Title: The estrogen-regulated lncRNA *H19*/miR-216a-5p axis alters stromal cell invasion and migration via *ACTA2* in endometriosis

Running title: Estrogen/*H19*/miR-216a-5p/*ACTA2* in endometriosis

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Abstract

Fibrotic tissue may contribute to the origin of some endometriosis-related symptoms, such as chronic pelvic pain and infertility. Alterations in the *H19*/miR-216a-5p/*ACTA2* pathway may mediate the regulation of eutopic endometrial stromal cell (euESC) invasion and migration and may represent a potential mechanism underlying fibrous tissue formation or fibrosis in women with endometriosis. In this study, we aimed to determine the expression of *H19* and ACTA2 in endometrial tissues of women with endometriosis. Two groups of 23 infertile women with endometriosis and 23 matched infertile women without endometriosis were investigated. Primary cultured cells of endometrial tissues were analysed using RT-PCR and western blotting (WB) to determine expression of *H19* and ACTA2. EdU, CCK8, and Transwell assays were used to study the functions of *H19* and ACTA2. HEK 293 cells were used for luciferase assays to study miR-216a-5p binding sites with *H19* and *ACTA2*. We found that *H19* and ACTA2 levels were significantly higher in endometriosis euESCs than in control euESCs ($P < 0.05$) and were positively correlated in endometriosis euESCs. Luciferase assays indicated that *H19* regulates *ACTA2* expression via competition for inhibitory miR-216a-5p binding sites. Our results indicate that alterations in the estrogen/*H19*/miR-216a-5p/*ACTA2* pathway regulated endometriosis euESC invasion and migration. Downregulation of *H19* or ACTA2 inhibited endometriosis euESC invasion and migration, however estrogen promoted endometriosis euESC invasion and migration via H19. The main limitation of our study was that experiments were conducted in vitro and further in-vivo studies are required in the future. However, our study showed that primary cultured cells represented endometriosis cells more clearly than cell lines.

Key words: endometriosis, lnc*H19*/miR-216a-5p/*ACTA2* pathway, fibrosis, invasion, migration, primary eutopic endometrial stromal cells

Introduction

Endometriosis is an estrogen-dependent, benign and chronic disorder characterised by the presence of endometrial glands and stroma outside of the uterine (Vercellini, Vigano et al. 2014). The disease affects ~10% of women of reproductive age, and ~50% of patients are infertile (Zondervan, Becker et al. 2018). Fibrotic tissue and pelvic adhesions may contribute to the origin of some endometriosis-related symptoms, such as chronic pelvic pain, infertility and amenorrhea (Somigliana, Vigano et al. 2012). Affected women are at a greater risk than the general female population of developing ovarian cancer, breast cancer and other cancers (Giudice and Kao 2004, Kajiyama, Suzuki et al. 2019). The pathogenesis of endometriosis remains unclear; the most widely accepted theory is the implantation theory(John 1927, J, M G et al. 1984), which involves endometrial tissue passing through patent fallopian tubes and being implanted outside uterine sites during the menstrual cycle (J, M G et al. 1984). According to implantation theory, two crucial endometrial cell processes are involved in the establishment of endometriosis: migration and invasion (Zeitvogel, Baumann et al. 2001, Giudice and Kao 2004, Hull, Escareno et al. 2008, King, Barbara et al. 2016). The endometrium is derived from the intermediate mesoderm in the mesenchymal to epithelial transition during the development of the urogenital system; that is to say, endometrial cells have some imprint of mesenchymal origin (Giudice 2010, Masuda, Matsuzaki et al. 2010). During wound healing, endometrial cells can gain invasion and migration capabilities and exhibit fibroblast-to-myofibroblast transdifferentiation (FMT) (Matsuzaki and Darcha 2012, Donnez, Van Langendonckt et al. 2013). During endometrial tissue repair, endometriotic lesions arise owing to repeated tissue injury and repair, with dense endometrial stromal fibrotic tissue in and surrounding the lesions (Bonte, Chapron et al. 2002, Itoga, Matsumoto et al. 2003, Giudice and Kao 2004, Guo 2015). Endometriotic lesions undergo FMT,

resulting in increased cellular contractility and collagen production, ultimately leading to fibrosis (Zhang, Duan et al. 2016, Duan, Liu et al. 2018).

ACTA2, also called alpha smooth muscle actin (α-SMA), is a critical marker of smooth muscle cells in fibrosis and is involved in vascular contractility and blood pressure homeostasis (Chen, Peters et al. 2017). Continuous exposure to activated platelets leads to the increased expression of ACTA2 and markers of differentiated smooth muscle cells in endometrial stromal cells, resulting in smooth muscle metaplasia. Zhang et al. (Zhang, Duan et al. 2016) showed that FMT, smooth muscle metaplasia and fibrogenesis are all involved in endometriotic lesion development. Herrera et al. (Herrera, Herrera et al. 2014) found that SNAI1 and ACTA2 are related to carcinoma-associated fibroblast cell invasion and migration, and Fu reported that ACTA2 is expressed in breast cancer stroma tissues (Fu, Song et al. 2014). ACTA2 is also positively expressed in endometriotic lesions (Khare, Martin et al. 1996, Anaf, Simon et al. 2000, Barcena de Arellano, Gericke et al. 2011).

Non-coding RNA has become a major focus of studies of various diseases (Ulitsky and Bartel 2013, Cech and Steitz 2014). The lncRNA *H19* was the first imprinted gene identified; it is 2.3 kb (11p15.5) and contains the reciprocally imprinted *IGF2* gene (Zhang and Tycko 1992). In the human and mouse endometrium, *H19* expression increases during the late proliferative phase in the menstrual cycle (Adriaenssens, Lottin et al. 1999, Korucuoglu, Biri et al. 2010). *H19* expression is positively regulated by estrogen and negatively regulated by progesterone in mice (Ivanga, Labrie et al. 2007). Furthermore, 17β-E2 promotes cell proliferation in endometriosis via the NF-kB pathway (Zhang, Zhao et al. 2010, Chowdhury, Banerjee et al. 2019). *H19* can act as an mRNA sponge to function as a competing endogenous mRNA (ceRNA) or bind to related proteins to regulate gene transcription and translation, thus inhibiting promoter and transcription factor interactions (Wang, Yang et al. 2017). Previous studies have shown that *H19* promotes tumour cell invasion and migration

via ceRNA pathways (Rainier, Johnson et al. 1993, Yan, Zhou et al. 2015). However, there was no significant difference in endometrial stromal cells. Considering the shared features between endometriosis and tumours, we hypothesised that in endometriosis, eutopic endometrial stromal cells invasion and migration are regulated by *H19* via ceRNA pathways. In this study, we show that the expression levels of *H19* and ACTA2 are significantly increased in endometrial stromal cells in endometriosis and provide evidence that *H19* acts as a ceRNA to regulate *ACTA2* expression by competing for miR-216a-5p binding sites. Thus, the dysfunction of *H19* and ACTA2 in endometriosis are important for endometrial stromal cell invasion and migration, contributing to fibrous tissue formation. This study presents the first evidence for crosstalk between *H19* miR-216a-5p and *ACTA2* in endometrial stromal cells, providing new molecular targets for the treatment of endometriosis.

