

Hypoxia Promotes Ectopic Adhesion Ability of Endometrial Stromal Cells via TGF- β 1/Smad Signaling in Endometriosis

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Hypoxia plays a vital role in the progression of endometriosis. Additionally, integrin-mediated aberrant adhesion is also essential for establishment of endometriotic lesions. In this study, we sought to determine the function of hypoxia in integrin-mediated adhesion of endometrial stromal cells (ESCs) in endometriosis. The expressions of adhesion molecule integrins (integrin α_5 , integrin α_v , integrin β_3 , and integrin β_5) were determined in 15 normal endometria and 15 paired eutopic and ectopic endometria by immunohistochemistry. Thirteen primary ESCs from patients with peritoneal endometriosis in the proliferative phase were cultured under a hypoxic (1% O₂) or normoxic (21% O₂) environment, and the expression levels of hypoxia-inducible factor (HIF)-1 α , transforming growth factor (TGF)- β 1, and integrins were detected by quantitative reverse transcription polymerase chain reaction and western blot. The alteration of integrins in endometriotic mouse models were also explored. Our results demonstrated that HIF-1 α and integrins were highly expressed in ESCs of endometriotic lesions compared with ESCs of eutopic and normal endometrium. Hypoxia treatment significantly increased ESC adhesion abilities and integrin expression, which were positively correlated with TGF- β 1 expression. Both TGF- β 1 and hypoxia enhanced ESC adhesion properties, whereas hypoxia combined with TGF- β 1 receptor inhibitor inhibited ESC adhesion. Knockdown of HIF-1 α attenuated TGF- β 1/Smad signaling activation and integrin expression and reduced ESC adhesion. Higher expression levels of HIF-1 α , TGF- β 1, and integrins were detected in endometriotic cysts from mice models. Our findings provide a novel insight of endometriosis that the hypoxic microenvironment stimulates ESCs to produce excessive TGF- β 1 and activates the TGF- β 1/Smad signaling pathway, thus enhancing integrin expression and the adhesion ability of ESCs. (*Endocrinology* 159: 1630–1641, 2018)

Endometriosis is a benign and estrogen-dependent gynecologic disorder characterized by the presence of endometrial-type gland and stroma outside the uterine cavity (1). Pelvic pain, dysmenorrhea, dyspareunia, and infertility are common symptoms in endometriosis that negatively impact a woman's quality of life (2).

Although there are many proposed theories of endometriosis, the most convincing one is the retrograde menstruation hypothesis. It is proposed that endometrial fragments flow backward to the pelvic cavity and undergo adhesion, proliferation, and angiogenesis, ultimately developing into ectopic cysts or nodules (3). The attachment of retrograde endometrial tissues to the pelvic cavity is

ISSN Online 1945-7170

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Received 13 December 2017. Accepted 31 January 2018.

First Published Online 8 February 2018

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Abbreviations: ANOVA, analysis of variance; EEC, endometrial epithelial cell; ELISA, enzyme-linked immunosorbent assay; ESC, endometrial stromal cell; HIF, hypoxia-inducible factor; hypoxia-SB431542, hypoxic culture condition supplemented with the TGF- β 1 receptor inhibitor SB431542; IF, immunofluorescence assay; IHC, immunohistochemistry; mRNA, messenger RNA; p-Smad, phosphorylated Smad; qRT-PCR, quantitative reverse transcription polymerase chain reaction; shRNA, short hairpin RNA; TGF, transforming growth factor.

regarded as the initiation stage of endometriosis, and many different adhesion molecules have been suggested to regulate this process (4). Cell adhesion molecules consist of four major groups: selectins, cadherins, members of the immunoglobulin superfamily, and integrins (5). Expression levels of integrin family members, including integrin $\alpha_3\beta_1$, integrin β_2 , and integrin $\alpha_v\beta_3$, are increased in endometriosis tissues (4, 6, 7), suggesting that integrin-associated adhesion plays an important role in the development of endometriotic lesions. Additionally, attachment of endometrial tissues to host peritoneum is mainly dependent on endometrial stromal cells (ESCs) in an integrin-dependent manner (8–10).

Growing evidence indicates that hypoxia participates in endometriosis, and the master regulation factor of hypoxia, hypoxia-inducible factor (HIF)-1 α , is overexpressed in endometriotic lesions (11, 12). Moreover, emerging evidence suggests a link between hypoxia and adhesion (13). However, the relationship between hypoxia and integrin-mediated adhesion in ESCs of endometriosis is unclear. Our study aims to clarify the mechanisms responsible for the formation of endometriotic lesions by considering the effects of hypoxia on the adherent ability of ESCs and the underlying signaling pathway.

Materials and Methods

Study approval

This study was initiated on 13 September 2015 and terminated on 20 May 2017. Ethics approval was obtained from the Medical Ethics Committee of Zhejiang University. Informed written consent was obtained from all patients prior to tissue collection. All animals were maintained in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, and the experiment was approved by the Experimental Animal Ethics Committee of Zhejiang University.

Collection of endometrium samples and endometriotic tissues

Eutopic and ectopic endometrium samples were collected from parous reproductive-aged women with peritoneal endometriosis. For each case, the diagnosis was initially considered as phase II of endometriosis according to the revised American Fertility Society classification of endometriosis. To avoid cellular heterogeneity, only histologically confirmed middle proliferative eutopic endometrial fragments were selected for extraction of ESCs and establishment of mice models of endometriosis (14). Detail inclusion criteria for patients and number of cases are presented in Supplemental Information.

Isolation, purification, culture, and authentication of ESCs

We performed primary cell culture to acquire highly pure ESCs according to previous reports (15, 16). More detail is

presented in Supplemental Information. ESCs from 13 individual patients representing a proliferating population of non-differentiated endometrial stromal cells were used between passages 3 and 5. Immunofluorescent staining was performed to determine the purity of isolated ESCs using monoclonal antibodies specific for human vimentin (ab92547; Abcam, Cambridge, U.K.) and pan-cytokeratin (ab215838; Abcam).

Normoxia, hypoxia, transforming growth factor- β 1, and hypoxic culture condition supplemented with the transforming growth factor- β 1 receptor inhibitor SB431542 treatment of ESCs

ESCs were cultured at 37°C in 21% O₂ as the normoxic culture group and 1% O₂ was used for the hypoxic culture group, as reported previously (17, 18). Hypoxic conditions were maintained using a hypoxia incubator chamber (27310; Stemcell Technologies, Vancouver, BC, Canada) with 5% CO₂ and 1% O₂ balanced with N₂. Transforming growth factor (TGF)- β 1 treatment was conducted at 37°C in 21% O₂ with 10 ng/mL TGF- β 1 (100-21; PeproTech, Rocky Hill, CT). For hypoxic culture condition supplemented with the TGF- β 1 receptor inhibitor SB431542 (hypoxia-SB431542), 10 μ M SB431542 (14775; Cell Signaling Technology, Beverly, MA) was directly added to the medium 2 hours before incubation in hypoxic conditions. The medium of all four groups was changed every 24 hours.

Adhesion and migration assays of ESCs

A CCK-8–based adhesion assay and transwell-based migration assay were performed to determine the adhesion and migration capacities of ESCs in different culture conditions, including normoxia or hypoxia, TGF- β 1 costimulation, and hypoxia-SB431542 treatment. Detailed procedures are provided in Supplemental Information.

RNA isolation and quantitative reverse transcription polymerase chain reaction

Total RNA was isolated from ESCs using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription, quantitative reverse transcription polymerase chain reaction (qRT-PCR), and measurement of target gene expression were performed as described in Supplemental Information. Primers sequences of target genes are listed in Supplemental Table 1.

Western blot assay

Western blotting was performed as described in Supplemental Information. Primary antibodies used in our study are listed in Supplemental Table 2. ImageJ analysis was applied to acquire the signal intensity of immunoblot target proteins.

Immunohistochemistry assay

Immunohistochemistry was performed according to common protocols as indicated in Supplemental Information. The H-score of staining results was calculated using the following equation: H-score = $\sum Pi (i + 1)$, where i indicates intensity of staining with a value of 1, 2, or 3 (weak, moderate, or strong, respectively) and Pi is the percentage of stained cells in total ESCs or endometrial epithelial cells (EECs), with each intensity ranging from 0% to 100%.

Immunofluorescence assay

To determine the biological mechanism of hypoxia on ESCs, an immunofluorescence assay (IF) was performed using a standard staining procedure as described in Supplemental Information to investigate the subcellular translocation of phosphorylated Smad (p-Smad) 2 under hypoxia.

Mouse model of endometriosis

We established a mouse model of endometriosis to clarify the alteration of integrins in endometriosis. Animal models were established by stitching eutopic endometrium onto the peritoneum of C57BL/6 female mice (19); lesions were harvested 48 hours after transplantation. Procedures for generation of mouse peritoneal lesions and subsequent tissue harvest are described in Supplemental Information.

