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ORIGINAL RESEARCH

Estrogen Regulates the Expression and Function of lncRNA-H19 in Ectopic Endometrium

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Background: Long noncoding RNAs (lncRNAs) are involved in the pathogenesis of endometriosis and can be regulated by estrogen. This study aimed to investigate the role of estrogen in regulating the expression and function of lncRNA-H19 in endometriosis.

Methods: Endometrial stromal cells (ESCs) were isolated from ectopic, eutopic endometrium with endometriosis and control endometrium without endometriosis, and lncRNA-H19 expression was detected using real-time polymerase chain reaction (RT-PCR). Ectopic endometrial stromal cells (ecESCs) were treated with 17β-estradiol at 10^{-8}mol/L for 0, 12, 24 and 48 hours, and lncRNA-H19 expressions of cells were evaluated using RT-PCR. After ecESCs were treated with 17β-estradiol for 48 hours, lncRNA-H19 expression was knocked down and cell proliferative and invasive abilities were compared.

Results: The expression of lncRNA-H19 in ecESCs was significantly higher than that in eutopic endometrial stromal cells (euESCs) and control ESCs. After treated with 17β-estradiol, ecESCshadupregulatedlncRNA-H19 expression with time-dependent manner. Cell proliferation and invasion increased when estrogen upregulated lncRNA-H19 expression in ecESCs, however, cell proliferation restored and cell invasion did not change when lncRNA-H19 was knocked down in ecESCs.

Conclusion: The expression and function of lncRNA-H19 was regulated by estrogen in ecESCs, which probably contributed to the pathogenesis of endometriosis.

Keywords: endometriosis, lncRNA-H19, estrogen, proliferation, invasion, ectopic endometrial stromal cells

Background

Endometriosis is one of the most prevalent gynecological disorders in women of reproductive age,[1,2] and is defined as the presence of endometrial-like tissue at sites other than the uterine cavity.[3–6] At present, the pathogenesis of endometriosis is still not understood well,[7] but ectopic endometrium will atrophy and the progress of endometriosis can be prevented by inhibiting ovarian function and decreasing estrogen level, suggesting that endometriosis is an estrogen-dependent disorder. At the same time, some previous studies showed that there was an increase estrogen level in endometriosis lesions, which explained the pathogenesis of postmenopausal endometriosis.[8,9] In summary, systemic or local estrogen is involved in the occurrence and development of endometriosis, however, it remains unclear how estrogen contributes to the pathogenesis.

Endometriosis has a high recurrence rate after surgery.[10] Gonadotropin-releasing hormone agonists (GnRH-a) is used widely, however, there is a high incidence of adverse events related to estrogen deficiency, including hot flashes, vaginal dryness, decreased libido and so on.[11] If the signaling pathway of estrogen in endometriosis is found and some targets are blocked to reduce the recurrence rate without the side-effects of systemic estrogen deficiency, which will be of great significance to improve the therapeutic effect and quality of life of patients with endometriosis.

Long noncoding RNAs (lncRNAs) are non-coding single-stranded RNAs with more than 200 nucleotides and have been found playing important roles in many biological processes in recent years.[12,13] LncRNA-H19 was one of the earliest lncRNAs to be

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discovered and has been increasingly recognized for its abilities to participate in cell function regulation, transcription and post transcriptional modification.[14,15] Our prior studies demonstrated that lncRNA-H19 regulated cell proliferation and invasion of ectopic endometrium by targeting ITGB3 via modulating miR-124-3p,[16] and it has also been reported that lncRNAs can be regulated by estrogen,[17,18] but it remains unclear whether estrogen regulates the expression and functions of lncRNA-H19 to participate in the pathogenesis of endometriosis.

In this study, ectopic endometrial stromal cells (ecESCs) were treated with estrogen and lncRNA-H19 expression was observed, after that, lncRNA-H19 was knocked down from these cells and cell proliferation and invasion was evaluated, and the effect of estrogen on the expression and functions of lncRNA-H19 was explored in endometriosis.

Methods

Flowsheet of the experiments is shown in Figure 1.