Materials and Methods

Patients and sample collection

Fresh endometrial eutopic tissues from 23 patients with endometriosis were collected during the late proliferative phase of the menstrual cycle (Day11~13) and from 23 women of reproductive-aged who underwent oviduct obstruction as controls. The diagnosis was confirmed by laparoscopy. Samples were collected from July 2017 to February 2018 at the Reproductive Hospital Affiliated to Shandong University. Women undergoing hysteroscope-laparoscopic surgery for suspected endometriosis were confirmed by histological examination. Both the endometriosis and control groups of women had no comorbidites such as PCOS, POI or IBD. Informed consent was obtained from patients before surgery. All patients received no hormonal treatments for three months prior to surgery. Patients data were collected, including age, body mass index and hormone levels. The study was approved by the Institutional Review Board (IRB) of the Reproductive Hospital Affiliated to Shandong

University (registration number 2014-39).

Human endometrial stromal cell isolation and culture

Eutopic endometrial stromal cells (euESCs) were isolated from fresh tissues and cultured following previously reported methods (Zhang, Li et al. 2009). Briefly, fresh tissues obtained during abdominal hysteroscopy combined with surgery were placed in 5 ml of sterile PBS. Tissues were washed twice with sterile PBS to remove blood, minced into small pieces using ophthalmic scissors, and incubated with 0.25% collagenase type I (Gibco, Gaithersburg, MD, USA) in a water bath for 1 h at 37°C. Collagenase activity was terminated by adding 3 volumes of PBS. The euESCs were obtained after filtering through a 100-μm monofilament nylon mesh and then through a 38.5-μm monofilament nylon mesh. The euESC suspension was collected and centrifuged at 1000 rpm for 5 min. Cells were maintained in phenol red-free DMEM/F12 (1:1) (11330-032; Gibco, Life Technologies) supplemented with 10% FBS (BI, Beit She'an, Israel) and 1% penicillin–streptomycin in a 5% CO_2 atmosphere at 37°C incubator. Culture medium was changed every 2 days. For passaging, when cells reached 80~90% density, 1ml trypsin (Gibco, Life Techenologies) was used to digest cells for 2 minutes in an incubator, then 3 ml culture medium added to stop the digestion. The primary cultured cells of passage 2 were used for subsequent experiments. The primary ESCs images are shown in Supplemental Figure 1.

HEK 293 cells were cultured with 90% DMEM (Gibco) supplemented with 10% FBS and 1% P/S in a 5% CO_2 atmosphere at 37°C. HEK 293 cells were used for Luciferase assays as tool cells based on previous studies (Lv, Tong et al. 2018).

Transfection assay and cell treatments

MiR-216a-5p mimics and si*ACTA2* were purchased from Genepharma (GenePharma, Co., Ltd., Shanghai, China). si*H19* was purchased from Life Technologies (Carlsbad, CA, USA). When endometriosis euESCs reached 70-80% confluence, transfection was conducted with

Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 24 h, the cells were harvested for RT-PCR. Cells were harvested after 48 h for other assays.

17β-Estradiol was dissolved in ethanol at a stock concentration of 10 mM. The treatment concentrations were determined according to published papers (Meng, Han et al. 2007, Song, Chi et al. 2015); euESCs were treated with 17β-estradiol for 1 h at 10 nM.

RNA extraction and qRT-PCR

TRIzol reagent (Invitrogen) was used to extract total RNA from primary cultured cells. A UV spectrophotometer was used to measure the total RNA concentration; an A260/280 ratio of 1.9-2.2 was considered for inclusion in our study. Total RNA (2μg) was used for the synthesis of cDNA according to PrimeScript RT Reagent Kit with gDNA Eraser (RR047,Takara) miRNA according to Mir-X™ miRNA First-Strand Synthesis Kit (638313, Takara). PCR primers were designed using Primer 3.0 web; the primer sequences are shown in Supplemental Table 1. The SYBR Fluorescent Quantitative Premix Kit (Takara, Shiga, Japan) was used for qRT-PCR. A total volume of 10 μL was used for the Light Cycler 480 real-time polymerase chain reaction system (Roche, Basel, Switzerland). PCR reactions were performed in a 10μl volume containing 1μl cDNA, 1 μl 20pmol of each primer, 5μl buffer supplied by the manufacturer and 3 μl ddH$_2$O. *U6* was as internal reference for miR-216a-5p, and *GAPDH* was used as a control for other mRNAs. The PCR reaction conditions were pre-denaturation at 95°C for 30s, 95°C for 5s, and 60°C for 30s for a total of 45 cycles. The data were analysed by using the $2^{-\Delta\Delta Ct}$ relative-expression method.

Protein extraction and western blotting

Radioimmunoprecipitation assay buffer (RIPA, Beyotime, Shanghai, China) was used to lyse cells after transfection for 48 h, followed by the determination of the protein concentration using bicinchoninic acid (BCA; Beyotime). The protein was separated by 10% sodium

dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P). After blocking in 5% non-fat milk at room temperature for 1 h, the primary antibody was added and incubated in a refrigerator at 4°C overnight. Tris-buffered saline-Tween (TBST) was used to wash the membrane for 15 min 3 times, and the corresponding secondary antibody was added and incubated for 1 h at room temperature(about 25°C). After washing each sample for 15 min 3 times with TBST, the protein was detected using the ECL detection system (Millipore, Billerica, MA, USA). Primary antibodies against rabbit anti-human α-SMA (1:1000 dilution, Abcam ab5695, Cambridge, UK) and mouse anti-human GAPDH (1:10000 dilution; Proteintech 60004-1-Ig, Rosemont, IL, USA) were used. Goat anti-rabbit and Goat anti-mouse HRP-conjugated secondary antibodies were obtained from ZSGB-BIO (1:10000 dilution, Beijing, China).

