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ENDOMETRIOSIS: ORIGINAL ARTICLE

# Linc-ROR Promotes EMT by Targeting miR-204-5p/SMAD4 in Endometriosis

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## Abstract

Endometriosis (EMs) is a systemic and chronic disease with cancer-like feature, namely, distant implantation, which caused heavy healthy burden of nearly 200 million females. LncRNAs have been proved as new modulators in epithelial–mesenchymal transition (EMT) and EMs. Quantitative real-time PCR was conducted to measure the expression level of long intergenic non-protein coding RNA, regulator of reprogramming (Linc-ROR), and miR-204-5p in ectopic endometrium ($n=25$), eutopic endometrium ($n=20$), and natural control endometrium ($n=22$). Overexpression of Linc-ROR, knockdown or overexpression of miR-204-5p in End1/E6E7 and Ishikawa cells, was conducted to detect the function of Linc-ROR and miR-204-5p in EMs. Furthermore, luciferase reports were used to confirm the combination of Linc-ROR and miR-204-5p and the combination between miR-204-5p and SMAD4. Cell-Counting Kit-8, EdU assay, transwell assays, and Western blotting were used to detect the function of Linc-ROR and miR-204-5p in EMs cancer-like behaviors and EMT process. Linc-ROR was up-regulated in ectopic endometrium. Overexpressed Linc-ROR promotes cell proliferation, invasion, and EMT process. Linc-ROR regulated the EMT process, cellular proliferation, and invasion of EMs via binding to miR-204-5p. In addition, overexpression of Linc-ROR up-regulated SMAD4, a target protein of miR-204-5p, with which regulated EMT process and cancer-like behaviors in EMs together. Linc-ROR/miR-204-5p/SMAD4 axis plays a vital role in regulation EMT process in EMs, which might become a novel therapeutic targets and powerful biomarkers in EMs therapy.

**Keywords** Linc-ROR · miR-204-5p · ceRNA · SMAD4 · Endometriosis · EMT

## Introduction

Endometriosis (EMs) is characterized by the abnormal existence of living endometrial tissue outside the uterus. It is associated with progressive dysmenorrhea, pelvic pain, and infertility [1]. Lesion resection helps some patients relieve from pain; however, there is a 5–15% risk of relapse [2]. Long-term medical management is required for pain relief and estrogen level reduction [3, 4]. Currently, researchers have proposed that immediate medicate pain relief treatment without histological definition is recommended to avoid central sensitivity [5]. However, a thorough pathogenesis assessment of the endometriosis is still the prerequisite for further research and treatment development. Importantly, the caner-like feature, namely, the invasion-metastasis cascade, was distinctive and significant characterize in EMs, as a benign disease [6]. Correspondingly, epithelial–mesenchymal transition (EMT) has been proved to play a significant role in EMs [6], during which, with faded epithelial characteristics and developed mesenchymal characteristics, endometrium cells are equipped with invasive and migratory capabilities for distant metastasis and implantation [7–9].

Long non-coding RNAs (LncRNAs) are longer than 200 nucleotides, and microRNAs (miRNAs) are a type of small RNA with a length of about 20–24 nucleotides. LncRNAs could act as competitive endogenous RNA (ceRNA) and absorb microRNAs, resulting in the segregation of miRNAs and their targeting mRNAs, therefore blocking the regulation of the target messenger RNA (mRNAs) [10–12]. EMs is regulated by multiple genetic and epigenetic mechanisms [13, 14]. Elevated or down-regulated expression of thousands of LncRNAs has been identified in EMs [15]. And it has been proven that various LncRNAs participated in an EMT process in EMs, as in the case of LncRNA actin

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filament associated protein 1 antisense RNA1, Linc01541, and LncRNA UBOX antisense RNA 1 [16–18]. Moreover, the functional ceRNAs were proved in EMs; for example, we and others demonstrated the contribution of MALAT1/miR-200c and the function of CDKN2B-SA1/miR-424-5p to EMT in EMs [19–21].

EMs is now considered a complicated chronic neuroinflammatory condition [9, 22]. Elevated levels of transforming growth factor-β (TGF-β) and the corresponding activation of the TGF-β/SMADs signal pathway was widely studied in EMs, which contributed to elevated cellular proliferation and implantation [9]. Consistently, the TGF-β/SMADs signal pathway was also identified to be involved in EMs in our previous study [23]. In the TGF-β/SMADs pathway, SMAD family member 4 (SMAD4) is a common binding partner of all receptor-regulated SMAD (R-SMADs) proteins[24], inactivation of SMAD4 abrogates TGF-β/SMADs-mediated changes [25]. Significantly, SMAD4's involvement in the EMT-regulation process, cell growth, and proliferation was identified in multiple studies [26, 27]. Importantly, SMAD4 mRNA expression was found to be up-regulated in the eutopic endometrium of patients with EMs compared to control patients using high-throughput sequencing [28]. Thus, it could be meaningful to discover more regulation on SMAD4 in EMs.

Recently, long intergenic non-protein coding RNA, regulator of reprogramming (Linc-ROR), was revealed to represent a high expression in adenomyosis [29]. Exploration of Linc-ROR' expression level in the EMs is significant, since the EMs and adenomyosis often share some molecular changes [30]. Moreover, Linc-ROR studies have identified the regulation of Linc-ROR on gene regulation in a ceRNA manner, and its participation in metastasis and EMT across multiple diseases [31–33]. However, whether Linc-ROR promotes EMT and participates in the etiology of EMs is still a topic that needs further research. To the best of our knowledge, this is the first study that attempts to reveal the expression level of Linc-ROR, identify its potential binding miRNA, and the regulation in EMs. As a result, this research would broaden the understanding of EMs and provide a novel biomarker for treatment.