Lentiviral-mediated interference of HIF-1 α in ESCs

Three different HIF-1 α interfering short hairpin RNA (shRNA) lentiviral vectors (shRNA1, shRNA2, and shRNA3) were purchased from Genechem (Shanghai, China). After stabilization of the primary culture of ESCs, cells were seeded in six-well plates and infected with lentivirus at a multiplicity of infection of 10 for 48 hours. ESCs were observed under fluorescence microscopy to confirm the infection efficiency after 24 hours of culture and harvested after 48 hours. The medium was changed every 24 hours. qRT-PCR and western blot analyses were performed to confirm the efficiency of HIF-1 α inhibition.

Enzyme-linked immunosorbent assay

Thirteen primary cultured ESCs were preseeded on 60-mm dishes at a density of 1×10^5 cells per dish. After normoxic or hypoxic treatments for 48 hours, the cell number was counted and the supernatant of culture medium was used to determine the concentration of activated TGF- β 1 using enzyme-linked immunosorbent assay (ELISA) kits from Multi Sciences Biotech (70-EK1812; Lianke, Hangzhou, China), according to the manufacturer's protocol.

Statistical analysis

SPSS program version 19.0 and GraphPad Prism 5 software were used for statistical analysis. Parametric tests of TGF- β 1, normoxia, hypoxia, and hypoxia-SB431542 groups and immunohistochemistry (IHC) results of human paraffin slides were analyzed by one-way analysis of variance (ANOVA). Other parametric comparison was conducted by a paired Student *t* test after confirming that the data obeyed normal distribution by a one-sample Kolmogorov–Smirnov test. Differences were considered to be statistically significant at $P \leq 0.05$.

Results

Expression of integrins and HIF-1 α was significantly higher in endometriotic lesions compared with normal endometrium

Although many studies suggested that an increased adhesive capacity of endometriosis may be due to dysregulation of integrins (20–22), little is known about the origin of integrins and the specific integrins that function in adhesive regulation of endometriosis. Therefore, we first analyzed the

expression of different integrin family members in normal endometrium, eutopic endometrium, and ectopic lesions by IHC (Fig. 1A). The H-scores of these proteins in EECs and ESCs are summarized in Fig. 1B and 1C, respectively ($P < 0.001$, one-way ANOVA). Integrin α_5 , integrin α_V , integrin β_3 , and integrin β_5 expression in stromal cells was higher in eutopic tissues and ectopic lesions than in normal tissues, with ectopic lesions showing the highest staining. Integrin α_5 , integrin α_V , and integrin β_5 in EECs showed weaker expression levels than in ESCs of eutopic tissues, whereas their expressions in EECs of eutopic tissues were higher than in EECs of normal tissues and ectopic issues. The high staining of integrin α_5 , integrin α_V , integrin β_3 , and integrin β_5 in ESCs of ectopic lesions indicates the importance of these integrins in aberrant adhesion of endometriosis.

HIF-1 α is a key regulator of hypoxia that was reported to participate in the progression of endometriosis. In this study, we found that HIF-1 α was strongly expressed in ectopic tissues in both ESCs and EECs, weakly expressed in eutopic tissues, and not expressed in EECs and ESCs of normal tissues.

Hypoxia increased the adhesion and migration abilities of ESCs

To our knowledge, adhesion studies in primary endometrial epithelial cells were unreproducible (23) and the peritoneal mesothelial cells mainly acted as a defensive barrier (24). Moreover, our IHC results of human tissues showed a significant upregulation of integrins in ESCs of ectopic lesions and eutopic endometrium compared with normal endometrium, whereas the expression of integrins in EECs of ectopic lesions was weak. Therefore, we focused on the integrins in ESCs.

We isolated 13 eutopic ESCs from 13 eutopic endometrium samples of endometriosis patients. All samples were characterized as vimentin-positive and pan-cytokeratin-negative (Fig. 2A). Then, we performed an adhesion assay of ESCs under hypoxia. As shown in Fig. 2B, the adhesion abilities of ESCs was significantly increased in hypoxia pretreated conditions compared with normoxic cultured conditions for 48 hours ($P < 0.001$, paired Student *t* test). A typical hallmark of endometriosis is the migration potential of ESCs. Using an uncoated transwell assay, we found that hypoxia significantly enhanced the migration ability of ESCs (Fig. 2C; $P < 0.001$, paired Student *t* test).

Hypoxic culture upregulated messenger RNA and protein expression of integrins in ESCs

Hypoxic culture for 48 hours significantly upregulated ESC messenger RNA (mRNA) expression of HIF-1 α , integrin α_5 , integrin α_V , integrin β_3 , and integrin β_5 compared with normoxic culture (Fig. 2D; $P < 0.001$, paired

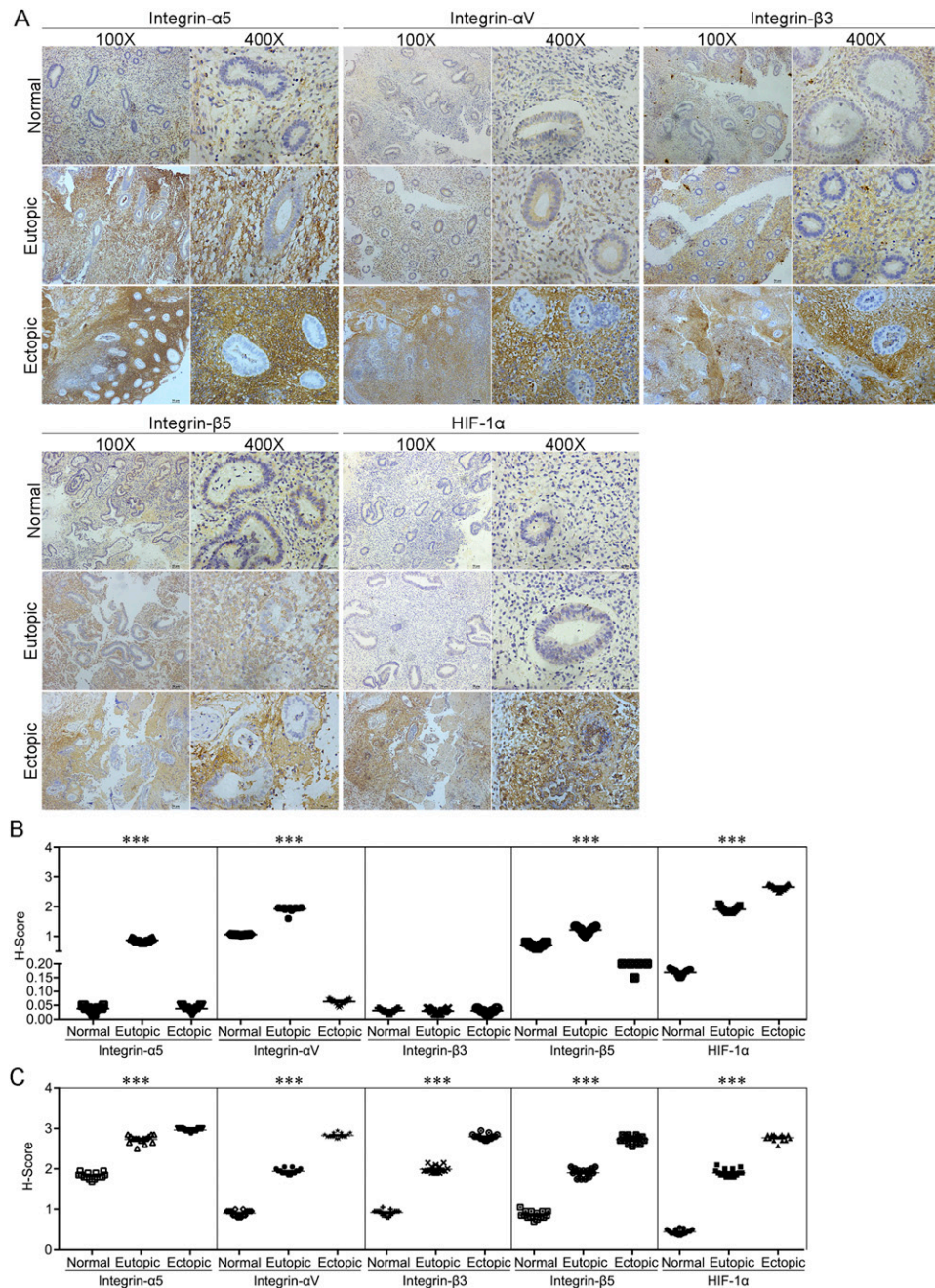


Figure 1. Ectopic lesions expressed higher levels of HIF-1 α and integrins than did normal endometrium. (A) Immunohistochemistry photomicrographs of integrin α_5 , integrin α_V , integrin β_3 , integrin β_5 , and HIF-1 α in normal endometrium and paired eutopic and ectopic tissues. Staining was developed by diaminobenzidine, and the cell nucleus was stained with hematoxylin. Summarized H-scores of protein expression in EECs and ESCs in the three types of sample are shown in (B) and (C), respectively. (B) Expression of integrin α_5 , integrin α_V , integrin β_3 , integrin β_5 , and HIF-1 α in EECs by immunohistochemistry represented by the H-score (y-axis) in all human tissues. H-scores of integrin α_5 , integrin α_V , and integrin β_5 in EECs in ectopic lesions were lowest ($n = 15$, $P < 0.001$, one-way ANOVA), and there was no difference in H-score for integrin β_3 in EECs ($n = 15$, $P > 0.05$, one-way ANOVA). In contrast, the H-score of HIF-1 α in EECs was higher in endometriosis samples than in normal endometrium, and ectopic lesions showed the strongest staining ($n = 15$, $P < 0.001$, one-way ANOVA). (C) Expression of integrin α_5 , integrin α_V , integrin β_3 , integrin β_5 , and HIF-1 α in ESCs by immunohistochemistry represented by the H-score (y-axis) in all human tissues. H-scores of integrin α_5 , integrin α_V , integrin β_3 , integrin β_5 , and HIF-1 α in ESCs were higher in ectopic lesions than in eutopic endometrium and weakest in normal endometrium ($n = 15$, $P < 0.001$, one-way ANOVA). *** $P < 0.001$.

