-rabbit horseradish peroxidase-conjugated IgG secondary antibodies (1:3,000; Abcam; cat. no. ab99702) at room temperature for 1 h. Protein bands were visualized using the Western Bright ECL kit (Bio-Rad Laboratories, Inc.). β -actin was used as the loading control. The protein expression levels were quantified with ImageJ software (version 1.8.0; National Institutes of Health).

Wound healing assay. To evaluate cell migration, ESCs were seeded into 12-well plates (0.1×10^6 cells per well) and cultured in DMEM supplemented with 10% FBS until reaching 100% confluence at 37°C. Next, DMEM was removed, and a P-200 pipette tip was used to scratch a straight line into the cell monolayer. Cells were subsequently washed with PBS to remove the cell debris. Cells were then cultured with serum-free DMEM supplemented with 10 μ M losartan potassium, 10 μ M AngII or 17.9 nM TPCA-1 depending on the treatment group at 37°C for 72 h. Cells were photographed at 0 and 72 h using a light microscope (Olympus Corporation), magnification x100, and the wound closure was quantitatively analyzed. The size of the regions with and without cells were quantitatively evaluated with ImageJ software (Version 1.8.0; National Institutes of Health) and used calculating the percentage wound closure.

Cell transfection. A total of 10^5 ESCs/well were seeded into 12-well culture plates and transfected with 40 nM small interfering RNA (siRNA) targeting AGTR1 (5'-CUGUAGAAUUGCAGAUUU dTdT-3', 3'-dTdT GACAUCUUAACGUCUAUAA-5) or a scrambled control (5'-UUCUCCGAACGUGACACGudTdT-3'; 3'-dTdT AAGAGGGUUGCACAGUGG A-5', negative control; Santa Cruz Biotechnology, Inc.) using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Following 24 h of transfection, RT-qPCR and western blotting were performed to prove the successful transfection, and then the ESCs were used for subsequent experiments.

Flow cytometric analysis of apoptosis. Flow cytometry was used to investigate the levels of apoptosis in ESCs *in vitro*. Following treatment, ESCs were treated with 0.25% trypsin and then gently centrifuged at 1,000 x g for 5 min at room temperature. The collected ESCs were washed with PBS. The cells were subsequently resuspended in 500 μ l binding buffer containing 5 μ l Annexin V-FITC and 10 μ l propidium iodide using the Annexin V-FITC Apoptosis Detection kit (Bio-Rad Laboratories, Inc.). Following incubation for 30 min in the dark at room temperature, apoptotic cells were subsequently analyzed using a BD FACScan™ flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (version 7.6.5; FlowJo, LLC).

Cell proliferation assay. A total of 5×10^3 cells/well were seeded into 96-well plates and were cultured for 72 h at 37°C. Cellular proliferation was subsequently analyzed using a WST-1 assay (Roche Diagnostics), according to the manufacturer's protocol. The absorbance was measured at 440 nm using a multimode plate reader.

ELISA. The hormone expression levels of estrogen (cat. no. K4267-100; BioVision, Inc.), progesterone (cat. no. MBS494530; MyBioSource, Inc.) and prolactin (cat. no. MBS580135; MyBioSource, Inc.) in endometrial tissue (1 g) were determined using ELISA kits, according to the manufacturers' protocols. Briefly, the frozen tissues were homogenized using a sonicator (20 kHz/s) and were subsequently centrifuged for 5 min at 5,000 x g at 37°C to obtain the supernatant, which was then suspended in PBS and added in the plates (100 μ l/well). A total of 0.1 ml biotinylated antibodies (1:100) against the target hormones and the extracts from tissues were then added to the plate. Standard reagents as the per the ELISA kits were diluted and added to the wells to generate a standard curve. Following incubation for 2 h at room temperature, the absorbance was measured at 450 nm using a multimode plate reader.