Cell Counting Kit-8 (CCK-8) experiments

Endometriosis euESCs were seeded in 96-well plates at 2×10^3 cells per well, with three replicate wells. They were then transfected, incubated for 0, 24, 48, 72, and 96 h, supplemented with 10 µL of CCK8 (Beyotime), and incubated at 37°C for 2 h. Cells reached about 90% confluence at 96 h. A microplate reader was subsequently used to measure absorbance (OD value) at 450 nm, and growth curves were drawn.

5-Ethyl-2′-Deoxyuridine Incorporation (EdU) assay

For the EdU assay, endometriosis euESCs were incubated with 50 µmol/L EdU (RiboBio, Guangzhou, China) for 6 h at 37°C and then fixed with 4% paraformaldehyde for 30 min at room temperature. After incubation with Apollo reaction reagent for 30 min, the cells were stained with Hoechst 33342. The Olympus 1X51 (Tokyo, Japan) was used to capture images of endometriosis euESCs, and cells in five random fields per group were counted. The EdU incorporation rate was estimated as the ratio of EdU-positive cells to Hoechst-positive cells.

Transwell assays

Transwell assays were performed using 24-well plates with 8-µm pore size inserts (Corning Life Sciences, Corning, NY, USA) according to the manufacturer's protocols. Endometriosis euESCs were plated into Transwell chambers after 48 h of transfection. The essential difference between the migration and invasion assays is that for migration assays, cells can migrate through the inserts directly but for invasion assays, they have to invade the matrix to reach the inserts. Details are shown as follows.

In the migration assay, the endometriosis euESCs (10^5 cells/well) were seeded into the upper chamber in 200 µL of serum-free phenol red-free DMEM/F12 and allowed to migrate for 24 h to the lower chamber, which contained phenol red-free DMEM/F12 with 10% FBS.

In the invasion assay, Matrigel (1 mg/mL; BD Biosciences, Franklin Lakes, NJ, USA), prepared in serum-free phenol red-free DMEM/F12 medium, was placed in the upper chamber and incubated for 8 h at 37°C. Similar to the migration assay, endometriosis euESCs (10^5 cells/well) were seeded in the upper chamber with 200 µL of serum-free medium and allowed to invade the lower chamber, which contained medium with 10% FBS, for 36 h. Transwell filters were fixed with 4% paraformaldehyde for 30 min, stained with hematoxylin for 30 min, and fixed on a glass slide. Cells that did not migrate or invade were wiped off with cotton swabs. The results were expressed as the number of cells that migrated or invaded per field, as counted using the Olympus 1X51 microscope in five random fields.

Dual Luciferase Reporter Assay

The *H19/ACTA2* wild-type (WT) fragment containing miR-216a-5p binding sites or *H19/ACTA2* mutant type (MUT) fragments were inserted into a dual-luciferase miRNA target expression vector (pmirGLO; Promega, Madison, WI, USA). HEK 293 cells were seeded on 24-well plates and co-transfected with a luciferase reporter plasmid (*H19/ACTA2* WT or *H19/ACTA2* MUT) and miR-216a-5p using Lipofectamine 3000 (Invitrogen), with negative

controls (NC). A dual-luciferase reporter assay system (Promega) was used to assess luciferase activity according to the manufacturer's protocols. Relative luciferase activity was measured as the ratio of firefly to *Renilla* luciferase activity.

Immunocytochemistry

Primary human endometrial stromal cells were fixed with 4% paraformaldehyde into slides. Immunocytochemistry according to the manufacturer's protocols (ZSGB-BIO PV9001). Cells were incubated with primary antibody rabbit anti-human vimentin (1:200 dilution; Proteintech, USA) at 4°C overnight. After being washed with PBS 3 times, slides were incubated with second antibody for 20 min at room temperature. The slides were conterstained with hematoxylin for 20 min. Finally, the slides were digitally photographed using an Olympus 1X51 microscope.

Statistical Analysis

Statistical analyses were performed using SPSS 21.0 (Statistical Package for the Social Sciences, SPSS, Inc., Chicago, IL, USA). Values were expressed as means ± SD, and comparisons were performed using Student's *t*-tests. Pearson's correlation coefficients were obtained to evaluate the relationship between the mRNA expression levels of *H19* and *ACTA2*. ANOVA test were used for estrogen stimulation three times. $P < 0.05$ was considered statistically significant. Each experiment was repeated three times.

Results

Characteristics of subjects in the endometriosis and control groups

The clinical characteristics of subjects in the endometriosis and control groups are shown in Supplementary Table 2. There were no significant differences in age, body mass index (BMI), follicle stimulating hormone (FSH), luteinizing hormone (LH) or serum prolactin

(PRL) between the endometriosis and control groups. Patients in endometriosis and control groups were all in the late proliferative phase from day 11-13 of the menstrual cycle. Estrogen levels were significantly higher in the endometriosis group than in the controls ($P < 0.05$). Based on The revised American Society for Reproductive Medicine (ASRM) classification, there were eight patients classified as Stage I, three as Stage II, ten as Stage III, and two as Stage IV.

The expression levels of *H19* and *ACTA2* were determined by quantitative RT-PCR using 23 endometriosis euESC samples and 23 control euESC samples. *H19* levels were significantly higher in euESCs of the endometriosis group than those from controls (0.3931 ± 0.06817 vs. 0.8935 ± 0.1500, *P=0.0119*) (Figure 1A). *ACTA2* mRNA levels were also higher in endometriosis euESCs than those from controls (0.4638 ± 0.06346 vs. 1.089 ± 0.1731, *P=0.0061*) (Figure 1B). A Pearson's correlation analysis suggested a positive relationship between *H19* and *ACTA2* mRNA in endometriosis euESCs (*P<0.0001*, R=0.6136), implying positive interactions between the two factors (Figure 1D). There was no such correlation in euESCs from the control group (*P=0.1587*, R=0.3698) (Figure 1E). Western blotting also indicated that the protein levels of ACTA2 were increased in the endometriosis euESCs compared to control euESCs (Figure 1C). These results suggested that increased expression of *H19* and ACTA2 might explain the development and function of the eutopic endometrium in patients with endometriosis.