Student t test). Western blotting results revealed that protein expression of HIF-1 α , integrin α_5 , integrin α_V , integrin β_3 , and integrin β_5 was also increased in hypoxic culture compared with normoxic culture (Fig. 2E; Supplemental Fig. 1), which was corresponded with qRT-PCR results.

Hypoxia induced integrin expression via autocrine TGF- β_1 in ESCs

Overexpression of TGF- β_1 in ESCs implies an autocrine mechanism in the development of fibrous adhesions and maintenance of endometriotic lesions (25, 26).

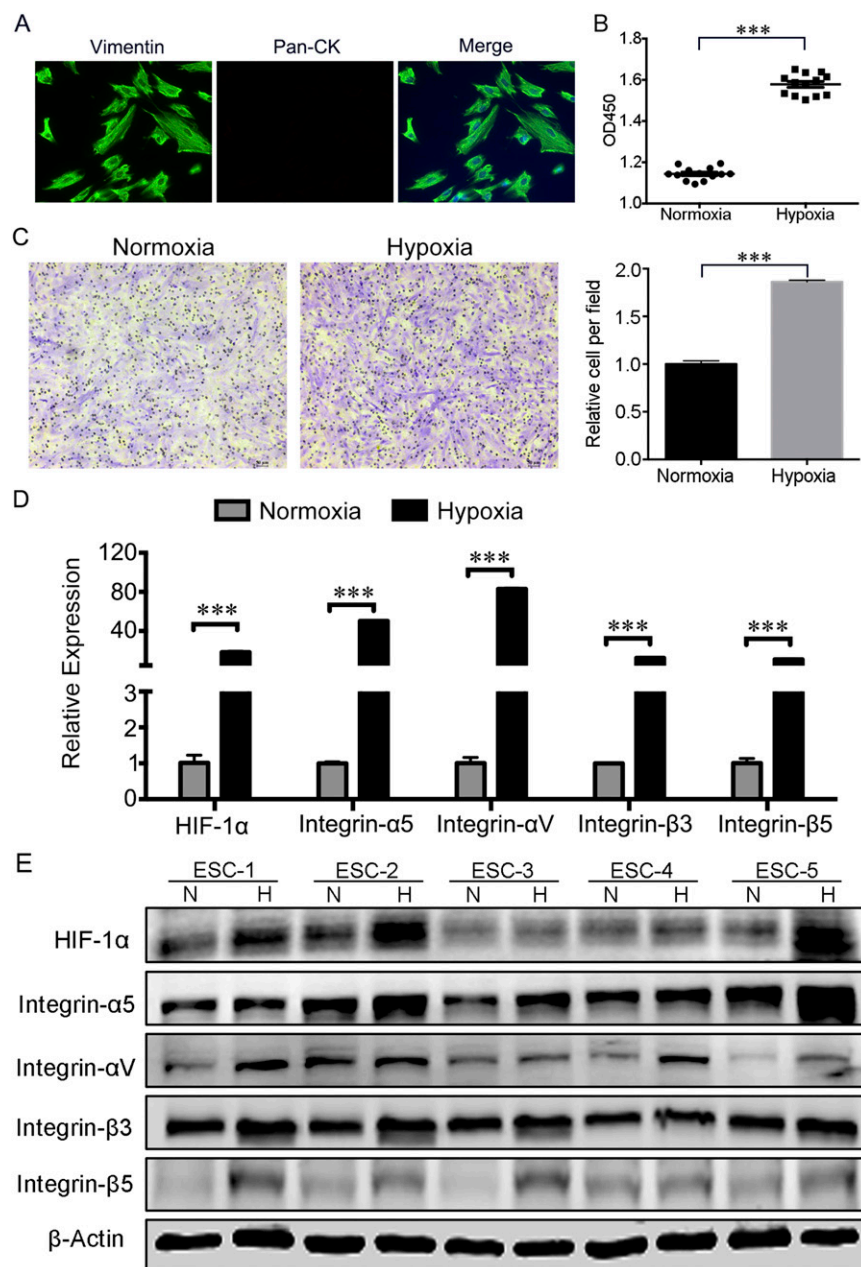


Figure 2. Hypoxia promoted adhesion and migration and increased mRNA and protein expression of integrins in ESCs. (A) Immunofluorescence assay of primary cultured cells showed positive staining for the stromal marker vimentin (green), and negative staining for the epithelial marker pan-cytokeratin (red). DAPI (blue) was used to stain the cellular nucleus. Original magnification, $\times 400$. (B) Cell adhesion of ESCs determined by CCK-8 assay in normoxic and hypoxic conditions ($n = 13$, $P < 0.001$ vs normoxia, paired Student t test). (C) Transwell-based cell migration assay under normoxia and hypoxia culture showed that hypoxia promoted the migration ability of ESCs ($n = 13$, $P < 0.001$ vs normoxia, paired Student t test). Cells were stained with crystal violet. A representative migration experiment of one ESC is shown in the left panel and the quantified results are presented as mean \pm standard deviation in the right panel. Original magnification, $\times 100$. (D) qRT-PCR assay of mRNA expression of HIF-1 α and integrins (integrin α_5 , integrin α_V , integrin β_3 , and integrin β_5) in ESCs under normoxic and hypoxic culture. HIF-1 α and integrin mRNA levels were dramatically higher under hypoxic culture compared with normoxic culture ($n = 13$, $P < 0.001$ vs normoxia, paired Student t test). (E) Western blot assay of five ESCs showed increased protein levels of HIF-1 α and integrins under hypoxic culture compared with normoxic culture ($n = 5$). All experiments were repeated three times and results of a representative experiment are shown. $***P < 0.001$. DAPI, 4',6-diamidino-2-phenylindole; H, hypoxic culture group; N, normoxic culture group; Pan-CK, pan-cytokeratin.

Interestingly, we found that both mRNA and protein levels of TGF- β 1 were significantly increased in ESCs after hypoxic treatment (Fig. 3A and 3B; Supplemental Fig. 2). Moreover, the ELISA results revealed that the supernatant of 13 ESCs under hypoxia culture contained higher levels of activated TGF- β 1 than did the supernatant of these cells under normoxic culture (Fig. 3C, $P < 0.001$, paired Student t test). Although many studies have reported the mechanism of hypoxia-induced high expression of TGF- β 1 (27), the influence of hypoxia on TGF- β 1 in endometriosis is obscure. Our *in vitro* results implied a potential role of hypoxia-induced TGF- β 1 in mediating ESC biofunction.

The IHC results of human tissues showed that TGF- β 1 exhibited the same trend as HIF-1 α (Fig. 3D; Supplemental Fig. 3; $P < 0.001$, one-way ANOVA) in both ESCs and EECs, with higher expression in eutopic tissues than in normal tissues and the highest staining in ectopic lesions. Classically, TGF- β ligands bind to the constitutively active transmembrane receptor TGF- β receptor II and recruit TGF- β receptor I, and then they activate Smad proteins through phosphorylation, subsequently regulating target gene expression in a transcriptional manner (28). To determine whether TGF- β 1 affects ESC biofunction, we further detected the expression and location of TGF- β receptor II in endometrium by immunohistochemistry. The results showed that the expression level of TGF- β receptor II in ESCs was higher in eutopic tissues than in normal tissues, with the highest staining in ectopic lesions (Fig. 3D; Supplemental Fig. 3; $P < 0.001$, one-way ANOVA). Although EECs showed weak and moderate expression of TGF- β receptor II in normal and eutopic endometrium, respectively, no expression of this protein was found in EECs of ectopic lesions. These results reinforced our hypothesis that hypoxia-induced TGF- β 1 mainly mediates ESC function via TGF- β receptor II in endometriosis.

