Original Article

Transforming Growth Factor-beta 1 Involved in the Pathogenesis of Endometriosis through Regulating Expression of Vascular Endothelial Growth Factor under Hypoxia

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Abstract

Background: Endometriosis (EMs) is a common gynecological disorder characterized by endometrial-like tissue outside the uterus. Hypoxia induces the expression of many important downstream genes to regulate the implantation, survival, and maintenance of ectopic endometriotic lesions. Transforming growth factor-beta 1 (TGF-β1) plays a major role in the etiology of EMs. We aimed to determine whether TGF-β1 affects EMs development and progression and its related mechanisms in hypoxic conditions.

Methods: Endometrial tissue was obtained from women with or without EMs undergoing surgery from October, 2015 to October, 2016. Endometrial cells were cultured and then exposed to hypoxia and TGF-β1 or TGF-β1 inhibitors. The messenger RNA (mRNA) and protein expression levels of TGF-β1, vascular endothelial growth factor (VEGF), and hypoxia-inducible factor-1α (HIF-1α) were measured. A Dual-Luciferase Reporter Assay was used to examine the effect of TGF-β1 and hypoxia on a *VEGF* promoter construct. Student's *t*-test was performed for comparison among groups (one-sided or two-sided) and a value of $P < 0.05$ was considered statistically significant.

Results: TGF-β1, VEGF, HIF-1α mRNA, and protein expression were significantly higher in EMs tissue than that in normal endometrial tissue ($t = 2.16$, $P = 0.042$). EMs primary cultured cells exposed to hypoxia expressed 43.8% higher *VEGF* mRNA and protein ($t = 6.84$, $P = 0.023$). *VEGF* mRNA levels increased 12.5% in response to TGF-β, whereas the combined treatment of hypoxia/TGF-β1 resulted in a much higher production (87.5% increases) of *VEGF*. The luciferase activity of the *VEGF* promoter construct was increased in the presence of either TGF-β1 (2.6-fold, $t = 6.08$, $P = 0.032$) or hypoxia (11.2-fold, $t = 32.70$, $P < 0.001$), whereas the simultaneous presence of both stimuli resulted in a significant cooperative effect (18.5-fold, $t = 33.50$, $P < 0.001$).

Conclusions: The data support the hypothesis that TGF-β1 is involved in the pathogenesis of EMs through regulating VEGF expression. An additive effect of TGF-β1 and hypoxia is taking place at the transcriptional level.

Key words: Endometriosis; Hypoxia-inducible Factor 1 Alpha Subunit; Transforming Growth Factor-beta 1; Vascular Endothelial Growth Factor

Introduction

Endometriosis (EMs) is an estrogen-dependent gynecological disorder characterized by ectopic endometrial tissue. It is frequently associated with pelvic pain and infertility. It affects 2–10% of reproductive age women.[1-3] Its pathogenesis and physiopathology remain widely debated, but we know it is polygenic and multifactorial. The most widely accepted hypothesis is the retrograde menstruation theory. Endometrial tissue flows back into the pelvic and

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implants through adhesion, invasion, angiogenesis, and proliferation.[4,5]

The local microenvironment must play modulatory roles in the pathogenesis of EMs. Hypoxia, as one of the most important local factors, is related to angiogenesis and is also a basic requirement for lesion formation.[6,7] Hypoxia activates signaling through hypoxia-inducible factor (HIF)-1α, which is a transcriptional complex with a crucial role in oxygen-regulated gene expression and it is upregulated by growth factors, cytokines, oncogenes, and hormones under hypoxic conditions.[8] HIF-1α helps cells survive hypoxic environments and is highly expressed in many tumors. It is a critical regulator of angiogenesis during tumor growth. Angiogenesis is regulated by numerous genes, but vascular endothelial growth factor (VEGF) is the most important one. A major hypoxia-responsive element was identified as a 28-base pair sequence located 900-base pairs upstream from the catabolite activator protein site of the *VEGF* promoter region. Deletion of this element significantly inhibits hypoxic induction of *VEGF* transcription.

