Biology of Reproduction, 2018, 99(5), 968–981
doi:10.1093/biolre/ioy128
Research Article
Advance Access Publication Date: 31 May 2018

Research Article

Autophagy contributes to hypoxia-induced epithelial to mesenchymal transition of endometrial epithelial cells in endometriosis†

Hengwei Liu[1], Yu Du[1], Zhibing Zhang[2], Liqun Lv[3], Wenqian Xiong[1], Ling Zhang[1], Na Li[1], Haitang He[1], Qi Li[1] and Yi Liu[1,*]

[1]Department of Obstetrics and Gynecology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China; [2]Department of Obstetrics and Gynecology, Virginia Commonwealth University, Richmond, USA and [3]Department of Obstetrics and Gynecology, Wu Han Kang Jian Fu Ying Hospital, Wuhan, China

*__Correspondence:__ Department of Obstetrics and Gynecology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1277 Jiefang Road, Hubei, Wuhan 430030, People's Republic of China.
Tel: +027-86-13971038710; E-mail: Liqun94@163.com

† **Grant Support:** This study was supported by the National Natural Science Foundation of China (NSFC No 81471439 to Yi Liu) and the NIH Award (Grant NIH HD076257 to Zhibing Zhang).
Edited by Dr. Romana Nowak, PhD, University of Illinois Urbana-Champaign

Received 2 December 2017; Revised 18 March 2018; Accepted 30 May 2018

Abstract

Endometriosis is a benign gynecologic disorder, and presents with malignant characteristics, such as migration and invasion. Hypoxia has been implicated in triggering epithelial–mesenchymal transition (EMT). Hypoxia is also known to induce autophagy. However, the relationship between autophagy and EMT under hypoxia conditions in endometriosis remains unknown. In the present study, we found that the expression of hypoxia-inducible factor-1α (HIF-1α), microtubule associated protein light chain 3 (LC3), and mesenchymal cell marker vimentin was significantly higher in ectopic endometrium from patients with endometriosis, along with decreased expression of epithelial cell marker E-cadherin. After hypoxia treatment, endometrial epithelial cells exhibited enhanced migration and invasion abilities, as well as promoted autophagy and the EMT phenotype. Our analyses also show that HIF-1α was responsible for induction of autophagy. Moreover, inhibition of autophagy by chemical or genetic approaches suppressed hypoxia triggered EMT and reduced cell migration and invasion. Collectively, our findings identify that autophagy is critical for the migration and invasion of endometrial cells through the induction of EMT and indicate that inhibition of autophagy may be a novel useful strategy in the treatment of endometriosis.

Summary Sentence

The autophagy, which is induced by peritoneal hypoxia, is essential for EMT initiation in endometriosis.

Key words: hypoxia, HIF-1α, autophagy, epithelial–mesenchymal transition, invasion, endometriosis.

Introduction

Endometriosis, characterized by the presence of the endometrial gland and stroma-like tissue in extra-uterine locations, is one of the most common and severe gynecological diseases [1]. It is estimated to affect 2—10% of all premenopausal women and is responsible for infertility and chronic pelvic pain [2]. Although endometriosis is a benign disease, it has migratory and invasive properties similar to malignant cancer [3]. However, so far very little is known about

the cellular and molecular mechanisms underlying the migration and invasion of endometrial cells.

Epithelial-to-mesenchymal transition (EMT), a complex biological process by which polarized epithelial cells change phenotype from epithelial to highly motile mesenchymal, plays a vital role in embryogenesis, fibrosis, and tumor metastasis [4]. EMT is characterized by the loss of epithelial markers such as E-cadherin together with the acquisition of mesenchymal markers including vimentin and N-cadherin. Furthermore, EMT is known to be a critical step during the process of cancer metastasis because it allows cells to migrate and invade surrounding tissues [5]. Recently, multiple researches have shown that EMT also plays a critical role in the formation of endometriotic lesions [6, 7].

Hypoxia is vital for the pathogenesis of endometriosis, and hypoxia-inducible factor-1α (HIF-1α) was upregulated with the development of endometriosis [8–10]. HIF-1α is the key mediator of the cellular response to hypoxia and is highly regulated by cellular oxygen tension. Under normoxic conditions, the HIF-1α subunit is rapidly degraded, whereas under hypoxic conditions, HIF-1α is stabilized and translocated to the nucleus where it initiates the transcription of downstream genes, which are involved in regulation of several biological processes including migration, invasion, and EMT [11, 12]. Hypoxia stimulates upregulation of HIF-1α, which has previously been shown to trigger EMT, as well as to facilitate metastasis of multiple types of cancer cells [13].

Autophagy is an evolutionarily conserved mechanism for eliminating and recycling nonessential cytoplasmic components to maintain cellular homeostasis [14]. During autophagy, a cup-shaped isolated membrane forms in the cytoplasm followed by formation of a double-membrane structure called the autophagosome. This then fuses with the lysosome to form an autolysosome which degrades the contents inside the autophagosome [15]. Autophagy is activated in response to metabolic stress, such as hypoxia [16, 17]. Accumulating evidence suggests that autophagy promotes cancer cell invasion by manipulating EMT process [18–20]. Our recent studies as well as others' have revealed that autophagy was higher in the ectopic endometrium of patients with ovarian endometriosis and autophagy could promote endometrial stromal cells migration and invasion under hypoxic condition [21, 22]. However, the correlation between autophagy induction and EMT has not been elucidated in the context of endometriosis. Therefore, considering the fact that the upregulated expression of autophagy in ovarian endometriosis and its connection with EMT, and both EMT and autophagy were triggered by hypoxia, we hypothesized that upregulation of autophagy in endometriosis may contribute to the progression of endometriosis by promoting EMT and enhancing cell migration and invasion under hypoxia conditions.

Materials and methods

Ethical approval

Ethics approval for the present study was obtained from the local Ethics Committee of Tongji Medical College, Huazhong University of Science (IORG No: IORG0003571). Written, informed agreement from each participant was obtained before surgery.

Patients and tissue samples

The patients recruited in this study were nonpregnant women of reproductive age (27–46 years) attending the Department of Obstetrics and Gynecology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology between September 2014 and December 2016. Each recruited patient had regular menstrual cycles ranging in length from 28 to 35 days, and no one received any kind of steroid medication within the past 6 months before the endometrial biopsy. All of the endometrial tissue samples were collected during the proliferative stage which was confirmed by experienced histopathologists, who examined hematoxylin-stained endometrial sections referring to established histological criteria.

As for the healthy control group, 31 cases of normal endometrium tissue samples were obtained from patients with tubal infertility. Thirty cases of eutopic endometrium of endometriosis and 30 cases of ovarian endometriotic cysts tissue samples (ectopic endometrium) were obtained from patients undergoing laparoscopy for pelvic endometriosis. The endometrial tissues were collected using the Nowak's curette just before the surgical procedure, and immediately transported to the laboratory. All of the ectopic endometrium was determined with the use of the American Society of Reproductive Medicine (ASRM) revised classification [23]. The collected endometrial biopsy was divided in two parts: the first part was fixed in 10% buffered formalin for immunohistochemistry analysis, and the second part was immediately submerged in liquid nitrogen to allow subsequent total protein extraction and western blotting analysis. Besides, another 68 cases of eutopic endometrium biopsies of patients with endometriosis were collected for isolation and cultivation of primary endometrial epithelial cells. The clinical data of the patients (age at the time of biopsy, the duration of menstrual cycle phase and rASRM stage of endometriosis) are summarized in Table 1.

Immunohistochemistry

Immunohistochemical staining was performed as described in our previous study [22]. Formalin-fixed paraffin-embedded samples were cut into 5 μm sections and mounted on alcohol-cleaned glass slides. The sections were dewaxed in xylene and rehydrated by passing through a graded series of alcohol to water, and antigen retrieval was performed by heating the samples in a 0.01 M citrate buffer (pH 6.0). Then the sections were incubated in 50% ethanol solution containing 3% H_2O_2 for 30 min to eliminate endogenous nonspecific peroxidase activity and blocked with bovine serum albumin for another 30 min. Subsequently, the samples were incubated with the following primary antibodies against HIF-1α (diluted 1:150; Affinity), LC3 (1:100; Abcam, Cambridge, UK), E-cadherin (1:100; Abcam), vimentin (1:100; Cell Signal Technology), and S100A4 (1:100; Cell Signal Technology) overnight at 4°C. After washing in PBS for three times, the samples were incubated with peroxidase-labeled anti-rabbit IgG (1:500; Boster Biotech, Wuhan, China) for 30 min. Finally, all slides were incubated with DAB-Substrate (Beyotime, Wuhan, China) and counterstained in hematoxylin before being dehydrated and mounted. The slides incubated with isotype-matched immunoglobulin G from normal rabbits were applied as a negative control. Antibody characteristics are listed in the Supporting information, Supplemental Table S1. Immunohistochemistry images were captured using a Nikon DXM1200C digital camera at ×200 (50 μm) or ×400 (25 μm) magnification.

Semiquantitative analysis of immunohistochemical staining

The immunohistochemistry slides were independently examined by two observers. We utilized immunohistochemical scores (IHS) based on the German Immuno-Reactive score [24]. The IHS was calculated by combining an estimate of the percentage of immunereactive cells (quantitative score) with an estimate of the staining

Table 1. Clinical characteristics of patients.

	Samples used for immunohistochemistry			
	Normal Endometrium	Eutopic Endometrium	Ectopic Endometrium	Samples used for primary cell cultures
Number of cases	31	30	30	68
[a]Age	32.5 (28–37)	35.5 (31–40)	37.5 (31–46)	35.5 (30–41)
Menstrual cycle phase	Proliferative	Proliferative	Proliferative	Proliferative
[2a]rASRM stage				
III	-	17	19	–
IV	–	13	11	–
Types of lesions	–	–	Bluish-black	–
Prior medication use	No	No	No	No

[a]Median (range).