## Methods

### Population

All the human ectopic, eutopic, and normal endometrium were obtained at the Second Xiangya Hospital of Central South University from September 2020 to April 2021 under laparoscopy and using a disposable intrauterine tissue suction tube from patients with or without EMs. Patients with benign ovarian or cervical disease who were scheduled for laparoscopic surgery were recruited and screened for inclusion in this study. Exclusion criteria were set as following: individuals who were unable to provide consent, age < 18 years, diagnosed with adenomyosis, or used hormonal therapy 6 months prior to surgery. Gynecological laparoscopic surgery and histologic assessment were used to determine the patient's disease (EMs in the experimental group; other benign ovarian or cervical diseases in the control group). The menstrual cycle was recorded according to pre-operative history and a histological assessment of the endometrium. Thirty-one patients who were diagnosed with endometriosis were enrolled in our study. Twenty-five cases of ectopic endometrium were collected from the patients' ovarian endometrioma cyst wall. Laparoscopic surgeries were performed by experienced operators to avoid damage to ovarian tissue as much as possible and then ascertained by pathological examination. Ectopic endometrium from the cyst walls of ovarian endometriomas was obtained immediately after surgery. One patient presented with ovarian EMs and deep infiltrating EMs and one patient with ovarian EMs and peritoneal EMs; only lesions from the endometrioma cyst wall were used in this study. Twenty cases of eutopic endometrium were collected with no strict match (14 patients rendered both ectopic and eutopic endometrium; both samples were used in this study). The stage of endometriosis was determined during surgery according to the revised Classification of the American Society of Reproductive Medicine (rASRM) [34]. Twenty-two cases of endometrium from the control group were collected.

Information including age, clinical information, menstrual cycle phase, type and stage of EMs were recorded (Table 1). According to the American College of Obstetricians and Gynecologists and the ReVITALize data definitions, in this study, we define chronic pelvic pain as pain symptoms perceived to originate from pelvic organs/structures typically lasting more than 6 months. All samples were collected during surgery, washed with phosphate-buffered saline (PBS), and then stored in liquid nitrogen for further RNA and protein extraction. This study was approved by the Institutional Ethics Review Committee of Second Xiangya Hospital of Central South University (#2016243). All tissue samples were obtained with full patient knowledge, and the patient consent process and all experiments followed the ethical principles set forth in the 1964 Declaration of Helsinki and its subsequent revisions.

### Cell Culture

Ishikawa (ISK) cell line and End1/E6E7 (E6E7) cell line were purchased from Cell Bank of Advanced Research Center (Changsha, China). ISK cells were cultured in DMEM (Gibco, Germany), supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin, and

**Table 1** Demographic variables of the study population

| | Control ($N=22$) | Cases ($N=31$) |
|---|---|---|
| Age, mean ± SD (%) | 35 ± 10 | 34 ± 8 |
| Infertile | - | 10 (32.26%) |
| Chronic pelvic pain # | - | 19 (61.29%) |
| Type (%) | | |
| Endometrioma | - | 31 |
| Peritoneal lesion | - | 1* |
| Deep-infiltrating lesion | - | 1* |
| Stage, *n* (%) | | |
| Stage 1 | - | 0 |
| Stage 2 | - | 0 |
| Stage 3 | - | 16 |
| Stage 4 | - | 15 |
| Cycle phase at biopsy, *n* (%) | | |
| Proliferative | 18 (81.82%) | 25 (80.64%) |
| Menstrual | 0 | 0 |
| Secretory | 4 (18.18%) | 6 (19.35%) |

*One study patient presented with ovarian EMs and deep infiltrating EMs, and one study patient presented with ovarian EMs and peritoneal EMs; only lesions from the endometrioma cyst wall were used in this study

100 μg/mL streptomycin (Gibco). E6E7 cell line was cultured in DMEM (Gibco), supplemented with human recombinant epidermal growth factor (EGF; 0.1 ng/mL), 10% FBS (Gibco), 100 U/ml penicillin, and 100 μg/mL streptomycin (Gibco). Cells were cultured at 37 °C and 5% CO2. Cells were inoculated into six well plates for further treatments.

## Cell Transfection

The cells were first inoculated in 6-well plates. To investigate the function of Linc-ROR, a Linc-ROR plasmid (plasmid map was shown in Fig. S1a) or an empty vector was transfected into cells, together with Lipofectamine 2000 (Thermo Fisher), respectively. Cells were divided into two groups: vector and Linc-ROR group. Briefly, 1 μg plasmid or vector, Lipofectamine 2000, was first added into DMEM. After 5 min, the liquid that contained Lipofectamine 2000 was added into the liquid that contained the plasmid or vector (ratio 1:1) for further balance (15 min). Finally, the mixed liquid was added into cells.

MiR-204-5p mimic, miR-204-5p inhibitor, and their respective negative control (mimic NC and Inhibitor NC) were synthesized (Rib-Bio, Guangzhou, China). To investigate the effect of miR-204-5p, cells were divided into four groups: mimic NC, miR-204-5p mimic, inhibitor NC, and miR-204-5p inhibitor group. Transfection was conducted using Lipofectamine 2000. The transfection results were verified using qRT-PCR (Fig. S1b, c). To investigate whether Linc-ROR conducted cell regulation via regulating miR-204-5p, rescue experiments were conducted and cells were divided into four groups: vector + mimic NC group (cells co-transfected with vector and mimic NC), vector + miR-204-5p mimic group (cells co-transfected with vector and miR-204-5p mimic), Linc-ROR + mimic NC group (cells co-transfected with Linc-ROR and mimic NC), and Linc-ROR + miR-204-5p mimic group (cells co-transfected with Linc-ROR and miR-204-5p mimic).