Transforming growth factor-beta 1 (TGF-β1), as an essential growth factor, is responsible for regulating cell proliferation, differentiation, angiogenesis, and immune responses.[9-11] TGF-β binding to the TGF-β Type II receptor (TβRII) activates the TGF-β signaling pathway and promotes dimerization with and activation of the TGF-β Type I receptor (TβRI). TβRI combines with TβRII and forms a TβRI-ligand-TβRII trimer that contains a phosphorylated kinase domain and combines with phosphorylated SMAD. It regulates target gene functions by binding to SMAD binding elements in the promoters of target genes.[12,13] In tumor tissue, TGF-β1 acts as activator of the SMAD2/3 pathway that inhibits cell apoptosis[14,15] and it activates the matrix metalloproteinases (MMPs) family that increases cell migration.[16,17]

The increasing evidences indicate that TGF-β1 expression is high in EMs lesions.[18-20] Many mechanisms must contribute to the development of EMs and TGF-β1 was hypothesized to play a key role in endometriotic lesion formation.[21,22] However, its role under hypoxic conditions is not clear. To investigate whether the hypoxia and TGF-β signaling pathways have additive effects on EMs through regulating the expression of VEGF, we analyzed the expression of TGF-β1, VEGF, and HIF-1α by quantitative reverse transcription polymerase chain reaction (RT-PCR) and western blotting in endometriotic tissues and in primary cultures of endometrial tissues incubated with TGF-β1 or the TGF-β1 signal pathway inhibitor galunisertib under normoxic or hypoxic (1% oxygen) atmospheres. A *VEGF* promoter construct containing the 5′-flanking region (−1200/+1) was transfected into cells and then the cells were treated with TGF-β1 or galunisertib under normoxic or hypoxic conditions. The reporter activities were measured to assess whether any collaborative effect was taking place at the transcriptional level.

Methods

Source of endometrial tissue

The study protocol was approved by the Ethical Review Board of investigation for humans of the Chinese People's Liberation Army 202 Hospital (No: 202H2015KPJ004). Tissues were collected after written informed consent was obtained. The endometrial tissues were obtained from 40 women with EMs who had not received hormone therapy or GnRH-α agonist treatment for at least half a year. The samples used as the normal controls were obtained from 40 women free of EMs who underwent hysterectomy due to other diseases. The fresh samples were immediately frozen at −80°C for future research.

Primary culture of endometrial tissue and *in vitro* hypoxia model establishment

The endometrial tissue samples used for the primary culture were removed and transported immediately to the laboratory. They were chopped to a size of 1 mm^3 and washed with PBS three times. After the cells were fully digested with trypsin/EDTA (TBD Science, Tianjin, China) in a humidified atmosphere of 5% CO_2 at 37°C, the cells were pelleted by centrifugation for 5 min, vigorously resuspended in RPMI 1640 (Gibco; Shanghai, China) supplemented with 10% fetal bovine serum (TBD Science, Tianjin, China) and 100 U/ml penicillin (Gibco, Shanghai, China) and 100 μg/ml streptomycin (Gibco, Shanghai, China), plated, and allowed to settle for up to 3 days. All the cells were identified to be epithelial cells and interstitial cells using immunofluorescence. The endometrial cells were exposed to 1% O_2 hypoxia in the presence or absence of 10 ng/ml TGF-β1 recombinant protein (Peprotech, NJ, USA) or 10 μmol/L of the TGF-β1 signal pathway inhibitor galunisertib (LY2157299) (Selleck Chemicals, Houston, USA). Hypoxic conditions were maintained using a modular incubator chamber (Hinasama, Tokyo, Japan) with 5% CO_2 and 1% O_2 balanced with N_2 gas. Data from cells cultured in 21% O_2 were used as normal controls.