[b]Revised American Society for Reproductive Medicine classification (rASRM: American Society for Reproductive Medicine, 1997).

intensity (staining intensity score). No staining was scored as 0; staining of 1–10% as 1; 11–50% as 2; 51–80% as 3; and 81–100% as 4. The staining intensity was rated on a scale of 0 to 3, with 0 representing no staining, 1 weak staining, 2 moderate staining, and 3 strong staining. The raw data were converted to IHS by multiplying the quantity score and the intensity score. A moderate to strong level (HIS > 4) was considered to be positive, and weak or absent expression (IHS 0–4) was considered to be negative.

Isolation and culture of primary endometrial epithelial cells and Ishikawa cell lines

Specifically, primary human endometrial epithelial cells were separated from stromal cells by digesting the tissue fragments with collagenase, as previously described [25]. Briefly, fresh endometrium tissues were washed with pre-warmed PBS several times to remove blood and debris and minced into 1 mm pieces with a sterile surgical scissor and incubated with PBS containing 2 mg/mL of type II collagenase (0.1%, Sigma-Aldrich) at 37°C for 45–60 min with constant agitation. Afterwards, the tissue suspension was filtered through a 140 μm wire sieve to remove undigested tissue pieces and mucous, and the cell suspension was then filtered through a 37 μm wire sieve to remove endometrial stromal cells. Then, the 37 μm wire sieve was washed thoroughly upside down with fresh pre-warmed culture medium to get the human endometrial epithelial cells. The epithelial cells were subsequently cultured in Dulbecco's modified Eagle's/F12 medium (DMEM/F12; HyClone) supplemented with 20% fetal bovine serum (FBS; HyClone), 100 U/mL penicillin, and 100 mg/mL streptomycin (HyClone) in a humidified atmosphere with 5% CO_2 at 37°C. The purity of isolated epithelial cells was >95% and epithelial cells were contaminated by less than 1% of stromal cells, as determined by cell immunofluorescence using E-cadherin (diluted 1:300; Abcam) and vimentin (diluted 1:300; Affinity) (see Supplemental Figure S1). The first passage cells were used for the subsequent experiments.

For the reason that primary human endometrial epithelial cell cannot be passaged and transfected. Therefore, Ishikawa cells (Shanghai Fuxiang Biotechnology Co. Ltd, China), a well-differentiated human endometrial epithelial adenocarcinoma cell line that is widely used in the studies of endometriosis [26, 27], were maintained in Roswell Park Memorial Institute-1640 culture media (RPMI-1640; HyClone) with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin (HyClone) in humidified atmosphere with 5% CO_2 at 37°C.

Hypoxia treatment

Cells were seeded in 60 mm culture dishes and fresh medium was used to keep the cells healthy by providing fresh nutrients before hypoxia treatment. For culture in hypoxic condition, the culture dishes were placed in a modular incubator chamber (Thermo Scientific) containing humidified hypoxic air (1% O_2, 5% CO_2, 94% N_2) for indicated period at 37°C. Cells incubated under normoxic conditions (21% O_2, 5% CO_2, 75% N_2) were used as controls.

Immunocytochemistry

Immunocytochemical staining was performed following a previous study. Isolated human endometrial epithelial cells were grown on coverslips in a six-well plate prior to treatment with hypoxia for 24 h, and washed three times with PBS. Treated cells were then fixed with 4% paraformaldehyde at room temperature for 15 min, and permeabilized with 0.5% Triton X-100 (Sigma, Belgium) for 15 min to increase their permeability to antibodies. After blocking with 5% bovine serum albumin (BSA) in PBS-T (0.1% Triton X-100/PBS) for 1 h, cells were incubated with E-cadherin (1:250; Abcam) and vimentin (1:300; Cell Signal Technology) primary antibodies overnight at 4°C. The next day cells were washed three times in PBS prior to incubation with horseradish peroxidase-conjugated (HRP) secondary antibody (1:5000 dilution, Boster Biotech) for another 1 h at room temperature. Cells were then washed with PBS and incubated with hematoxylin for 15 min at room temperature. Finally, the cells were washed three times with ice-cold PBS and images were taken using an Eclipse TE2000-S microscope system (Nikon UK Ltd, Surrey) at ×200 magnification. The slides incubated with isotype-matched immunoglobulin G from normal rabbits were applied as a negative control. Characteristics of antibody used for immunohistochemistry are listed in Supplemental Table S1.

Western blot assays

Collected endometrial tissues and cultured cells were washed three times with ice-cold PBS and lysed in radio immunoprecipitation assay (RIPA) buffer (Beyotime Biotechnology, China) containing protease inhibitors (Sigma). The cells were scraped in this lysis buffer, kept on ice for at least 30 min, centrifuged at 12 000 g at 4°C for 15 min, and diluted in 5x sample buffer (Beyotime Biotechnology). BCA protein assay kit (Beyotime) was used to determine the protein concentrations. Equal amounts of proteins (30 μg) were mixed with the sample buffer (4% SDS, 10% β-mercaptoethanol, and 20% glycerol in 0.125 M Tris, pH 6.8) containing bromophenol blue, and were boiled for10 min at 95°C. The samples were loaded

and separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels (SDS-PAGE) with running buffer. The proteins separated by SDS-PAGE were transferred to polyvinylidene difluoride membranes (Immobilon-P transfer membrane). The membranes were incubated with 5% fat-free milk in Tris-buffered saline containing 0.05% Tween 20 for 1 h, and were then incubated overnight at 4°C with the following primary antibodies: HIF-1α (diluted 1:1000; Affinity), LC3 (diluted 1:1000, Abcam), Beclin1 (diluted 1:1000, Abcam), E-cadherin (diluted 1:1000; Abcam), vimentin (diluted 1:1000; Cell Signal Technology), N-cadherin (diluted 1:1000; Cell Signal Technology), and GAPDH (diluted 1:1000; Affinity). The membranes were washed three times with TBST for a total of 15 min, and then incubated with an HRP-labeled secondary antibody at room temperature for 1 h. The membranes were washed again and treated with ECL-Western blot detecting reagent (Millipore) according to the manufacturer's recommendations. The band intensity on western blots was quantified by imaging system (Gel Doc 2000; Bio-Rad) and analysis with Image J software (NIH) (version 1.5, USA) and normalized to GAPDH. Characteristics of antibody used for western blot are listed in Supplemental Table S1.

Transmission electron microscopy

To identify autophagosomes at the ultrastructural level, human endometrial epithelial cells were cultured under hypoxic or normoxic conditions for 24 h. After the indicated treatment, human endometrial epithelial cells were washed three times with PBS and incubated with trypsin for 2 min. Cells were collected by centrifugation at 1000 × g for 5 min. Samples were fixed with 2% glutaraldehyde-paraformaldehyde in 0.1 M Na phosphate buffer (pH 7.4) for 12 h at 4°C and washed three times for 30 min in 0.1 M Na phosphate buffer (pH 7.4) and post-fixed with 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.4) for 3 h. After being washed by 0.1 M Na phosphate buffer, the cells were then dehydrated at 25°C with a graded series of ethanol and gradually infiltrated with epoxy resin mixture (812 resin embedding kit). The samples were sequentially polymerized at 37°C for 12 h, 45°C for 12 h, and 60°C for 24 h. Ultrathin sections (50–70 nm) were cut by using LKB microtome and mounted on single-slot copper grids. The sections were subjected to double staining with uranyl acetate and lead citrate and examined using a transmission electron microscope (FEI Tecnai G20, Super twin, Double tilt, LaB6 Gun, USA).

Cell transfection assay

HIF-1α small interfering RNA (siRNA), Beclin1 siRNA, and scrambled negative control siRNA were purchased from Shanghai GenePharma (China). The sense sequence of HIF-1α siRNA (HIF-1α-homo-1612) was 5′-GCUGGAGACAAUCAUAUTT-3′, and the antisense was 5′-AUAUGAUUGUGUCUCCAGCTT-3′; the sense sequence of Beclin1 siRNA was 5′-CGGGAAUACAGUGAAUUUATT-3, and the antisense was 5′-UAAAUUCACUGUAUUCCCGTT-3′. HIF-1α overexpression plasmid (pG/CMV/HIF-1α/IRES/EGFP) and negative control (NC) plasmid were purchased from Gemma Pharmaceutical Technology (China). For knockdown, Ishikawa cells (2×10^5 cells/well) were seeded in six-well plates and grown to 60–80% confluence, and transfected with the above plasmids or siRNA using lipofectamine2000 (Invitrogen Life Technologies) according to the manufacturer's protocol. The transfection mixture was replaced 6 h later with DMEM/F-12 with 20% FBS. Then Ishikawa cells were incubated in normoxic or hypoxic conditions for another 24 h and subjected to western blot analysis.

Transient transfection GFP-LC3 adenoviral vector and identification of autophagy

GFP-tagged LC3 adenoviral vector has recently been utilized to demonstrate the occurrence of autophagy. Ishikawa cells were seeded (1×10^4 cells/well) in 12-well plates overnight and allowed to reach 50–70% confluence at the time of transfection. Then GFP-LC3 adenoviral vector was transiently transfected into the cells according to the manufacturer's instructions. After being cultured for 24 h to ensure the expression of GFP-LC3, the cells were subjected to hypoxia for another 24 h. At the end of the treatment, cell was incubated with DAPI for 15 min at room temperature and autophagy was detected by counting the percentage of cells with GFP-LC3-positive dots under laser scanning confocal microscope (Olympus America Inc, Center Valley, PA). Autophagic level was determined by evaluating the number of GFP-LC3 puncta (puncta/cell were counted) in triplicate for each experiment.