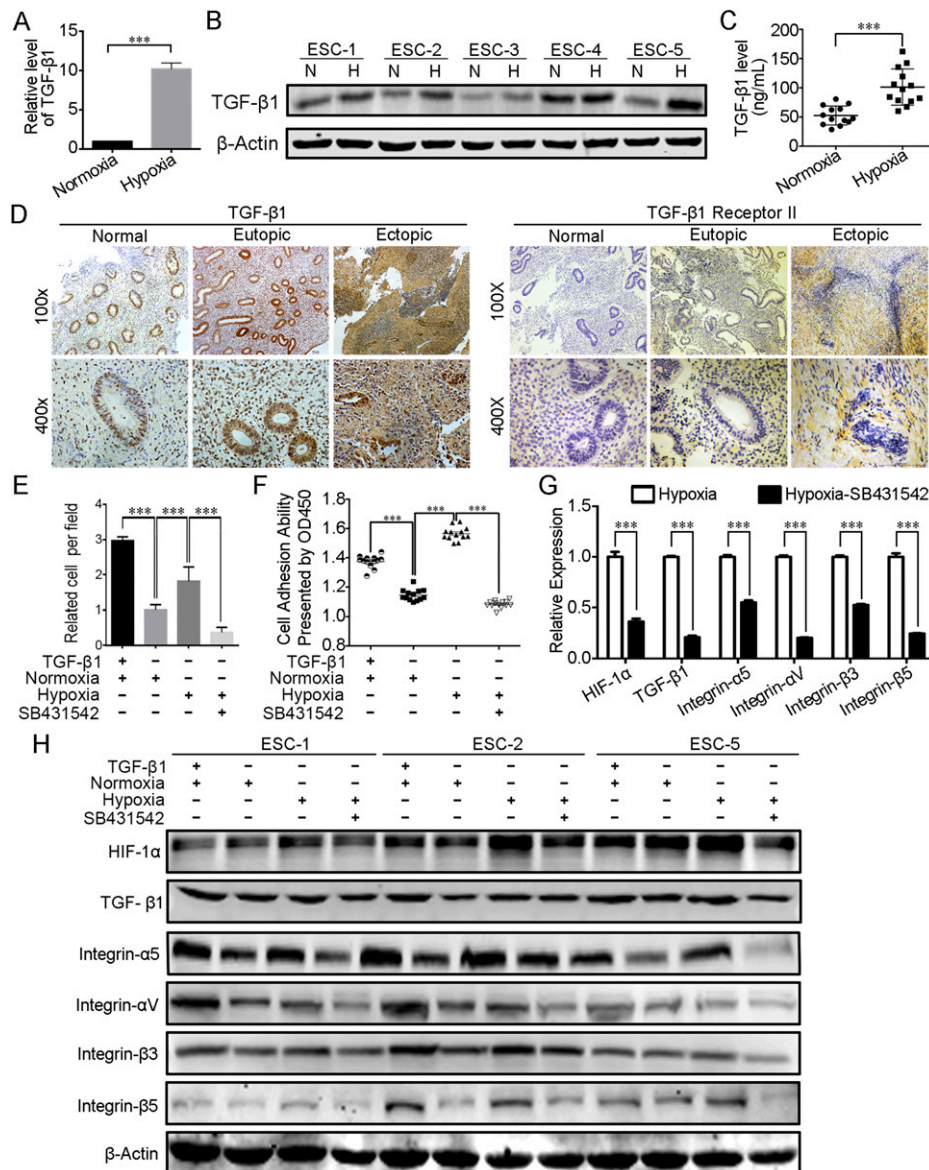


Figure 3. High expression of TGF- β 1 detected under hypoxic culture played a vital role in the increased adhesion and migration ability of ESCs. (A) A high level of TGF- β 1 mRNA in ESCs was detected under hypoxic culture by qRT-PCR assay ($n = 13$, $P < 0.001$ vs normoxia, paired Student t test). (B) High level of TGF- β 1 protein in ESCs was detected under hypoxic culture by western blot analysis ($n = 13$). Representative western blot results of five ESCs are shown. (C) ELISA of supernatant from 13 ESCs under normoxic and hypoxic culture showed a significantly high concentration of activated TGF- β 1 under hypoxia culture ($n = 13$, $P < 0.001$ vs normoxia, paired Student t test). (D) IHC photomicrographs of TGF- β 1 and TGF- β 1 receptor II in normal endometrium and paired eutopic and ectopic tissues. Staining was developed using diaminobenzidine, and the cell nucleus was stained with hematoxylin. TGF- β 1 expression was higher in both ESCs and EECs of eutopic tissues than that of normal tissues, with the highest staining in both ESCs and EECs of ectopic lesions. The expression level of TGF- β receptor II in ESCs was higher in eutopic tissues than normal tissues, with the highest staining in ectopic lesions ($n = 15$, $P < 0.001$, one-way ANOVA). Although EECs showed weak and moderate expression of TGF- β receptor II in normal and eutopic endometrium, respectively, no expression of TGF- β receptor II was found in EECs of ectopic lesions ($n = 15$, $P < 0.001$, one-way ANOVA). (E) Transwell migration assay was conducted to test the effects of TGF- β 1, normoxia, hypoxia, and hypoxia-SB431542 costimulation on cell migration. The migration ability of ESCs was significantly stronger under TGF- β 1 costimulation and hypoxic culture than under normoxia culture, whereas hypoxia-SB431542 costimulation under hypoxia decreased ESC migration ability compared with hypoxic culture alone. The quantified results are presented as mean \pm standard deviation in the right panel ($n = 13$, $P < 0.001$, one-way ANOVA). (F) Adhesion assay showing absorbance of ESCs under TGF- β 1 costimulation, normoxia, hypoxia, and hypoxia-SB431542 costimulation via a cell counting kit-8 colorimetric assay. Optical density at 450 nm (OD450) was significantly higher in the TGF- β 1 costimulation group and hypoxic culture group, whereas the hypoxia-SB431542 group showed dramatically decreased OD450 compared with hypoxic culture alone ($n = 13$, $P < 0.001$, one-way ANOVA). (G) qRT-PCR analysis of HIF-1 α , TGF- β 1, and integrins (integrin α_5 , integrin α_V , integrin β_3 , and integrin β_5) in ESCs under hypoxia and culture condition supplemented with the TGF- β 1 receptor inhibitor SB431542 (hypoxia-SB431542) costimulation showed that mRNA levels of HIF-1 α , TGF- β 1, and integrins were dramatically decreased under hypoxia-SB431542 costimulation compared with hypoxic culture alone ($n = 13$, $P < 0.001$, paired Student t test). (H) Western blot analysis of HIF-1 α , TGF- β 1, and integrins (integrin α_5 , integrin α_V , integrin β_3 , and integrin β_5) in ESCs under TGF- β 1 costimulation, normoxia, hypoxia, and hypoxia-SB431542 costimulation for 48 hours. The protein expression of TGF- β 1 and integrins was dramatically increased under TGF- β 1 costimulation and hypoxic culture ($n = 5$), whereas hypoxia-SB431542 treatment decreased protein expression of TGF- β 1 and integrins compared with hypoxic culture ($n = 5$). A representative experiment of three ESCs is shown. *** $P < 0.001$. H, hypoxic culture group; N, normoxic culture group.

Next, recombinant TGF- β 1 or its inhibitor SB431542 was added to ESCs for 2 hours before hypoxic treatment and the cells were subsequently subjected to migration and adhesion assays. As shown in Fig. 3E and 3F, the percentage of migrated and adhered ESCs was dramatically increased in the hypoxia culture group and TGF- β 1 costimulation group compared with the normoxic culture group, whereas migration and adhesive abilities were decreased in the hypoxia-SB431542 group relative to the hypoxia culture group ($P < 0.001$, one-way ANOVA). Addition of TGF- β 1 to normoxic culture markedly increased ESC mRNA expression levels of integrin α_5 , integrin α_V , integrin β_3 , and integrin β_5 compared with normoxic culture (Supplemental Fig. 4, $P < 0.001$, paired Student t test). In contrast, mRNA expression of integrins was dramatically inhibited when ESCs were pretreated with SB431542 in hypoxic condition compared with that in hypoxic culture alone (Fig. 3G; $P < 0.001$, paired Student t test). Moreover, the alterations in integrin protein levels corresponded to changes in mRNA, with significantly increased integrin protein expressions in ESCs after incubation with TGF- β 1 compared with the normoxic group (Fig. 3H; Supplemental Fig. 5). In contrast, combination treatment with SB431542 under hypoxic culture blocked the upregulation of integrins induced by hypoxia, suggesting that the increase in adhesion ability and integrin expression in ESCs under hypoxia depended on the overexpression of TGF- β 1.

Hypoxia-induced TGF- β 1 stimulated Smad2 signaling

Increased TGF- β 1 in peritoneal fluid and adjacent peritoneum of endometriosis has been reported to stimulate the Smad2/3 signaling pathway and promote the establishment of endometriotic lesions (29, 30); therefore, we next investigated whether Smads could be activated by hypoxic treatment in ESCs. After culture of ESCs in hypoxic conditions for several hours the level of p-Smad2 was increased, accompanied by an increase in HIF-1 α (Supplemental Fig. 6). Further experiments revealed that p-Smad2, p-Smad3, and Smad4 protein expression levels were remarkably elevated in ESCs cultured in hypoxic condition compared with normoxic condition, with no influence on total Smad2 level (Fig. 4A; Supplemental Fig. 7). In contrast to the increase in p-Smad2, p-Smad3, and Smad4 proteins in the TGF- β 1 costimulation group and hypoxia group (Fig. 4B; Supplemental Fig. 8), the hypoxia-SB431542 group showed inhibition of upregulated protein levels that were stimulated by hypoxia alone (Supplemental Fig. 9).