Quantitative reverse transcription polymerase chain reaction analysis

Total RNA was extracted from cells and endometrial tissues by Trizol Reagent (TakaRa, Dalian, China). The isolated total RNA (1 μg) was reverse transcribed into complementary DNA with the one-step Prime Script RT Reagent Kit (TaKaRa; Dalian, China) following the manufacturer's protocol, and real-time (RT) quantitative PCR was performed in an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, USA) by a SYBR green-based RT-PCR assay. The primers were: sense 5′-GCAAGTTCAACGGCACAG-3′ and antisense 5′-GCCAGTAGACTCCACGACATA-3′ for the internal control *GAPDH*; sense 5′CCCACTGATACGCCTGAG-3′and antisense 5′-TGAAGCGAAAGCCCTGTA-3′ for *TGF-β1*; sense 5′-ATCCATGTGTGACCATGAGGAAATG-3′ and antisense 5′TCGGCTAGTTAGGGTACACTTC-3′ for *HIF-1α*; sense 5′-TGCATTCACATTGTGCTGCTGTAG-3′

and antisense 5′-GCAGATTATGCGGATCAAACC-3′ for *VEGF*. The 25 μl reaction mixtures contained 10 mmol Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 μmol/L dNTPs, 1.25 U Taq polymerase, 40 ng template cDNA, and 150 mol/L primers (400 nmol/L of each). All PCR reagents were purchased from TaKaRa Biotech (Dalian, China). The reaction conditions were 95°C for 10 min followed by 40 cycles at 95°C for 15 s, 60°C for 50 s, and 72°C for 30 s. Cycle threshold values (Ct) were analyzed using SDS version 1.4 software (Applied Biosystems, CA, USA.) and relative quantification of gene expression was determined using the comparative Ct method (ABI PRISM 7500, SDS User Bulletin; Applied Biosystems, CA, USA.). Each sample was analyzed three times to obtain an average Ct value.

Western blot analysis

Cell and tissue protein samples were collected using radioimmunoprecipitation buffer (Beyotime, Shanghai, China) and then mixed with SDS sample buffer (62.5 mmol/L Tris-HCL pH 6.8, 10% glycerol, 1% SDS, 0.1% 2-mercaptoethanol, 1 mmol/L phenylmethylsulfonyl fluoride) and heated for 15 min at 65°C. The lysates were loaded onto polyacrylamide gels, subjected to electrophoresis, and transferred to a polyvinylidene difluoride membrane. The membranes were incubated in blocking buffer (5% skim milk/TBS-Tween) for 30 min at room temperature and incubated with a 1:100 dilution of the primary antibody (Santa Cruz, CA, USA) in blocking buffer overnight at 4°C. The membranes were incubated with the appropriate secondary antibody (Beverly, MA, USA) in blocking buffer for 1 h at room temperature. After washing, the membrane was subjected to ECL hypersensitive reagents. β-actin was used as the internal reference. The protein bands on the membrane were scanned and analyzed with a molecular biological image analysis system to determine the protein levels.

pGL3-vascular endothelial growth factor construction and Dual-Luciferase Reporter Assay

A luciferase reporter plasmid lacking a eukaryotic promoter and enhancer, pGL3-Basic (Promega, Wisconsin, USA) was used. A PCR fragment comprising −1200 to +1 of the *VEGF* gene was generated from human genomic DNA. For reporter construction, the PCR product was cloned into the pMD18-T vector and subcloned into the unique Kpn I/Mlu I sites of the pGL3-Basic vector. The construct was confirmed by sequencing to ensure no coding frame shift in the luciferase gene. Mid Pre Plasmid kits (Qiagen, Germany) were used as plasmids for transient transfection. The endometrial carcinoma cell line Ishikawa was seeded and grown to 80–90% confluence in 24-well plates. They were transfected by Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol with 1 μg of the reporter construct. Cotransfection of 0.01 μg pRL-TK (Promega, USA), a plasmid encoding for renillaluciferase, was performed to normalize transfection efficiencies. After culturing for 24 h, cells were placed in the 1% O_2 hypoxia environment in the presence or absence of 10 ng/ml TGF-β1 recombinant protein or 10 μmol/L TGF-β1 galunisertib and incubated for another 24 h. Cells were then harvested and luciferase activities were measured using the Dual-Luciferase Reporter Assay system (Promega, USA) on a Lumat LB9507 luminometer (Bethold Technologies, Bad Wildbad, Germany).