## RNA Extraction

RNAiso Plus (Takara, Kusatsu, Japan) was used to extract total RNA from tissue or cells according to the manufacturer's instructions. Briefly, RNAiso Plus was added to tissue or adherent cells, and the supernatants were collected. Chloroform was added to the supernatants. Samples were then centrifugated and the supernatants were harvested in a tube containing isopropyl alcohol. The sediment was harvested after centrifugation, washed with 75% ethanol, and finally centrifugated and dissolved in DNase & RNase free water (Beyotime, Shanghai, China). The RNA concentration was detected using NanoDrop 2000/2000c UV–Vis (Thermo Fisher Scientific, Waltham, MA, USA).

## Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

The Mir-X miRNA First-Strand Synthesis Kit (Takara) was used for reverse transcription of microRNA. cDNA was obtained according to the manufacturer's instructions. The TB Green Premix Ex Taq™ II (Takara) was used for cDNA amplification. For target microRNAs, U6 was used as internal reference gene. The program was set as: pre-denaturation at 95 °C for 10 s, 1 cycle; PCR reaction at 95 °C for 5 s and 60 °C for 30 s, 40 cycles; melting at 95 °C for 10 s, 60 °C for 1 s, and 95 °C for 15 s, 1 cycle.

The PrimeScript™ RT reagent Kit with gDNA Eraser (Takara) was used for reverse transcription of mRNA and LncRNA. GAPDH was used as internal reference gene. Program was set as following for mRNA or LncRNA amplification: pre-denaturation at 95 °C for 30 s, 1 cycle; PCR reaction at 95 °C for 5 s and 60 °C for 34 s, 40 cycles; melting at 95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s, 1 cycle.

U6 primers were brought from Takara, other primers in our study were brought from RIB-BIO, and the primers were designed for Tailing method. Data was collected and analyzed using a Lightcycler96 fluorescence quantitative PCR instrument and software correspondingly using the $2^{-\Delta\Delta Ct}$ method. The relative expression level of miRNAs was normalized to that of internal gene U6 and the control group. The relative expression level of mRNAs and LncRNA was normalized to that of internal control GAPDH and the control group. (GAPDH: F1, GGAAATCCCATCACCATC