Primary cultured cells used were from the second passage, with an euESC purity of >98% (Zhang, Li et al. 2010). Cells were stained with Rabbit anti-human vimentin antibody to identify human endometrial stromal cells (Supplemental Figure 2). *H19* expression in eutopic endometrial epithelial cells of the endometriosis and control groups were not significantly different (0.3974 ± 0.06131 vs. 0.5009 ± 0.06397, *P=0.2491*, Supplemental Figure 3).

H19 and ACTA2 were essential for euESC function in endometriosis

The effects of *H19* and *ACTA2* on endometriosis euESC proliferation were determined by CCK8 and EdU assays after the knockdown of *H19* (si*H19*) or *ACTA2* (si*ACTA2*) by siRNA transfection. The results of the CCK8 and EdU assays indicated that low *H19* expression significantly decreased endometriosis euESC proliferation (Figures 2A, 2C). The EdU-positive cell ratios compared with the negative controls in the si*H19* group were 23.83 ± 1.628 vs. 17.18 ± 1.034, respectively (*P*= 0.001; Figure 2C). However, based on the CCK8 assay and EdU assay, the low expression of *ACTA2* had no significant effect on endometriosis euESC proliferation (Figures 2B, 2D). The EdU-positive cell ratios compared with the negative controls in the si*ACTA2* group were 31.37 ± 2.699 vs. 32.79 ± 1.225, respectively (*P = 0.556;* Figure 2D). There was no difference in proliferation between non-transfected (basal) euESCs from endometriosis and control patients, with EdU-positive cell ratios of the control and endometriosis groups being 16.73±0.965 vs. 15.14±0.734, respectively (*P* = 0.201; Supplemental Figure 4).

We also assessed the basal difference in migration and invasion in the endometriosis and control groups and found that migration and invasion were significantly greater in the euESCs of the endometriosis group compared to the control group (*P* < 0.0001) (Supplemental Figure 5). Furthermore, the effects of *H19* and *ACTA2 o*n endometriosis euESC invasion and migration were determined by Transwell assays after the knockdown of *H19* (si*H19*) or *ACTA2* (si*ACTA2*), as summarised in Figure 2E and2F. In invasion assays, si*H19* significantly decreased the invasion of endometriosis euESCs compared to that in the NC group (352.4 ± 9.822 vs. 159.1 ± 5.864, *P* < 0.0001; Figure 2E). si*ACTA2* also significantly decreased the invasion of endometriosis euESCs compared to that in the NC group (299.0 ± 8.462 vs. 102.7 ± 2.696, *P* < 0.0001; Figure 2F). In migration assays, si*H19* significantly decreased the migration of endometriosis euESCs compared to that in the NC group (290.1 ± 10.99 vs. 164.7 ± 6.372, *P* < 0.0001; Figure 2E). si*ACTA2* also significantly

decreased the migration of endometriosis euESCs compared to that in the NC group (405.8 ± 10.03 vs. 177.8 ± 9.043, $P < 0.0001$; Figure 2F). Together, these results suggest that the increased expression of *H19* and *ACTA2* might be essential for the migratory and invasive functions of euESCs in patients with endometriosis.

H19 regulated ACTA2 expression via competition for miR-216a-5p binding sites

A Pearson's correlation analysis indicated that there was a positive correlation between the *H19* and *ACTA2* levels in endometriosis euESCs (*R = 0.6136, P < 0.0001*; Figure 1D). When *H19* was knocked down by siRNA, the *ACTA2* mRNA and ACTA2 protein levels were decreased in endometriosis euESCs (Figure 1G and 1H). The knockdown efficiency of *H19* was demonstrated by RT-PCR (Figures 1F).

A bioinformatics analysis showed that miR-216a-5p can bind to *H19* and to *ACTA2* in the 3′ untranslated region (3′UTR). The binding sites are shown in Figures 3C and 3D (www.targetscan.org; www.microrna.gr/microT; bibiserv.techfak.uni-bielefeld.de/rnahybrid; cbcsrv.watson.ibm.com/rna22.html). Therefore, miR-216a-5p levels were significantly lower in euESCs in the endometriosis group than in the control group (1.208 ± 0.09437 vs. 0.5921 ± 0.07995, $P < 0.0001$) (Figure 3A) and negatively correlated with *H19* in the endometriosis group (*P = 0.0031, R = 0.5893*; Figure 3B).

We investigated cells overexpressing miR-216a-5p compared with endometriosis euESCs transfected with control (NC) mimics and observed downregulated *H19* and *ACTA2* expression ($P < 0.01$, Figures 3C, 3D). To further test whether *H19* and *ACTA2* are direct targets of miR-216a-5p, dual-luciferase reporter vectors were used in HEK 293 cells using fragments of *H19* or *ACTA2* containing the miR-216a-5p binding sites. Compared to the NC mimics, miR-216a-5p mimic transfection significantly reduced the luciferase activity of *H19* and *ACTA2*; however, this effect was eliminated when the targeting sites were mutated (*P = 0.0035*; $P = 0.001$; Figures 3E and 3F).

To verify the role of the *H19*/miR-216a-5p axis in regulating endometriosis euESC invasion and migration, we performed *H19* knockdown experiments combined with the inhibitor i216a-5p. Endometriosis euESCs were transfected with a control mixture or a mixture containing si*H19* with or without i216a-5p, followed by an analysis of gene expression and cell function. *H19* knockdown inhibited the expression of *ACTA2* in endometriosis euESCs, but co-transfection with i216a-5p rescued this inhibition ($P < 0.05$; Figure 3G), suggesting negative feedback regulation between *H19* and i216a-5p. Western blotting showed similar results (Figure 3H). Functional rescue by i216a-5p was further supported by endometriosis euESC invasion and migration assays ($P < 0.001$; Figure 3I).

Estrogen promoted invasion and migration via H19

The effects of estrogen on the expression of *H19* and ACTA2 were evaluated by RT-PCR and western blot analyses respectively. We used estrogen to treat endometriosis euESCs for 0, 30, and 60 min. H19 levels reached a peak at 30 min (P=0.0004), while miR-216a-5p were lower at 30 min and 60 min (P=0.0006), and *ACTA2* levels reached a peak at 60 min (Figures 4A, 4B, 4C) The invasion and migration of endometriosis euESCs were determined by Transwell assays. Estrogen significantly increased the invasion of endometriosis euESCs transfected with si*H19* ($P < 0.0001$, Figure 4D) after treatment for 60 min. Similar data were obtained in migration assays. Estrogen significantly increased the migration of endometriosis euESCs after *H19* knockdown ($P < 0.01$, Figure 4D) after treatment for 60 min. The effect of estrogen treatment on the *H19* knockdown cells was not due to a rescue of proliferation in these cells. A short time for estrogen stimulation could not significantly increase the proliferation of endometriosis euESCs after *H19* knockdown ($P > 0.05$) (Figure 4E).