Statistical analysis

All results are expressed as mean ± standard error (SE) of three independent experiments. All statistical results were generated using SPSS software version 17.0 (IBM, Chicago, IL, USA). Student's *t*-test and paired *t*-test were performed for comparison among the groups (one-sided or two-sided). Each experiment was repeated three times and a value of $P < 0.05$ was considered statistically significant.

Results

Expression of transforming growth factor-beta 1, vascular endothelial growth factor, and hypoxia-inducible factor-1α in endometrial tissue

We examined the expression of TGF-β1, VEGF, and HIF-1α in EMs samples and normal control endometrial tissue samples using real-time RT-PCR and western-blot assays. In agreement with previous studies' results, we found a significant elevation of TGF-β1, VEGF, and HIF-1α messenger RNA (mRNA) and protein expression. TGF-β1 and HIF-1α expression were positively correlated with VEGF expression [$t = 2.16$, $P = 0.042$, Figure 1].

Expression of transforming growth factor-beta 1, vascular endothelial growth factor, and hypoxia-inducible factor-1α in primary culture of endometrial tissue with different stimuli

The primary cultures of endometrial tissue cells were incubated in hypoxic or normoxic conditions, with or without TGF-β1 and TGF-β1 signal pathway inhibitor galunisertib. Quantitative RT-PCR and western blot assays were used to analyze the expression of TGF-β1, VEGF, and HIF-1α. Hypoxia enhanced the production of VEGF. The *VEGF* mRNA levels increased 43.8% in hypoxia compared with the untreated cells under normoxic conditions. *VEGF* mRNA levels also increased 12.5% in response to TGF-β1. There was a much higher production (87.5% increases) of *VEGF* in the combined treatment group with hypoxia and TGF-β1 [Figure 2]. *VEGF* mRNA levels were found to be almost unaffected by the TGF-β1 signal pathway inhibitor alone but increased 41.2% with combined treatment of hypoxia/TGF-β1 inhibitor. Hypoxia increased the expression of HIF-1α significantly. HIF-1α was induced by TGF-β1 at both the mRNA and protein level ($t = 6.84$, $P = 0.023$), and the expression of TGF-β1 was also increased in 1% O_2 hypoxic conditions.

Reporter activity of vascular endothelial growth factor promoter construct

To identify whether this additive effect was taking place at the transcriptional level, we analyzed the activity of the

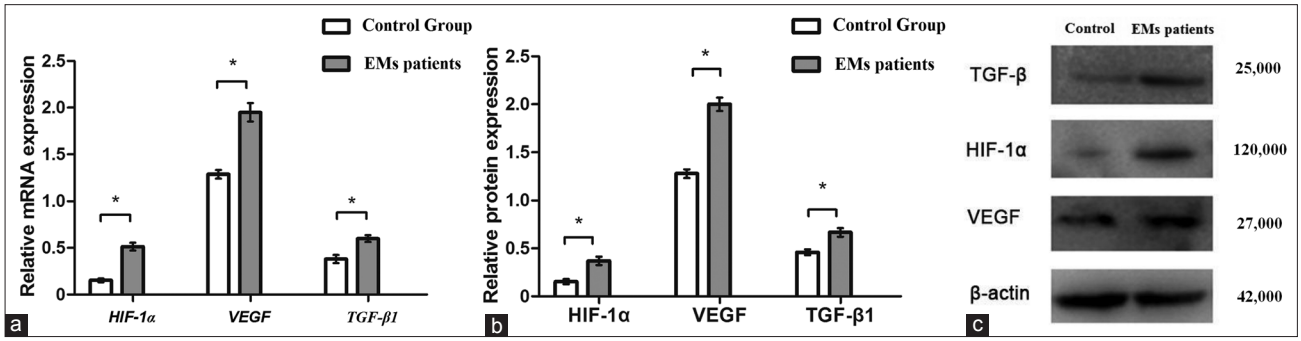

Figure 1: Expression of transforming growth factor-beta 1 (TGF-β1), vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1α (HIF-1α) in endometrial tissues ($n = 40$). (a) Relative messenger RNA expression of the three genes; (b) Relative protein density of the three proteins. (c) Western blotting of the three proteins *$P < 0.05$. EMs: Endometriosis.

