TET1 may contribute to hypoxia-induced epithelial to mesenchymal transition of endometrial epithelial cells in endometriosis

Jingni Wu 1, Xidie Li 1, Hongyan Huang 1, Xiaomeng Xia 1, Mengmeng Zhang 1, Xiaoling Fang 1

1 Department of Obstetrics and Gynecology, The Second Xiangya Hospital, Central South University, Changsha, Hunan, China

Corresponding Author: Xiaoling Fang
Email address: fxfxl0510@csu.edu.cn

Background. Endometriosis (EMs) is a non-malignant gynecological disease, whose pathogenesis remains to be clarified. Recent studies have found that hypoxia induces epithelial-mesenchymal transition (EMT) as well as epigenetic modification in EMs. However, the relationship between EMT and demethylation modification under hypoxia status in EMs remains unknown.

Methods. The expression of N-cadherin, E-cadherin and TET1 in normal endometria, eutopic endometria and ovarian endometriomas was assessed by immunohistochemistry and immunofluorescence double staining. 5-hmC was detected by fluorescence-based ELISA kit using a specific 5-hmC antibody. Overexpression and inhibition of TET1 or hypoxia-inducible factor 2α (HIF-2α) were performed by plasmid and siRNA transfection. The expression of HIF-2α, TET1 and EMT markers in Ishikawa (ISK) cells (widely used as endometrial epithelial cells) was evaluated by western blotting. The interaction of HIF-2α and TET1 was analyzed by chromatin immunoprecipitation.

Results. Demethylation enzyme TET1 (ten-eleven translocation1) was elevated in glandular epithelium of ovarian endometrioma, along with the activation of EMT (increased expression of N-cadherin, and decreased expression of E-cadherin) and global increase of epigenetic modification marker 5-hmC(5-hydroxymethylcytosine). Besides, endometriosis lesions had more TET1 and N-cadherin co-localized cells. Further study showed that ISK cells exhibited enhanced EMT, and increased expression of TET1 and HIF-2α under hypoxic condition. Hypoxia-induced EMT was partly regulated by TET1 and HIF-2α. HIF-2α inhibition mitigated TET1 expression changes provoked by hypoxia.

Conclusions. Hypoxia induces the expression of TET1 regulated by HIF-2α, thus may promote EMT in endometriosis.
TET1 may contribute to hypoxia-induced epithelial to mesenchymal transition of endometrial epithelial cells in endometriosis

Jingni Wu¹, Xidie Li¹, Hongyan Huang¹, Xiaomeng Xia¹, Mengmeng Zhang¹, Xiaoling Fang¹*

1 Department of Obstetrics and Gynecology, The Second Xiangya Hospital, Central South University, Changsha 410011, China

Corresponding Author: Email: fxlfxl0510@csu.edu.cn

Abstract

Background. Endometriosis (EMs) is a non-malignant gynecological disease, whose pathogenesis remains to be clarified. Recent studies have found that hypoxia induces epithelial-mesenchymal transition (EMT) as well as epigenetic modification in EMs. However, the relationship between EMT and demethylation modification under hypoxia status in EMs remains unknown.

Methods. The expression of N-cadherin, E-cadherin and TET1 in normal endometria, eutopic endometria and ovarian endometriomas was assessed by immunohistochemistry and immunofluorescence double staining. 5-hmC was detected by fluorescence-based ELISA kit using a specific 5-hmC antibody. Overexpression and inhibition of TET1 or hypoxia-inducible factor 2α (HIF-2α) were performed by plasmid and siRNA transfection. The expression of HIF-2α, TET1 and EMT markers in Ishikawa (ISK) cells (widely used as endometrial epithelial cells) was evaluated by western blotting. The interaction of HIF-2α and TET1 was analyzed by chromatin immunoprecipitation.
Results. Demethylation enzyme TET1 (ten-eleven translocation1) was elevated in glandular epithelium of ovarian endometrioma, along with the activation of EMT (increased expression of N-cadherin, and decreased expression of E-cadherin) and global increase of epigenetic modification marker 5-hmC (5-hydroxymethylcytosine). Besides, endometriosis lesions had more TET1 and N-cadherin co-localized cells. Further study showed that ISK cells exhibited enhanced EMT, and increased expression of TET1 and HIF-2α under hypoxic condition. Hypoxia-induced EMT was partly regulated by TET1 and HIF-2α. HIF-2α inhibition mitigated TET1 expression changes provoked by hypoxia.

Conclusions. Hypoxia induces the expression of TET1 regulated by HIF-2α, thus may promote EMT in endometriosis.

Keywords
Endometriosis, TET1, EMT, HIF-2α

Introduction
Endometriosis is a chronic and non-malignant gynecological disease characterized by the growth of endometrial glands and stroma outside the uterus (Giudice, 2010). It exhibits cancer-like features, such as cell proliferation and metastatic invasion. Endometriosis is a major contributor to pelvic pain and infertility (Mahmood & Templeton, 1991). Although the etiology of endometriosis is still unclear, retrograde menstrual reflux is the widely accepted hypothesis for the mechanism of endometriosis. This theory states that retrograded endometrial tissues must migrate, invade and
survive outside the cavity of uterus, then establish new endometriosis lesions. However, little is known about the molecular events that lead to the development of endometriosis.

Due to lack of hormone and blood supply, the retrogradated endometrial debris during menstruation is in hypoxic status (Maybin & Critchley, 2015). Hypoxia plays a role in the migration and invasive of endometrial epithelial cells in endometriosis (Xiong et al., 2016). It probably induces the survival of retrogradated endometrial debris and angiogenesis in implanted ectopic endometrial lesions (Wu et al., 2007). That is, the hypoxia microenvironment may contribute to the migration, invasion, and ectopic implant formation of the eutopic endometrial epithelial cells. Therefore, hypoxia has been regarded as an important stimulus of the pathological process of endometriosis. Hypoxia can stabilize hypoxia-inducible factors (HIFs, including HIF-1α and HIF-2α), which are the most significant and sensitive mediators of hypoxia-induced cellular responses. Stabilized HIFs dimerize with their constitutively stabilized partner, HIF-1β, and regulate the expression of the target gene, thereby leading to hypoxia-induced phenotypes (Hsiao, 2015; Jain et al., 2018). However, few studies have investigated the role of the transcription factor HIF-2α in endometriosis.