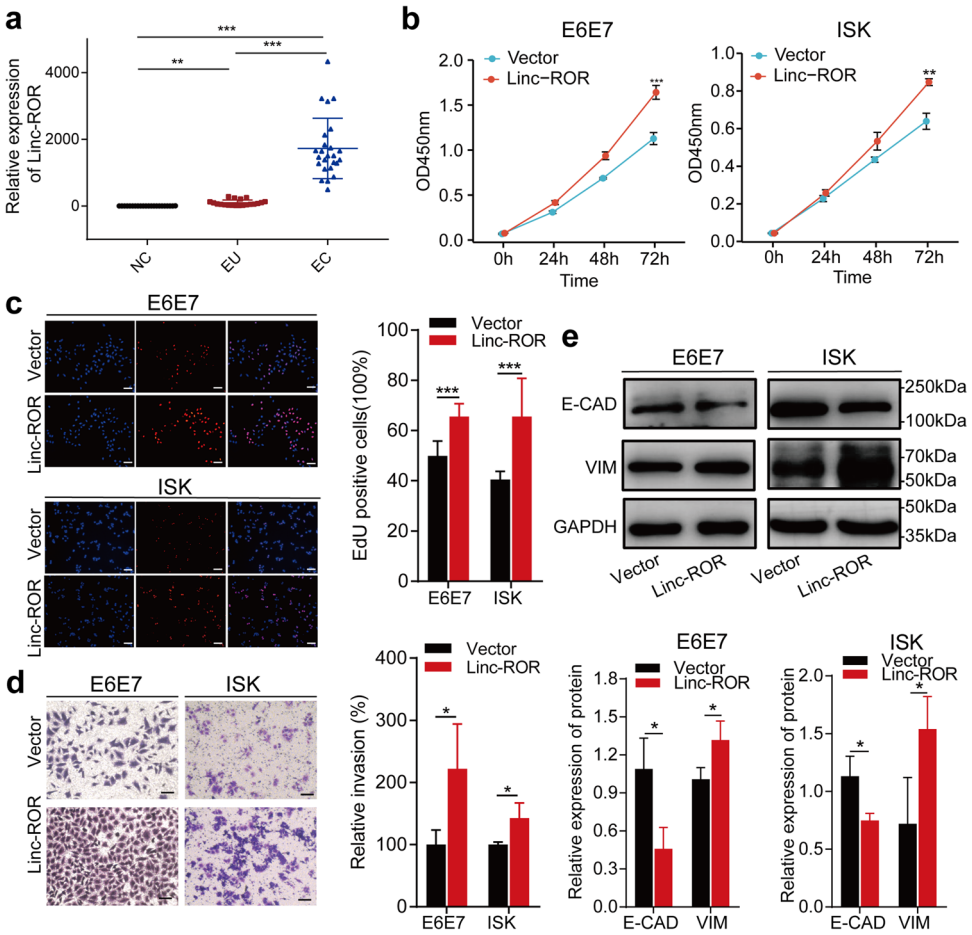



**Fig. 1** Over-expression of long intergenic non-protein coding RNA, regulator of reprogramming (Linc-ROR) promotes cellular proliferation, invasion, and epithelial-mesenchymal transition (EMT). **a** Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was used to detect the expression levels of Linc-ROR in ectopic endometrium (EC, $n=25$), eutopic endometrium (EU, $n=20$) and control group (NC, $n=22$). Kruskal–Wallis test followed by pot-hoc Dunn's test was conducted for statistical analysis. **b** Cell-counting-kit-8 (CCK-8) assay showed the effect of Linc-ROR on End1/E6E7 and Ishikawa cells proliferative capacity, the measurement time points were set at 0, 24, 48, and 72 h, the wavelength was set at 450 nm. Two-way repeated measures ANOVA was conducted for statistical analysis. **c** Represented images of 5-ethynyl-2′-deoxyuridine (EdU) assay showed the effect of Linc-ROR on End1/E6E7 and Ishikawa cell proliferative capacity. Nuclei were stained blue with Hoechst3342 solution for DNA staining; proliferative cells were stained red with Apollo solution. Two-tailed Student's *t*-test was conducted for statistical analysis. **d** Transwell invasion assay evaluated cell invasion capacity of End1/E6E7 and Ishikawa cells transfected with Linc-ROR plasmid or control vector. Two-tailed Student's *t*-test was conducted for statistical analysis. **e** Immunoblot analysis revealed that Linc-ROR decreased the protein expression level of E-cadherin and increased the protein expression level of vimentin in End1/E6E7 and Ishikawa cells. Two-tailed Student's *t*-test was conducted for statistical analysis. E6E7, End1/E6E7; ISK, Ishikawa; E-CAD, E-cadherin; VIM, vimentin; scale bars in white, 100 μm; scale bars in black, 50 μm

TTC; R1, TGGACTCCACGACGTACTCAG; SMAD4: F1, GCTTCCACTTGAATGCTGCTCT; R1, CTCCAGACA ACGGTATTGCCTC; LINC-ROR: F1, GAAGGTTCAACA TGGAAACTGG; R1: TGAGACCTGCTGATCCCATTC).

## Protein Extraction and Concentration Determinations

Radio Immunoprecipitation Assay (RIPA, Beyotime) lysis buffer and 1% phenylmethanesulfonyl fluoride (PMSF, Beyotime) were added to tissue or cells for total protein extraction for 30 min with oscillation every 10 min. The solution was centrifuged and the supernatant was collected. The BCA method (Beyotime) was used to detect the concentration of protein, after which the protein was boiled at 100 °C for 5 min with loading buffer (Beyotime). Samples were stored at − 20 °C for future applications.

## Immunoblot Analysis

To perform immunoblot analysis, 10% sodium dodecyl sulfate polyacrylamide gels were prepared to load the protein samples. After electrophoresis, the protein was transferred from the gel onto a polyvinylidene fluoride (PVDF)

membrane, followed by blocking for 2 h in 5% milk in PBST (PBS with 0.1% tween). Primary antibodies were added to the membranes and incubated overnight at 4 °C: SMAD4 (1:1500; Proteintech, Chicago, USA), Vimentin (1:2000; Cell signaling technology, Boston, USA), and E-Cadherin (1:20,000; Proteintech). Horseradish peroxidase-conjugated goat anti-rabbit IgG (H + L) secondary antibody (1:5000, Proteintech) was added to the PVDF membrane for 1 h. Finally, immunoassay of the PVDF membrane was performed using SuperSignal West Atto (Thermo Scientific) in the Amersham Imager 600. The integrated density was analyzed using ImageJ software.

## Cell Proliferation Assays

The cells were inoculated in six-well plates, and the cells were transfected with plasmid or vector, or cells were transduced with microRNA gene products 24 h after cell adherent as described before. Approximately 24 h later, the cell suspensions were prepared.

For cell-counting assay, suspensions were inoculated in a 96-well plate at 1500 cells per well and cultured in an incubator for another 0, 24, 48 and 72 h after adherence. Then, a 10 µL-cell-counting-kit-8 (CCK-8, Dojindo, Tokyo) solution was added into each well and incubated for 2 h in the cellular incubator. Finally, the absorbance was measured at 450 nm using a Multiskan GO (ThermoFisher).

A 5-ethynyl-2′-deoxyuridine (EdU) assay was conducted according to the manufacturer's instruction using Cell-Light EdU Apollo567 in vitro Kit (RIBBIO). Briefly, pre-transfected cells were inoculated in a 96-well plate at 6000 cells per well, and 24 h later, 100 µL DMEM containing 50 nM EdU reagent was added and further incubated for 2 h. Then, the cells were fixed with 4% paraformaldehyde for 15 min and washed with PBS. Then the cells were dyed using Apollo solution for 30 min in the dark. Finally, after washed with PBS, the cells were dyed with Hoechst3342 solution for 30 min for DNA staining in the dark. Finally, cells were washed with PBS and a fluorescence microscope AXIO Vert A1 (Carl Zeiss AG, Germany) and ZEN software were used for photography.