Statistical analysis. All the data were analyzed and graphics produced using R (version 3.6.2). Data are presented as the mean \pm SEM of three independent experimental repeats. Statistical differences between >2 groups were determined using one-way ANOVA with Tukey's post hoc test, whereas differences between 2 groups were determined using Student's t-test. Correlation analysis was performed using Pearson's correlation test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Increased AGTR1 and NF- κ B expression levels are present in human EM tissues. AGTR1 expression levels in EM tissues were determined using IHC. Compared with that of normal endometrial tissues (control), Immunohistochemical staining showed that there were numerous brown red immunoreactive particles of AGTR1 distributed densely in stromal cells and glandular epithelial cells in the endometrium. The expression levels of AGTR1 in EM tissues were significantly increased compared with those of the control tissues (Fig. 1A). Subsequently, the mRNA expression levels of AGTR1 in EM tissues were evaluated using RT-qPCR, and it was demonstrated that AGTR1 expression levels were significantly increased in EM tissue compared with those in control tissue (Fig. 1B).

Previously, multiple studies have reported that estrogen serves a critical role in the development of EM (20). In the present study, the levels of estrogen, progesterone and prolactin in both EM and normal tissues were investigated, and it was observed that the levels of estrogen in EM tissues were significantly decreased compared with those in control tissues (Fig. S1). Moreover, the levels of estrogen in EM were negatively correlated with the expression levels of AGTR1 (Fig. 1C). However, the results also demonstrated that there was no significant difference between the levels of progesterone or prolactin found in EM tissues and those found in control