Studies have shown that hypoxia promotes the epithelial to mesenchymal transition (EMT) and enhances endometrial cell migration and invasion in endometriosis (Liu et al., 2017, 2018). EMT is a process by which epithelial cells lose polarity and cell-to-cell contacts and transform into mesenchymal cells with high motility. This process is characterized by the downregulation of the epithelial marker, E-cadherin, and the upregulation of mesenchymal markers, N-cadherin and vimentin (Lamouille, Xu & Derynck, 2014), which may occur in EMs (Matsuzaki & Darcha,
EMT endows cells with migratory and invasive properties, a prerequisite for the establishment of endometriotic lesions (Matsuzaki & Darcha, 2012; Xiong et al., 2016). Epigenetic alterations of chromatin (including DNA methylation, histone modifications, and non-coding RNAs regulation) are proposed to facilitate EMT in many diseases. Aberrations in DNA methylation are associated with EMT and tumorigenesis under hypoxia conditions (Camuzi et al., 2019). However, in endometriosis, the relationship between EMT and DNA methylation under hypoxia status remains largely unknown. The dynamic balance between methylation and demethylation is crucial for various biological processes (Jones, 2012). The altered expression of the demethylation enzyme ten-eleven translocation (TET1) disrupts this balance (Lorsbach et al., 2003), leading to aberrant DNA methylation patterns, which is seen in many human diseases, such as cancer (Baylin & Jones, 2011). TET1 enzymes iteratively oxidize 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC), thereby contributing to CpG island demethylation in specific gene promoters (Wu & Zhang, 2017). TET1 is involved in cell migration, differentiation, and oncogenesis (Yang et al., 2015). A recent study has shown that TET genes are dysregulated in endometriosis (Roca et al., 2016). However, the function of TET1 in endometriosis is not yet fully understood.

Our study investigated for the first time the role and mechanism of TET1 in regulating the EMT process of endometrial epithelial cells under hypoxic conditions. We hypothesized that hypoxia may increase TET1 expression, mediated by HIF-2α, thus potentially inducing the EMT of endometrial epithelial cells and contributing to the development of endometriosis. An infographic of this work is shown in Figure 1.
Materials and methods

Patients and sample collection

The patients recruited in the study were women of childbearing age from the Second Xiangya Hospital. Patients with any hormonal-dependent disease, irregular menstrual cycles and those who took steroids and GnRH agonists for the past six months were excluded. The endometrial samples were obtained from these patients and were all confirmed in the early proliferation stage by pathological examination. This study was approved by the Human Ethics Committee of Second Xiangya Hospital, Central South University. Written informed consent was obtained from all the patients (Ref. No. 2016-243).

Different types of endometriotic lesions may have different pathogeneses (Nisolle & Donnez, 1997). Ovarian endometriosis lesions are more correlated with hypoxia and angiogenic factors (Filippi et al., 2016). Hence, we focused on the ovarian endometrioma in this study. Fifteen ovarian endometriomas and fifteen eutopic endometria (from the same group of women with ovarian endometriosis), and fifteen samples of normal endometria were used for the immunohistochemistry. The stages of endometriosis were classified according to the rASRM classification criteria during the procedure of laparoscopy. Clinical characteristics of the patients are shown in Table 1. Besides, these samples were fixed in 10% buffered formalin and embedded in paraffin for immunohistochemistry analysis.

Immunohistochemistry (IHC)

Immunohistochemistry staining was performed on paraffin-embedded blocks of eutopic endometria, ovarian endometriomas and normal endometria. 5-μm-thick tissue sections were
deparaffinized in xylene and rehydrated in graded ethanol, and antigen retrieval was performed in 0.01mol/l sodium citrate buffer (pH 6.0) using a pressure cooker. Sections were then treated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase. After rinsing in PBST, sections were blocked in 5% bovine serum albumin for 30 min, then incubated overnight at 4°C with primary antibodies. The second day, sections were incubated with horseradish peroxidase (HRP)-conjugated secondary anti-rabbit IgG for 30 min after rinsing for three times. Antigen-antibody reactions were detected by the HRP-catalyzed reaction with 3,3’-diaminobenzidine (DAB) (Beyotime Biotechnology, Shanghai, China). Finally, the sections were counterstained with hematoxylin and analyzed by optical microscopy. Tyramide signal amplification (TSA) technique was used to enhance the sensitivity of IHC. The following primary antibodies were applied: N-cadherin (#66219-1-AP, proteintech, Wuhan, China, 1:1000), E-cadherin (#20874-1-AP, proteintech, Wuhan, China, 1:2000) and TET1 (ab191698, Abcam, Cambridge, UK, 1:1000).

**DNA isolation and determination of global 5-hmC levels**

Genomic DNA from human endometrium and endometriosis lesions were isolated and purified using the CWBIO Genomic DNA kit according to the manufacturer’s instructions. The concentration and quality of the DNA were estimated by agarose electrophoresis and UV absorption analysis. Global 5-hmC levels of this isolated genomic DNA were determined with a fluorescence-based Enzyme Linked Immunosorbent Assay (ELISA) kit using a specific 5-hmC antibody (Epigentek, Farmingdale, NY, USA), according to the manufacturer’s recommendations. 200 ng of total genomic DNA was applied for this assay. The 5-hmC quantities in these DNA samples were analyzed based on the OD value generated by a microplate reader compared to
control DNA in the kit. All samples were run in duplicate. These data were obtained from six
normal endometria and six eutopic endometria and six ovarian endometriomas.

**Immunofluorescence double staining**

Paraffin-embedded sections were deparaffinised, rehydrated, heated in 0.01 mol/l sodium citrate
buffer (pH 6.0) for antigen retrieval, and treated with 3% hydrogen peroxide for 10 min to block
endogenous peroxidase activity. After washing with PBS three times and blocking in 5% bovine
serum albumin for 30 min, the sections were incubated in the primary antibody solution containing
the rabbit anti-TET1 antibody (1:1000), rabbit anti-N-Cadherin antibody (1:200), and rabbit anti-
E-cadherin antibody (1:50). Because we used TSA technique to enhance the fluorescent signal,
antibodies of the same species were acceptable in our experiment. The biotinylated secondary anti-
rabbit antibody was used to detect primary antibodies. The detection was performed with
Streptavidin-HRP D (DAB Map kit, Ventana Medical Systems), followed by incubation with 488
(cat# T20922) or 594 (cat# T20935) Tyramide Alexa Fluors (Invitrogen) prepared in the dark
according to the manufacturer’s instructions. Sections were counterstained with DAPI.

**Image analysis**

Immunohistochemistry and immunofluorescence double staining experiments were analyzed with
a PerkinElmer Quantitative Pathology Imaging System in the Hunan Epigenetics Laboratory. This
imaging system includes the Mantra Quantitative Pathology Workstation (PerkinElmer,
CLS140089) for collecting staining data and PerkinElmer inForm software (PerkinElmer,
Hopkinton, MA, USA) for analysis. The scanned images were visually examined. The samples
with staining artifacts or with low quality were excluded. Image processing comprised the training
session and analysis session. InForm software was trained to identify regions of interest and used to analyze the whole image. Three fields of view were randomly selected per slide to calculate the expression levels and distribution of TET1, E-cadherin, and N-cadherin in the glandular epithelium. The proportion of positive cells is calculated as the number of positive-staining cells divided by the number of total glandular epithelial cells. The correlation between TET1 and EMT markers expression in the endometrial epithelium (positive cell proportion) was analyzed by Pearson correlation test.