Discussion

In the current study, we detected increased expression of lncRNA *H19* and its positively related protein-coding gene *ACTA2* in endometriosis euESCs. Moreover, we demonstrated that *H19* post-transcriptionally modulates the expression of *ACTA2* by competitively binding to miR-216a-5p. These results indicated that the estrogen/*H19*/miR-216a-5p/*ACTA2* pathway plays a critical role in endometriosis by regulating euESC invasion and migration. The process is briefly described in Figure 4F.

The pathogenesis of endometriosis is still not clear. Endometriosis is characterised by the presence of endometrial glands and stroma with surrounding fibrous tissue (Giudice and Kao 2004, Zondervan, Becker et al. 2018). ACTA2 is the most commonly used molecular marker for smooth muscle cells, which are important for fibrosis. Khare et al. (Khare, Martin et al. 1996) first reported that ACTA2 is positively expressed in peritoneal endometriosis. In 2000, Anaf et al. (Anaf, Simon et al. 2000) demonstrated that 21 endometriotic peritoneal lesions were positive for ACTA2. Barcena de Arellano et al. (Barcena de Arellano, Gericke et al. 2011) showed that all 60 endometriotic lesions exhibited *ACTA2 ex*pression. Therefore, smooth muscle seems to represent an important and consistent feature of endometriosis (Yan, Liu et al. 2018, Ibrahim, Sillem et al. 2019). However, the above-mentioned studies have evaluated ACTA2 in ectopic tissues or endometriotic lesions, and few studies have focused on euESCs from endometriosis patients, which are equally important in the pathogenesis of endometriosis. In this study, we explored endometriosis euESCs and detected significantly higher *ACTA2* expression in women with endometriosis than in those without endometriosis. Furthermore, ACTA2 stimulated endometriosis euESC invasion and migration.

A few studies have shown that the lncRNA *H19* enhances fibrous tissue formation, cell proliferation, invasion, and migration (Lottin, Adriaenssens et al. 2005, Ghazal, McKinnon et al. 2015, Yan, Zhou et al. 2015, Song, Liu et al. 2017). Lottin et al. (Lottin, Adriaenssens et

al. 2005) detected *H19* overexpression in the stroma of women with myometrium, similar to our results in which *H19* was highly expressed in endometriosis euESCs. *H19* and *ACTA2* levels were positively correlated in endometriosis euESCs in this study, but negatively correlated in the euESCs from the controls. Previous studies have demonstrated that lncRNAs regulate the expression of target genes via various mechanisms, including transcription as antisense RNAs, the production of endogenous small interference RNAs, regulation of mRNA stability, etc. (Tutar 2012, Johnsson, Morris et al. 2014). The emerging ceRNA paradigm indicates that pseudogenes can regulate parental RNA expression as competitive RNAs.

In this study, miR-216a-5p was compromised when *H19* expression was high, leading to the decreased bioavailability of miR-216a-5p and increased expression of *ACTA2*, which in turn activated endometriosis euESC invasion and migration. Downregulated *H19* decreased *ACTA2* expression via increased miR-216a-5p. Our luciferase assay also demonstrated that *H19* and *ACTA2* are direct targets for miR-216a-5p. *H19* regulated the expression of *ACTA2* by acting as a ceRNA in endometriosis euESCs. Wang et al. (Wang, Xu et al. 2014) indicated that miR-216a is a cancer suppressor and inhibits tumour cell progression, consistent with our results, showing that miR-216a inhibited cell invasion and migration.

Endometriosis has traditionally been viewed as an estrogen-dependent, proliferative and invasive disorder involving endometrial cells in ectopic locations (Giudice and Kao 2004). However, previous data have shown ectopic endometrium defects in women with endometriosis, and not in women without endometriosis (Zhang, Zhao et al. 2010). During the proliferative phase of the menstrual cycle, estrogen stimulates the expression of *H19* in the endometrium (Ivanga, Labrie et al. 2007). Our 23 patients showed significantly higher estrogen levels compared to controls, and the *H19*/miR-216a-5p/*ACTA2* pathway appeared to be altered in women with endometriosis with persistent high-level estrogen exposure.

Normal euESCs grow quickly during the proliferative phase of the menstrual cycle when estrogen levels are high. Moreover, our findings suggested that alterations in the *H19*/miR-216a-5p/*ACTA2* pathway impair euESC functions in women with endometriosis. High levels of estrogen increased *H19*, which acted as a sponge to downregulate miR-216a-5p, thereby stimulating *ACTA2* and promoting cell invasion and migration.

Our results have unveiled novel gene regulatory pathways involved in dysfunctional endometrial development in women with endometriosis. Based on our results, we proposed a model for the *H19*/miR-216a-5p/*ACTA2*-mediated regulation of endometriosis euESC invasion and migration. Patients with endometriosis with high levels of estrogen have increased *H19* expression in endometriosis euESCs, which leads to decreased miR-216a-5p bioavailability, increased ACTA2 expression and endometriosis euESC invasion and migration.

Our approach had some advantages. First, we cultured 23 primary euESC samples from women with endometriosis and found that second passage cells expressed ACTA2 and *H19*. These primary cultured cells represented the physiology of patients with endometriosis. Second, we found for the first time that an important smooth muscle cell marker, ACTA2, regulated endometriosis euESC invasion and migration, which may explain fibrous formation in endometriosis. Third, *H19*/miR-216a-5p/*ACTA2* seemed to be a new pathway for the regulation of endometriosis. We found that this pathway influenced endometriosis euESC invasion and migration, two critical processes for endometriosis progression. However, some limitations of our study cannot be neglected. First, *H19* and ACTA2 were highly expressed in endometriosis euESCs, but primary cultured cells could not be used for persistent overexpression experiments as the viruses and plasmids were too long for transfection. Accordingly, we used siRNAs to knock down *H19* and *ACTA2*. Secondly, Ghazal et al. (Ghazal, McKinnon et al. 2015) claimed that *H19* is downregulated in the eutopic

endometrium of patients with endometriosis compared to controls but exhibited no significant difference in the endometriosis euESCs of 10 patients. However, we used a larger sample size of 23 and found higher *H19* expression in primary cultured endometriosis euESCs than in controls. These inconsistent results may be explained by demographic differences among studies, including differences in race. Therefore, further genetic experiments are needed. Ghazal concluded that *H19* promotes ESC euESC proliferation, similar to our findings. Third, our conclusions were based on in-vitro experiments, and further in-vivo studies are needed.