Moreover, IF results showed that p-Smad2 was mainly located in the cytoplasm of ESCs with faint staining in the

nucleus under normoxic conditions (hypoxia at 0 hour). After hypoxic incubation for 4 hours, p-Smad2 started to enter the nucleus of ESCs. The accumulation of p-Smad2 in the nucleus peaked after 8 hours of incubation in hypoxic conditions (Fig. 4C). Given that SB431542 blocked the hypoxia-induced integrin expression, these results suggested that hypoxia induced excessive p-Smad2 translocation into the nucleus by stimulating TGF- β 1 signaling, which subsequently regulated the expression of integrins in ESCs under hypoxia.

HIF-1 α participated in hypoxia induction of TGF- β 1/Smad/integrins in ESCs

Because HIF-1 α is abundantly expressed in ESCs extracted from ectopic endometrium of endometriosis compared with eutopic endometrium (31), we next investigated whether knockdown of HIF-1 α by shRNAs in ESCs could influence cell adhesion and integrin expression. As shown in Fig. 5A, the HIF-1 α mRNA expression level was notably downregulated by shRNAs in both normoxic and hypoxic groups ($P < 0.01$, one-way ANOVA). Inhibition of HIF-1 α expression significantly decreased the adhesion abilities of ESCs that were induced by hypoxia (Fig. 5B, $P < 0.001$, paired Student t test). Moreover, mRNA levels of integrins, including α_5 , α_V , β_3 , and β_5 , were remarkably inhibited in culture conditions of either normoxia or hypoxia (Fig. 5C, one-way ANOVA). Furthermore, RNA interference of HIF-1 α under hypoxia decreased both TGF- β 1 and p-Smad2 protein levels without any influence on total Smad2 (Fig. 5D; Supplemental Fig. 10B–D). Consistent with mRNA expression of integrins, levels of integrin proteins were all decreased by HIF-1 α interference under hypoxia (Fig. 5D; Supplemental Fig. 10E–H). These results demonstrated that HIF-1 α participated in the induction of TGF- β 1/Smad/integrins in ESCs under hypoxic conditions.

High expression of integrins in endometriotic cysts from mouse model

As the above results proved that hypoxia promoted the adhesion and migration of ESCs *in vitro* through induction of TGF- β 1, we further assessed the alteration of HIF-1 α , TGF- β 1, and integrins *in vivo* using mouse model of peritoneal endometriosis. Eleven C57BL/6 mice were euthanized 48 hours after implantation; all mice developed endometriotic lesions containing typical endometrial glandular and stroma cells. The lesions were collected for IHC assays to determine the expression of HIF-1 α , TGF- β 1, and integrins. As shown in Fig. 6A, HIF-1 α was expressed weakly in ESCs of eutopic endometrium (0 hour), but was strongly expressed in ESCs of ectopic lesions (48 hours). TGF- β 1-positive staining was distributed in ESCs from

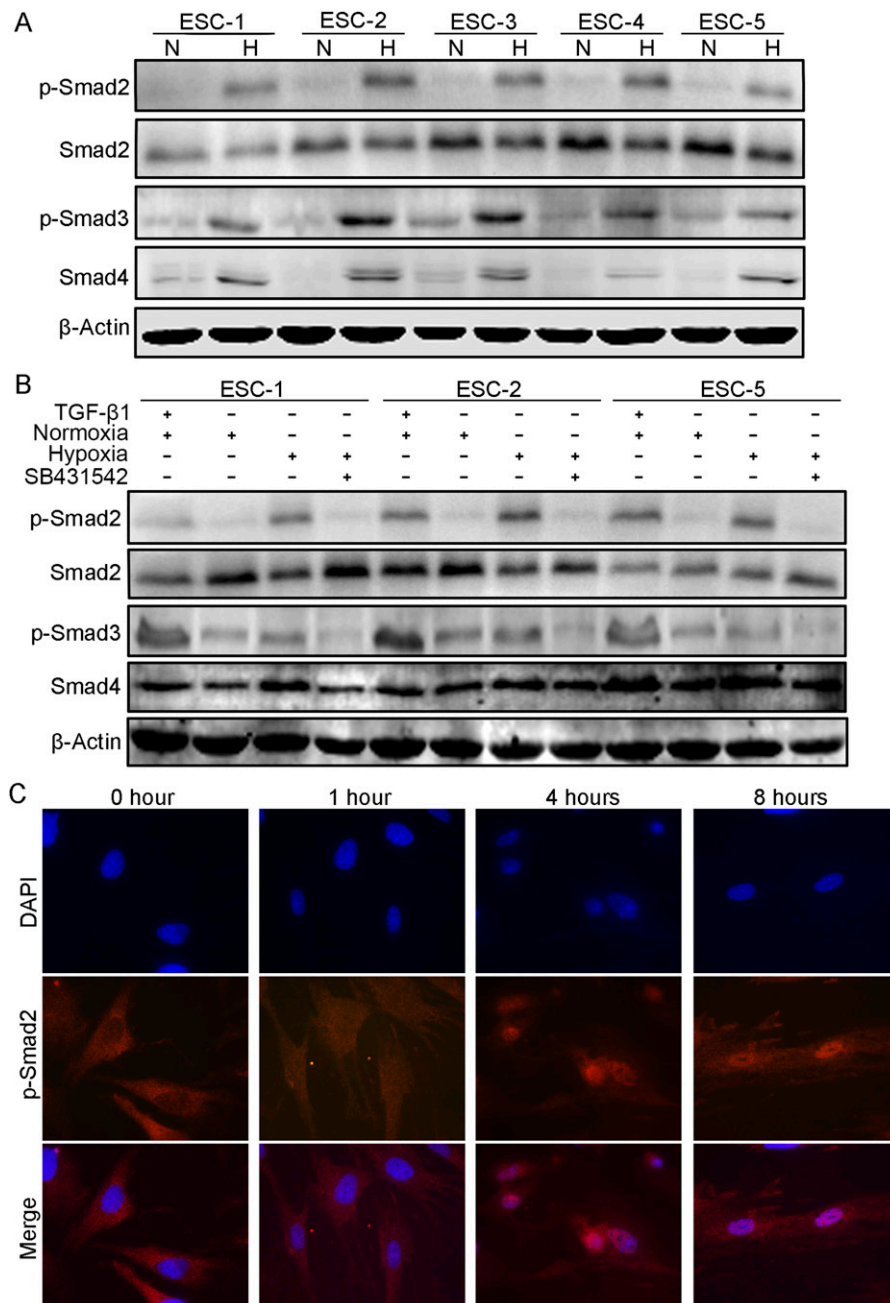


Figure 4. Hypoxia stimulated the TGF- β 1/Smad signaling pathway in ESCs. (A) Western blot results showed significant activation of p-Smad2 and p-Smad3 under hypoxic culture with no influence on total Smad2, and the total protein level of Smad4 was remarkably increased under hypoxic culture ($n = 5$). (B) Western blot results showing p-Smad2, total Smad2, p-Smad3, and total Smad4 in ESCs under TGF- β 1 costimulation, normoxia, hypoxia, and hypoxia-SB431542 costimulation for 48 hours. Levels of p-Smad2, p-Smad3, and total Smad4 protein were remarkably increased under TGF- β 1 costimulation and hypoxic culture with no influence on total Smad2 ($n = 5$), whereas hypoxia-SB431542 costimulation decreased protein expression of p-Smad2, p-Smad3, and total Smad4 compared with hypoxic culture alone ($n = 5$). Representative western blot results of three ESCs are shown. (C) Immunofluorescence assay showed that p-Smad2 protein was mainly localized in the cytoplasm of ESCs under normoxic conditions (hypoxia 0 hour), started to enter the nucleus at 4 hours of hypoxia treatment (hypoxia 4 hours), and then peaked at 8 hours of hypoxia ($n = 5$). Original magnification, $\times 400$. All experiments were repeated three times and representative IF results of one ESC at 0 hour, 4 hours, and 8 hours are shown. DAPI, 4',6-diamidino-2-phenylindole; H, hypoxic culture group; N, normoxic culture group.

both eutopic endometrium and ectopic lesions, but the intensity was much stronger in ectopic lesions than in eutopic endometrium. Staining signals for integrin α_5 , integrin α_V , integrin β_3 , and integrin β_5 were significantly higher in ectopic lesions than eutopic endometrium in stromal cells. Although expression of HIF-1 α and TGF- β 1

in EECs of ectopic lesions was higher than that in eutopic endometrium, expression levels of integrins in EECs did not change in the two groups. The H-scores of HIF-1 α , TGF- β 1, integrin α_5 , integrin α_V , integrin β_3 , and integrin β_5 in glandular cells and stromal cells are summarized in Fig. 6B and 6C, respectively (paired Student t test).