**Cell culture and hypoxia treatment**

Ishikawa (ISK) cells (ZQ0472, Zhong Qiao Xin Zhou Biotechnology Co., Ltd, Shanghai, China) come from well-differentiated human endometrial adenocarcinoma. The human endometrial epithelial cell is hard to passage and transfect; endometrial stromal cells are the only cells to survive after 3–4 generations *in vitro*. Therefore, ISK cells are widely used instead of human endometrial glandular epithelial cells in studies of endometriosis (Guay & Akoum, 2007; Cho et al., 2016). ISK cells were cultured in Dulbecco’s Modified Eagle’s Medium/Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS) and 1% penicillinstreptomycin in 5% CO₂ incubators at 37°C until 70% confluence was reached. To ensure adequate nutrients and growth factors, the medium was renewed 1 h prior to hypoxia treatment. Thereafter, ISK cells were cultured under hypoxia (5% O₂) or normoxia (20% O₂) conditions for 4, 8, and 24 h.

**Gene transfection**

ISK cells were seeded in six-well plates for 24 h to reach approximately 70% confluence and
transfected with TET1 or the negative control plasmid (GenScript Biotech Corporation; Piscataway, NJ, USA) using Lipofectamine (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. In Brief, we used the QIAGEN® kit to purify and extract the plasmid. Then, 1–4 µg of plasmid DNA and Lipofectamine solution were mixed in serum-reduced medium and added to the ISK cells. After 6 h of incubation, fresh medium was added. For TET1 downregulation, TET1-targeting siRNAs and the negative control (NC) were obtained from Ribobio (Guangzhou, China). The RNA interference (RNAi) experiments were performed following the manufacturer’s protocol. First, the TET1 siRNA and NC siRNAs were each mixed with RNA transfection buffer (Ribo FECT CP) and then added to ISK cell medium. After 48 h, western blotting was performed to determine the transfection efficiency.

**Protein extraction and western blotting analysis**

Total proteins were extracted from cells using RIPA lysis buffer and phosphatase inhibitors. All lysates were centrifuged at 12,000 × g for 20 min. The supernatants were collected, and the protein concentrations were determined by the Bicinchoninic Acid (BCA) protein assay kit (Beyotime Biotechnology, Shanghai, China). Proteins were mixed with the same amount of loading buffer and boiled for 5 min. The samples were then resolved by Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membranes, incubated with 5% fat-free milk, and hatched with the following primary antibodies at 4°C overnight: N-cadherin, E-cadherin, TET1, HIF-2α, and HIF-1α (Abcam, Cambridge, UK). The membranes were washed with TBST three times for 15 min, and then incubated in a secondary antibody at 37°C for 1.5 h. The blots were analyzed by a chemiluminescence system (Millipore, USA). These experiments were
repeated three times, and the average values of the blot bands were calculated.

Co-immunoprecipitation

Co-immunoprecipitation was carried out to identify protein–protein interactions. Cells were collected after exposure to hypoxia. Cell pellets were lysed in RIPA lysis buffer containing phosphatase inhibitors. 750 μl of the cell lysates were incubated with 2 μg of antibodies (rabbit HIF-2α, mouse HIF-1α, and rabbit TET1) overnight at 4°C. Meanwhile, β-actin, rabbit IgG, and mouse IgG (Proteintech, Wuhan, China) were used as the input and negative controls, respectively, for the experiments. On the second day, each group was mixed with 20 μl of Protein G Plus/Protein A Agarose Suspension (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the solution was softly shook at 4°C for 2 h. To separate agarose beads, these mixtures were centrifuged at 3000 rpm for 3 min at 4°C. The supernatant was transferred to a new tube on ice, and the beads were eluted again with lysis buffer. The total eluted supernatants were washed 5 times, resuspended in 1× loading buffer, and boiled for 5 min at 100°C before western blotting.

Statistical analysis

Statistical analysis of data was performed by one-way ANOVA using SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). All experiments were repeated three times. The results are shown as the mean ± standard deviation (SD). A P value of <0.05 was considered statistically significant.

Results

Accompanied with TET1 upregulation, EMT might occur in endometrial epithelial cells of ovarian endometriosis
To evaluate the expression and localization of TET1 and EMT markers, E-cadherin and N-cadherin, in endometriosis, we performed IHC and immunofluorescence double staining in human endometrial tissues. Since the morphology of stromal cells in the ovarian endometriomas is much different from that in the normal endometria and eutopic endometria, we only analyzed the glandular epithelial cells in the three groups.

The expression of TET1 and EMT markers in the glandular epithelium of normal endometria, eutopic endometria, and ovarian endometriomas was analyzed by IHC. Figure 2A-R show the representative IHC results. Table 2 illustrates the expression of TET1 and EMT markers. The proportions of cells positively stained for TET1 or N-cadherin in the glandular epithelium of the eutopic endometria and ovarian endometriomas were significantly higher than those in the normal endometria, and the number of E-cadherin–positive cells was significantly lower in the glandular epithelium from eutopic endometria and ovarian endometriomas than those in the normal endometria (Figure 2S-U). The normal ovary tissue has a low expression of TET1 (supplementary Figure 1). Moreover, to demonstrate whether the demethylation enzyme TET1 was activated in endometriosis, we explored the expression of 5-hmC, which is an important marker for the activated demethylation process. Figure 2V shows that the ovarian endometriomas have higher 5-hmC levels (5.15±1.488% of total DNA) than eutopic endometria (3.33±0.876% of total DNA, p<0.05) and normal endometria (3.5±0.881% of total DNA, p<0.05). All in all, these data suggest that TET1 is upregulated and EMT may occur in the glandular epithelium of endometriosis.

Immunofluorescence double staining was performed to compare the localization of TET1 and EMT markers. Consistent with the IHC results, in eutopic endometria and normal endometria,
TET1 and N-cadherin were mainly expressed in the stromal cells and barely expressed in the glandular epithelial cells. However, in the ovarian endometriomas, TET1 and N-cadherin were both expressed in the glandular epithelial cells. Furthermore, the distribution of TET1 was more obvious in the cytoplasm or nuclear of N-cadherin–positive cells (Figure 2FF-NN), not E-cadherin–positive cells (Figure 2W-EE). Ovarian endometriotic lesions (Figure 2LL-NN) had more TET1 and N-cadherin co-localized cells compared to eutopic endometria (Figure 2II-KK) and normal endometria (Figure 2FF-HH). The large amount of co-localized cells suggests a close relationship between TET1 and N-cadherin. In addition, a correlation analysis was applied to the proportions of cells that were positively stained for TET1 and N-cadherin/E-cadherin in the epithelium (Figure 2OO-PP), which showed that TET1 was positively correlated with N-cadherin and negatively correlated with E-cadherin. Our findings suggest that the demethylation enzyme TET1 may play a role in the EMT pathological process of endometriosis.