Study Population and Samples

Forty cases and 20 controls were enrolled in this study from January 2018 to December 2018. Cases were diagnosed with endometriosis by histological confirmation, and controls underwent surgery for other diseases, including ovarian benign tumor and high-grade squamous intraepithelial lesion of cervix, and was ruled out endometriosis during the procedures. There was no significant differences in age, body mass index, gravidity and parity between cases and controls and the sociodemographic characteristics are summarized in Table 1. The clinical characteristics of the patients with endometriosis are shown in Table 2. All participants did not receive any hormonal treatments for three months prior to surgery and underwent laparoscopic surgery in the late proliferation phase of the menstrual cycle (days 11–13). During surgery, ectopic endometrial tissues (cyst walls of ovarian endometriomas) were obtained, and an endometrial sampler (Saipujiuzhou, Peking, China) was used to absorb the eutopic and control endometrial tissues carefully.

Institutional review board approval was received from Zhenjiang Maternal and Child Health Hospital and all patients provided the informed consent for the investigation and experiments.

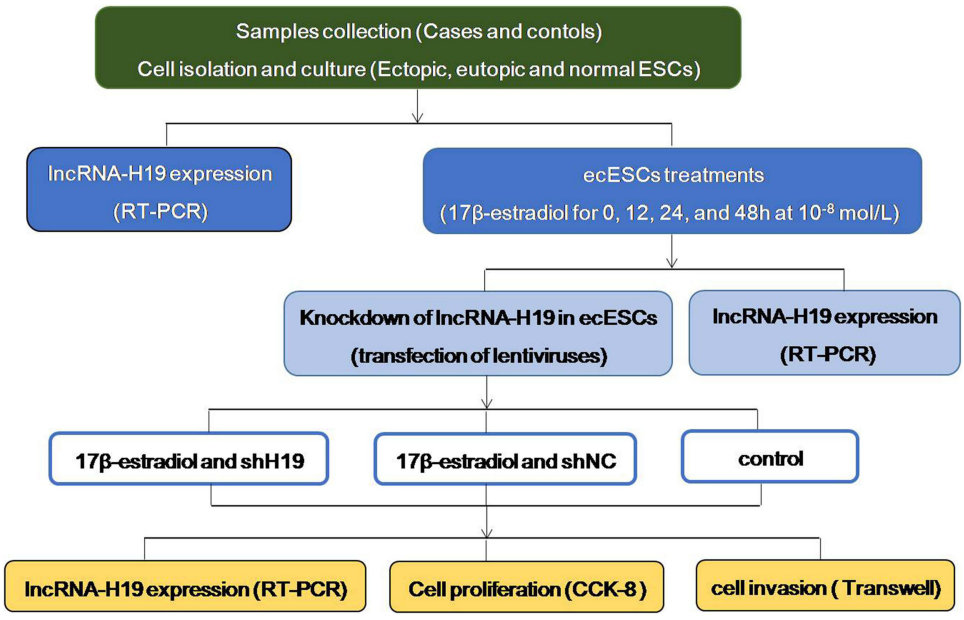

Figure 1 Flowsheet of the experiments.
Abbreviations: ESCs, endometrial stromal cells; ecESCs, ectopic endometrial stromal cells; shH19, lentiviruses of lncRNA-H19 shRNA; shNC, lentiviruses of shRNA negative control.

Table 1 Sociodemographic Characteristics of the Endometriosis and Control Patients

Variables Median (Range)	Endometriosis Group (n=40)	Control Group (n=20)	*P*
Age, years	33 (23–48)	34 (25–51)	0.265
Gravidity, n	1 (0–3)	1 (0–4)	0.127
Parity, n	1 (0–2)	1 (0–3)	0.264
BMI, kg/m^2	22.5 (19.1–28.3)	22.7 (19.7–29.2)	0.352

Abbreviation: BMI, body mass index.