Immunofluorescence assay

Human endometrial epithelial cells were immunostained to detect the biomarker expression (E-cadherin and vimentin protein). Briefly, the isolated primary cells were seeded and grown on coverslips in six-well plates. After incubation overnight, cells were washed with ice-cold PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. Then cells were permeabilized with 0.5% Triton X-100 (Sigma, Belgium) at 4°C for 30 min, and blocked in 5% BSA for 1 h to block nonspecific binding at room temperature. After that, cells were incubated with rabbit monoclonal antibody against E-cadherin (1:300; Abcam) and rabbit monoclonal antibody vimentin (1:100; Cell Signal Technology) overnight at 4°C. The next day, the cells were incubated with goat FITC-conjugated anti-rabbit IgG (diluted 1:100, Wuhan Servicebio Technology, China) and goat Cy3-conjugated anti-rabbit IgG (diluted 1:100, Wuhan Servicebio Technology) for 2 h in dark room and then incubated with DAPI for 15 min at room temperature. Finally, the cells were washed three times with ice-cold PBS, and immediately observed using a laser scanning confocal microscope (Olympus America Inc). The slides incubated with isotype-matched immunoglobulin G from normal rabbits were applied as a negative control.

Transwell migration and invasion assays

In vitro transwell migration and invasion assays were performed using a 24-well transwell insert (8 mm pore size, Corning Costar, Tewksbury, MA) as described previously. For invasion assay, the upper surface of the chambers was pre-coated with 50 μl of Matrigel (2 mg/mL) (Becton, Dickinson and Company) and incubated at 37°C for at least 4 h. After rinsing with pre-warmed PBS, human endometrial epithelial cells were harvested and washed, and 200 μl of cell suspension containing 50 μM chloroquine (CQ) or 10 mM 3-Methyladenine (3-MA) was added to the matrigel of the upper compartments, and 600 μl of DMEM/F12 culture medium containing 40% FBS was added into the lower chamber. The human endometrial epithelial cells cultured in normoxic condition for 48 h were used as the control groups. The cells cultured under hypoxic condition with or without 50 μM CQ or 10 mM 3-MA for 48 h were used as experimental groups. To evaluate the migration potential, cells were allowed to migrate towards medium over a period of 24 h.

Table 2. Immunostaining score for HIF-1α, LC3, E-cadherin, vimentin, and S100A4.

	Immunostaining score (mean ± SD)				
	HIF-1α	LC3	E-cadherin	Vimentin	S100A4
Normal endometrium (N)	3.210 ± 1.273	1.517 ± 2.146	6.734 ± 1.165	3.720 ± 2.839	1.937 ± 1.332
Eutopic endometrium (U)	4.165 ± 2.305	2.236 ± 1.737	6.227 ± 2.762	4.122 ± 3.537	2.282 ± 2.004
Ectopic endometrium (C)	8.772 ± 2.732	7.978 ± 4.237	2.175 ± 2.260	6.421 ± 3.449	5.815 ± 4.513
Statistical analysis					
N versus U	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$
N versus E	$^{**}P < 0.01$	$^{**}P < 0.01$	$^{*}P < 0.05$	$^{**}P < 0.01$	$^{*}P < 0.05$
U versus E	$^{*}P < 0.05$	$^{**}P < 0.01$	$^{*}P < 0.05$	$^{*}P < 0.05$	$^{*}P < 0.05$

$^{*}P < 0.05$, $^{**}P < 0.01$.
All data are expressed as mean ± SD.
Statistical significance (one-way ANOVA analysis).

Ishikawa cells transfected with or without si HIF-1α or si Beclin1 were resuspended in RPMI-1640 culture medium without FBS and then plated in the matrigel of the upper compartments. RPMI-1640 culture medium (600 μl) plus 10% FBS was added to the lower chamber, and the plate was incubated in hypoxia or normoxia conditions for 24 h (for migration assay) or 48 h (for invasion assay). After indicated time, cells were fixed in methanol for 20 min and stained with 0.5% Coomassie brilliant blue for another 20 min. Then the cells on the upper surface of the chambers were removed by wiping with a cotton swab, and the filters were washed three times with ice-cold PBS. The cells on the underside of the chambers were immediately observed and counted under an inverted microscope at ×200 magnification. Duplicate wells per condition were tested in three independent experiments.

Statistical analysis

Statistical calculations were performed using GraphPad Prism software (version 6.01; GraphPad Software). The Kruskal–Wallis test was used for statistical significance of differences in variables with nonnormal distribution. The Student *t* test and one-way analysis of variance followed by the Tukey post hoc test was used to measure the comparisons between groups in normal distribution. All data sets were shown as mean ± standard deviations from at least three independent experiments. For all tests, differences of *P* values of <0.05 were considered statistically significant.

Results

HIF-1α, LC3, E-cadherin, vimentin, and S100A4 protein expression and localization in ectopic endometrium of endometriosis patients

To investigate the functional relevance of HIF-1α, autophagy, and EMT during endometriosis, we first performed immunohistochemistry staining to detect the protein expression level of HIF-1α, autophagy marker LC3, E-cadherin, vimentin, and S100A4in normal endometrium, eutopic and ectopic endometrium from women with endometriosis. HIF-1α was predominantly localized in the nuclei of epithelial and stromal cells; however, LC3 was expressed within the cytoplasm of both cell types. The expression levels of HIF-1α and LC3 in the ectopic endometrium were significantly greater than those in normal endometrium and eutopic endometrium from women with endometriosis. E-cadherin was distributed strongly in the membranes of epithelial cells of normal and eutopic endometrium, and exhibited a dramatic decrease in the ectopic endometrium. The vimentin protein was abundantly expressed in the cytoplasm of epithelial and stromal cells of normal and eutopic endometrium and was significantly increased in ectopic endometrium sections (Figure 1A and Table 2). No S100A4 expression was detected in epithelial cells of normal and eutopic endometrium. Immunostaining scores for S100A4 expression in epithelial cells were significantly higher in ovarian endometriosis compared with those of normal and eutopic endometrium. Nonspecific rabbit IgG was used as a negative control and showed no immunostaning (Figure 1A, fifth row). Afterwards, we detected the protein levels of HIF-1α, LC3, E-cadherin, and vimentin by western blot analysis. The results showed that the protein expression levels of HIF-1α, total accumulation of LC3-II, and vimentin were significantly increased in ectopic endometrium compared to normal and eutopic endometrium, while the expression level of E-cadherin was significantly decreased in ectopic endometrium (Figure 1B and C). Taken together, the data provided evidence that EMT process occurred during endometriosis, and HIF-1α and autophagy may be involved in the pathological process.

Hypoxia induces epithelial-to-mesenchymal transition in human endometrial epithelial cells

To investigate the potential for hypoxia to induce EMT in human endometrial epithelial cells, we conducted several investigations. We first examined the expression levels of EMT-related protein (E-cadherin, vimentin, and N-cadherin) using western blot analysis. With increasing incubation time under hypoxia condition (0, 4, 8, 16, and 24 h), expression levels of the epithelial marker E-cadherin were downregulated; however, vimentin and N-cadherin, the mesenchymal biomarkers, were increased in human endometrial epithelial cells (Figure 2A and B). We next monitored the morphologic appearance of human endometrial epithelial cells in response to hypoxia under microscopy. After exposure to hypoxia for 24 h, typical morphological changes occurred in human endometrial epithelial cells. The cells became elongated, spindle-shaped, and fibroblast-like, together with loss of tight junctions between cells (Figure 2C). In addition, immunocytochemical staining showed that after hypoxia treatment for 24 h, E-cadherin protein was reduced in majority of human endometrial epithelial cells. In contrast, expression of vimentin was extensively detected in these cells (Figure 2D). The migration and invasion abilities of hypoxic endometrial epithelial cells were further tested. Compared with cells under normoxic condition, hypoxia treatment remarkably increased the number of migrated and invaded cells, and endometrial epithelial cells in transwell assay