Hypoxia alters the expression of HIFs, TET1 and EMT markers

Although various epigenetic mechanisms have been demonstrated to regulate EMT under hypoxia status (Wu et al., 2012; Tsai & Wu, 2014), the role of the DNA demethylation enzyme TET1 in regulating hypoxia-induced EMT in endometriosis remains largely unknown. Therefore, we investigated the expression of TET1, HIFs (HIF-1α and HIF-2α), and EMT markers (E-cadherin, N-cadherin, and vimentin) in ISK cells exposed to 5% O₂ for 0 h, 4 h, 8 h and 24 h. As shown in Figure 3, the expression of TET1, HIF-1α, HIF-2α, and vimentin increased significantly and the expression of E-cadherin decreased significantly after hypoxia treatment for 8 h and 24 h, especially for 24 h. N-cadherin expression peaked at 8 h after hypoxic treatment and went down
at 24 h, we presumed N-cadherin might be transiently triggered by hypoxia. Homeostasis should work to reverse the hypoxia condition, and genes may be activated in different time period of hypoxia during this process (Deret et al., 2004; Javaid et al., 2013; Song et al., 2019). Hence, the time point with the maximal response was selected, and vimentin was chosen as a mesenchymal marker in the following experiences. Our findings indicate that hypoxia activates TET1 expression and the EMT.

TET1 overexpression may promote EMT, and knockdown of TET1 mitigates hypoxia-induced EMT in ISK cells

To evaluate the role of TET1 in hypoxia-induced endometrial EMT, we successfully established TET1-overexpressing ISK cells under normal conditions and TET1 down-regulation ISK cells under hypoxia conditions. As seen in Figure 4, western blotting indicated that the expression of vimentin was increased and the expression of E-cadherin was decreased by upregulation of TET1 under normoxia. In addition, hypoxia-induced EMT, characterized by the repression of E-cadherin and the upregulation of vimentin, was abolished by TET1 knockdown. These results reveal that TET1 plays a crucial role in hypoxia-induced EMT.

Knockdown of HIF-2α inhibits hypoxia-induced EMT and TET1 expression in ISK cells

To investigate the mechanism of TET1 in the regulation of hypoxia-induced EMT, hypoxia-induced HIF-2α expression was inhibited by a specific siRNA for 48 h. Western blotting analysis confirmed the down-expression of HIF-2α. Knockdown of HIF-2α mitigated the activation of TET1 and EMT under hypoxia in ISK cells (Figure 5A and Figure 5C-F), indicating that HIF-2α is a regulator of TET1 expression and the EMT under hypoxia. Xiong et al. reported that HIF-
1α also induced EMT in endometrial epithelial cells under hypoxia (Xiong et al., 2016). Therefore, we conducted chromatin immunoprecipitation experiments, which showed that the anti-TET1 antibody pulled down HIF-2α and TET1, not HIF-1α, and the anti-HIF-2α antibody pulled down both TET1 and HIF-2α. This implied that HIF-2α, not HIF-1α, is directly or indirectly bound to the TET1 protein (Figure 5B). Reporter gene assay demonstrated that the promoter region of the TET1 gene was activated by hypoxia/HIF-2α (Tsai et al., 2014). All these results indicate that HIF-2α may serve as a transcription activator in ISK cells to regulate TET1 involving hypoxia-induced EMT.

Discussion
Aberrant DNA methylation may be associated with the pathogenesis of endometriosis (Arosh et al., 2015; Koukoura, Sifakis & Spandidos, 2016; Li et al., 2017; Juanqing et al., 2019). TET1-mediated DNA hydroxymethylation is a crucial mechanism of DNA demethylation (Jeschke, Collignon & Fuks, 2016). The balance between methylation and demethylation is important for gene expression and cellular environmental homeostasis (Song & He, 2012; Yang et al., 2015). However, the demethylation modification in endometriosis has not yet been well studied. Our study shows for the first time that the elevated expression of the demethylation enzyme TET1 may be associated with the activated EMT phenotype in the epithelia of endometriotic lesions, which may provide a novel insight into the pathogenesis of EMs.

Genome-wide DNA hypomethylation occurs in many diseases (Ehrlich, 2009; Song & He, 2012; Lim et al., 2015). 5-hmC is a critical epigenetic modification marker that plays a noteworthy
role in regulating gene expression (Lorsbach et al., 2003; Mariani et al., 2014; Jeschke, Collignon & Fuks, 2016). Our study had some consistency with the report by Roca (Roca et al., 2016).

Although Roca et al. showed that TET1 was down-regulated in endometriotic tissues, the contribution of TET1 expression from each component of the endometrium (epithelium or stroma) is unknown. Endometriosis is an estrogen-dependent disease. Roca et al. found that global 5-hmC was upregulated in endometriotic tissues and TET1 was upregulated in endometrial epithelial cell line treated with estradiol, which supports our study and suggests that TET1 in epithelial cells may affect the expression levels of 5-hmC globally or locally. It is worth noting that the stromal cells are more loosely arranged in the ovarian endometriomas compared to the normal endometria and eutopic endometria. In addition, the endometriotic stromal and epithelial cells have different molecular expression profiles (Logan, Yango & Tran, 2018; Noë et al., 2018), and the endometriotic epithelial cells might originate from endometrial epithelial cells, whereas the origin of endometriotic stromal cells remains to be investigated (Matsuzaki & Darcha, 2012). Hence, we focused on the endometrial epithelium, which might play a specific and dynamic role in the pathogenesis of endometriosis (Logan, Yango & Tran, 2018; Noë et al., 2018). A recent study showed that decreased TET1 expression led to 5-hmC loss in ISK cells (Lv et al., 2017). We need to explore the distribution of 5hmC in endometriosis, especially in the epithelia, and the specific gene whose promoter is enriched with 5-hmC in future studies.