Table 2 Clinical Characteristics of the Patients with Endometriosis

Variables	Number (%)
Pelvic pain	
Absent	23 (57.5)
Present	17 (42.5)
Infertility	
Absent	26 (65.0)
Present	14 (35.0)
CA125 level (U/mL)	
<100	22 (55.0)
100–200	15 (37.5)
≥200	3 (7.5)
ASRM stage	
I	1 (2.5)
II	6 (15.0)
III	15 (37.5)
IV	18 (45.0)

Abbreviation: ASRM, American Society for Reproductive Medicine.

Cell Isolation and Culture

Endometrial stromal cells (ESCs) were isolated and cultured according to published papers.[19,20] Fresh tissues were washed twice with sterile phosphate buffer saline to remove blood, minced into small pieces using ophthalmic scissors, and incubated with 0.25% collagenase type IV (Sigma, MO, USA) in a water bath at 37°C (eutopic and control endometrium for 40min, ectopic endometrium for 90min). After terminating collagenase activity, ESCs were obtained by filtering with a 40-μm monofilament nylon mesh. The process of digestion and filtration was repeated (eutopic and control endometrium for 1 time, ectopic endometrium for 3 times) and the collected ESCs suspension was centrifuged at 1000 rpm for 10min. ESCs were grown in high glucose Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum (GIBCO, MA, USA) and 1% antibiotic (Beijing Solarbio Science & Technology, Beijing, China). Cells were cultured in an incubator (Thermo Fisher Scientific, MA, USA) at 37°C with 5% CO2 and the media was replaced every two days.

Construction and Transfection of Lentiviruses

Three interference sequences targeting lncRNA-H19 were synthesized (Table 3) and inserted each into the AgeI-EcoRI site of the pLKO.1-Puro vector. Subsequently, pLKO.1-Puro-shH19 or pLVX-Puro-FKBP3 were co-transfected into 293T cells with viral packaging plasmids, psPAX2 and pMD2.G (Cambridge, MA, USA), using Lipofectamine 2000 (Invitrogen, CA, USA). After incubated for 48 h, viral particles were collected by ultracentrifugation.

EcESCs were infected with lentiviruses of short hairpin RNA (shRNA) negative control (shNC) and lentiviruses of lncRNA-H19 shRNA (shH19), including shH19-1, shH19-2, or shH19-3, and real-time polymerase chain reaction (RT-PCR) assay was used to determine the transfection efficiency of shH19.

Table 3 lncRNA-H19 Interference and Negative Control Targets

Gene	Sequences (5' to 3')
shH19−1(2357–2375)	CCAAGTAGGGACAACCCTT
shH19-1-F	**CCGGT**CCAAGTAGGGACAACCCTTCTCGAGAAGGGTTGTCCCTACTTGGTTTTT**G**
shH19-1-R	**AATTC**AAAAACCAAGTAGGGACAACCCTTCTCGAGAAGGGTTGTCCCTACTTGG**A**
shH19-2 (2753–2771)	GGTGACTGTCCCTTTCCAA
shH19-2-F	**CCGGT**GGTGACTGTCCCTTTCCAACTCGAGTTGGAAAGGGACAGTCACCTTTTT**G**
shH19-2-R	**AATTC**AAAAAGGTGACTGTCCCTTTCCAACTCGAGTTGGAAAGGGACAGTCACC**A**
shH19-3 (2965–2983)	TCCCATTCCTTAGCTCCAT
shH19-3-F	**CCGGT**TCCCATTCCTTAGCTCCATCTCGAGATGGAGCTAAGGAATGGGATTTTT**G**
shH19-3-R	**AATTC**AAAAATCCCATTCCTTAGCTCCATCTCGAGATGGAGCTAAGGAATGGGA**A**
shNC	AAGTCGAATTGTTCCCATT
shNC-F	**CACCG**AAGTCGAATTGTTCCCATTCCGAAGAATGGGAACAATTCGACTT**C**
shNC-R	**AAAAG**AAGTCGAATTGTTCCCATTCTTCGGAATGGGAACAATTCGACTT**C**

Note: The bold in the sequences were endonuclease sites.
Abbreviations: shH19, lentiviruses of lncRNA-H19 shRNA; shNC, lentiviruses of shRNA negative control.