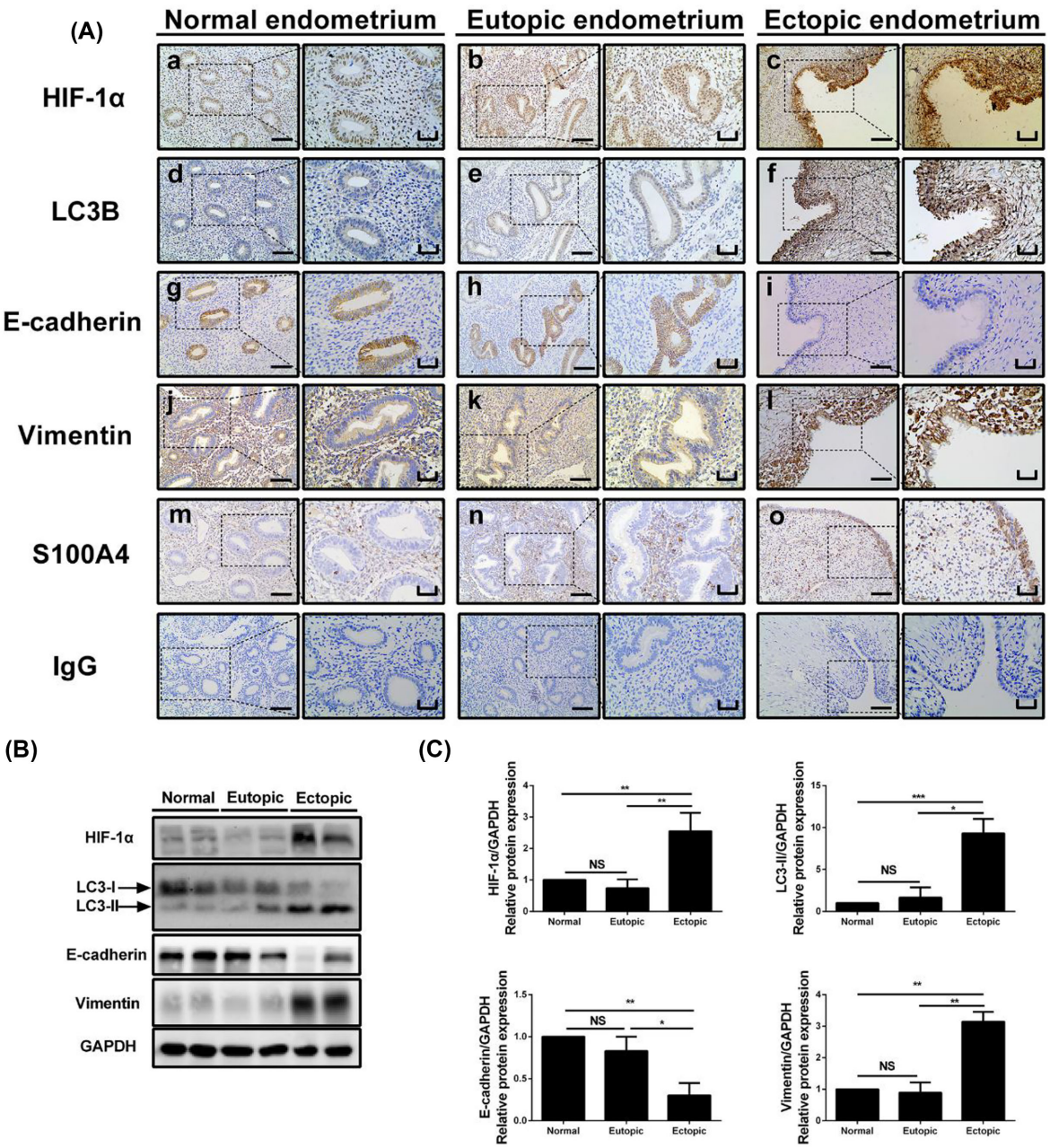

Figure 1. HIF-1α, LC3, E-cadherin, vimentin, and S100A4 localization and expression in different groups of endometria. (**A**) Representative immunohistochemical analysis of HIF-1α, LC3, E-cadherin, vimentin, and S100A4 protein localization in normal endometrium (a, d, g, j, m), eutopic endometrium (b, e, h, k, n), and ectopic endometrium (c, f, i, l, o). Photographs were taken at magnifications of $\times$200 (left panels) and $\times$400 (right panels), respectively. Left bar = 50 μm, right bar = 25μm. Normal IgG is used as negative control. (**B**) Representative western blots analysis of HIF-1α, LC3, E-cadherin, and vimentin protein levels in normal endometrium, eutopic endometrium, and ectopic endometrium. (**C**) Representative quantitative data of densitometric analyses. GAPDH was used as a loading control. The data are presented as the means $\pm$ SD of three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

(Figure 6A–D). Collectively, these results suggested that hypoxia could sufficiently trigger EMT in endometrial epithelial cells.

Hypoxia upregulates autophagy in human endometrial epithelial cells

To examine whether hypoxia induces autophagy in human endometrial epithelial cells, the cells were cultured in hypoxia condition for increasing time points (0, 4, 8, 16, and 24 h). Western blot analysis displayed that protein expression levels of HIF-1α, Beclin1, a ubiquitin E1-like enzyme essential for autophagosome formation, and LC3-II were gradually increased by hypoxia treatment (Figure 3A and B). Transmission electron microscopy examination revealed that the number of autophagosomes was also increased (Figure 3C). For the reason that the increased LC3-II protein level could be resulted from increased autophagosomes formation or decreased autophagosome degradation. We further evaluated autophagy flux with or without the presence of lysosomal degradation inhibitor CQ. [28].

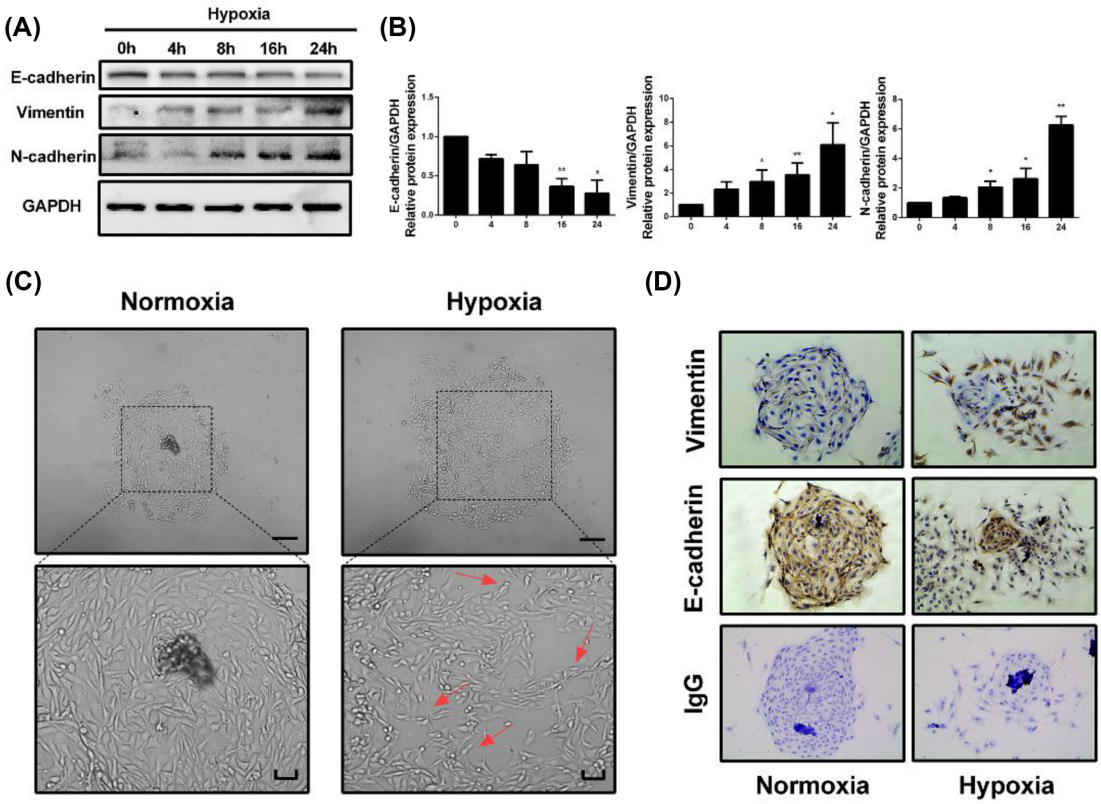

Figure 2. Hypoxia induces EMT in human endometrial epithelial cells. (**A**) Human endometrial epithelial cells were cultured under hypoxic condition for the indicated times (0, 4, 8, 16, and 24 h), and the EMT-related protein E-cadherin, vimentin, and N-cadherin were monitored by western blotting analysis. GAPDH used as the loading control. (**B**) Quantitative comparison of the fold difference in the expression of E-cadherin, vimentin, and N-cadherin proteins. The data are presented as the means ± SD of three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by one-way ANOVA). (**C**) The figure shows the changed cellular morphologies of normoxic- and hypoxic-treated treated endometrial epithelial cells. Normal IgG is used as negative control. Photographs were taken at magnifications of ×200 (scale bar = 50 μm) and ×400 (scale bar = 25μm). (**D**) The figure shows the immunocytochemistry staining of E-cadherin and vimentin protein in endometrial epithelial cells following hypoxia treatment for 24 h.

As shown in Figure 3D and E, a blockade of the autophagosome–lysosome fusion using CQ significantly increased the accumulation of endogenous LC3-II protein, and hypoxia apparently augmented this effect, indicating that hypoxia-induced elevation of LC3-II was due to increased autophagosomes formation rather than a blockage of lysosomal degradation. Altogether, our data indicate that hypoxia is able to induce autophagy in human endometrial epithelial cells.

HIF-1α mediates hypoxia-induced autophagy in Ishikawa cells

Next, to explore the role of HIF-1α in hypoxia-induced autophagy, we conducted the following experiments. Ishikawa cells were first treated by hypoxia for 24 h, then upregulation of HIF-1α, Beclin1, and LC3-II protein was observed by western blot (Figure 4A and B). In addition, we found that compared to the normoxia control group, the Ishikawa cells cultured under hypoxic conditions for 24 h showed dense accumulation of GFP-LC3 puncta in the perinuclear region (Figure 4C and D). Ishikawa cells were then transfected with a HIF-1α expression plasmid under normoxic condition. Compared to control group and NC group, the cells transfected with the HIF-1α expression plasmid significantly increased the protein expression of HIF-1α, Beclin1, and LC3-II (Figure 4E and F). To further validate the role of HIF-1α, Ishikawa cells were transiently transfected with an siRNA containing an HIF-1α targeting sequence. Compared to the Ishikawa cells transfected with control siRNA, Ishikawa cells transfected with HIF-1α siRNA under hypoxia condition showed decreased expression of HIF-1α, Beclin1, and LC3-II (Figure 4G and H). These results indicated that hypoxia-induced autophagy is mediated through HIF-1α.

Inhibition of autophagy attenuates hypoxia-induced endometrial cells migration and invasion

To explore the relationship between autophagy and EMT under hypoxic conditions, human endometrial epithelial cells were treated with hypoxia combined with autophagy inhibitor 3-MA or CQ. Our results showed that both 3-MA and CQ clearly inhibited autophagy in hypoxia-treated human endometrial epithelial cells, accompanied with the downregulation of mesenchymal markers vimentin and the upregulation of epithelial marker E-cadherin (Figure 5A–D).

Furthermore, siRNA to knock down Beclin 1 was used to inhibit autophagy in hypoxia-treated Ishikawa cells. The results showed that hypoxia-induced EMT was significantly suppressed by Beclin1 siRNA (Figure 5E and F). Taken together, these results demonstrated that autophagy facilitates hypoxia-triggered endometrial cells EMT.