TET1 was mainly expressed in stromal cells but not epithelial cells in the normal and eutopic endometria, whereas the epithelial expression of TET1 expression was ubiquitous and stromal staining was scattered in ovarian endometriotic lesions. What’s more, we showed a significant
inverse expression between TET1 and the epithelial marker E-cadherin and a positive correlation
between the expression of TET1 and the mesenchymal marker N-cadherin. Therefore, our study
demonstrates for the first time that the enhanced epithelial expression of TET1 in ectopic lesions
may play a crucial role in the EMT phenotype of endometriosis while the role of TET1 in stromal
cells needs to be further investigated. Our research may lay the foundation for this new
demethylation mechanism of endometriosis. The localization of TET1 in both the nucleus and
cytoplasm was surprising since TET1 is a nuclear protein. However, the similar subcellular
localization of TET1 was also shown in prostate, gastric cancer, hippocampus neurons and so on
(Hsu et al., 2012; Kaas et al., 2013; Fu et al., 2014; Han et al., 2017). TET genes localize in the
cytoplasm of neurons to sustain cell survival (Mi et al., 2015) and localize in the cytoplasm of
colorectal cancer cells to promote tumor metastasis (Huang et al., 2016). The variations of TET1
immunostaining results indicate a possible regulation of TET1 subcellular localization by still
unclear signaling pathways.

Hypoxia is an important factor of the endometriosis microenvironment (Becker et al., 2008;
Wu, Hsiao & Tsai, 2019). Hypoxia can induce epigenetic changes in tumor cells, whereas hypoxia-
induced epigenetic changes in endometriosis haven’t been reported yet (Zhou et al., 2006;
Shahrzad et al., 2007). In our study, we showed for the first time that hypoxia/HIF-2α activated
TET1 expression, and TET1 knockdown mitigated hypoxia-induced EMT in ISK cells. Co-
immunoprecipitation revealed that HIF-2α, not HIF-1α, was bound to the TET1 protein in ISK
cells. Therefore, we did not further explore the role of HIF-1α in our study. This co-
immunoprecipitation result of TET1 and HIF-1α is different from the data reported by Tsai et al.
(Tsai et al., 2014) and Cheng et al. (Cheng et al., 2018). Tsai et al. showed the interaction of TET1 and HIF-1a based on the 293 T cell line while Cheng et al. showed the same interaction based on the cells in mouse prefrontal cortex. Whereas, our study was performed on the ISK cells which could have different protein-protein interactions. What’s more, Tsai et al. concluded that HIF-2α was the major regulator of TET1 expression under hypoxia (Tsai et al., 2014). In this study, the HIF-2a knockdown didn’t eliminate the expression of TET1, which might indicate the existence of other regulators. Hu et al. reported microRNA-210 was involved in the regulation of TET1 under hypoxia status (Hu et al., 2018). Lin et al. showed HIF-1a could regulate the hypoxia-induced expression of TET in hepatoblastoma HepG2 cells (Lin et al., 2017), whereas Tsai et al. showed the HIF-1a knockdown had no effect on the TET1 expression in cancer cell lines (Tsai et al., 2014). It will be explored in future studies. Combined with the reporter gene assay of HIF-2α and TET1 (Tsai et al., 2014), we speculate that the hypoxia microenvironment may induce the EMT of endometrial epithelial cells via the activation of TET1 partly regulated by HIF-2α. These epithelial cells may pass through oviducts and implant into the ovarian surfaces, thus contributing to the development of endometriosis.

Our study had several limitations. First, we used ISK cells for in vitro experiments instead of the primary endometrial epithelial cells. The primary endometrial epithelial cells could hardly survive in primary culture. Therefore, ISK cells were used in numerous studies of endometriosis (Cho et al., 2016b; Lee et al., 2018; Matson et al., 2018; Choi et al., 2018). The ISK cell is a well-differentiated human endometrial adenocarcinoma cell line. It retains the phenotype of endometrial epithelial cells, bears estrogen and progesterone receptors, and displays a similar molecular
expression profile as endometrium (Du et al., 2018). However, our study will be more convincing if we use more cell lines. With the advancement of technology and improvement of experimental approaches, a deep understanding of the roles of TET1 in endometriosis will be possible. Secondly, the precise role and mechanism of TET1 in regulating the EMT remain to be investigated. The TET1 target gene and the correlation of TET1 with the EMT transcriptional factors slug, snails, and twist need to be elucidated in future studies. The interaction between TET1 and HIF-2α needs to be demonstrated by additional experiments, such as GST pull-down and yeast two-hybrid experiments.Thirdly, although 5-hmC detection is very expensive, analyzing more samples for overall 5-hmC expression in the epithelia and identifying the specific gene enriched with 5-hmC are still needed to study the pathogenesis of endometriosis.

Conclusion

To our knowledge, we are the first to explore the relationship between EMT and the demethylation enzyme TET1 in endometriosis. Hypoxia induces the expression of TET1, mediated by transcription factor HIF-2α, which may promote the EMT of endometriosis. These data provide a new understanding of the pathological process of endometriosis, which may advance knowledge of the epigenetic mechanism as well as the therapeutic approach towards endometriosis.

Authors’ Contribution

J.W., X.L., X.F. conceived and designed the experiments. J.W., X.L., H.H., M.Z., H.H., X.F.
performed and acquired the data. J.W., X.X., X.F. analyzed the data. J.W., X.L., H.H., X.F. drafted and critically evaluated the article.

**Funding**

This work was supported by the National Natural Science Foundation of China [grant number 81671437, 81771558]. Natural Science Foundation of Hunan Province [grant number 2020JJ4814] and the Postgraduate Independent Exploration and Innovation Project of Central South University, China [grant number 2020zzts285].

**Conflict of interest**

The authors declare no conflict of interest regarding this paper.

**Acknowledgments**

None

**References**

Arosh JA, Lee J, Starzinski-Powitz A, Banu SK. 2015. Selective inhibition of prostaglandin E2 receptors EP2 and EP4 modulates DNA methylation and histone modification machinery proteins in human endometriotic cells. *Molecular and cellular endocrinology* 409:51–58.

Baylin SB, Jones PA. 2011. A decade of exploring the cancer epigenome — biological and translational implications. *Nature Reviews. Cancer* 11:726–734. DOI: 10.1038/nrc3130.
Becker CM, Rohwer N, Funakoshi T, Cramer T, Bernhardt W, Birsner A, Folkman J, D’Amato RJ. 2008. 2-Methoxyestradiol Inhibits Hypoxia-Inducible Factor-1α and Suppresses Growth of Lesions in a Mouse Model of Endometriosis. *The American Journal of Pathology* 172:534–544. DOI: 10.2353/ajpath.2008.061244.

Camuzi D, de Amorim ÍSS, Ribeiro Pinto LF, Oliveira Trivilin L, Mencalha AL, Soares Lima SC. 2019. Regulation Is in the Air: The Relationship between Hypoxia and Epigenetics in Cancer. *Cells* 8:300.