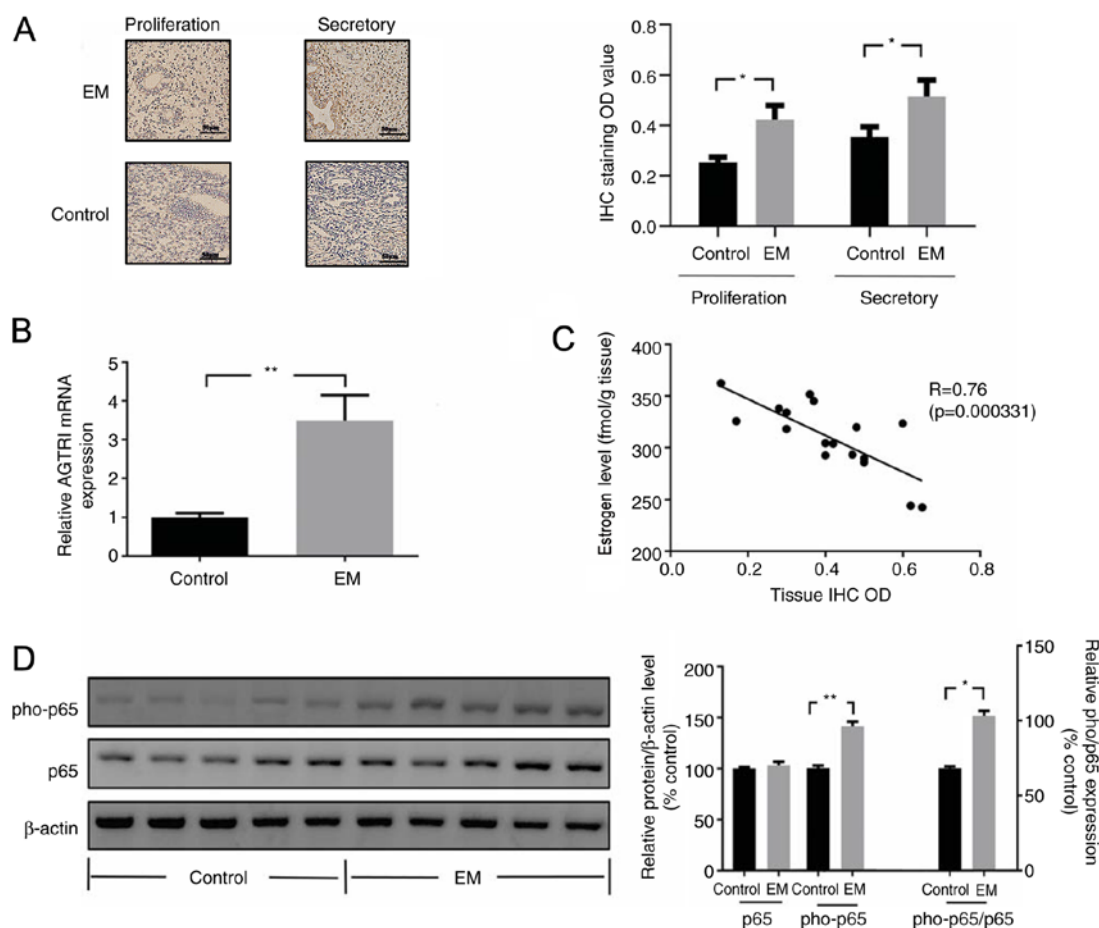


Figure 1. Expression levels of AGTR1, NF- κ B activity and estrogen levels in human EM tissues. (A) IHC staining was performed to determine the expression levels of AGTR1 in human EM tissues (n=17) and normal endometrium tissues (n=21) in the proliferative phase and secretory phase. AGTR1 expression levels were significantly increased in EM compared with those in normal tissues during the menstrual cycle. The expression levels were semi-quantified according to the regular IHC staining grade system (17). Scale bar, 50 μ m. (B) The expression levels of AGTR1 were assessed using reverse transcription-quantitative PCR; AGTR1 mRNA expression levels were increased in EM tissue compared with those in control tissue. The expression of AGTR1 was normalized to that of β -actin. (C) Correlation analysis of AGTR1 protein expression levels with estrogen levels in EM. A significant, negative correlation between AGTR1 protein expression levels (IHC OD value) and estrogen levels were observed in the tissues. The level of estrogen was evaluated by ELISA. (D) The activity of NF- κ B was analyzed using western blotting. The expression levels of pho-p65 and the pho-p65/p65 ratio were increased in EM compared with the values in control tissues. Data are presented as the mean \pm SEM. *P<0.05, **P<0.01. EM, endometriosis; AGTR1, angiotensin II receptor type 1; IHC, immunohistochemistry; OD, optical density; pho, phosphorylated.

tissues (Fig. S1). Notably, the ratio of estrogen/progesterone was significantly decreased in EM tissues compared with that of control tissues (Fig. S1).

In addition, NF- κ B activity in EM was investigated using western blotting, and the data revealed that pho-p65 expression levels (the ratio of pho-p65:p65) were significantly increased in EM tissues compared with those in control tissues (Fig. 1D).

AGTR1 promotes the activity of NF- κ B. The effect of AGTR1 on the NF- κ B signaling pathway in ESCs was subsequently investigated. *In vitro*, losartan treatment significantly decreased the expression levels of pho-p65 (the ratio of pho-p65:p65) in ESCs compared with those of untreated control cells, which suggested that AGTR1 antagonist may exert an inhibitory effect on the NF- κ B signaling pathway (Fig. 2A). By contrast, ESCs treated with AngII, an activator of AGTR1 (21), exhibited significantly increased pho-p65 expression levels (the ratio of pho-p65:p65) compared with those of control cells, and the upregulation effect of AngII on pho-p65 was attenuated by losartan (Fig. 2A) when cells were

treated with AngII in combination with losartan. In addition, following the genetic knockdown of AGTR1 expression in ESCs using siRNA (Fig. 2B and C), the expression levels of pho-p65 (the ratio of pho-p65:p65) were observed to be significantly decreased compared with those of scramble-treated cells (Fig. 2C).

Estrogen inhibits the expression levels of AGTR1. This study found that estrogen and AGTR1 levels were negatively correlated in EM. It was subsequently identified that the loss of estrogen expression could significantly increase AGTR1 expression levels; upon culturing ESCs and subsequently treating them with estrogen *in vitro*, it was discovered that estradiol treatment significantly decreased the expression levels of AGTR1 compared with those of the control, whereas the inhibition of AGTR1 expression by estrogen could be alleviated using the estrogen receptor modulator tamoxifen (Fig. 3), when cells were treated with estrogen combination with tamoxifen. Tamoxifen also increased the expression levels of AGTR1 compared with those of the control.