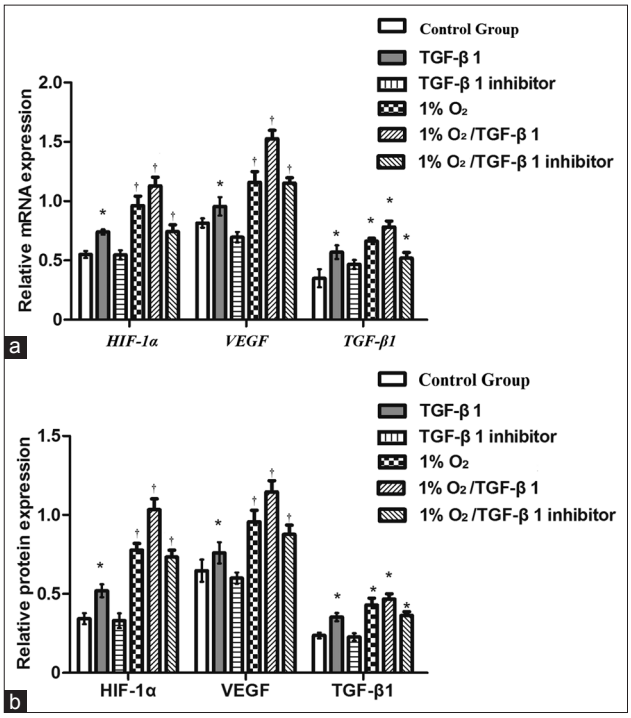

Figure 2: Expression of TGF-β1, VEGF and HIF-1α in primary culture of endometrial cells with different stimuli ($n = 40$). (a) Relative mRNA expression of the three genes. The *VEGF* mRNA levels increased in hypoxia and response to TGF-β1 compared with the untreated cells under normoxic conditions. There was a much higher production of *VEGF* in the combined treatment group with hypoxia and TGF-β1; (b) Relative protein density of the three proteins. The VEGF expression levels increased in hypoxia and response to TGF-β1 compared with the untreated cells under normoxic conditions. There was a much higher expression of VEGF in the combined treatment group with hypoxia and TGF-β1. *$P < 0.05$; †$P < 0.01$. HIF-1α: Hypoxia-inducible factor-1α VEGF: Vascular endothelial growth factor; TGF-β1: Transforming growth factor-beta 1; mRNA: Messenger RNA.