In summary, we demonstrated that *H19* and ACTA2 play important roles in endometriosis euESC invasion and migration, and increased *H19* and ACTA2 expression levels are associated with endometriosis. Alterations in the *H19*/miR-216a-5p/*ACTA2* pathway mediated the regulation of endometriosis euESC invasion and migration in women with endometriosis. These findings indicate that a novel regulatory network involving *H19*, ACTA2 and miR-216a-5p might be a molecular target for the treatment of endometriosis.

Authors' roles

Zhen Xu, Liping Zhang, Qian Yu and Yanan Zhang contributed to the conception and design of the work or the acquisition, analysis and interpretation of the data. Zhen Xu drafted the manuscript and revised it critically for important intellectual content. Lei Yan approved of final the version to be published. Lei Yan and Zi-Jiang Chen agree to be accountable for all

aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of interest

The authors declare no competing interests.

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Figure Legends

Figure 1. The expression levels of *H19* and *ACTA2* were increased in endometrial stromal cells (euESCs) from endometriosis patients compared to that from controls. A and B, The mRNA expression levels of *H19* and *ACTA2* in endometriosis euESCs were higher than in that from controls (23 vs 23), 0.3931 ± 0.06817 vs. 0.8935 ± 0.1500, *P=0.0119*; 0.4638 ± 0.06346 vs. 1.089 ± 0.1731, **P=0.0061*. C, Western blotting showed that the protein level of ACTA2 were increased in endometriosis euESCs. D and E, Pearson correlation showed that *H19* and *ACTA2* RNA levels of endometriosis euESCs were positively correlated, ****P<0.0001, R=0.6136*;, , yet there was no such correlation in the controls, *P*=0.1587, *R=0.3698*. F and G, mRNA expression levels of *H19* in endometriosis euESCs were downregulated in *H19* knockdown cells (si*H19*), ***P=0.0005*; mRNA expression levels of *ACTA2* in endometriosis euESCs were downregulated in *H19* knockdown cells (si*H19), ***P=0.0004*. H, Western blotting showed the protein level of ACTA2 was downregulated in si*H19* cells. Data were presented as mean ± SD of three independent experiments.