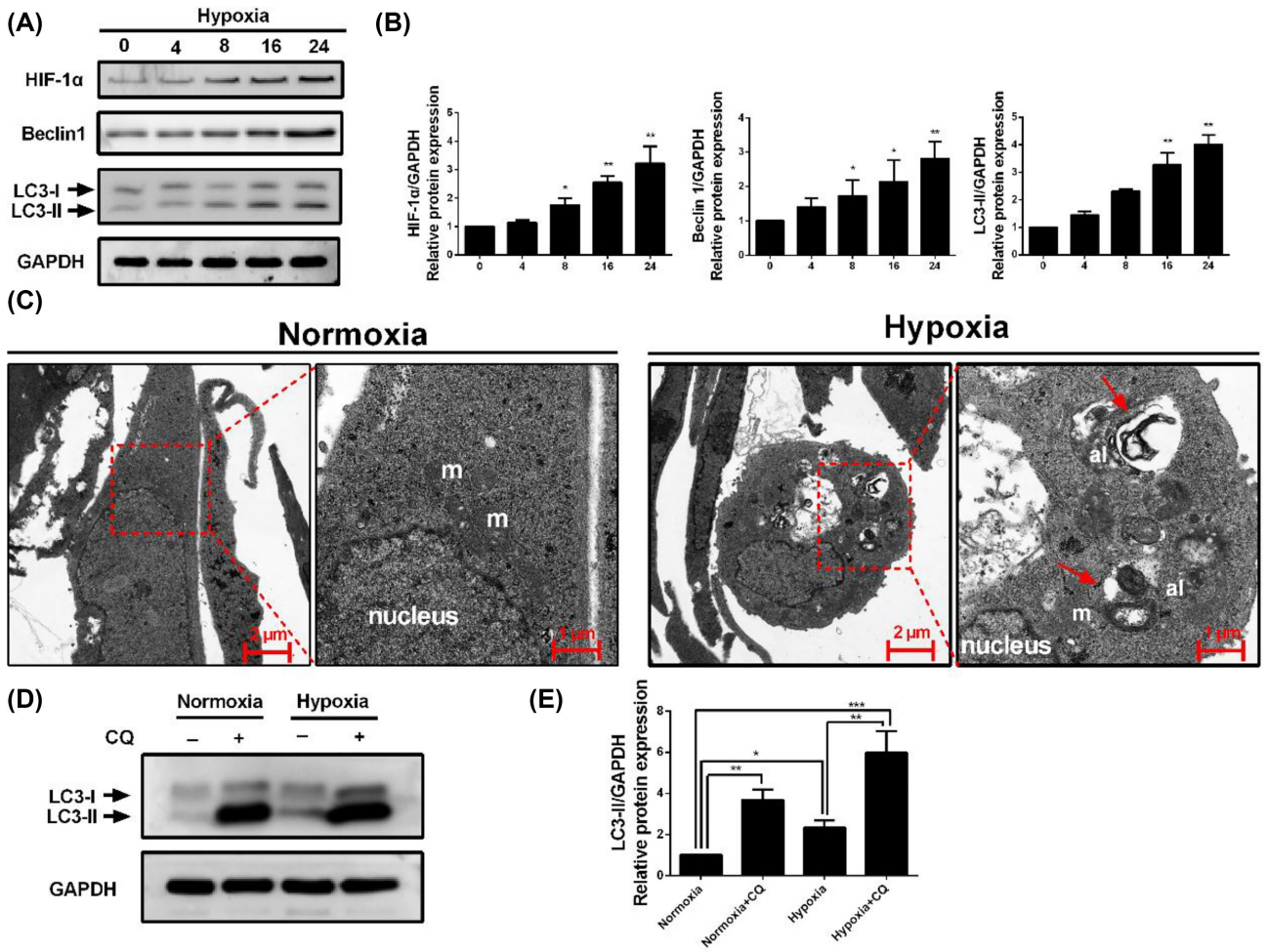

Figure 3. Hypoxia induces autophagy in human endometrial epithelial cells. (**A**) Human endometrial epithelial cells were cultured under hypoxic condition for the indicated times (0, 4, 8, 16, and 24 h), and then the expression of HIF-1α and autophagy-related protein Beclin1 and total accumulation of LC3-II were detected by western blotting analysis. GAPDH used as the loading control. (**B**) Quantitative comparison of the fold difference in the expression of HIF-1α, Beclin1, and total accumulation of LC3-II proteins. The data are presented as the means ± SD of three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by one-way ANOVA). (**C**) Human endometrial epithelial cells were incubated under hypoxic condition for 24 h, and then were analyzed by transmission electron microscopy (TEM). Autophagical vacuoles containing organelle remnants were highlighted by red arrows. Al: autophagolysosomes; m: mitochondria. In each group of figures, calibration bars in the left figure of each group = 2 μm, while those in the right figure of each group = 1 μm. (**D**) The difference in LC3-II expression levels between normoxic and hypoxic conditions with or without CQ was compared by western blotting. (**E**) Quantitative comparison of the fold difference in the expression of the total accumulation of LC3-II protein. The data are presented as the means ± SD of three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by one-way ANOVA).

Inhibition of autophagy attenuated hypoxia-induced migration and invasion of endometrial cells

To determine the effect of autophagy on cell migration and invasion, human endometrial epithelial cells were treated by hypoxia for 24 h with and without autophagy inhibitors and then assessed by performing transwell migration and invasion assay. The results showed that human endometrial epithelial cells treated by hypoxia displayed a higher ability of migration and invasion compared to the control cells; however, when co-treated with autophagy inhibitors 3-MA and CQ, the migratory and invasive potential were dramatically decreased (Figure 6A–D).

To further examine whether autophagy contributes to endometrial cells migration and invasion, we reduced Beclin1 expression by using the RNA interference approach in Ishikawa cells. We found that reduced Beclin1 expression by siRNA also significantly attenuated migratory and invasive capability of Ishikawa cells under hypoxia conditions (Figure 6E–H). These observations indicated that autophagy facilitates the hypoxia-triggered migration and invasion of endometrial cells in vitro.

Discussion

The present study demonstrates for the first time that autophagy induction is involved in hypoxia-induced human endometrial epithelial cells EMT, which in turn enhanced cell migration and invasion in endometriosis.

Endometriosis is a very common disorder associated with certain biological characteristics reminiscent of malignant tumors, including local tissue invasion and spread to adjacent and distant organs [3]. The migration and invasion of viable endometrial tissues outside the uterine cavity are crucial steps for the development of endometriosis [29]. Although the etiology of endometriosis remains obscure,