## Transwell Invasion Assay

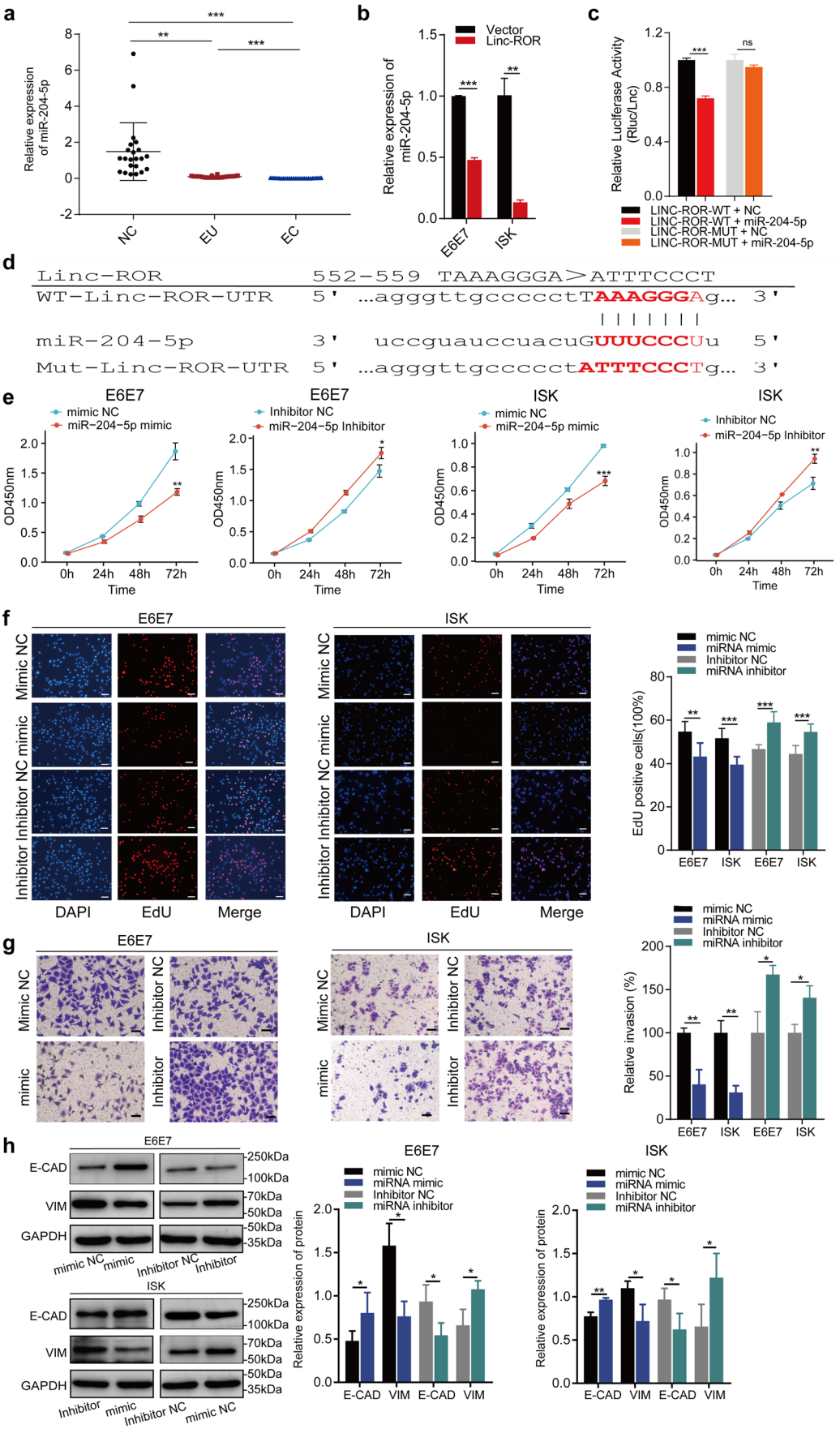
A Matrigel was precoated 12 h before usage, culture medium with serum was added in the lower chamber, 200 µL pre-transfected cells suspensions at a density of $1 \times 10^5$ without serum were placed in the upper chamber of the 24-well insert with 8 mm pore size and incubated for 48 h. The cells invaded to the lower side were fixed by 4% paraformaldehyde at room temperature (RT) for 20 min and washed. Crystal violet staining was performed at RT for 15 min (in the dark), non-invasive cells in upper chambers were scraped off with cotton swabs. The remaining invasive cells were recorded using EVOS XL Core Instrument (AMEX1000, Thermo Fisher).

## Dual Luciferase Report

Potential miR-204-5p binding sites of Linc-ROR and SMAD4 3′-UTR were predicted by miRanda database (http://www.mirbase.org/) and Target Scan (http://www.targetscan.org/). Sequences containing the presumed binding sites of Linc-ROR or *SMAD4* mRNA with miR-204-5p were designed. To test the binding specificity, the binding sequences expected to interact with the miR-204-5p seed sequence were mutated, and the mutant Linc-ROR 3′-UTR (sequences are shown in Fig. 2d) or *SMAD4* mRNA 3′-UTR (sequences are shown in Fig. 3a, b) was inserted into an equivalent luciferase reporter. For the luciferase reporter assays, HEK293 cells were used to verify the combinations between RNA transcripts owing to its relatively high transfection efficiency according to the former studies [35, 36]. HEK293 cells were cultured in 24-well plates. MiR-204-5p mimics, wild type of Linc-ROR or *SMAD4* mRNA, or a mutant form of Linc-ROR or *SMAD4* mRNA were transfected with Lipofectamine 6000 (Thermo Fisher). The cells were assayed using a luciferase assay kit (Rio-Bio) 24 h after transfection.

## Endometriosis Nude Mice Model

Five-week-old specific pathogen-free, non-pregnant female BALB/c nude mice were purchased from Chengdu Yaokang Biotechnology Co., Ltd (China). The animals were housed and given free access to food and water. Xenotransplantation of human endometrium have been widely used for murine EMs models [37], and EMs nude mice were established according to the former study [38]. Fresh endometrium from patients without endometriosis was collected. Patient 1 was 32 years old with a regular menstruation; patient 2 was 26 years old with a regular menstruation; patient 3 was 37 years old with a regular menstruation. Briefly, the 5% pentobarbital was intraperitoneally used for anesthesia before construction of EMs. The endometrium fragment of 0.5 $cm^2$ diameter was washed with sterile PBS solution and transplanted onto the surface of the abdominal wall; the abdominal pockets were secured with mouse suture. The mice were subcutaneously injected with exogenous estrogen every three days. Three days after surgery, the mice with subcutaneous EMs lesions were randomly selected for subcutaneous injection using in vivo si-NC (RIB-BIO, control group) and in vivo si-Linc-ROR (treatment group). All mice