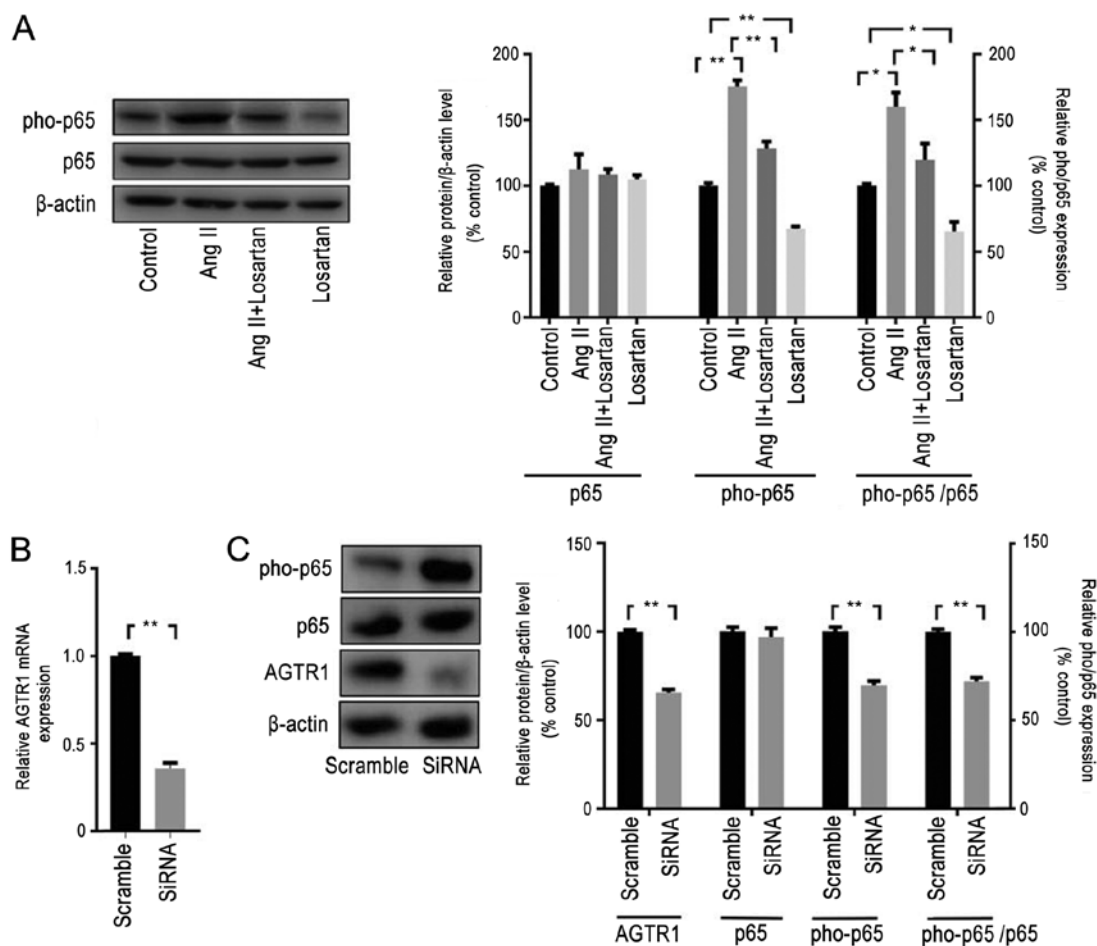


Figure 2. AGTR1 expression promotes the activity of NF- κ B in ESCs. (A) ESCs were exposed to AngII, losartan or DMSO for 72 h, and expression levels of pho-p65 were analyzed using western blotting. Activation of AGTR1 by AngII increased the expression levels of pho-p65, which was inhibited by losartan treatment. (B) AGTR1 expression was knocked down using siRNA. The expression levels of AGTR1 were analyzed using reverse transcription-quantitative PCR; siRNA significantly decreased AGTR1 expression. (C) The expression levels of ATGR1 and pho-p65 were determined using western blotting. siRNA decreased the phosphorylation of p65 and the pho-p65/p65 ratio, as well as the expression levels of ATGR1. All experiments were performed in triplicate, and data are presented as the mean \pm SEM. *P<0.05, **P<0.01. AGTR1, angiotensin II receptor type 1; pho, phosphorylated; siRNA, small interfering RNA; ESCs, endometrial stromal cells; AngII, angiotensin II.