A

B

C

D

Correlation

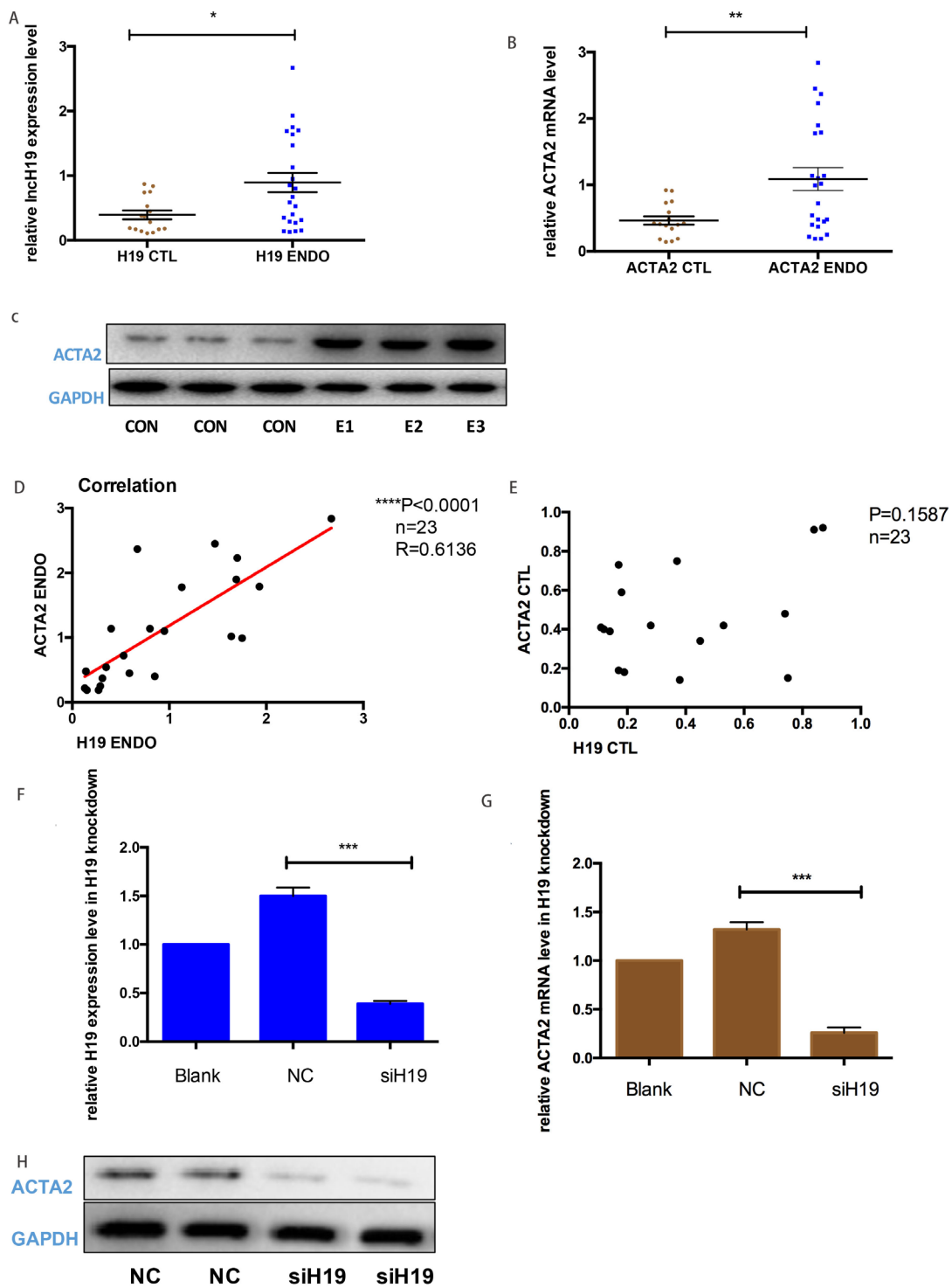****P<0.0001
n=23
R=0.6136

E

P=0.1587
n=23

F

G

H

Figure 2. *H19* and ACTA2 were essential for endometriosis eutopic endometrial stromal cell functions. A and B, Cell counting kit-8 assay showed that si*H19* treatment inhibited the proliferation of endometriosis euESCs. However, si*ACTA2* treatment showed no significant difference on endometriosis euESCs viability. C and D, 5-Ethynyl-2′- deoxyuridine incorporation assays (EdU) showed that si*H19* transfection restrained endometriosis euESCs proliferation 23.83 ± 1.628 vs. 17.18 ± 1.034, [**]*P = 0.0010*, yet si*ACTA2* showed no significant difference on it 31.37 ± 2.699 vs. 32.79 ± 1.225, *P=0.6355*. E and F, Transwell assays demonstrated that si*H19* or si*ACTA2* transfection inhibited endometriosis euESCs invasion and migration. Si*H19* downregulated cells invasion (352.4 ± 9.822 vs. 159.1 ± 5.864, [****]*P<0.0001*) and migration (290.1 ± 10.99 vs. 164.7 ± 6.372, [****]*P < 0.0001*). Si*ACTA2* downregulated cells invasion (299.0 ± 8.462 vs. 102.7 ± 2.696, [****]*P < 0.0001*) and migration (405.8 ± 10.03 vs. 177.8 ± 9.043, [****]*P < 0.0001*). Data were presented as mean ± SD of three independent experiments.
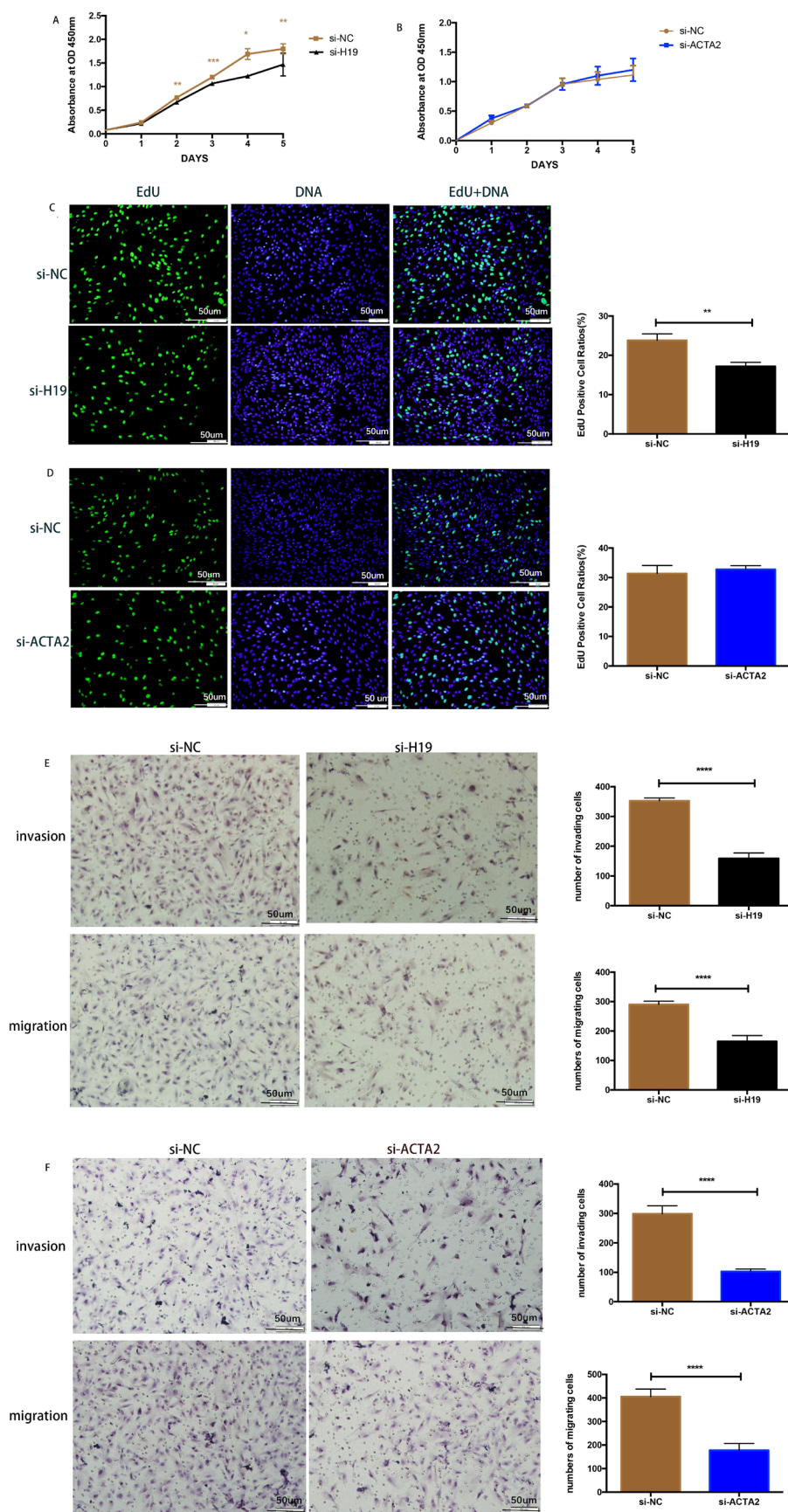
Figure 3. *H19* regulated *ACTA2* expression through competition for miR-216a-5p.