a
Relative expression of miR-204-5p
NC
EU
EC
b
Vector
Linc-ROR
Relative expression of miR-204-5p
E6E7
ISK
c
Relative Luciferase Activity (Rluc/Lnc)
ns
LINC-ROR-WT + NC
LINC-ROR-WT + miR-204-5p
LINC-ROR-MUT + NC
LINC-ROR-MUT + miR-204-5p
d
Linc-ROR 552-559 TAAAGGGA>ATTTCCCT
WT-Linc-ROR-UTR 5' ...agggttgccccctTAAAGGGAg... 3'
miR-204-5p 3' uccguauccuacuGUUUCCCUu 5'
Mut-Linc-ROR-UTR 5' ...agggttgccccctATTTCCCTg... 3'
e
E6E7
mimic NC
miR-204-5p mimic
Inhibitor NC
miR-204-5p Inhibitor
ISK
OD450nm
Time
0h
24h
48h
72h
f
E6E7
ISK
Mimic NC
Inhibitor NC
mimic
Inhibitor
DAPI
EdU
Merge
mimic NC
miRNA mimic
Inhibitor NC
miRNA inhibitor
EdU positive cells(100%)
g
Relative invasion (%)
h
E-CAD
VIM
GAPDH
250kDa
100kDa
70kDa
50kDa
35kDa
Relative expression of protein

◂**Fig. 2** Linc-ROR sponged miR-204-5p and miR-204-5p attenuated cell proliferation, invasion, and EMT. **a** Expression levels of miR-204-5p were validated in ectopic endometrium (EC, $n=25$), eutopic endometrium (EU, $n=20$), and control group (NC, $n=22$) by qRT-PCR. Kruskal–Wallis test followed by post hoc Dunn's test was conducted for statistical analysis. **b** The down-regulation of Linc-ROR on expression level of miR-204-5p in End1/E6E7 and Ishikawa cells was proved by qRT-PCR. Two-tailed Student's *t*-test was conducted for statistical analysis. **c** Luciferase reporter assay was used to confirm the combination between Linc-ROR and miR-204-5p. Two-tailed Student's *t*-test was conducted for statistical analysis. **d** Binding site between Linc-ROR and miR-204-5p was predicted by miRanda database. **e** CCK-8 assay showed the effect of miR-204-5p on End1/E6E7 and Ishikawa cells proliferative capacity in indicated groups, the measurement time points were set at 0, 24, 48 and 72 h, the wavelength was set at 450 nm. Two-way repeated measures ANOVA was conducted for statistical analysis. **f** Represented images and bar graph of EdU assay shows cell proliferative capacity of End1/E6E7 and Ishikawa cells transfected with mimic NC, miR-204-5p mimic, inhibitor NC or miR-204-5p Inhibitor. Nuclei were stained blue with Hoechst3342 solution for DNA staining; proliferative cells were stained red with Apollo solution. Two-tailed Student's *t*-test was conducted for statistical analysis. **g** Transwell invasion assay evaluated cell invasion capacity of End1/E6E7 and Ishikawa cells transfected with mimic NC, miR-204-5p mimic, inhibitor NC or miR-204-5p Inhibitor. Two-tailed Student's *t*-test was conducted for statistical analysis. **h** Immunoblot analysis revealed that miR-204-5p mimic increased the protein expression level of E-cadherin, and decreased the protein expression level of vimentin in End1/E6E7 and Ishikawa cells; and miR-204-5p Inhibitor decreased the protein expression level of E-cadherin, and increased the protein expression level of vimentin in End1/E6E7 and Ishikawa cells, respectively. Two-tailed Student's *t*-test was conducted for statistical analysis. E6E7, End1/E6E7; ISK, Ishikawa; miRNA mimic, miR-204-5p mimic; miRNA inhibitor, miR-204-5p inhibitor; E-CAD, E-cadherin; VIM, vimentin; scale bars in white, 100 μm; scale bars in black, 50 μm

were sacrificed after three weeks, and the lesions were collected for further experiments.

### Immunohistochemistry (IHC)

Microscope slides with the targeted tissues were heated and sequentially hydrated and boiled with citrate solution. Next, $H_2O_2$ was added for incubation (10 min), and primary antibody was added for incubation overnight under 4 °C (SMAD4 [1:100, proteintech]; Ki-67 [1:10000, proteintech]; E-cadherin [1:1000, proteintech]; Vimentin [1:1000, proteintech]). Next, the second antibody was added, and the slides were stained with DAB solution and hematoxylin. The slides were dehydrated and sealed with neutral gum. Images were taken using AXIO Vert A1 (Carl Zeiss AG) and ZEN software at magnifications of 100 × and 200 ×.

### Statistical Analysis

In our study, $\alpha=0.05$. Firstly, all the data was tested for homogeneity of variances by Bartlett's test. For unequal variances (or non-normal distributions), two-tailed Mann–Whitney (two independent groups) or Kruskal–Wallis test followed by post hoc Dunn's test (when $P<0.05$, multiple independent groups), respectively, was used. If variances are similar, we applied unpaired and two-tailed Student's *t*-test (two independent groups), one-way ANOVA test (multiple independent groups), or two-way ANOVA test (two independent variables) followed by post hoc Turkey's test (when $P<0.05$). Data was presented as mean ± standard deviation (SD). Statistical analysis was performed with GraphPrism version 7.0 (GraphPad Software Inc., San Diego, CA, USA). For all panels, not significant: (ns) $P$ value > 0.05; $P$ value < 0.05 = *; $P$ value < 0.01 = **; $P$ value < 0.001 = ***.