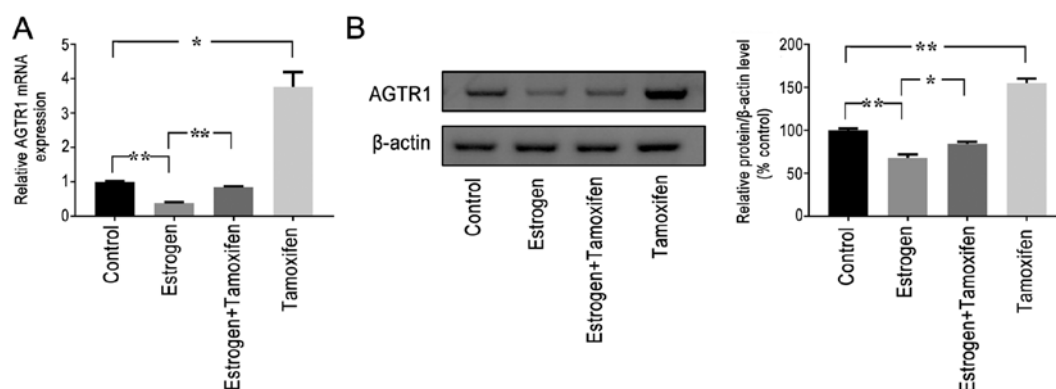


Figure 3. Estrogen regulates the expression levels of AGTR1 in ESCs. (A and B) ESCs were treated with estrogen or tamoxifen for 72 h and then collected to determine the expression levels of AGTR1 using (A) reverse transcription-quantitative PCR and (B) western blotting. The results revealed that the expression levels of AGTR1 were significantly decreased in cells treated with estrogen but increased in cells treated with the estrogen receptor modulator tamoxifen both at the mRNA and protein level. All experiments were performed in triplicate, and data are presented as the mean \pm SEM. *P<0.05, **P<0.01. AGTR1, angiotensin II receptor type 1; ESCs, endometrial stromal cells.

AGTR1 promotes cell migration and proliferation, and inhibits apoptosis in ESCs through the NF- κ B signaling pathway. The effects of AGTR1 on the migration of ESCs

were investigated using wound healing assays. Pre-treatment with AngII for 72 h resulted in a significant increase in cell migratory ability compared with that of the control group;