Cheng Y, Sun M, Chen L, Li Y, Lin L, Yao B, Li Z, Wang Z, Chen J, Miao Z, Xin N, Huang L, Allen EG, Wu H, Xu X, Jin P. 2018. Ten-Eleven Translocation Proteins Modulate the Response to Environmental Stress in Mice. *Cell Reports* 25:3194-3203.e4. DOI: 10.1016/j.celrep.2018.11.061.

Cho S, Mutlu L, Zhou Y, Taylor HS. 2016a. Aromatase inhibitor regulates let-7 expression and let-7f-induced cell migration in endometrial cells from women with endometriosis. *Fertility and Sterility* 106:673–680. DOI: 10.1016/j.fertnstert.2016.05.020.

Cho S, Mutlu L, Zhou Y, Taylor HS. 2016b. Aromatase inhibitor regulates let-7 expression and let-7f–induced cell migration in endometrial cells from women with endometriosis. *Fertility and Sterility* 106:673–680. DOI: 10.1016/j.fertnstert.2016.05.020.

Choi YS, Park JH, Lee JH, Yoon J-K, Yun BH, Park JH, Seo SK, Sung H-J, Kim H-S, Cho S, Lee BS. 2018. Association Between Impairment of DNA Double Strand Break Repair and Decreased Ovarian Reserve in Patients With Endometriosis. *Frontiers in Endocrinology* 9. DOI: 10.3389/fendo.2018.00772.

Deret S, Voegelin J, Lelong-Rebel IHC, Rebel G. 2004. Effects of culture conditions on taurine uptake by various variants of human endometrial carcinoma cells in culture. *Amino Acids* 26:183–195. DOI: 10.1007/s00726-003-0035-7.
Du Y, Zhang Z, Xiong W, Li N, Liu H, He H, Li Q, Liu Y, Zhang L. 2018. Estradiol promotes EMT in endometriosis via MALAT1/miR200s sponge function. *Reproduction (Cambridge, England)*. DOI: 10.1530/REP-18-0424.

Ehrlich M. 2009. DNA hypomethylation in cancer cells. *Epigenomics* 1:239–259. DOI: 10.2217/epi.09.33.

Filippi I, Carrarelli P, Luisi S, Batteux F, Chapron C, Naldini A, Petraglia F. 2016. Different Expression of Hypoxic and Angiogenic Factors in Human Endometriotic Lesions. *Reproductive Sciences* 23:492–497. DOI: 10.1177/193371915607978.

Fu H-L, Ma Y, Lu L-G, Hou P, Li B-J, Jin W-L, Cui D-X. 2014. TET1 exerts its tumor suppressor function by interacting with p53-EZH2 pathway in gastric cancer. *Journal of Biomedical Nanotechnology* 10:1217–1230. DOI: 10.1166/jbn.2014.1861.

Giudice LC. 2010. Endometriosis. *New England Journal of Medicine* 362:2389–2398. DOI: 10.1056/NEJMcp1000274.

Guay S, Akoum A. 2007. Stable inhibition of interleukin 1 receptor type II in Ishikawa cells augments secretion of matrix metalloproteinases: possible role in endometriosis pathophysiology. *Reproduction* 134:525–534.

Han X, Zhou Y, You Y, Lu J, Wang L, Hou H, Li J, Chen W, Zhao L, Li X. 2017. TET1 promotes cisplatin-resistance via demethylating the vimentin promoter in ovarian cancer: TET1 in ovarian cancer. *Cell Biology International* 41:405–414. DOI: 10.1002/cbin.10734.

Hsiao K-Y. 2015. Pathological functions of hypoxia in endometriosis. *Frontiers in Bioscience* 7:352–366. DOI: 10.2741/e736.

Hsu C-H, Peng K-L, Kang M-L, Chen Y-R, Yang Y-C, Tsai C-H, Chu C-S, Jeng Y-M, Chen Y-T, Lin F-M,
Huang H-D, Lu Y-Y, Teng Y-C, Lin S-T, Lin R-K, Tang F-M, Lee S-B, Hsu HM, Yu J-C, Hsiao P-W, Juan L-J. 2012. TET1 suppresses cancer invasion by activating the tissue inhibitors of metalloproteinases. *Cell Reports* 2:568–579. DOI: 10.1016/j.celrep.2012.08.030.

Hu X-Q, Dasgupta C, Xiao J, Yang S, Zhang L. 2018. Long-term high altitude hypoxia during gestation suppresses large conductance Ca2+-activated K+ channel function in uterine arteries: a causal role for microRNA-210. *The Journal of Physiology* 596:5891–5906. DOI: 10.1113/JP276058.

Huang Y, Wang G, Liang Z, Yang Y, Cui L, Liu C-Y. 2016. Loss of nuclear localization of TET2 in colorectal cancer. *Clinical Epigenetics* 8:9. DOI: 10.1186/s13148-016-0176-7.

Jain T, Nikolopoulou EA, Xu Q, Qu A. 2018. Hypoxia inducible factor as a therapeutic target for atherosclerosis. *Pharmacology & Therapeutics* 183:22–33. DOI: 10.1016/j.pharmthera.2017.09.003.

Javaid S, Zhang J, Anderssen E, Black JC, Wittner BS, Tajima K, Ting DT, Smolen GA, Zubrowski M, Desai R, Maheswaran S, Ramaswamy S, Whetstine JR, Haber DA. 2013. Dynamic Chromatin Modification Sustains Epithelial-Mesenchymal Transition following Inducible Expression of Snail-1. *Cell reports* 5:1679–1689. DOI: 10.1016/j.celrep.2013.11.034.

Jeschke J, Collignon E, Fuks F. 2016. Portraits of TET-mediated DNA hydroxymethylation in cancer. *Current Opinion in Genetics & Development* 36:16–26. DOI: 10.1016/j.gde.2016.01.004.

Jones PA. 2012. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nature Reviews. Genetics* 13:484–492. DOI: 10.1038/nrg3230.

Juanqing L, Hailan Y, Xiangwei F, Tao Z, Junyan M, Jianhong Z, Jun L, Jianhua Y. 2019. Relationship between the methylation levels of Twist gene and pathogenesis of endometriosis. *Cellular and Molecular Biology (Noisy-Le-Grand, France)* 65:94–100.
Kaas GA, Zhong C, Eason DE, Ross DL, Vachhani RV, Ming G-L, King JR, Song H, Sweatt JD. 2013. TET1 controls CNS 5-methylcytosine hydroxylation, active DNA demethylation, gene transcription, and memory formation. *Neuron* 79:1086–1093. DOI: 10.1016/j.neuron.2013.08.032.

Koukoura O, Sifakis S, Spandidos DA. 2016. DNA methylation in endometriosis. *Molecular medicine reports* 13:2939–2948.