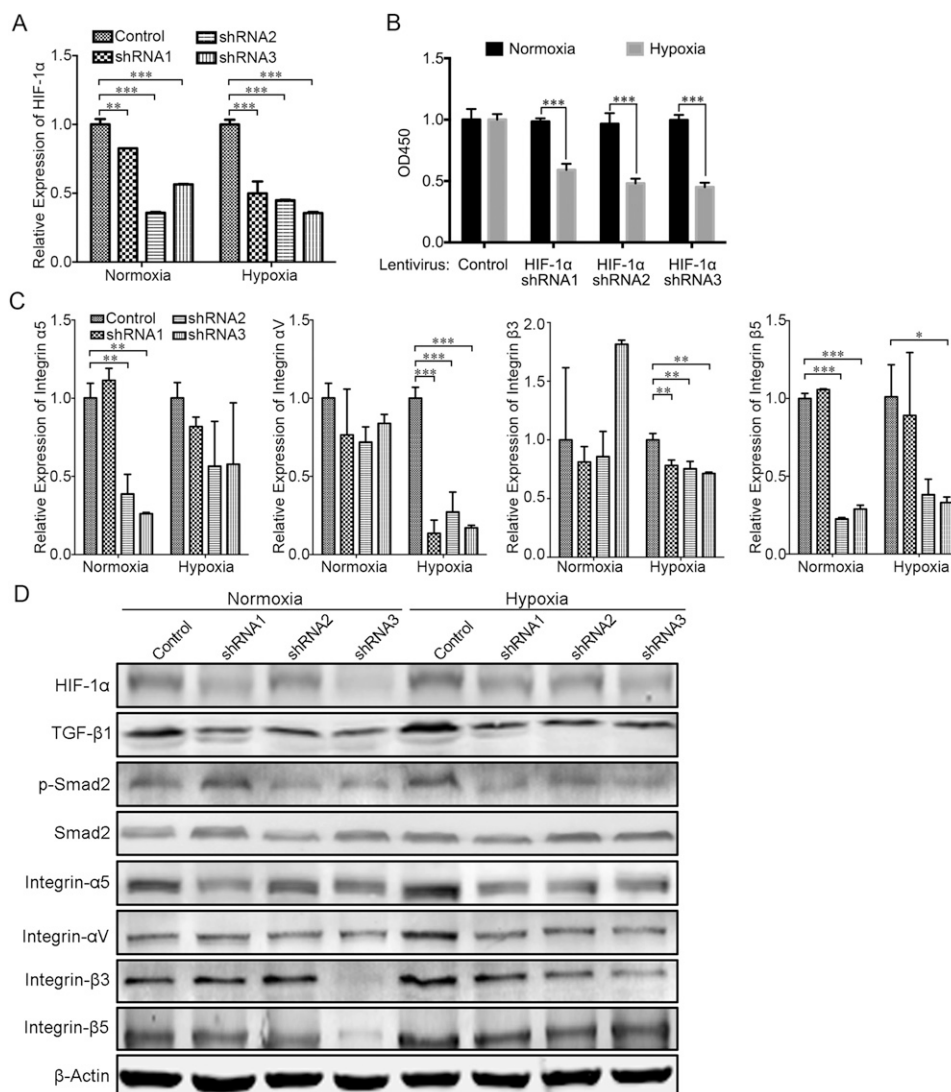


Figure 5. Knockdown of HIF-1 α in ESCs blocked adhesion ability and inhibited TGF- β 1/Smad signaling and integrin expression. (A) qRT-PCR results of HIF-1 α expression in ESCs after knockdown of HIF-1 α with shRNA1, shRNA2, or shRNA3 under normoxic and hypoxic culture conditions. mRNA expression of HIF-1 α was reduced in knockdown groups (shRNA1, shRNA2, and shRNA3) compared with the negative control group (control) under both normoxic and hypoxic culture conditions ($n = 3$, $P < 0.01$ vs control, Student t test). (B) Adhesion assay showed decreased absorbance of ESCs after knockdown of HIF-1 α with shRNA1, shRNA2, and shRNA3 under hypoxic culture compared with that under normoxic culture ($n = 3$, $P < 0.001$, Student t test). (C) qRT-PCR analysis of integrin expression in ESCs after knockdown of HIF-1 α with shRNA1, shRNA2, and shRNA3 under normoxic and hypoxic culture conditions. mRNA expression of integrin α _v, integrin β ₃, and integrin β ₅ in HIF-1 α interference groups was decreased compared with negative control group under either hypoxia culture ($n = 3$, $P < 0.05$), whereas expression of integrin α ₅ and integrin β ₅ was decreased in HIF-1 α interference groups compared with negative control group under normoxia culture ($n = 3$, $P < 0.05$). (D) Western blot results of HIF-1 α , TGF- β 1, p-Smad2, Smad2, and integrins from negative control ESCs and HIF-1 α interference ESCs under normoxia or hypoxia culture. Decreased protein expression of HIF-1 α , TGF- β 1, p-Smad2, integrin α ₅, integrin α _v, integrin β ₃, and integrin β ₅ was detected in HIF-1 α interference groups compared with the negative control group under hypoxic culture ($n = 3$). A representative experiment is shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Discussion

Integrins are cell adhesion molecules that participate in cell–cell and cell–substratum interactions. Integrins exhibit dynamic expression patterns in human endometrium and are restricted spatially to specific cell types or segregated temporally within the menstrual cycle (32). Our IHC results in control groups (0 hour group of mouse model and normal endometrium group) complement previously reported data on the expression of integrins in proliferative

endometrium (33, 34). Results from clinical samples showed that levels of integrins (integrin α ₅, integrin α _v, integrin β ₃, and integrin β ₅) were elevated in ESCs of both eutopic and ectopic endometrial tissues compared with those in normal endometrium, with strongest expression in ectopic tissues. The results of an *in vivo* model also showed upregulation of all of these integrins in ESCs 48 hours after peritoneal transplantation of endometrial fragments. These results are concordant with increased adhesion of ESCs via integrins in endometriosis.