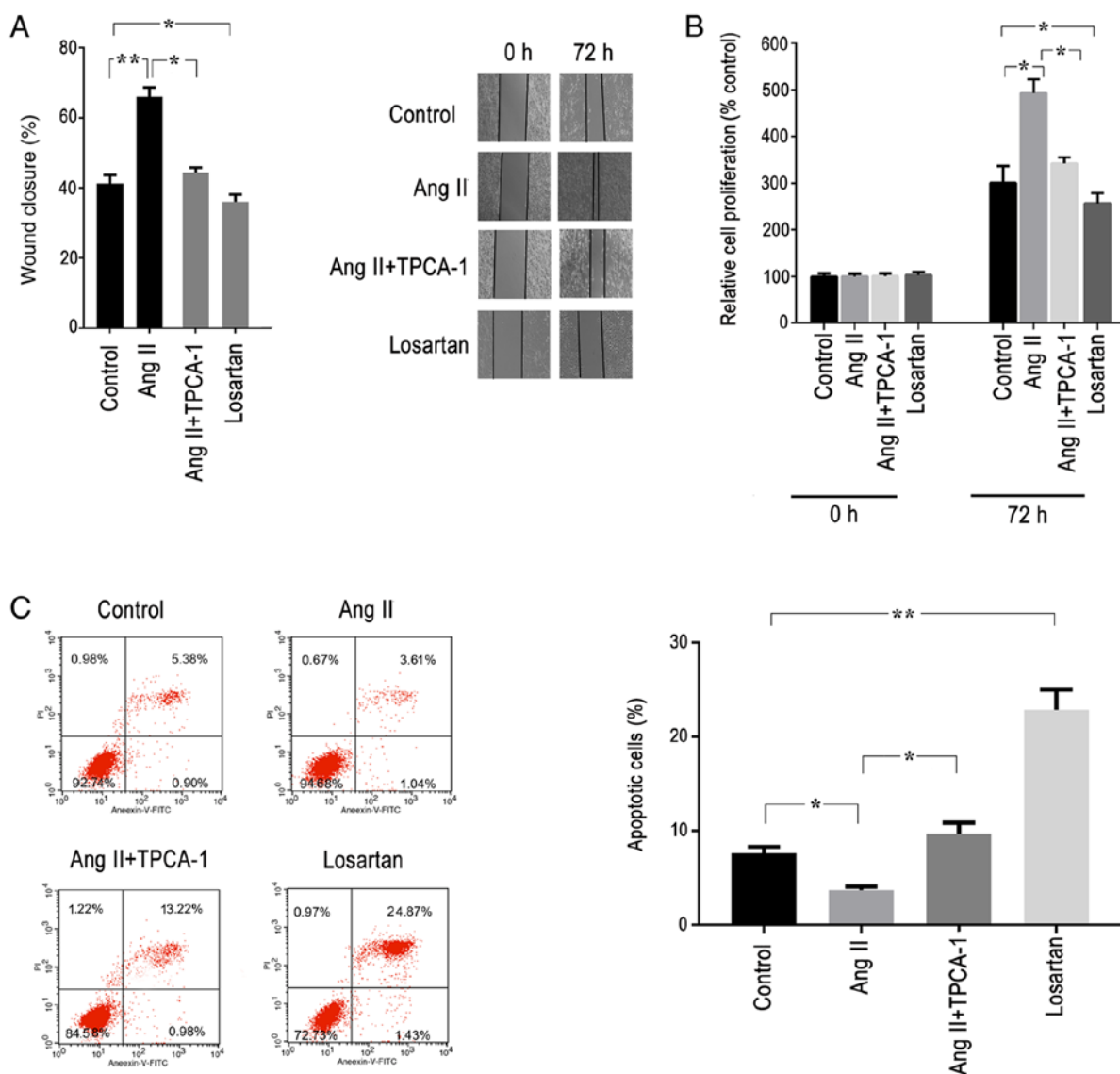


Figure 4. Activation of AGTR1 promotes cell proliferation and migration, and prevents apoptosis in ESCs *in vitro* via the NF- κ B signaling pathway. ESCs were treated with AngII (AGTR1 activator), TPCA-1 (selective inhibitor of I κ B kinase β), losartan (AGTR1 antagonist) or DMSO (control) for 72 h *in vitro*, and cell proliferation, apoptosis and migration were analyzed. (A) Wound healing assay was performed, and images were acquired using light microscopy (magnification, 100x). The activation of AGTR1 promoted wound closure, which was blocked by TPCA-1. Losartan significantly inhibited cell migration compared with that of the control. (B) Cell proliferation assay. The activation of AGTR1 by AngII increased cell proliferation compared with that of the control, whereas TPCA-1 inhibited the cell proliferation induced by AngII. Losartan also inhibited the cell proliferation. (C) Cell apoptosis assay. Following treatment for 72 h *in vitro*, ESCs were collected, stained with Annexin-V and PI, and analyzed by flow cytometry. The number of apoptotic cells (Annexin V-positive cells) was determined as the percentage of gated cells at upper-right and lower-right quadrants. Representative images and relative quantifications are shown. The results indicated that the activation of AGTR1 by AngII inhibited cell apoptosis, whereas the anti-apoptotic effect was inhibited by TPCA-1. Losartan promoted the apoptosis of cells. All experiments were performed in triplicate, and the data are presented as the mean \pm SEM. * P <0.05, ** P <0.01. ESCs, endometrial stromal cells; AGTR1, angiotensin II receptor type 1; AngII, angiotensin II; PI, propidium iodide.

however, this effect was significantly prevented following treatment with TPCA-1, an antagonist of the signaling NF- κ B pathway (Figs. 4A and S2A). In addition, losartan treatment also significantly decreased the migratory ability of ESCs compared with that of the control group (Fig. 4A).