Lamouille S, Xu J, Derynck R. 2014. Molecular mechanisms of epithelial–mesenchymal transition. *Nature reviews Molecular cell biology* 15:178.

Lee M-Y, Kim SH, Oh YS, Heo S-H, Kim K-H, Chae HD, Kim C-H, Kang BM. 2018. Role of interleukin-32 in the pathogenesis of endometriosis: in vitro, human and transgenic mouse data. *Human Reproduction* 33:807–816. DOI: 10.1093/humrep/dey055.

Li Y, An D, Guan Y, Kang S. 2017. Aberrant Methylation of the E-Cadherin Gene Promoter Region in Endometrium and Ovarian Endometriotic Cysts of Patients with Ovarian Endometriosis. *Gynecologic and Obstetric Investigation* 82:78–85. DOI: 10.1159/000445293.

Lim YW, Sanz LA, Xu X, Hartono SR, Chédin F. 2015. Genome-wide DNA hypomethylation and RNA:DNA hybrid accumulation in Aicardi-Goutières syndrome. *eLife* 4. DOI: 10.7554/eLife.08007.

Lin G, Sun W, Yang Z, Guo J, Liu H, Liang J. 2017. Hypoxia induces the expression of TET enzymes in HepG2 cells. *Oncology letters* 14:6457–6462.

Logan PC, Yango P, Tran ND. 2018. Endometrial Stromal and Epithelial Cells Exhibit Unique Aberrant Molecular Defects in Patients With Endometriosis. *Reproductive Sciences (Thousand Oaks, Calif.)* 25:140–159. DOI: 10.1177/1933719117704905.

Lorsbach RB, Moore J, Mathew S, Raimondi SC, Mukatira ST, Downing JR. 2003. TET1, a member of a novel
protein family, is fused to MLL in acute myeloid leukemia containing the t (10; 11)(q22; q23). Leukemia 17:637.

Lv Q-Y, Xie B-Y, Yang B-Y, Ning C-C, Shan W-W, Gu C, Luo X-Z, Chen X-J, Zhang Z-B, Feng Y-J. 2017. Increased TET1 Expression in Inflammatory Microenvironment of Hyperinsulinemia Enhances the Response of Endometrial Cancer to Estrogen by Epigenetic Modulation of GPER. Journal of Cancer 8:894–902. DOI: 10.7150/jca.17064.

Mahmood TA, Templeton A. 1991. Prevalence and genesis of endometriosis. Human Reproduction 6:544–549. DOI: 10.1093/oxfordjournals.humrep.a137377.

Mariani CJ, Vasanthakumar A, Madzo J, Yesilkanal A, Bhagat T, Yu Y, Bhattacharyya S, Wenger RH, Cohn SL, Nanduri J. 2014. TET1-mediated hydroxymethylation facilitates hypoxic gene induction in neuroblastoma. Cell reports 7:1343–1352.

Matson BC, Quinn KE, Lessey BA, Young SL, Caron KM. 2018. Elevated levels of adrenomedullin in eutopic endometrium and plasma from women with endometriosis. Fertility and Sterility 109:1072–1078. DOI: 10.1016/j.fertnstert.2018.02.004.

Matsuzaki S, Darcha C. 2012. Epithelial to mesenchymal transition-like and mesenchymal to epithelial transition-like processes might be involved in the pathogenesis of pelvic endometriosis†. Human Reproduction 27:712–721. DOI: 10.1093/humrep/der442.

Maybin JA, Critchley HOD. 2015. Menstrual physiology: implications for endometrial pathology and beyond. Human Reproduction Update 21:748–761. DOI: 10.1093/humupd/dmv038.

Mi Y, Gao X, Dai J, Ma Y, Xu L, Jin W. 2015. A Novel Function of TET2 in CNS: Sustaining Neuronal Survival. International Journal of Molecular Sciences 16:21846–21857. DOI: 10.3390/ijms160921846.
Nisolle M, Donnez J. 1997. Peritoneal endometriosis, ovarian endometriosis, and adenomyotic nodules of the rectovaginal septum are three different entities. *Fertility and Sterility* 68:585–596. DOI: 10.1016/s0015-0282(97)00191-x.

Noë M, Ayhan A, Wang T-L, Shih I-M. 2018. Independent development of endometrial epithelium and stroma within the same endometriosis. *The Journal of Pathology* 245:265–269. DOI: 10.1002/path.5082.

Roca FJ, Loomans HA, Wittman AT, Creighton CJ, Hawkins SM. 2016. Ten-eleven translocation genes are downregulated in endometriosis. *Current molecular medicine* 16:288–298.

Shahrzad S, Bertrand K, Minhas K, Coomber B. 2007. Induction of DNA hypomethylation by tumor hypoxia. *Epigenetics* 2:119–125.

Song C-X, He C. 2012. Balance of DNA methylation and demethylation in cancer development. *Genome Biology* 13:173. DOI: 10.1186/gb-2012-13-10-173.

Song J, Wang W, Wang Y, Qin Y, Wang Y, Zhou J, Wang X, Zhang Y, Wang Q. 2019. Epithelial-mesenchymal transition markers screened in a cell-based model and validated in lung adenocarcinoma. *BMC Cancer* 19:680. DOI: 10.1186/s12885-019-5885-9.

Tsai Y-P, Chen H-F, Chen S-Y, Cheng W-C, Wang H-W, Shen Z-J, Song C, Teng S-C, He C, Wu K-J. 2014. TET1 regulates hypoxia-induced epithelial-mesenchymal transition by acting as a co-activator. *Genome Biology* 15:513. DOI: 10.1186/s13059-014-0513-0.

Tsai Y-P, Wu K-J. 2014. Epigenetic regulation of hypoxia-responsive gene expression: Focusing on chromatin and DNA modifications. *International Journal of Cancer* 134:249–256. DOI: 10.1002/ijc.28190.

Wu M-H, Chen K-F, Lin S-C, Lgu C-W, Tsai S-J. 2007. Aberrant expression of leptin in human endometriotic stromal cells is induced by elevated levels of hypoxia inducible factor-1α. *The American journal of...*
pathology 170:590–598.

Wu M-H, Hsiao K-Y, Tsai S-J. 2019. Hypoxia: The force of endometriosis. Journal of Obstetrics and Gynaecology Research 45:532–541. DOI: 10.1111/jog.13900.

Wu C-Y, Tsai Y-P, Wu M-Z, Teng S-C, Wu K-J. 2012. Epigenetic reprogramming and post-transcriptional regulation during the epithelial–mesenchymal transition. Trends in Genetics 28:454–463. DOI: 10.1016/j.tig.2012.05.005.

Wu X, Zhang Y. 2017. TET-mediated active DNA demethylation: mechanism, function and beyond. Nature Reviews Genetics 18:517.