Cell Treatments

17β-estradiol was dissolved in ethanol at a stock concentration of 10^{-2}mol/L.The treatment concentration was determined according to published papers.[21–23] EcESCs were treated with 17β-estradiol for 0, 12, 24, and 48h at 10^{-8}mol/L and lncRNA-H19 expressions of cells were detected using RT-PCR.

After treated with 17β-estradiol at 10^{-8}mol/Lfor 48h, ecESCs were separated into the following treatment groups: 17β-estradiol and shH19, 17β-estradiol and shNC, control, and the proliferative and invasive ability of cells was assessed using CCK-8 and Transwell respectively.

RT-PCR Assay

Total RNA was isolated from ESCs using Trizol reagent (Invitrogen, CA, USA), followed by quantification and confirmation of RNA integrity. A Reverse Transcription Kit (Fermentas, CA, USA) was used to reverse transcribe 1 μg of RNA into complementary DNA (cDNA). A SYBR Green PCR kit (Fermentas, CA, USA) was used to perform RT-PCR and generation of cDNA templates on an ABI-7300 (Thermo Fisher Scientific, CA, USA) and the following thermocycler parameters were used: 10 min at 95°C, followed by 40 cycles of 15/45 sec at 95/60°C, 15/60 sec at 95/60°C, or 15/15 sec at 95/60°C.[24]

Subsequently, lncRNA-H19 mRNA expression relative to the internal reference, GAPDH, was calculated using the $2^{-\Delta\Delta Cq}$ method.[25]

The primers used include as shown in Table 4.

Cell Proliferation Assay

Cells in the logarithmic growth phase were trypsinized and 3000 cells were seeded per well in 96-well plates (TRUELINE, MA, USA) and cultured overnight. After 0, 24, 48, and 72-hour of treatment, 100 μL of Cell Counting Kit-8 (CCK-8) (SAB Biotherapeutics, SD, USA) solution (CCK-8: serum-free medium = 1:10) was added to each group

Table 4 The Primers Sequences of PCR

Gene	Primer	Sequence (5' to 3')
lncRNA H19	Forward	GCGGGTCTGTTTCTTTACTTCC
	Reverse	CTTTGATGTTGGGCTGATGAGG
GAPDH	Forward	AATCCCATCACCATCTTC
	Reverse	AGGCTGTTGTCATACTTC

and incubated at 37°C for 1 h. A microplate reader was used to measure cell proliferation as the absorbance value (OD) at 450 nm.

Cell Invasion Assay

Transwell chambers (Cambridge, MA, USA) and 24-well plates were soaked in PBS for 5 min before adding 80 μL of Matrigel (Corning, New York, USA) and allowed to coagulate for 30 min at 37°C. After treatment, cells were cultured for 24 h in serum-free media, then trypsinized and seeded into Transwell chambers and 24-well plates. Following the addition of 0.7 mL of RPMI-1640 medium containing 10% fetal bovine serum, which acted as a chemoattractant, to 24-well plates, the cells were incubated for 48 h in a 37°C incubator. After fixation with 4% formaldehyde (Sinopharm, Shanghai, China) for 10 min, cells were stained for 30 min with 0.5% crystal violet (Beijing Solarbio Science & Technology, Beijing, China). Invading cells were counted at 100× magnification.

Statistical Analysis

All statistical analyses in this study were carried out with SPSS 20.0 (Statistical Package for the Social Sciences, SPSS, Inc., Chicago, IL, USA). Based on triplicate experiments, values were presented as mean ± standard deviation (SD). The statistical significance among multiple groups was tested by one-way analysis of variance (ANOVA) and Tukey's post-hoc test. A P value <0.05 was considered to be statistically significant.