## Results

### Up-regulated Expression of Linc-ROR Was Observed in Endometriosis

To explore the possible role of Linc-ROR in EMs, 25 ectopic and 20 eutopic endometrium from endometriosis patients and 22 normal endometrium from control cases were collected. We performed qRT-PCR analysis to determine the differential expression of Linc-ROR among groups. As shown in Fig. 1a, the up-regulated expression of Linc-ROR was observed in the EC (ectopic endometrium, relative expression: 1728.238 ± 904.330, $P<0.001$) group compared to the NC (natural control) group and EU group (eutopic endometrium, relative expression: 96.408 ± 85.444, $P=0.001$). The significant increase in endometriosis lesions suggested that Linc-ROR might play a role in the pathogenesis that is underlying EMs.

### Overexpression of Linc-ROR Promotes Cell Proliferation, Invasion and Promotes EMT Process In Vitro

To detect the potential functions of Linc-ROR, a Linc-ROR plasmid was transfected into cells (Fig. S1a), and a set of functional assays was performed. Elevated expression of Linc-ROR was identified using qRT-PCR (Fig. S1b, $P<0.001$). As shown in Fig. 1b–c, CCK-8, EdU assay ($P<0.001$) verified the induced proliferation after Linc-ROR overexpression. The results of the transwell invasion assay revealed the significant increased invasion capability of E6E7 ($P=0.049$) and ISK cells ($P=0.041$) that contained the plasmid Linc-ROR compared to the control vector group (Fig. 1d). The enhanced proliferation and invasion ability related to EMT was further explored by immunoblot analysis, the expression level was normalized to GAPDH, and significant up-regulated vimentin (E6E7, $P=0.038$; ISK, $P=0.044$) and decreased E-cadherin (E6E7, $P=0.021$; ISK, $P=0.023$) were detected, which reflected the promotion of EMT by overexpressed Linc-ROR (Fig. 1e). Thus,