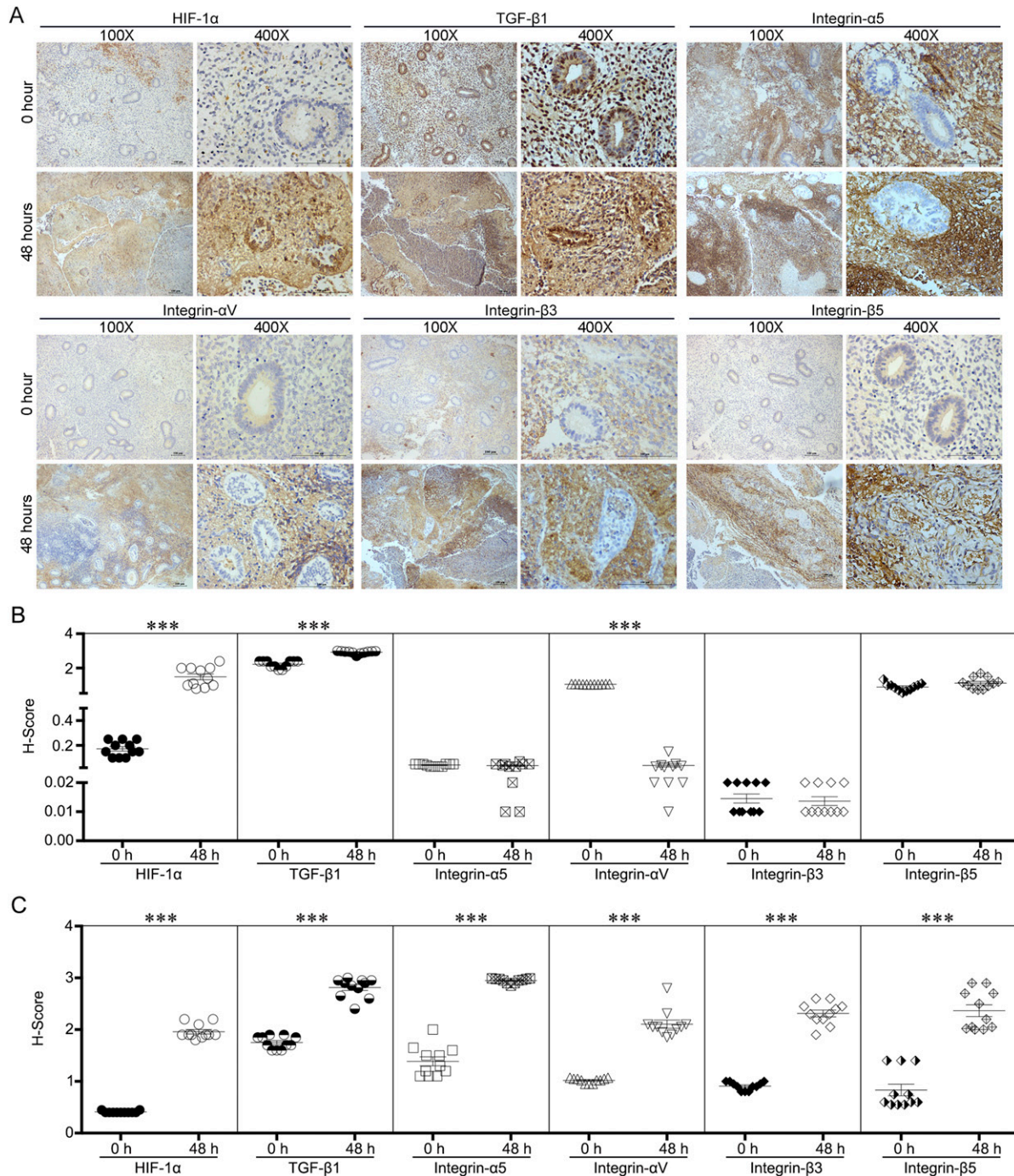


Figure 6. ESCs in lesions from mouse models of endometriosis expressed high levels of HIF-1 α , TGF- β 1, and integrins. (A) Animal models were established by stitching eutopic endometria onto the peritoneum of C57BL/6 female mice and lesions were harvested after 48 hours of transplantation. Staining was developed using diaminobenzidine, and the cell nucleus was stained with hematoxylin. Protein expression levels of HIF-1 α , TGF- β 1, integrin α ₅, integrin α _V, integrin β ₃, and integrin β ₅ in endometrium before transplantation (hour 0) and after transplantation (hour 48) were compared. Immunohistochemistry assay photomicrographs showed higher expression of HIF-1 α , TGF- β 1, integrin α ₅, integrin α _V, integrin β ₃, and integrin β ₅ in ESCs of hour 48 lesions ($n = 11$, $P < 0.001$ vs ESCs in hour 0 endometrium, paired Student t test), whereas only HIF-1 α and TGF- β 1 were overexpressed in EECs in hour 48 lesions ($n = 11$, $P < 0.001$ vs EECs in hour 0 endometrium, Student t test), whereas integrin α _V was decreased in EECs in hour 48 lesions ($n = 11$, $P < 0.001$ vs EECs in hour 0 endometrium, paired Student t test). (B) Expression levels of HIF-1 α , TGF- β 1, integrin α ₅, integrin α _V, integrin β ₃, and integrin β ₅ in EECs by immunohistochemistry assay represented by the H-score (y -axis) according to hour 0 or hour 48 tissues in all mice. H-scores of HIF-1 α and TGF- β 1 in EECs were higher in hour 48 lesions than in hour 0 endometrium ($n = 11$, $P < 0.001$ vs EECs in hour 0 endometrium, Student t test); H-score of integrin α _V in EECs was lower in hour 48 lesions than that in hour 0 tissues ($n = 11$, $P < 0.001$ vs EECs in hour 0 endometrium, paired Student t test), whereas there was no difference in H-score for integrin α ₅, integrin β ₃, and integrin β ₅ in EECs between hour 0 and hour 48 tissues ($n = 11$). (C) Expression levels of HIF-1 α , TGF- β 1, integrin α ₅, integrin α _V, integrin β ₃, and integrin β ₅ in ESCs by immunohistochemistry assay represented by the H-score (y -axis) according to hour 0 or hour 48 tissues in all mice. H-scores of HIF-1 α , TGF- β 1, integrin α ₅, integrin α _V, integrin β ₃, and integrin β ₅ in ESCs were higher in hour 48 lesions than in hour 0 endometrium ($n = 11$, $P < 0.001$ vs ESCs in hour 0 endometrium, paired Student t test). *** $P < 0.001$.

HIF-1 α was not only suggested to contribute to the progression of endometriosis, but was also reported to be one of the regulators of integrin-induced adhesion in many different cancers (13). Interestingly, HIF-1 α expression in ESCs of clinical samples and a mouse model showed a trend of upregulation corresponding to that of integrins. Consistent with IHC findings, our *in vitro* studies showed that hypoxic treatment enhanced adhesion and migration abilities of ESCs, which were subsequently found to be associated with upregulation of integrin α_5 , integrin α_V , integrin β_3 , and integrin β_5 . However, little is known about how hypoxia influences the increase of these integrins in ESCs during the development of ectopic foci.

Our *in vitro* studies showed that hypoxic treatment of ESCs also stimulated TGF- β 1 production. The results for clinical samples and the results of mouse model studies showed that TGF- β 1 expression in ESCs was upregulated with a trend corresponding to HIF-1 α expression. Because HIF-1 α can bind to the hypoxia response element of TGF- β 1 promoter and stimulate TGF- β 1 production in different cells (35), we explored the relationship between HIF-1 α and TGF- β 1 in ESCs. By shRNA interference of HIF-1 α expression, we found that the adhesion capacity of ESCs was markedly reduced in hypoxic culture conditions when HIF-1 α expression was inhibited, which was accompanied by decreased protein expression of TGF- β 1 and decreased integrin expression at both RNA and protein levels. This indicates that adhesion regulation by integrins in hypoxia may at least in part depend on HIF-1 α via increased TGF- β 1 protein expression in ESCs. Moreover, exposure to TGF- β 1 was suggested to stimulate intraperitoneal adhesion formation of endometriosis through upregulation of integrins (30, 36, 37). In our study, overexpression of integrin α_5 , integrin α_V , integrin β_3 , and integrin β_5 in ESCs was accompanied by high levels of TGF- β 1 under hypoxia culture, and addition of SB431542 to hypoxia pretreated ESCs remarkably blocked the upregulation of integrins by hypoxia. Our results of HIF-1 α , TGF- β 1, and integrin expression in endometriosis imply that upregulation of integrins might be induced by hypoxia-mediated overexpression of HIF-1 α and TGF- β 1, although we could not rule out the possibility that this might be simply a coincidental phenotype with endometriosis progression.

Transduction of TGF- β 1 signals to the nucleus depends on the activation of p-Smad2, p-Smad3, and a common partner Smad4 (30). In our research, hypoxia upregulated TGF- β 1 accompanied by activation of p-Smad2 with no change in total Smad2, whereas TGF- β receptor antagonist showed an opposite effect on ESCs. Moreover, p-Smad3 and Smad4 protein levels were increased in hypoxic culture and these changes were reversed by TGF- β receptor antagonist. RNA interference

of HIF-1 α under hypoxia not only decreased TGF- β 1 protein levels, but also decreased p-Smad2 protein levels without an influence on total Smad2. Collectively, these results indicated functional activation of the TGF- β 1/Smad signaling pathway in hypoxia-induced expression of integrins and increased adhesion behavior in ESCs.

Our study showed that HIF1- α and TGF- β 1 were overexpressed in EECs of eutopic endometrium and ectopic lesions, whereas expression of TGF- β receptor II in EECs of ectopic lesions was decreased compared with eutopic endometrium. The different expression trends of TGF- β receptor II in eutopic and ectopic EECs indicated that the influence of hypoxia on EECs may not depend on the TGF- β /Smad signaling pathway. However, further research is needed.

Taken together, these data provide a novel mechanistic view for endometriosis development in which hypoxia upregulates the adhesion properties of ESCs via the HIF-1 α /TGF- β 1/Smad pathway. Our study also provides further evidence that the pelvic hypoxic microenvironment is a key participant in endometriosis progression via dysregulated adhesion.

Acknowledgments

We are most grateful to all of the patients who participated in our study. We thank Jing Li and Yanlin Zhang for collecting samples and Yunbin Yao for housing animals. We deeply thank Mary Derry (from Liwen Bianji, Edanz Group China) for editing the English text of a draft of this manuscript.

Financial Support: This study was supported by National Natural Science Foundation of China Grants 81402131 (to Y.D.), 81671435 (to S.Z.), 81471505 (to S.Z.), and 81601308 (to Y.P.); Key Research and Development Program of Zhejiang Province Grant 2017C03022 (to S.Z.); and Natural Science Foundation of Zhejiang Province Grants LY18H040008 (to Y.D.) and LY15H040004 (to F.Z.).

Author Contributions: Xiang Lin and Y.D. performed experiments. Xiang Lin, W.X., F.Z., and Xiaona Lin collected the clinical samples. Xiang Lin, Y.D., L.S., X.J., and C.L. analyzed the data. Xiang Lin and Y.D. made the figures and drafted the article. Y.P., Y.Z., and Xiaona Lin critically reviewed the article. Y.D. and S.Z. conceived and supervised the project. All authors read and approved the final version of the paper.

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Disclosure Summary: The authors have nothing to disclose.

References

1. Bulun SE. Endometriosis. *N Engl J Med*. 2009;360(3):268–279.
2. Moradi M, Parker M, Sneddon A, Lopez V, Ellwood D. Impact of endometriosis on women's lives: a qualitative study. *BMC Womens Health*. 2014;14(1):123.