Results

Isolation and Identification of ESCs in Endometriosis and Control Group

Under the inverted microscope, the newly isolated cells were different in size and shape, and most of them were round. There were only a few adherent cells 2 hours after isolation, and almost all cells adhered to the wall 24 hours after isolation. As shown in Figure 2A–C, ecESCs and eutopic endometrial stromal cells (euESCs) had similar morphologywithcontrol ESCs,

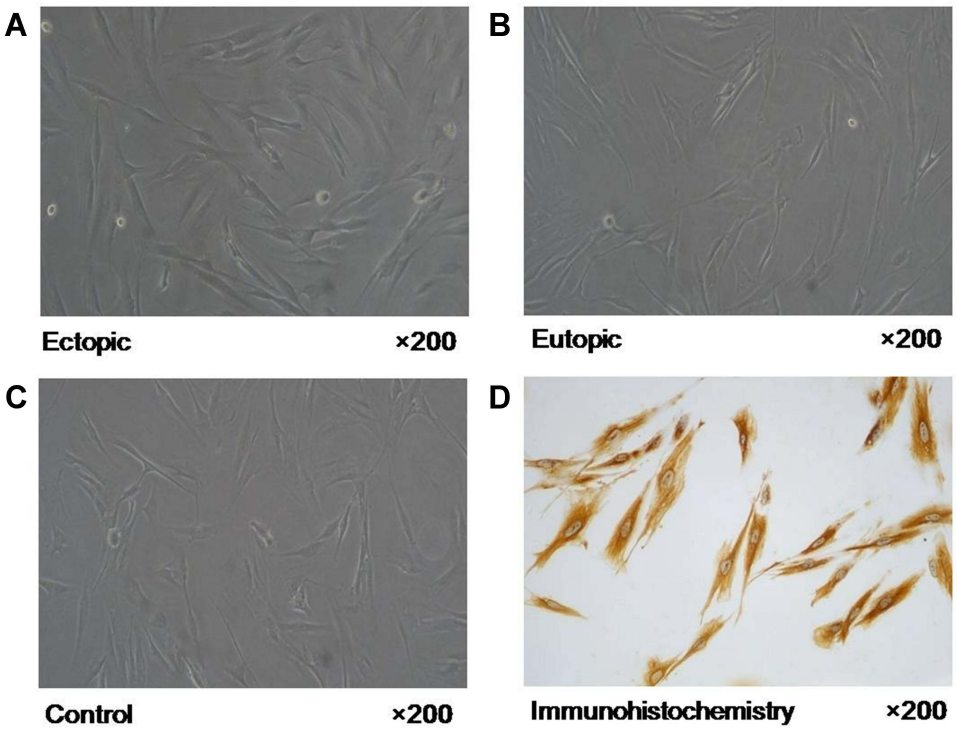

Figure 2 Characteristics of endometrial stromal cells. (**A**) Morphology of ectopic endometrial stromal cells. (**B**) Morphology of eutopic endometrial stromal cells. (**C**) Morphology of control endometrial stromal cells. (**D**) Immunohistochemical identification of endometrial stromal cells.

and they were spindle, scattered, arranged parallelly or radially between cells. As shown in Figure 2D, immunohistochemical results indicated that vimentin staining was positive, confirming the isolated cells were ESCs.

lncRNA-H19 Expression of ESCs in Endometriosis and Control Group

lncRNA-H19 expression was detected in the isolated ESCs of endometriosis and control patients using RT-PCR. As shown in Figure 3, lncRNA-H19 levels increased in ecESCs as compared to euESCs and controlled ESCs, suggesting that lncRNA-H19 maybe involved in the progression of endometriosis.

Knockdown of lncRNA-H19 in ecESCs

EcESCs were infected with shH19, including shH19-1, shH19-2 and shH19-3. The expression of lncRNA-H19 in ecESCs after transfection was detected using RT-PCR. The application of shH19 to ecESCsleaded to decreased lncRNA-H19 expression, with shH19-1 and shH19-2 showing the highest knockdown efficiency.The results suggested that shH19 transfection could cause the knockdown of lncRNA-H19 effectively in ecESCs.

Estrogen Upregulated lncRNA-H19 Expression in ecESCs

We next investigated the potential interaction of estrogen and lncRNA-H19 in ecESCs. After ecESCs were treated with 17β-estradiol at 10^{-8}mol/L for 0, 12, 24, and 48 hours, cellular lncRNA-H19 expression at different time was detected using RT-PCR. As shown in Figure 4, 17β-estradiol promoted significantly lncRNA-H19 expression in ecESCs, with time-dependent manner.