A and B, miR-216a-5p expression was lower in endometriosis euESCs compared to the controls (1.208 ± 0.09437 vs. 0.5921 ± 0.07995, *****P<0.0001*), and negatively correlated with *H19* in endometriosis euESCs (***P=0.031, R=0.589*). C, endometriosis euESCs transfected mimics of miR-216a-5p (***P=0.0063*) inhibited *H19* (***P=0.0023*) and *ACTA2* (****P=0.0010*) expression in RT-PCR. D, Western blotting showed the protein level of ACTA2 decreased with miR-216a-5p mimics. E and F, The binding sites of miR-216a-5p in *H19* and *ACTA2* 3'UTR. Treatment with miR-216a-5p mimics in HEK 293 cells reduced the luciferase activity of *H19* (***P=0.0035*) and *ACTA2* (****P=0.0010*). There was no change in luciferase activity when the binding sites were mutated. Western blotting showed the same results. G, H and I, Endometriosis euESCs transfected with control mixture siNC+iNC, si*H19*+iNC, si*H19*+i216a-5p, RT-PCR (G: *H19* siNC+iNC vs. si*H19*+iNC, **P=0.038*; si*H19*+iNC vs. si*H19*+i216a-5p, **P=0.0426*; *ACTA2*: siNC+iNC vs. si*H19*+iNC, **P=0.013*; si*H19*+iNC vs. si*H19*+i216a-5p, **P=0.015*). Western blotting (H: showd si*H19*+iNC expressed lower expression of ACTA2, while si*H19*+i216a-5p rescued *ACTA2* expression). Transwell assays (I: invasion siNC+iNC vs. si*H19*+iNC, ****P=0.0001*; si*H19*+iNC vs. si*H19*+i216a-5p, ****P=0.0003*; ACTA2: siNC+iNC vs. si*H19*+iNC, ****P=0.0001*; si*H19*+iNC vs. si*H19*+i216a-5p, ****P=0.0005*) showed consistent results with the RNA rescue experiments for *ACTA2*. Data were presented as mean ± SD of three independent experiments.
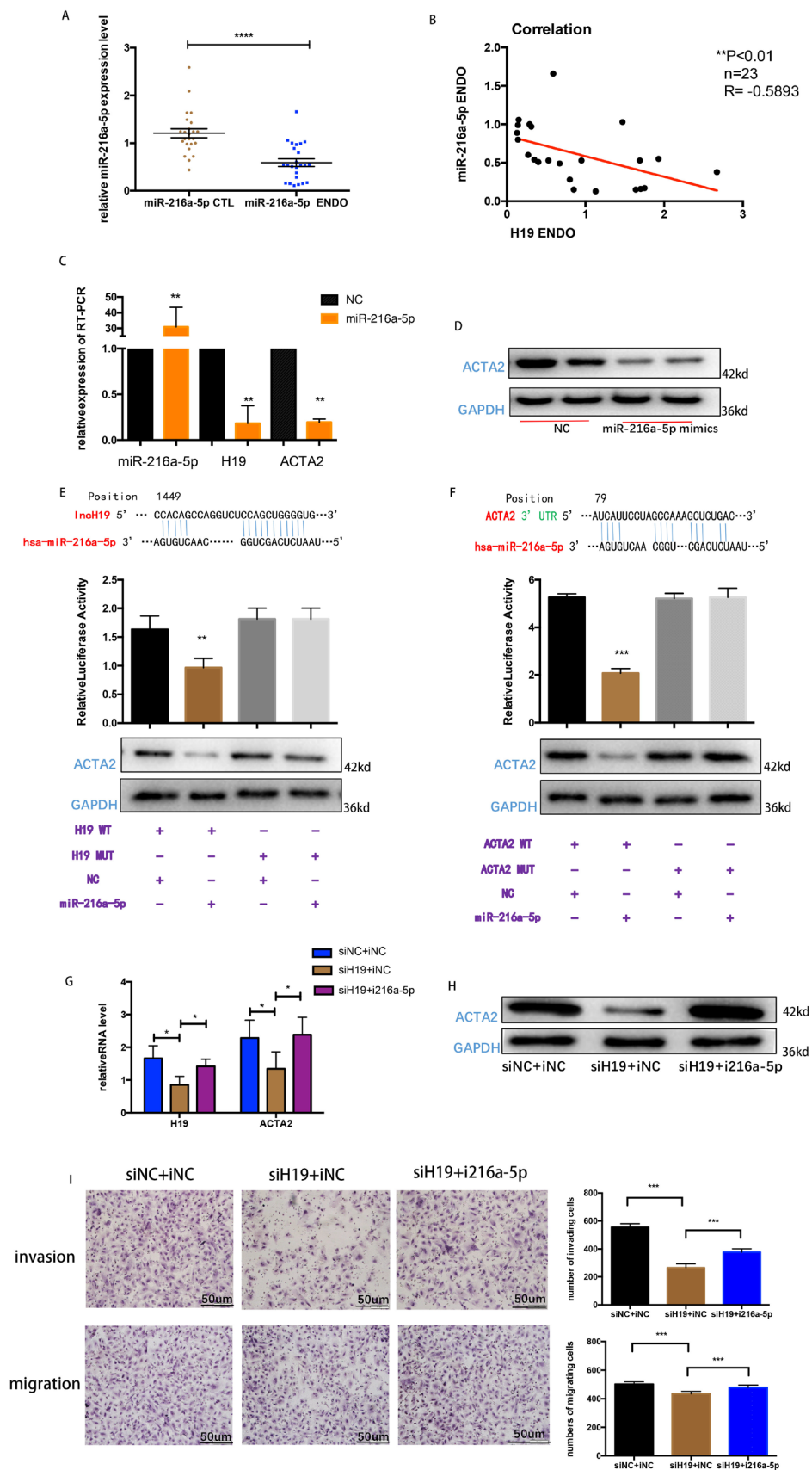
Figure 4. Estrogen promoted endometriosis euESCs invasion and migration through

H19. A, B and C, In endometriosis euESCs treated with estrogen for 0, 30 min and 60 min, H19 reached peak at 30 min (A, ***P=0.0004*), while miR-216a-5p was lower at 30 min and 60 min compared to 0 min (B, ***P=0.0006*). Western blotting (C) showed higher ACTA2 expression at 60min. D, Endometriosis euESCs treated with estrogen for 60min, E2-si*H19* increased cell invasion (si*H19* vs. siNC, *****P<0.0001*; E2-si*H19* vs. E2-siNC, *P=0.0355*; si*H19* vs. E2-si*H19*, *****P<0.0001*) and migration (si*H19* vs. siNC, ***P=0.0012; E2-si*H19* vs. E2-siNC, ****P=0.0003*;si*H19* vs. E2-si*H19*, ***P=0.0041*) compared with si*H19*. E, si*H19* endometriosis euESCs treated with estrogen 60min did not increase cells proliferation compared to the si*H19* (si*H19* vs. siNC, ***P=0.0071*; E2-si*H19* vs. E2-siNC, ***P=0.0012*; si*H19* vs. E2-si*H19*, *P=0.1697*). Data were presented as mean ± SD of three independent experiments. F. The process of estrogen/*H19*/miR-216a-5p/*ACTA2* pathway plays a critical role in endometriosis by regulating eutopic endometrial stromal cell invasion and migration.

A

B

C

D

E

F