3. Vercellini P, Viganò P, Somigliana E, Fedele L. Endometriosis: pathogenesis and treatment. *Nat Rev Endocrinol*. 2014;10(5):261–275.
4. Sundqvist J, Andersson KL, Scarselli G, Gemzell-Danielsson K, Lalitkumar PG. Expression of adhesion, attachment and invasion markers in eutopic and ectopic endometrium: a link to the aetiology of endometriosis. *Hum Reprod*. 2012;27(9):2737–2746.
5. Elangbam CS, Qualls CW Jr, Dahlgren RR. Cell adhesion molecules—update. *Vet Pathol*. 1997;34(1):61–73.
6. Giannelli G, Sgarra C, Di Naro E, Lavopa C, Angelotti U, Tartagni M, Simone O, Trerotoli P, Antonaci S, Loverro G. Endometriosis is characterized by an impaired localization of laminin-5 and $\alpha 3\beta 1$ integrin receptor. *Int J Gynecol Cancer*. 2007;17(1):242–247.
7. Marianowski P, Szymusik I, Malejczyk J, Hibner M, Wielgos M. Proteomic analysis of eutopic and ectopic endometriotic tissues based on isobaric peptide tags for relative and absolute quantification (iTRAQ) method. *Neuroendocrinol Lett*. 2013;34(7):717–721.
8. Hull ML, Escareno CR, Godsland JM, Doig JR, Johnson CM, Phillips SC, Smith SK, Tavaré S, Print CG, Charnock-Jones DS. Endometrial-peritoneal interactions during endometriotic lesion establishment. *Am J Pathol*. 2008;173(3):700–715.
9. Adachi M, Nasu K, Tsuno A, Yuge A, Kawano Y, Narahara H. Attachment to extracellular matrices is enhanced in human endometriotic stromal cells: a possible mechanism underlying the pathogenesis of endometriosis. *Eur J Obstet Gynecol Reprod Biol*. 2011;155(1):85–88.
10. Lessey BA, Young SL. Integrins and other cell adhesion molecules in endometrium and endometriosis. *Semin Reprod Endocrinol*. 1997;15(3):291–299.
11. Becker CM, Rohwer N, Funakoshi T, Cramer T, Bernhardt W, Birsner A, Folkman J, D'Amato RJ. 2-Methoxyestradiol inhibits hypoxia-inducible factor-1 α and suppresses growth of lesions in a mouse model of endometriosis. *Am J Pathol*. 2008;172(2):534–544.
12. Zhan L, Wang W, Zhang Y, Song E, Fan Y, Wei B. Hypoxia-inducible factor-1 α : a promising therapeutic target in endometriosis. *Biochimie*. 2016;123:130–137.
13. Ata R, Antonescu CN. Integrins and cell metabolism: an intimate relationship impacting cancer. *Int J Mol Sci*. 2017;18(1):189.
14. Fassbender A, Rahmioglu N, Vitonis AF, Viganò P, Giudice LC, D'Hooghe TM, Hummelshoj L, Adamson GD, Becker CM, Missmer SA, Zondervan KT; WERF EPHeCT Working Group. World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonisation Project: IV. Tissue collection, processing, and storage in endometriosis research. *Fertil Steril*. 2014;102(5):1244–1253.
15. Hirata T, Osuga Y, Hamasaki K, Yoshino O, Ito M, Hasegawa A, Takemura Y, Hirota Y, Nose E, Morimoto C, Harada M, Koga K, Tajima T, Saito S, Yano T, Taketani Y. Interleukin (IL)-17A stimulates IL-8 secretion, cyclooxygenase-2 expression, and cell proliferation of endometriotic stromal cells. *Endocrinology*. 2008;149(3):1260–1267.
16. Li J, Dai Y, Zhu H, Jiang Y, Zhang S. Endometriotic mesenchymal stem cells significantly promote fibrogenesis in ovarian endometrioma through the Wnt/ β -catenin pathway by paracrine production of TGF- $\beta 1$ and Wnt1. *Hum Reprod*. 2016;31(6):1224–1235.
17. Hsiao KY, Wu MH, Chang N, Yang SH, Wu CW, Sun HS, Tsai SJ. Coordination of AUF1 and miR-148a destabilizes DNA methyltransferase 1 mRNA under hypoxia in endometriosis. *Mol Hum Reprod*. 2015;21(12):894–904.
18. Hsiao KY, Chang N, Lin SC, Li YH, Wu MH. Inhibition of dual specificity phosphatase-2 by hypoxia promotes interleukin-8-mediated angiogenesis in endometriosis. *Hum Reprod*. 2014;29(12):2747–2755.
19. Pelch KE, Schroder AL, Kimball PA, Sharpe-Timms KL, Davis JW, Nagel SC. Aberrant gene expression profile in a mouse model of endometriosis mirrors that observed in women. *Fertil Steril*. 2010;93(5):1615–1627.e18.
20. Regidor PA, Vogel C, Regidor M, Schindler AE, Winterhager E. Expression pattern of integrin adhesion molecules in endometriosis and human endometrium. *Hum Reprod Update*. 1998;4(5):710–718.
21. Klemmt PA, Carver JG, Koninckx P, McVeigh EJ, Mardon HJ. Endometrial cells from women with endometriosis have increased adhesion and proliferative capacity in response to extracellular matrix components: towards a mechanistic model for endometriosis progression. *Hum Reprod*. 2007;22(12):3139–3147.
22. Delbandi AA, Mahmoudi M, Shervin A, Akbari E, Jeddi-Tehrani M, Sankian M, Kazemnejad S, Zarnani AH. Eutopic and ectopic stromal cells from patients with endometriosis exhibit differential invasive, adhesive, and proliferative behavior. *Fertil Steril*. 2013;100(3):761–769.
23. Liu YG, Tekmal RR, Binkley PA, Nair HB, Schenken RS, Kirma NB. Induction of endometrial epithelial cell invasion and *c-fms* expression by transforming growth factor beta. *Mol Hum Reprod*. 2009;15(10):665–673.
24. Dunselman GA, Groothuis PG, de Goeij AF, Evers JL. The mesothelium, Teflon or Velcro? Mesothelium in endometriosis pathogenesis. *Hum Reprod*. 2001;16(4):605–607.
25. Chegini N, Gold LI, Williams RS. Localization of transforming growth factor beta isoforms TGF- $\beta 1$, TGF- $\beta 2$, and TGF- $\beta 3$ in surgically induced endometriosis in the rat. *Obstet Gynecol*. 1994;83(3):455–461.
26. Küpker W, Schultze-Mosgau A, Diedrich K. Paracrine changes in the peritoneal environment of women with endometriosis. *Hum Reprod Update*. 1998;4(5):719–723.
27. Ji RC. Hypoxia and lymphangiogenesis in tumor microenvironment and metastasis. *Cancer Lett*. 2014;346(1):6–16.
28. Shi Y, Massagué J. Mechanisms of TGF- β signaling from cell membrane to the nucleus. *Cell*. 2003;113(6):685–700.
29. Young VJ, Brown JK, Saunders PT, Duncan WC, Horne AW. The peritoneum is both a source and target of TGF- β in women with endometriosis. *PLoS One*. 2014;9(9):e106773.
30. Young VJ, Ahmad SF, Duncan WC, Horne AW. The role of TGF- β in the pathophysiology of peritoneal endometriosis. *Hum Reprod Update*. 2017;23(5):548–559.
31. Wu MH, Lin SC, Hsiao KY, Tsai SJ. Hypoxia-inhibited dual-specificity phosphatase-2 expression in endometriotic cells regulates cyclooxygenase-2 expression. *J Pathol*. 2011;225(3):390–400.
32. Lessey BA, Castelbaum AJ, Buck CA, Lei Y, Yowell CW, Sun J. Further characterization of endometrial integrins during the menstrual cycle and in pregnancy. *Fertil Steril*. 1994;62(3):497–506.
33. Lessey BA, Damjanovich L, Coutifaris C, Castelbaum A, Albelda SM, Buck CA. Integrin adhesion molecules in the human endometrium. Correlation with the normal and abnormal menstrual cycle. *J Clin Invest*. 1992;90(1):188–195.
34. Aplin JD, Spanswick C, Behzad F, Kimber SJ, Vičovic L. Integrins $\beta 5$, $\beta 3$, and αv are apically distributed in endometrial epithelium. *Mol Hum Reprod*. 1996;2(7):527–534.
35. Hung SP, Yang MH, Tseng KF, Lee OK. Hypoxia-induced secretion of TGF- $\beta 1$ in mesenchymal stem cell promotes breast cancer cell progression. *Cell Transplant*. 2013;22(10):1869–1882.
36. Dou Q, Williams RS, Chegini N. Inhibition of transforming growth factor- $\beta 1$ alters the growth, anchor-dependent cell aggregation and integrin mRNA expression in human promonocytes: implications for endometriosis and peritoneal adhesion formation. *Mol Hum Reprod*. 1997;3(5):383–391.
37. Beliard A, Noël A, Goffin F, Frankenne F, Foidart JM. Adhesion of endometrial cells labeled with 111 indium-tropolonate to peritoneum: a novel in vitro model to study endometriosis. *Fertil Steril*. 2003;79(Suppl 1):724–729.