Estrogen Promoted the Proliferation and Invasion of ecESCsvia Modulating lncRNA-H19

We further explored the influences and mechanism of estrogen on the fuctions of ecESCsin endometriosis. EcESCs were infected with shH19 and shNC after cultured with 17β-estradiol at 10^{-8}mol/L, as shown in Figure 5A, estrogen upregulated the expression of lncRNA-H19 and the knockdown of lncRNA-H19 eliminated the effect of estrogen. Next, the proliferative and invasive ability of ecESCs was assessed. As shown in Figure 5B and C, cell proliferation and invasion increased when estrogen upregulated lncRNA-H19 expression in ecESCs, however, cell proliferation restored and invasive ability did not change significantly when lncRNA-H19 was knocked down in ecESCs. These results show that lncRNA-H19-regulated cell proliferation and invasion in ecESCs is modulated, at least in part, by estrogen.

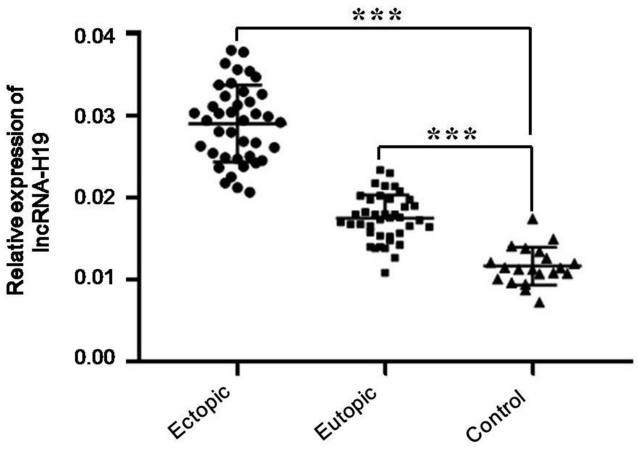

Figure 3 lncRNA-H19 expressions of endometrial stromal cells in endometriosis and control group.***P< 0.001 compared to normal endometrium.

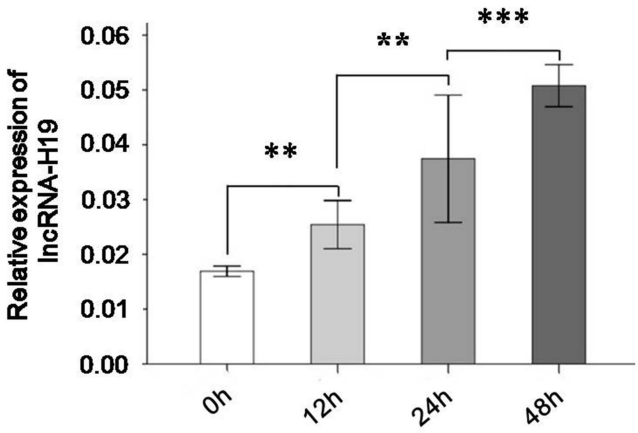

Figure 4 Estrogen upregulated lncRNA-H19 expressions in endometrial stromal cells. **P< 0.01, ***P< 0.001 compared to 0h, 12h and 24h.