To investigate the role of AGTR1 on the proliferative activity of ESCs, cell proliferation and apoptosis assays were conducted. The cells were cultured for 72 h in the presence of AngII, TPCA-1 or losartan. ESCs treated with AngII exhibited a significantly increased proliferative rate compared with that of the control group, alongside a significantly decreased apoptotic rate (Fig. 4B and C). By contrast, TPCA-1 treatment significantly

blocked the effects of AngII on ESCs (Figs. 4B, C, S2B and C). ESCs treated with losartan or TPCA-1 displayed significantly decreased proliferation rates and increased apoptotic rates compared with those of the control group (Fig. 4B and C).

Discussion

AGTR1 is a component of the RAS, and has been reported to be upregulated in EM (6,21). In the present study, decreased levels of estrogen in EM tissues were found to be associated with increased expression levels of AGTR1, which in turn promoted cell proliferation and prevented cell apoptosis

through the activation of the NF- κ B signaling pathway. In the RAS, bioactive effector molecules, AngI and AngII serve antagonistic roles by binding to different receptors, namely AGTR1, AGTR2, respectively (22), which subsequently exert vasoactive or proliferative roles. In recent years, increasing attention has been paid to the local activity of the RAS in ovarian and endometrial tissues, which may subsequently contribute to physiological and pathological processes such as follicle maturation, regulation of reproduction, angiogenesis and tumor cell proliferation (23-25). Notably, the dysregulation of the RAS has been observed in EM; for example, Abraham *et al* (26) reported that, in rat endometrial stromal cells, AngII induced Cyclooxygenase-2 gene expression by activating the calcineurin/NFAT signaling pathway. In another study, Kowalczyńska *et al* (27) investigated the polymorphisms in the angiotensin I converting enzyme (ACE) gene and AGTR1 in women with EM, and their results indicated that the A2350G polymorphism in the ACE gene was associated with the development of EM. Nakao *et al* (28) discovered that the expression of AGTR1 was largely located in endometrial glandular epithelium and stromal cells, and AGTR1 expression levels were markedly increased in EM compared with those of normal tissue. Therefore, these findings suggested that the increased expression levels of AGTR1 may increase RAS sensitivity in endothelium tissue, and the subsequent activation of the RAS-AGTR1 system may promote the pathogenesis of EM.

The NF- κ B family represents a family of transcription factors that serve vital roles in various processes such as cellular survival, proliferation and differentiation. Furthermore, the NF- κ B signaling pathway has been found to regulate menstruation (20). King *et al* (29) reported that I κ B kinase α and TANK Binding Kinase 1 mRNA expression levels were increased in the human endometrium during the perimenstrual phase of the menstrual cycle in response to premenstrual progesterone withdrawal. In addition, a previous study have demonstrated the constitutive activation of NF- κ B in endometriotic lesions (30). The activation of the NF- κ B signaling pathway induces the production of pro-inflammatory cytokines such as IL-8 and matrix metalloproteinases, which subsequently induce tissue breakdown (31). Nie *et al* (32) reported the expression of NF- κ B in the eutopic endometrium of patients with adenomyosis, which reportedly increased the expression of nuclear p65 and p52, whilst decreasing the expression of progesterone receptor B and cytoplasmic I κ B α . Park *et al* (33) reported that the expression levels of NF- κ B p65 were increased in the eutopic endometrium and adenomyosis nodules of women with adenomyosis, which strongly suggested that NF- κ B served a critical role in the pathogenesis and pathophysiology of adenomyosis. Finally, Wei and Shao (11) observed that blocking NF- κ B activity with nobiletin reduced the lesion size and pain in a mouse model of EM. In the present study, the activation of AGTR1 in EM increased the activity of NF- κ B, and subsequently promoted cell proliferation and migration. The interaction between AGTR1 and NF- κ B has been reported in several studies; Li *et al* (34) found that the treatment of hepatic stellate cells with AngII activated its receptor AGTR1, which subsequently increased the activity of NF- κ B; Du *et al* (35) reported that an AGTR1 antagonist inhibited the expression of NF- κ B,