Xiong Y, Liu Y, Xiong W, Zhang L, Liu H, Du Y, Li N. 2016. Hypoxia-inducible factor 1α-induced epithelial–mesenchymal transition of endometrial epithelial cells may contribute to the development of endometriosis. Human Reproduction 31:1327–1338. DOI: 10.1093/humrep/dew081.

Yang R, Qu C, Zhou Y, Konkel JE, Shi S, Liu Y, Chen C, Liu S, Liu D, Chen Y. 2015. Hydrogen sulfide promotes Tet1-and Tet2-mediated Foxp3 demethylation to drive regulatory T cell differentiation and maintain immune homeostasis. Immunity 43:251–263.

Zhou J, Schmid T, Schnitzer S, Brüne B. 2006. Tumor hypoxia and cancer progression. Cancer letters 237:10–21.
Hypoxia may increase TET1 expression, mediated by HIF-2α, thus potentially inducing the EMT of endometrial epithelial cells and contributing to the development of endometriosis.
Figure 2

Accompanied with the TET1 upregulation, EMT might occur in endometrial epithelial cells of ovarian endometriosis.

**Fig. 1** Accompanied with TET1 upregulation, EMT might occur in endometrial epithelial cells of ovarian endometriosis. **(A-R)** Immunohistochemistry for TET1 and EMT markers (E-cadherin and N-cadherin) in epithelial gland cells of the normal endometria (Normal), eutopic endometria (Eutopic) and ovarian endometrioma (Ectopic). Left: Magnification×100, Right: Magnification×200. **(S-U)** The proportions of cells positively stained for TET1, E-cadherin or N-cadherin in Normal, Eutopic and Ectopic groups (*p< 0.05 versus normal endometria; #p< 0.05 versus eutopic endometria). **(V)** Expression of global 5-hmC in the total genomic DNA (*p< 0.05 versus normal endometria; #p< 0.05 versus eutopic endometria). **(W-EE)** Representative double immunofluorescence images for TET1 and E-cadherin in Normal (W-CC), Eutopic (Z-BB) and Ectopic (CC-EE) groups. **(FF-NN)** Representative double immunofluorescence images for TET1 and N-cadherin in Normal (FF-HH), Eutopic (II-KK) and Ectopic (LL-NN) groups. **(OO-PP)** The correlation between TET1 and EMT markers expression in the endometrial epithelium is confirmed by quantitative analysis of the double staining immunofluorescence. Magnification×200 (Left: red luminescence represents TET1. Yellow luminescence represents E-cadherin. Right: red luminescence represents TET1. Green luminescence represents N-cadherin). Normal: normal endometria, Eutopic: eutopic endometria, Ectopic: ovarian endometriomas.
Figure 3

Hypoxia alters the expression of HIFs, TET1 and EMT markers.

(A–B) Western blotting analysis showed the expression of HIF-1α, HIF-2α, TET1 and EMT markers (E-Cad: E-cadherin, N-Cad: N-cadherin and Vim: Vimentin) in Ishikawa(ISK) cell cultured under hypoxia at different time point (0 h, 4 h, 8 h and 24 h) (*p< 0.05 versus normoxia; #p< 0.05 versus hypoxia for 4 h; & p< 0.05 versus hypoxia for 8 h).
Figure 4

TET1 overexpression may promote EMT and knockdown of TET1 mitigates hypoxia-induced EMT.

(A, B) Western blotting analysis showed the expression of vimentin (Vim) and E-cadherin (E-Cad) in cells transfected with TET1 expression plasmid (TET1) or empty vectors (NC) under normoxia conditions, and cells transfected with TET small interfering RNA (si-TET1) or negative control (si-NC) under hypoxia conditions. (*p< 0.05 versus normoxia+NC; #p< 0.05 versus normoxia+TET1; & p< 0.05 versus hypoxia+si-NC).
Figure 5

Knockdown of HIF-2α inhibits hypoxia-induced EMT and TET1 expression.

(A, C) Western blotting showed the expression of TET1, HIF-2α, vimentin (Vim) and E-cadherin (E-Cad) in cells under normoxia conditions and cells transfected with HIF-2α small interfering RNA (si-HIF-2α) or negative control (si-NC) under hypoxia conditions (*p< 0.05 versus normoxia or normoxia+si-NC; #p< 0.05 versus hypoxia or hypoxia+si-NC). (B) Co-immunoprecipitation of TET1, HIF-2α or HIF-1α. Hypoxia treated ISK cells were lysed and immunoprecipitated by TET1, HIF-2α, HIF-1α and normal rabbit/mouse IgG antibodies, respectively. Cell lysates (input) and immunoprecipitated proteins (IP) were analyzed by Western blotting.
Table 1 (on next page)

Clinical characteristics of patients
**Table 1 Clinical characteristics of patients.**

| Immunohistochemistry samples | Normal endometria | Eutopic endometria | Ectopic endometria |
|------------------------------|-------------------|--------------------|--------------------|
| case number                  | 20                | 15                 | 15                 |
| *age                         | 35(27-41)         | 29(27-38)          | 29(27-38)          |
| menstrual cycle phase        | Early proliferation | Early proliferation | Early proliferation |
| # rASRM stage                |                   |                    |                    |
| III                          | -                 | 9                  | 9                  |
| IV                           | -                 | 6                  | 6                  |

Eutopic endometria were paired with ectopic endometria.

*Median (interquartile range).

# rASRM (Revised American Society for Reproductive Medicine classification, 1997).
Table 2 (on next page)

Expression profiles of TET1 and EMT markers were detected by immunohistochemistry in the epithelium of normal endometria, eutopic endometria, and ectopic endometria.
Table 2 Expression profiles of TET1 and EMT markers were detected by immunohistochemistry in the epithelia of normal endometria, eutopic endometria, and ectopic endometria.

| Marker    | Normal endometria, % (group1,n=15) | Eutopic endometria, % (group2,n=15) | Ectopic endometria, % (group3,n=15) | p-value (1 versus 2) | p-value (2 versus 3) | p-value (1 versus 3) |
|-----------|------------------------------------|-------------------------------------|-------------------------------------|----------------------|----------------------|----------------------|
| E-cadherin| 91.66±3.196                        | 77.75±4.024                         | 2.668±1.174                        | <0.05*               | <0.01**              | <0.01**              |
| N-cadherin| 2.235±0.339                        | 24.11±2.237                         | 60.98±8.858                        | <0.01**              | <0.01**              | <0.01**              |
| TET1      | 1.668±0.438                        | 16.57±3.308                         | 59.03±6.042                        | <0.01**              | <0.01**              | <0.01**              |

*P<0.05, **P<0.01

All data are expressed as mean ± SD.