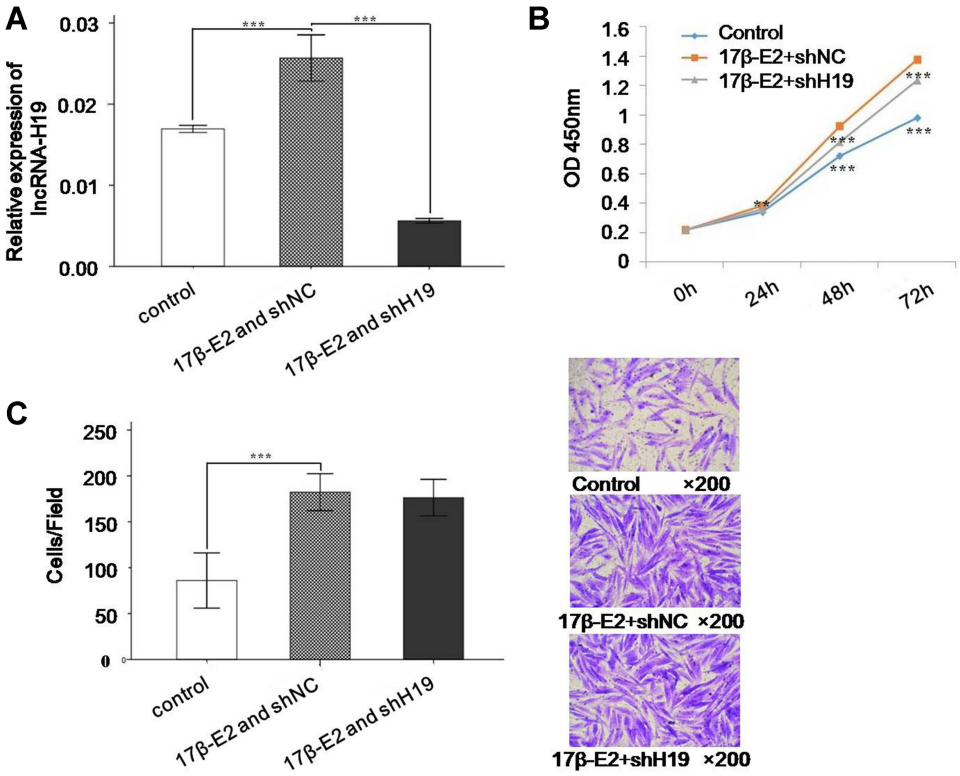

Figure 5 Estrogen promoted the proliferation and invasion of ectopic endometrial stromal cells via lncRNA-H19. (**A**) The expression of lncRNA-H19 was detected by RT-PCR. (**B**) The proliferation of cells following knockdown of lncRNA-H19 was assessed at 0, 24, 48 and 72 hours by CCK-8. (**C**) Invasion cells after knockdown of lncRNA-H19 were tested by Transwell. **P< 0.01, ***P< 0.001 17β-E_2 + shNC was compared to control, 17β-E_2 + shH19 was compared to 17β-E_2 + shNC.
Abbreviation: 17β-E2, 17β-estradiol.

Discussion

In this study, we found that compared to euESCs of endometriosis patients and control ESCs, lncRNA-H19 levels elevated significantly in ecESCs, whereas estrogen upregulated the expression of lncRNA-H19 with time-dependent manner. Cell proliferation and invasion increased when estrogen upregulated lncRNA-H19 expression in ecESCs, and cell proliferation and invasion decreased to some extend and estrogen phenotype was reversed partly when lncRNA-H19 was knocked down in ecESCs. Together, these results suggested that estrogen-regulated lncRNA-H19 may play a crucial role in endometriosis.

Panir K et al reviewed the literatures related to non-coding RNA (ncRNA) in endometriosis in PubMed, MEDLINE and Google Scholar from 2000 to 2016, and found that the disorder of ncRNA regulation was an important factor in the occurrence and development of endometriosis although there were also some different conclusions in these studies, and predicted the ncRNA-related biomarkers and therapeutic techniques may change the treatment mode of endometriosis in the future.[26]

Earlier studies suggested that lncRNA-H19 was involved in the pathogenesis of disease by regulating cell proliferation and invasion.[27–29] It was reported that the expression levels of lncRNA-H19 were upregulated in cutaneous squamous cell carcinoma tissues and cell lines, and the knockdown of lncRNA-H19 expression inhibited cell proliferation, migration and invasion, but induced cell apoptosis.[27] In hepatocellular carcinoma, lncRNA-H19 knockdown inhibited cell proliferation, migration, invasion and increase apoptosis via modulating miR-15b/CDC42/PAK1 axis.[28] In addition, lncRNA H19 expression was down-regulated in patients with asthma andlncRNA H19 inhibits proliferation and migration of airway smooth muscle cells induced by PDGF-BB through miR-21/PTEN/Akt axis.[29] In line with these reports, our prior study[10,16] foundlncRNA-H19 may be involved in the pathogenesis of endometriosis and regulated ITGB3 via miR24-3p to affect cell proliferation and invasion in endometriosis.