which then decreased the proliferation of breast cancer cells; and Ekambaram *et al* (36) suggested that AGTR1 overexpression may activate the NF- κ B signaling pathway, and may promote cell proliferation, migration and invasion, as well as angiogenesis. In addition, AngII/AGTR1 exacerbated vascular calcification following the activation of NF- κ B, which induced the inflammatory response in human vascular smooth muscle cells (37). Therefore, it was hypothesized that, in EM, the upregulation of AGTR1 expression may activate the NF- κ B signaling pathway, which may promote cell migration and proliferation to contribute to EM development, as well as the inflammatory response to induce symptoms of EM.

The present study also discovered that the expression of AGTR1 was regulated by estrogen. EM is known to be an estrogen-dependent disease; Galvankar *et al* (38) reported that estrogen was essential for the induction of EM, whereas Wang *et al* (39) suggested that estradiol may promote inflammation, and increasing expression of both C-X-C motif chemokine 12(CXCL12) and C-X-C Motif Chemokine Receptor 4 in human endometrial stromal cells, which contributes to EM pathogenesis. CXCL12 is a chemokine and plays a crucial role inflammatory reaction and cell migration (40). Moreover, low expression levels of estrogen and progesterone have been found in the serum and urine of women with EM (41,42). In the present study, it was suggested that the estrogen/AGTR1 signaling pathway may be involved in EM pathogenesis. In stromal cells derived from human endometrial tissue, estrogen treatment decreased the expression levels of ATGR1, which subsequently inhibited NF- κ B activity, whereas the estrogen receptor modulator tamoxifen increased the expression levels of components of the ATGR1/NF- κ B signaling pathway. The regulatory effect of estrogen on AGTR1 expression has been found in other studies; Kooptiwut *et al* (43) suggested that, under high-glucose conditions, estradiol could decrease the mRNA expression levels of AGTR1 in pancreatic β -cells, while Gao *et al* (44) reported that estradiol could decrease the expression levels of AGTR1 in the uterine artery. Nickenig *et al* (45) also found that the AGTR1 protein density in rat aortic tissue was increased during estrogen deficiency. Therefore, during menstruation, the low levels of estrogen may induce an increase in AGTR1 expression, and then activate its downstream signaling pathway. However, in a study on ischemic injury of the heart in rats, Xue *et al* (46) argued that treatment with estradiol increased the expression levels of AGTR1 in the heart. This paradoxical result suggests that these effects may be organ specific, and further research is required to understand the role of AGTR1 in EM. In addition, the present study was limited by the fact that it did not involve *in vivo* studies to verify the effect of AGTR1; thus, further investigations are required to understand the pathogenesis of EM.

In conclusion, the present study suggested that AGTR1 may contribute to the development of EM through the NF- κ B signaling pathway, and the increased expression levels of AGTR1 observed in EM tissue may be due to the low levels of estrogen during menstruation.

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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 on reasonable request.

Authors' contributions

ZZ and YY performed the experiments. ZZ, YY, XY and LH collected and analyzed the data. ZZ and JC designed the study and analyzed the data, drafted and reviewed the manuscript and supervised the entire study. YY designed the study, revised the manuscript and provided material support. All the authors have read and approved the final manuscript.

Ethics approval and consent to participate

Study protocols involving human subjects were approved by the Institutional Ethics Committee of The Fourth Hospital of Shijiazhuang City, and written informed consent was obtained from all subjects.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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