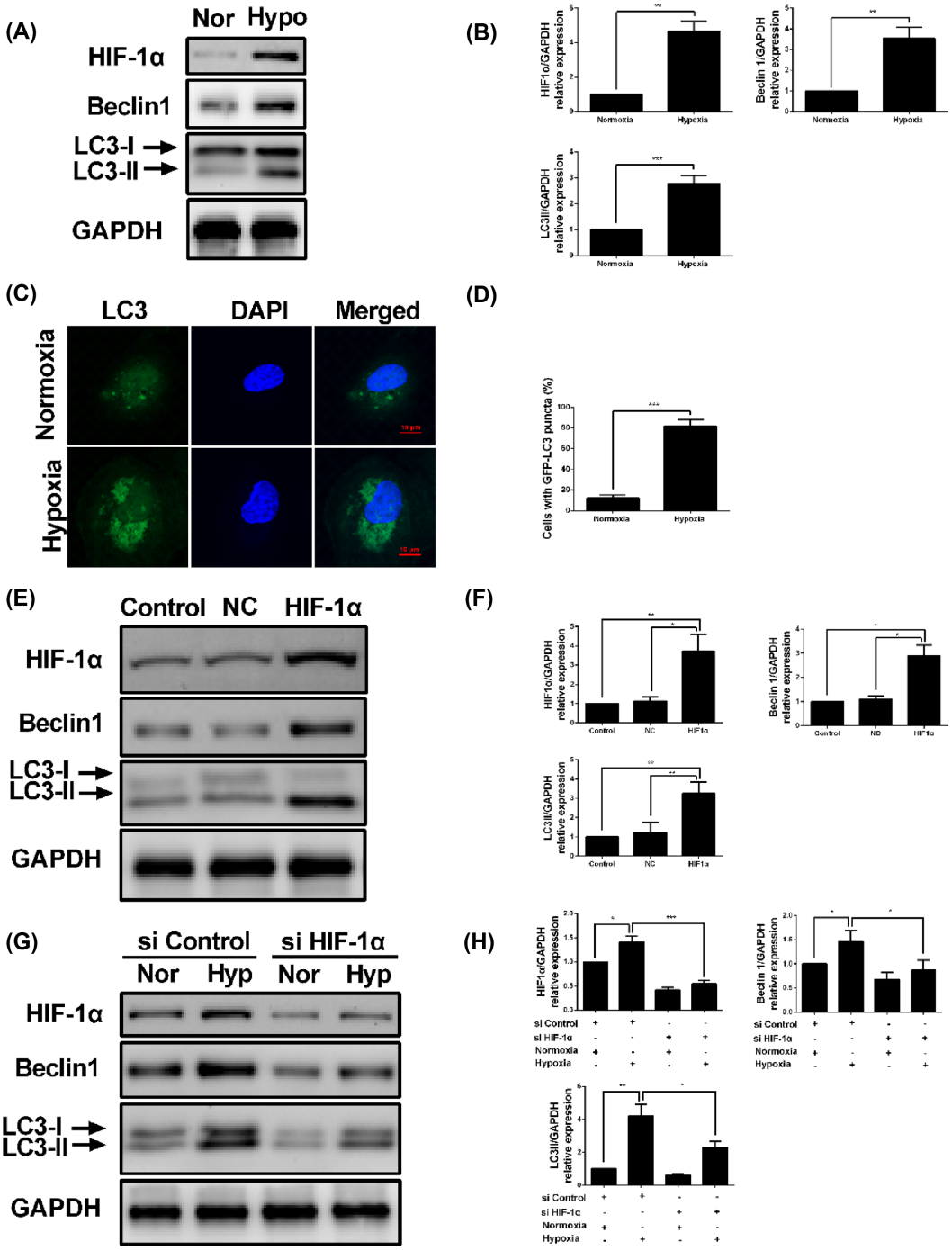

Figure 4. HIF-1α mediated hypoxia-induced autophagy in endometrial cells. (**A**) Ishikawa cells were incubated under normoxic and hypoxic condition for 24 h, and then the expression of HIF-1α and autophagy-related protein Beclin1 and total accumulation of LC3-II were detected by western blotting analysis. GAPDH used as the loading control. (**B**) Quantitative comparison of the fold difference in the expression of HIF-1α, Beclin1, and total accumulation of LC3-II proteins. The data are presented as the means ± SD of three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by one-way ANOVA). (**C**) Ishikawa cells are transfected with GFP-MAP-LC3 adenoviral vector for 24 h and then treated with normoxia or hypoxia for another 24 h. Punctate distribution of LC3 was visualized under the laser scanning confocal fluorescence microscopy at ×800 magnification, and representative images were presented. Scale bar: 10 μm. (**D**) Quantification of the percentage of cells displaying punctate GFP-MAP-LC3 at the indicated time after hypoxia treatment. The data are presented as the means ± SD of three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). (**E**) Ishikawa cells are transfected with negative control (NC) plasmid or HIF-1α expression plasmid under normoxic condition for 24 h and incubated under normoxic condition for another 24 h. The expression of HIF-1α, Beclin1, and LC3-II was monitored by western blot. GAPDH was used as the loading control. (**G**) Ishikawa cells are transfected with control or HIF-1α siRNA for 24 h and incubated with or without hypoxia for 24 h. The expression of HIF-1α, Beclin1, and LC3-II was monitored by western blot. GAPDH was used as the loading control. (**F and H**) Quantitative comparison of the fold difference in the expression of HIF-1α, Beclin1, and total accumulation of LC3-II proteins. The data are presented as the means ± SD of three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by one-way ANOVA).

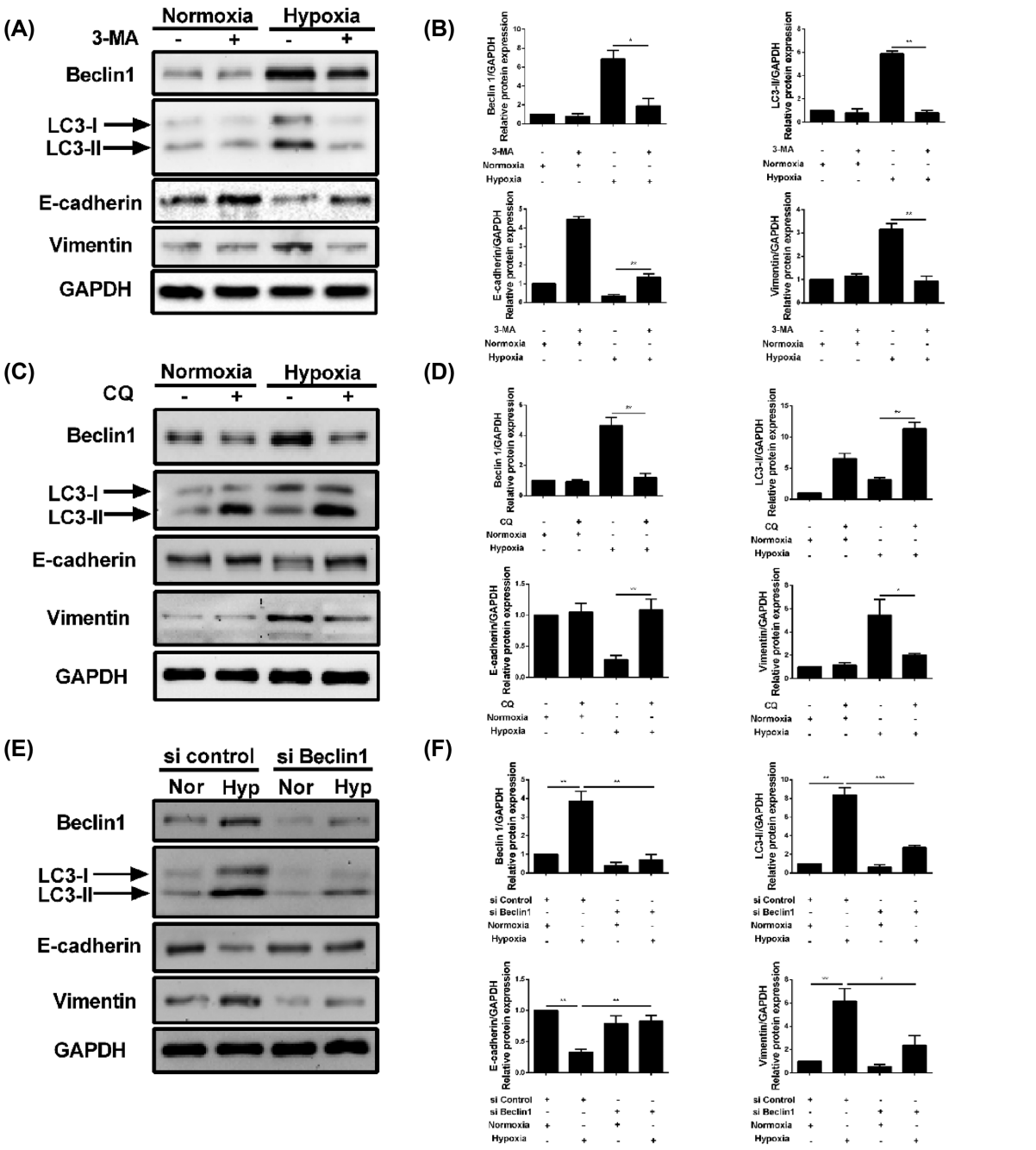

Figure 5. Autophagy promotes hypoxia-triggered EMT in endometrial cells. (**A**) Human endometrial epithelial cells were treated with 3-MA (10 m M) for 24 h and incubated with or without hypoxia for 24 h. The expression of Beclin1, LC3-II, E-cadherin, and vimentin was monitored by western blot. GAPDH was used as the loading control. (**B**) Quantitative comparison of the fold difference in the expression of Beclin1, LC3-II, E-cadherin, and vimentin proteins. The data are presented as the means ± SD of three independent experiments (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ by one-way ANOVA). (**C**) Human endometrial epithelial cells were treated with CQ (50 μM) for 24 h and incubated with or without hypoxia for 24 h. The expression of Beclin1, LC3-II, E-cadherin, and vimentin was monitored by western blot. GAPDH was used as the loading control. (**D**) Quantitative comparison of the fold difference in the expression of Beclin1, LC3-II, E-cadherin, and vimentin proteins. The data are presented as the means ± SD of three independent experiments (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ by one-way ANOVA). (**E**) Ishikawa cells are transfected with Beclin1 siRNA or with scrambled negative control siRNA for 24 h and incubated with or without hypoxia for 24 h. The expression of Beclin1, LC3-II, E-cadherin, and vimentin was monitored by western blot. GAPDH was used as the loading control. (**F**) Quantitative comparison of the fold difference in the expression of Beclin1, LC3-II, E-cadherin, and vimentin proteins. The data are presented as the means ± SD of three independent experiments (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ by one-way ANOVA).