Endometriosis is an estrogen-dependent disorder, however, the signaling pathway of estrogen involves in endometriosis retains unclear. Earlier studies have suggested that lncRNAscan be regulated by estrogen. A prior study identified an intergenic lncRNA ERINA (estrogen inducible lncRNA) as a novel lncRNA highly expressed in multiple cancer types, especially in estrogen receptor-positive (ER+) breast cancers, which may serve as a novel biomarker and potential therapeutic target in breast cancer.[17] It was also reported estrogen promoted lncRNA H19 expression to regulate osteogenic differentiation of bone marrow mesenchymal stem cells and reduce osteoporosis.[18] However, it has not been reported the effect of estrogen on the expression and function of lncRNA-H19 in endometriosis. Our study suggested that estrogen could promoted the proliferation and invasion of ecESCs via modulating lncRNA-H19, and lncRNA-H19 may be involved in the signaling pathway of estrogen in endometriosis, which is consistent with the conclusion of a published paper with euESCs as subjects.[30] However, cell proliferation and invasion increased when estrogen upregulated lncRNA-H19 expression in ecESCs, but cell proliferation restored and invasion ability did not change significantly when lncRNA-H19 was knocked down in ecESCs, which showed lncRNA-H19 regulated cell proliferation and invasion in ecESCS by estrogen partly.

In addition, according to our prior study,[16] alterations in the lncRNA-H19/miR-124-3p/ITGB3 pathway regulated the proliferation and invasion of ecESCs in endometriosis, and downregulation of lncRNA-H19or ITGB3, or upregulation of miR-124-3p inhibited the proliferation and invasion of ecESCs in endometriosis. Combining the results of our prior study with this study, we can inferred blocking lncRNA-H19/miR-124-3p/ITGB3 pathway may affect the effect of estrogen to some extend, and these molecules are expected to become new therapeutic targets of endometriosis.

In conclusion, this study presents the first evidence for crosstalk between lncRNA-H19 and estrogen in ecESCs, providing new molecular targets for the treatment of endometriosis. Furthermore, primary cultured ecESCs represented endometriosis cells better. Our study showed the expression and function of lncRNA-H19 was regulated by estrogen in ecESCs, which probably contributed to the pathogenesis of endometriosis.

There were also some limitations that need to be addressed. (1) We only collected ovarian endometrioma samples, so it remains unclear whether there are fluctuations in regulation mode for different types of lesions. The profiles that estrogen regulates the expression and function of lncRNA-H19 in non-ovarian endometriotic lesions should be determined in the future. (2) In our study primary cultured ectopic endometrial epithelial cells (ecEECs) were not isolated and cultured successfully and experiments were conducted in vitro. In the future estrogen/lncRNA-H19/miR-124-3p/ITGB3 pathway studies should be conducted in ecEECs and further in-vivo studies are also required. (3) The study had a relatively small sample size, especially in the control group, due to time and funding constraints. More studies with larger sample sizes are needed to confirm the conclusion.

Abbreviations

lncRNAs, long noncoding RNAs; ESCs, endometrial stromal cells; ecESCs, ectopic endometrial stromal cells; euESCs, eutopic endometrial stromal cells; RT-PCR, real-time polymerase chain reaction; GnRH-a, gonadotropin-releasing

hormone agonists; shRNA, short hairpin RNA; shNC, lentiviruses of shRNA negative control; shH19, lentiviruses of lncRNA-H19 shRNA; ncRNA, non-coding RNA.

Data Sharing Statement

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

Written consent was obtained from each participant. The study protocol was approved by the Medical Ethics Committee of Zhenjiang Maternal and Child Health Hospital and conducted in accordance with the Declaration of Helsinki.

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Disclosure

The authors report no conflicts of interest in this work.

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