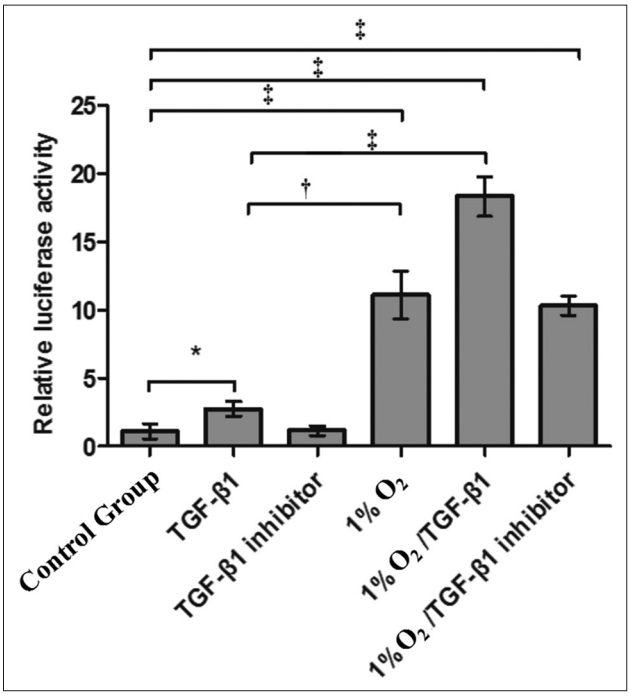

Figure 3: Relative luciferase activities of the *VEGF* promoter construct with different stimuli. Induction of *VEGF* promoter activity was shown in either TGF-β1 or hypoxia, whereas both stimuli resulted in a significant cooperative effect ($n = 40$). In the presence of the TGF-β1 signal pathway and circumstance of hypoxia, *VEGF* promoter activity was increased. These results suggest that the collaboration between TGF-β1 and hypoxia occurs at the transcriptional level. *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$. *VEGF*: Vascular endothelial growth factor; TGF-β1: Transforming growth factor-beta 1.

human *VEGF* promoter region. For this purpose, we used a reporter construct containing the 5′-flanking region of the human *VEGF* gene promoter (−1200/+1) fused to the luciferase gene. This *VEGF* promoter construct contains the hypoxia-response element (*HRE*) and other elements. As shown in Figure 3, transient transfection experiments demonstrated induction of *VEGF* promoter activity in the presence of either TGF-β1 (2.6-fold, $t = 6.08$, $P = 0.032$) or hypoxia (11.2-fold, $t = 32.70$, $P < 0.001$), whereas both stimuli resulted in a significant cooperative effect (18.5-fold, $t = 33.50$, $P < 0.001$). In the presence of the TGF-β1 signal pathway, *VEGF* promoter activity was increased 1.15-fold and 10.4-fold in combination with hypoxia. These results suggest that the collaboration between TGF-β and hypoxia occurs at the transcriptional level.

Discussion

EMs is a non-malignant disease with the presence of ectopic endometrial tissue-like chocolate cysts[23] and is

associated with pelvic pain and infertility.[24] In recent studies, EMs could also affect obstetric outcomes.[25] However, the etiology of this disease is unclear. Recently, there has been increasing evidence that hypoxia plays an important role by inducing gene expression to regulate the implantation, survival, and maintenance of ectopic endometriotic lesions. Hypoxia pretreatment promoted the proliferation and angiogenesis of EMs in an animal study.[26] Hypoxia is a critical factor that potentiates the sensitivity of the *COX-2* gene in ectopic endometriotic stromal cells. Since COX-2 regulates some particular enzymes such as PGs that have been linked to the development of EMs,[27-31] it could be assumed that hypoxia could affect EMs through inducing aberrant COX-2 expression. The level of leptin is elevated either in serum or peritoneal fluid from women with EMs.[32,33] Leptin could promote cell proliferation and angiogenesis in EMs lesions.[34] Interestingly, leptin is not expressed in normal endometrial stromal cells unless the cells are cultured in hypoxic conditions.[35,36] Taken together, hypoxia could induce EMs lesion formation.

Hypoxia, as a known master regulator of angiogenesis, can induce many angiogenic factors such as VEGF. The expression of VEGF protein and mRNA in ectopic endometrial tissue is significantly higher than that in normal endometrium.[37,38] Recently, a study demonstrated that suppression of VEGF expression and blockade of angiogenesis reduced the size of endometriotic lesions in a mouse model.[39] The study of Song *et al*.[40] showed that VEGF might be involved in the pathogenesis of EMs by regulating angiogenesis, and the expression of VEGF has a relationship with the severity of EMs.

HIF-1α, a transcription factor that responds to hypoxic stress, is known to regulate VEGF expression and mediate cell adaption to the hypoxic environment.[41-43] Many studies have shown that HIF-1α expression in endometrial cancer and other malignant tumors is significantly increased.[34,44] A study by Lu *et al*.[26] showed elevated levels of HIF-1α mRNA and protein in ectopic endometriotic lesions. In addition, several papers reported a role of HIF-1α during the development of EMs.[38,45-48]