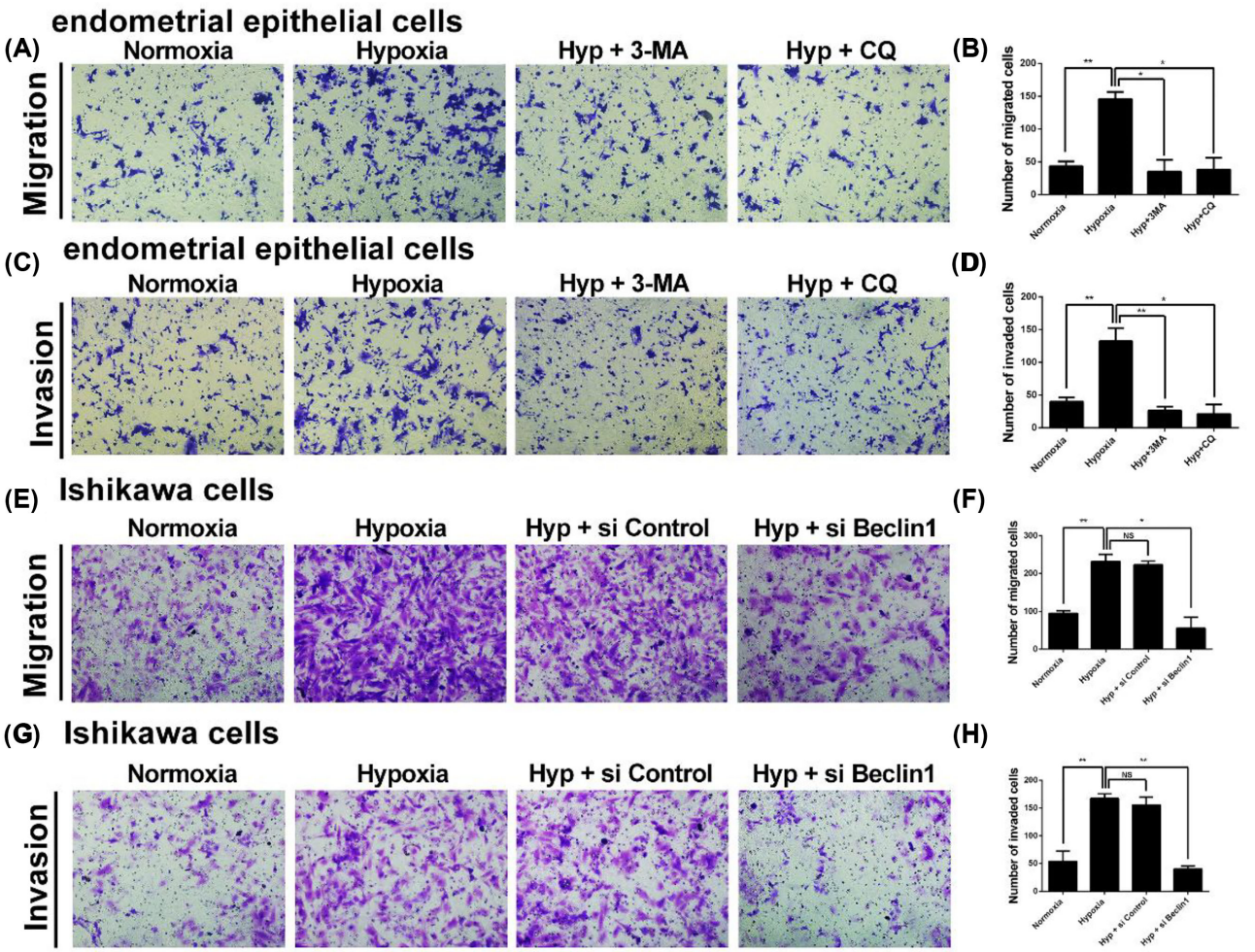

Figure 6. Inhibition of autophagy attenuates hypoxia-induced endometrial cells migration and invasion. (**A and C**) Human endometrial epithelial cells cultured in normoxic or hypoxiac condition were treated with CQ (50 μM) and 3-MA (10 mM) for 24 h, and then cell migration and invasion abilities were examined by transwell assay. (**E and G**) Ishikawa cells cultured in normoxic or hypoxic condition were transfected with Beclin1 siRNA or with scrambled negative control siRNA. The migration and invasion abilities were examined by transwell assay. (**B, D, F, and H**) The migrated and invaded cells were counted in five random fields from each treatment. The data are presented as the means ± SD of three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). Photographs were taken at magnifications of ×200.

multiple hypotheses have been brought forward in the literature to explain the pathogenesis of this disease. Among these, the most widely accepted is the theory of retrograde menstruation proposed by Sampson [30]. However, retrograde menstruation occurs in up to 90% of women and thus cannot account for the only 5%–10% of women that develop endometriosis. Researchers have found that other factors like local hypoxia may contribute to the development of endometriosis. According to Sampson's theory, when shed endometrial debris retrogrades to the peritoneal cavity, the first stress faced is the local altered hypoxic microenvironment. Accumulating evidence indicated that hypoxia may be important for the pathogenesis of endometriosis [8, 9, 31]. In addition, our previous work has established that the HIF-1α level is elevated in ectopic endometrium of endometriosis and hypoxia promotes invasion of endometrial stromal cells [32].

Recently, increasing evidence suggests that the aberrant activation of EMT process, which is necessary for the invasion–metastasis of cancer, was also involved in the initial formation of endometriosis [6, 7, 33]. Previous studies have shown that hypoxia can trigger the EMT process by upregulating the expression of HIF-1α and other EMT-related transcription factors, thereby inducing a more aggressive tumor phenotype and conferring on tumor cells the ability to invade to distant locations [34, 35]. For example, HIF-1α promoted EMT through activation of Snail1 transcription in hepatocellular carcinoma cells [36] and direct upregulation of Zeb1 by hypoxia response element 3 in colorectal cancer [37]. Consistent with other studies [33], the present study showed that EMT-like process occurs during ovarian endometriosis with decreased expression of E-cadherin but increased expression of vimentin in the epithelial cells of endometriotic lesions. HIF-1α was highly expressed in ectopic endometrium tissue and positively correlated with EMT process. Moreover, hypoxia in endometrial epithelial cells promoted EMT phenotype and facilitates cell migration and invasion. Taking our current data together, we demonstrate that hypoxia can trigger EMT of endometriosis.

Autophagy is a highly regulated process which exerts a critical influence on multiple human pathophysiological processes, including neurodegeneration, cancer, and immune-related disorders [38].

Both autophagy and EMT can, for example, be triggered by adverse stresses, such as starvation and hypoxia [39, 40]. Hypoxia leads to HIF-1α stabilization, and subsequently activates the downstream gene BNIP3 that competes with Bcl-2 and Bcl-XL for interaction with Beclin1 to trigger autophagy [16]. Recently, upregulated autophagy was observed in ovarian endometriomas and this process possibly contributing to survival of endometriotic cells in ectopic sites and to lesion maintenance [21]. In this study, we demonstrated that levels of LC3 were elevated in ectopic endometrium tissues of endometriosis. Our data further demonstrated that hypoxia upregulates autophagy activity in both endometrial epithelial cells and Ishikawa cells, and HIF-1α is required for hypoxia-induced autophagy. Taking these data together, we demonstrate that hypoxia can upregulate endometrial cell autophagy level in a HIF-1α-dependent manner.

As described above, both autophagy and EMT could be induced by hypoxia. Yet the relationship between autophagy and EMT under hypoxia conditions is unclear. The complex reciprocal interplay between autophagy and EMT has gradually emerged in the past few years [19, 41]. During the metastatic spreading process, autophagy activation is essential for the activation of EMT. Li et al. reported that autophagy enhances the invasion abilities of hepatocellular carcinoma through activation of EMT [42]. Inhibition of autophagy by either genetic or pharmacological means prevented the acquisition of the mesenchymal phenotype in breast cancer cells [43]. Conversely, however, it was also reported that autophagy can inhibit the EMT process. Induction of autophagy has been shown to attenuate EMT by selectively promoting the degradation of the Snail and Twist in hepatocyte [44] and human breast cancer [45]. In glioblastoma cells, autophagy activation was able to impair migration and invasion by reversing EMT process [46]. This discrepancy could be explained by the fact that autophagy is a highly regulated process, which can be perceived as acting like a double-edged sword in tumor metastasis, either through promoting or reversing EMT process, depending on the different cellular type and/or on stage of disease progression. In the present study, we found the elevated LC3 expression is positively and strongly correlated with EMT process in the same cohort of ectopic endometrium samples. Our data also demonstrate that inhibition of autophagy dramatically mitigated hypoxia-enhanced EMT and attenuated the migration and invasion of endometrial cells. These results suggest that autophagy positively regulates the hypoxia induced EMT and invasion of endometrial cells. However, it is unclear at present how autophagy exactly regulates EMT in human endometriosis under hypoxia conditions. Considered that many EMT-related transcription factors, such as Snail and Twist, account for the initiation of EMT process, it is possible that autophagy may regulate their expression indirectly promoting the EMT in endometriosis [18, 47]. Therefore, this point needs to be further investigated.

However, on a cautionary note, it should be pointed out that some limitations of our present study should be mentioned. First, the endometrium samples were only collected during the proliferative phase and the sample size is relatively small, which may limit the interpretable power of the current results. Thus, in future studies, the larger sample sizes collected from the entire menstrual cycle should be used. Second, Ishikawa cells were used instead of primary endometrial epithelial cells, which are very difficult to grow and passage for transfection and part of the transwell experiments. Third, only late stage (stages III and IV) endometrioic cyst lesions were employed and thus results may not translate to lesions from stages I and II. Fourth, the results may be unique to ovarian endometriomas and provide little insight into peritoneal and deep infiltrating lesions. Thus, additional investigation is still needed.

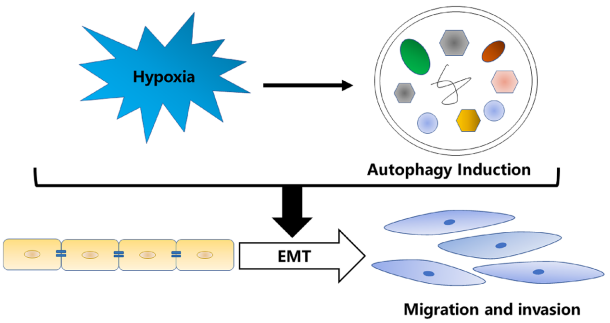

Figure 7. Proposed model illustrating the effect of autophagy on hypoxia-induced endometrial epithelial cells EMT.

In summary, to our knowledge, this is the first report to show that autophagy promotes hypoxia-triggered EMT, which is required for enhanced migration and invasion of endometrial cells (Figure 7). These findings not only improve our understanding of the molecular mechanisms underlying EMT induction in endometriosis, but also provide evidence that inhibition of autophagy may be new potential therapeutic targets for the treatment of endometriosis.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplemental Figure S1. Identification of markers for isolation of human endometrial epithelial cells. Immuofluorescence microscopy staining of (**A**) vimentin in red represents and (**B**) E-cadherin in green represents. (**C**) The slides incubated with isotype-matched immunoglobulin G from normal rabbits were applied as a negative control. Blue signal represents nuclear DNA staining by DAPI. Human endometrial epithelial cells were judged by negative cellular staining for vimentin and positive staining for E-cadherin. Photographs were taken at magnifications of $\times$1600. Data presented are from three independent experiments.

Supplemental Table S1. Commercial sources and characteristics of antibodies used.

Acknowledgments

We gratefully thank our Central Laboratory for Experimental Medicine, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. We also would like to thank Pei Zhang and An Na Du from the Core Facility and Technical Support, Wuhan Institute of Virology, for her help with producing TEM micrographs.

Conflict of interest: The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

References

1. de Ziegler D, Borghese B, Chapron C. Endometriosis and infertility: pathophysiology and management. *Lancet* 2010; **376**:730–738.
2. Dunselman G, Vermeulen N, Becker C, Calhaz-Jorge C, D'Hooghe T, De Bie B, Heikinheimo O, Horne A, Kiesel L, Nap A. ESHRE guideline: management of women with endometriosis. *Hum Reprod* 2014; **29**:400–412.
3. Bassi MA, Podgaec S, Dias Junior JA, Sobrado CW, N DAF. Bowel endometriosis: a benign disease?. *Rev Assoc Med Bras* 2009; **55**:611–616.

4. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest* 2009; **119**:1420–1428.
5. Yang J, Weinberg RA. Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. *Dev Cell* 2008; **14**:818–829.
6. Yang YM, Yang WX. Epithelial-to-mesenchymal transition in the development of endometriosis. *Oncotarget* 2017; **8**:41679–41689.
7. Bilyk O, Coatham M, Jewer M, Postovit LM. Epithelial-to-mesenchymal transition in the female reproductive tract: from normal functioning to disease pathology. *Front Oncol* 2017; **7**:145.
8. Hsiao KY, Lin SC, Wu MH, Tsai SJ. Pathological functions of hypoxia in endometriosis. *Front Biosci (Elite Ed)* 2015; **7**:309–321.
9. Zhan L, Wang W, Zhang Y, Song E, Fan Y, Wei B. Hypoxia-inducible factor-1alpha: a promising therapeutic target in endometriosis. *Biochimie* 2016; **123**:130–137.
10. Lin SC, Lee HC, Hou PC, Fu JL, Wu MH, Tsai SJ. Targeting hypoxia-mediated YAP1 nuclear translocation ameliorates pathogenesis of endometriosis without compromising maternal fertility. *J Pathol* 2017; **242**:476–487.
11. Semenza GL. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *J Appl Physiol* 2000; **88**:1474–1480.
12. Semenza GL. Oxygen sensing, hypoxia-inducible factors, and disease pathophysiology. *Annu Rev Pathol Mech Dis* 2014; **9**:47–71.
13. Yang SW, Zhang ZG, Hao YX, Zhao YL, Qian F, Shi Y, Li PA, Liu CY, Yu PW. HIF-1alpha induces the epithelial-mesenchymal transition in gastric cancer stem cells through the Snail pathway. *Oncotarget* 2017; **8**:9535–9545.
14. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. *Nature* 2008; **451**:1069–1075.
15. Yang Z, Klionsky DJ. An overview of the molecular mechanism of autophagy. *Curr Top Microbiol Immunol* 2009; **335**:1–32.
16. Bellot G, Garcia-Medina R, Gounon P, Chiche J, Roux D, Pouysségur J, Mazure NM. Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains. *Mol Cell Biol* 2009; **29**:2570–2581.
17. Kroemer G, Mariño G, Levine B. Autophagy and the integrated stress response. *Mol Cell* 2010; **40**:280–293.
18. Mowers EE, Sharifi MN, Macleod KF. Autophagy in cancer metastasis. *Oncogene* 2017; **36**:1619–1630.
19. Gugnoni M, Sancisi V, Manzotti G, Gandolfi G, Ciarrocchi A. Autophagy and epithelial–mesenchymal transition: an intricate interplay in cancer. *Cell Death Dis* 2016; **7**:e2520.
20. Zou M, Zhu W, Wang L, Shi L, Gao R, Ou Y, Chen X, Wang Z, Jiang A, Liu K, Xiao M, Ni P et al. AEG-1/MTDH-activated autophagy enhances human malignant glioma susceptibility to TGF-β1-triggered epithelial-mesenchymal transition. *Oncotarget* 2016; **7**:13122–13138.
21. Allavena G, Carrarelli P, Del Bello B, Luisi S, Petraglia F, Maellaro E. Autophagy is upregulated in ovarian endometriosis: a possible interplay with p53 and heme oxygenase-1. *Fertil Steril* 2015; **103**:1244–1251.e1. e1241.
22. Liu H, Zhang Z, Xiong W, Zhang L, Xiong Y, Li N, He H, Du Y, Liu Y. Hypoxia-inducible factor-1alpha promotes endometrial stromal cells migration and invasion by upregulating autophagy in endometriosis. *Reproduction* 2017; **153**:809–820.
23. Canis M, Donnez JG, Guzick DS, Halme JK, Rock JA, Schenken RS, Vernon MW. Revised American Society for Reproductive Medicine classification of endometriosis: 1996. *Fertil Steril* 1997; **67**:817–821.
24. Remmele W, Schicketanz KH. Immunohistochemical determination of estrogen and progesterone receptor content in human breast cancer. *Pathol Res Pract* 1993; **189**:862–866.
25. Xiong Y, Liu Y, Xiong W, Zhang L, Liu H, Du Y, Li N. Hypoxia-inducible factor 1alpha-induced epithelial-mesenchymal transition of endometrial epithelial cells may contribute to the development of endometriosis. *Hum Reprod* 2016; **31**:1327–1338.
26. Nishida M, Kasahara K, Kaneko M, Iwasaki H, Hayashi K. Establishment of a new human endometrial adenocarcinoma cell line, Ishikawa cells, containing estrogen and progesterone receptors. *Nihon Sanka Fujinka Gakkai Zasshi* 1985; **37**:1103–1111.
27. Holinka CF, Hata H, Kuramoto H, Gurpide E. Effects of steroid hormones and antisteroids on alkaline phosphatase activity in human endometrial cancer cells (Ishikawa line). *Cancer Res* 1986; **46**:2771–2774.
28. Mizushima N, Yoshimori T. How to Interpret LC3 Immunoblotting. *Autophagy* 2007; **3**:542–545.
29. Moggio A, Pittatore G, Cassoni P, Marchino GL, Revelli A, Bussolati B. Sorafenib inhibits growth, migration, and angiogenic potential of ectopic endometrial mesenchymal stem cells derived from patients with endometriosis. *Fertil Steril* 2012; **98**:1521–1530.e2. e1522.
30. Sampson JA. Peritoneal endometriosis due to the menstrual dissemination of endometrial tissue into the peritoneal cavity. *Am J Obstet Gynecol* 1927; **14**:422–469.
31. Lu Z, Zhang W, Jiang S, Zou J, Li Y. Effect of oxygen tensions on the proliferation and angiogenesis of endometriosis heterograft in severe combined immunodeficiency mice. *Fertil Steril* 2014; **101**:568–576.
32. Xiong W, Zhang L, Xiong Y, Liu H, Liu Y. Hypoxia promotes invasion of endometrial stromal cells via hypoxia-inducible factor 1α upregulation-mediated β-catenin activation in endometriosis. *Reprod Sci* 2016; **23**:531–541.
33. Matsuzaki S, Darcha C. Epithelial to mesenchymal transition-like and mesenchymal to epithelial transition-like processes might be involved in the pathogenesis of pelvic endometriosis†. *Hum Reprod* 2012; **27**:712–721.
34. Cooke VG, LeBleu VS, Keskin D, Khan Z, O'Connell JT, Teng Y, Duncan MB, Xie L, Maeda G, Vong S, Sugimoto H, Rocha RM et al. Pericyte depletion results in hypoxia-associated epithelial-to-mesenchymal transition and metastasis mediated by met signaling pathway. *Cancer Cell* 2012; **21**:66–81.
35. Duan Y, He Q, Yue K, Si H, Wang J, Zhou X, Wang X. Hypoxia induced Bcl-2/Twist1 complex promotes tumor cell invasion in oral squamous cell carcinoma. *Oncotarget* 2017; **8**:7729–7739.
36. Zhang L, Huang G, Li X, Zhang Y, Jiang Y, Shen J, Liu J, Wang Q, Zhu J, Feng X, Dong J, Qian C. Hypoxia induces epithelial-mesenchymal transition via activation of SNAI1 by hypoxia-inducible factor -1alpha in hepatocellular carcinoma. *BMC Cancer* 2013; **13**:108.
37. Zhang W, Shi X, Peng Y, Wu M, Zhang P, Xie R, Wu Y, Yan Q, Liu S, Wang J. HIF-1alpha promotes epithelial-mesenchymal transition and metastasis through direct regulation of ZEB1 in colorectal cancer. *PLoS One* 2015; **10**:e0129603.
38. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. *Cell* 2008; **132**:27–42.
39. Mazure NM, Pouyssegur J. Hypoxia-induced autophagy: cell death or cell survival? *Curr Opin Cell Biol* 2010; **22**:177–180.
40. Jiang J, Tang YL, Liang XH. EMT: a new vision of hypoxia promoting cancer progression. *Cancer Biol Ther* 2011; **11**:714–723.
41. Marcucci F, Ghezzi P, Rumio C. The role of autophagy in the cross-talk between epithelial-mesenchymal transitioned tumor cells and cancer stem-like cells. *Mol Cancer* 2017; **16**:3.
42. Li J, Yang B, Zhou Q, Wu Y, Shang D, Guo Y, Song Z, Zheng Q, Xiong J. Autophagy promotes hepatocellular carcinoma cell invasion through activation of epithelial–mesenchymal transition. *Carcinogenesis* 2013; **34**:1343–1351.
43. Cufi S, Vazquez-Martin A, Oliveras-Ferraros C, Martin-Castillo B, Vellon L, Menendez JA. Autophagy positively regulates the CD44(+) CD24 (-/low) breast cancer stem-like phenotype. *Cell Cycle* 2011; **10**:3871–3885.
44. Grassi G, Di Caprio G, Santangelo L, Fimia GM, Cozzolino AM, Komatsu M, Ippolito G, Tripodi M, Alonzi T. Autophagy regulates hepatocyte identity and epithelial-to-mesenchymal and mesenchymal-to-epithelial transitions promoting Snail degradation. *Cell Death Dis* 2015; **6**: e1880–.
45. Lv Q, Wang W, Xue J, Hua F, Mu R, Lin H, Yan J, Lv X, Chen X, Hu ZW. DEDD Interacts with PI3KC3 to activate autophagy and attenuate epithelial-mesenchymal transition in human breast cancer. *Cancer Res* 2012; **72**:3238–3250.

46. Catalano M, D'Alessandro G, Lepore F, Corazzari M, Caldarola S, Valacca C, Faienza F, Esposito V, Limatola C, Cecconi F, Di Bartolomeo S. Autophagy induction impairs migration and invasion by reversing EMT in glioblastoma cells. *Mol Oncol* 2015; **9**:1612–1625.

47. Pang M, Wang H, Rao P, Zhao Y, Xie J, Cao Q, Wang Y, Wang YM, Lee VW, Alexander SI, Harris DC, Zheng G. Autophagy links beta-catenin and Smad signaling to promote epithelial-mesenchymal transition via upregulation of integrin linked kinase. *Int J Biochem Cell Biol* 2016; **76**:123–134.