The epithelial-to-mesenchymal transition (EMT) plays a key role in metastasis of many kinds of tumors such as colorectal cancer, bladder cancer, and breast cancer[49-51] and EMT induced by HIF-1α has been reported to play a role in the development of many tumor types.[52-54] Hypoxia induces the EMT of endometrial cells, resulting in cellular characteristics changes, which may be a prerequisite for the establishment of endometriotic lesions.[55]

TGF-β1 is a polypeptide growth factor that plays an important role in angiogenesis. It can induce VEGF expression. TGF-β1 can promote cell migration by inhibiting the expression of cytokines that act to maintain the integrity of blood vessels, thus allowing for increased permeability,[56,57] favoring the migration of cells. TGF-β1 is also able to promote cell migration by up-regulating the activity of MMPs.[58,59] Other researchers showed that TGF-β1/SMAD2/3 signaling can stimulate human granulosa cell migration by up-regulating connexin 43 expression[60] and regulate insulin gene transcription and pancreatic islet cell function through SMAD2/3 signaling,[61] and this pathway plays a critical role in renal fibrosis and chronic liver disease.[62] However, the effect of TGF-β1/SMAD2/3 signaling on EMs pathogenesis is unclear.

In agreement with previous reports, the data showed higher HIF-1α and VEGF expression in endometrial cells under hypoxia through western blotting and RT-PCR. We showed that the expression of TGF-β1 was significantly higher than that in normal endometrial tissues. After hypoxic treatment of normal endometrial cells or the addition of TGF-β1 recombinant protein or TGF-β1 specific inhibitor, compared with the control group, VEGF expression was increased and the highest level was present in the combined hypoxia and TGF-β1 group.

We used endometrial cells in primary culture to detect the response of TGF-β1 and HIF-1α to VEGF, and we found that HIF-1α could regulate VEGF expression directly. We also found that TGF-β1 could regulate VEGF expression through the SMAD2/3 pathway, and interestingly, if we combined TGF-β1 and hypoxia, the expression of VEGF was more than two times higher, suggesting that HIF-1α and TGF-β1 have a synergistic action in regulating VEGF expression. TGF-β1 and hypoxia are acting independently of each other because TGF-β1 inhibitors did not inhibit the induction of VEGF by hypoxia.

In this study, we found TGF-β1 could not only affect *VEGF* expression but also HIF-1α. TGF-β1 and HIF-1α are not acting as upstream and downstream proteins. We hypothesize that endometrial fragments reflux to the pelvis during menstruation and when exposed to hypoxic stress release large amounts of VEGF and settle in the peritoneal cavity. This, in turn, would induce local angiogenesis and aid revascularization of the endometrium at the ectopic site. Transient transfection experiments demonstrated the presence of either TGF-β1 (2.6-fold, $t = 6.08$, $P = 0.032$) or hypoxia (11.2-fold) could induce *VEGF* promoter activity ($t = 32.07$, $P < 0.001$), whereas the simultaneous presence of both stimuli resulted in a significant cooperative effect (18.5-fold, $t = 33.50$, $P < 0.001$). These results suggest that the additive and synergistic effect of TGF-β1 and hypoxia on *VEGF* expression has a transcriptional basis.

This study is subject to some limitations. First, the sample size is small and the data might, therefore, not be representative. Second, the women with EMs who we selected chose to undergo surgery, which means patients with mild conditions were not included in the study. Accordingly, there might have been selection bias. The results demonstrated TGF-β1 and hypoxia can regulate *VEGF* transcription by targeting the promotor region of the *VEGF* gene independently with a synergistic action. Because an increased level of TGF-β1

was found in a hypoxic environment and EMs lesions are also in a hypoxic environment, we focus on the effect of hypoxia and TGF-β1 on VEGF expression in EMs.

In conclusion, the altered expression of VEGF observed in endometrial primary culture cells through regulating TGF-β1 and HIF-1α expression suggests both TGF-β1 and hypoxia affect ectopic endometrial cells by inducing angiogenesis. The results suggest hypoxia and TGF-β1 could promote EMs formation through synergistic action by regulating *VEGF* at the transcriptional level. These findings might explain angiogenesis progression allowing for lesion formation after endometrial tissue flows back into the pelvis.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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