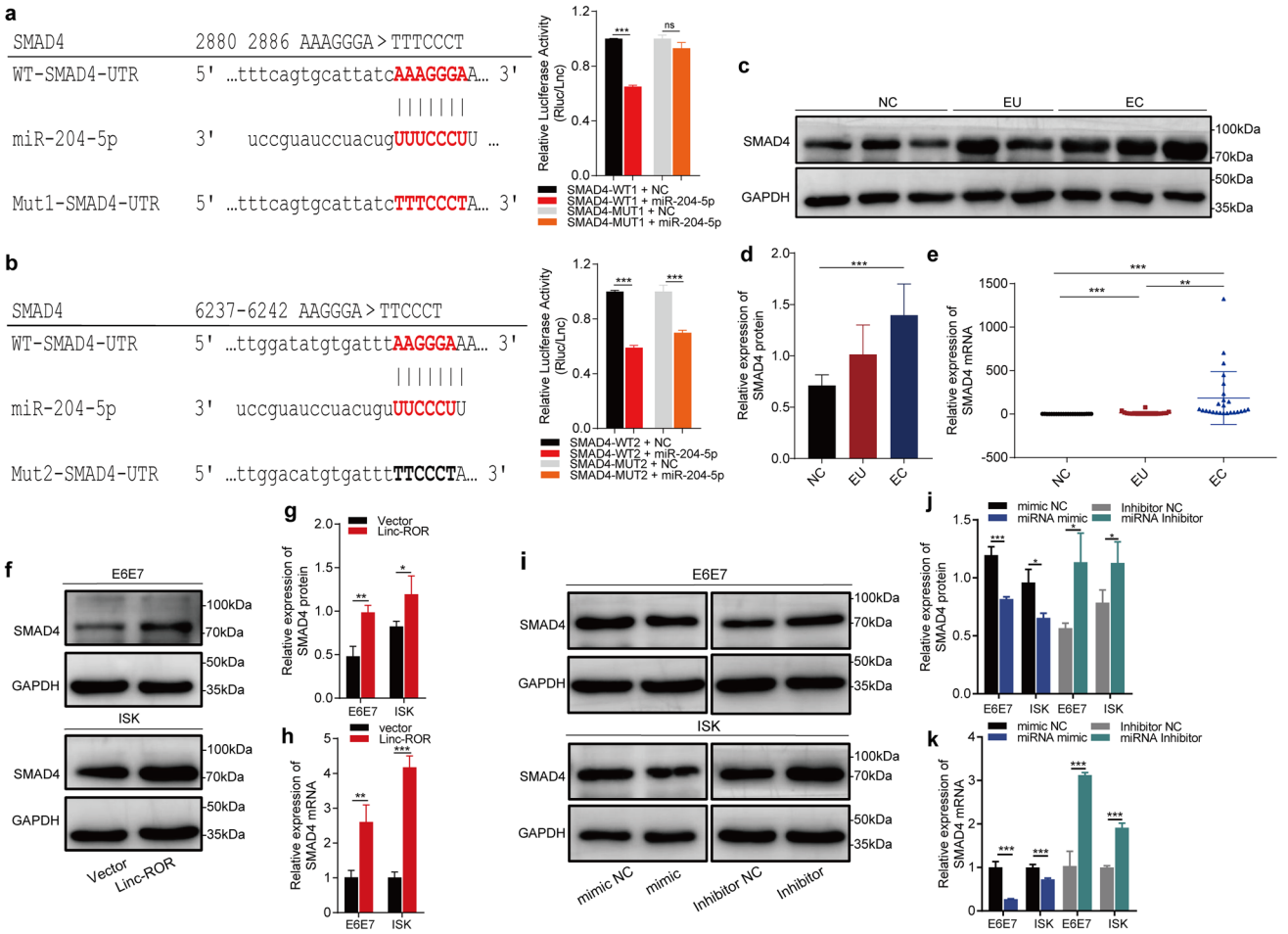



**Fig. 3** miR-204-5p sponged SMAD family member 4 *(SMAD4)* mRNA. **a**–**b** Binding sites between *SMAD4* mRNA and miR-204-5p were predicted by TargetScan database; luciferase report detected the combination of miR-204-5p and *SMAD4* mRNA in the predicted sites. **c** Represented image of immunoblot analysis of the expression level of SMAD4 protein in different tissues. **d** Bar graph showing relative expression of protein in ectopic endometrium (EC, $n=9$), eutopic endometrium (EU, $n=6$), and control group (NC, $n=9$). Kruskal–Wallis test followed by pot-hoc Dunn's test was conducted for statistical analysis. **e** qRT-PCR was used to validated the expression level of *SMAD4* mRNA extracted from ectopic endometrium (EC, $n=25$), eutopic endometrium (EU, $n=20$) and control group (NC, $n=22$). Kruskal–Wallis test followed by pot-hoc Dunn's test was conducted for statistical analysis. **f**–**h** The effect of Linc-ROR on SMAD4 expression was detected using immunoblot analysis and qRT-PCR in End1/E6E7 and Ishikawa cells. Two-tailed Student's *t*-test was conducted for statistical analysis. **i**–**k** The effect of miR-204-5p on SMAD4 expression in End1/E6E7 and Ishikawa cells was detected using qRT-PCR and immunoblot analysis, respectively. Two-tailed Student's *t*-test was conducted for statistical analysis. E6E7, End1/E6E7; ISK, Ishikawa; mimic, miR-204-5p mimic; inhibitor, miR-204-5p Inhibitor; E-CAD, E-cadherin; VIM, Vimentin

Linc-ROR might increase cell proliferation and invasion and promote EMT process.

## Linc-ROR Functions as ceRNA and Sponged miR-204-5p

To investigate the Linc-ROR's latent-binding miRNAs, we first explored the expression levels of miR-124-3p, miR-204-5p, miR-195-5p, miR-205-5p, and miR-308-5p in endometrium tissues of the subjects in the EC, EU, and NC groups. The results are shown in Figs. 2a and S2; compared with the NC group, the expression of miR-124-3p (relative expression: $70.329 \pm 91.795$), miR-138-5p (relative expression: $50.551 \pm 58.292$), and miR-195-5p (relative expression: $48.174 \pm 32.809$) was up-regulated in EC tissues, while miR-204-5p (relative expression: $0.010 \pm 0.012$) and miR-205-5p (relative expression: $0.028 \pm 0.015$) showed significant down-regulated changes. We further investigated the regulation of Linc-ROR towards the candidate microRNAs in vitro. As shown in Figs. 2b and S2, we observed a significant decrease in miR-204-5p expression after Linc-ROR overexpression in cells, while miR-124-3p, miR-138-5p, miR-195-5p, and miR-205-5p showed a noticeable,

up-regulated change. As such, we finally selected miR-204-5p as the optimal selection. To further explore the functional mechanism underlying Linc-ROR and miR-204-5p, the miRanda database was used to predict the binding site, and an effective binding site emerged (Fig. 2d). We then used a dual-luciferase reporter assay and proved the combination between Linc-ROR and miR-204-5p (Fig. 2c). Most importantly, the same cellular function assays and immunoblotting for EMT markers were conducted in a gain-and-loss-of-function manner to identify the function of miR-204-5p, the transfection efficiency of mimic RNA or inhibitor was detected using qRT-PCR, and the expression altered level of miR-204-5p in cells was identified and shown in Fig. S1c. The results in Fig. 2e–g demonstrated that up-regulated miR-204-5p significantly inhibited cell proliferation and invasion as measured by the CCK-8, EdU (E6E7, $P = 0.005$; ISK, $P < 0.001$), and invasion assays (E6E7, $P = 0.004$; ISK, $P = 0.002$), while down-regulation of miR-204-5p enhanced cell proliferation and invasion in vitro. Elevated expression of E-cadherin (E6E7, $P = 0.048$; ISK, $P = 0.003$) and decreased expression of Vimentin (E6E7, $P = 0.010$; ISK, $P = 0.034$) were confirmed in the miR-204-5p mimic group compared to the mimic NC group. Meanwhile, decreased levels of E-cadherin (E6E7, $P = 0.018$; ISK, $P = 0.023$) and elevated Vimentin (E6E7, $P = 0.026$; ISK, $P = 0.025$) in the miR-204-5p inhibitor group presented a promotion of EMT after down-regulation of miR-204-5p (Fig. 2h). These results further suggested that Linc-ROR promoted cellular proliferation and invasion, as well as the EMT process via modulation of miR-204-5p by functioning as a ceRNA.

## SMAD4 is Target of miR-204-5p

MicroRNAs are often involved in post-transcriptional regulation of their target protein. Thus, to determine the role of miR-204-5p, we examined the combination of miR-204-5p and SMAD4 mRNA. After prediction of binding sites using TargetScan, potent binding sites (site1 and site2) between miR-204-5p and SMAD4 mRNA were identified. The results of dual-luciferase reporter assay indicated that regulation of miR-204-5p on SMAD4 mRNA occurs on the binding site 1 (Fig. 3a, b). Furthermore, the expression level of SMAD4 was detected in tissues from ectopic endometrium, eutopic endometrium, and endometrium from patients without endometriosis; elevated expression of SMAD4 in both mRNA and protein level merged in the EC group compared with the NC groups (Fig. 3c-e, $P < 0.001$). To further confirm the regulation of Linc-ROR and miR-204-5p on SMAD4, experiments in vitro were conducted. As shown in Fig. 3f–h, elevated SMAD4 mRNA and protein expression were determined with Linc-ROR overexpression. Meanwhile, up-regulated miR-204-5p repressed the expression of SMAD4, decreased miR-204-5p promoted expression of SMAD4 (Fig. 3i-k). The above results suggested that SMAD4 was involved in the regulation of Linc-ROR and miR-204-5p.

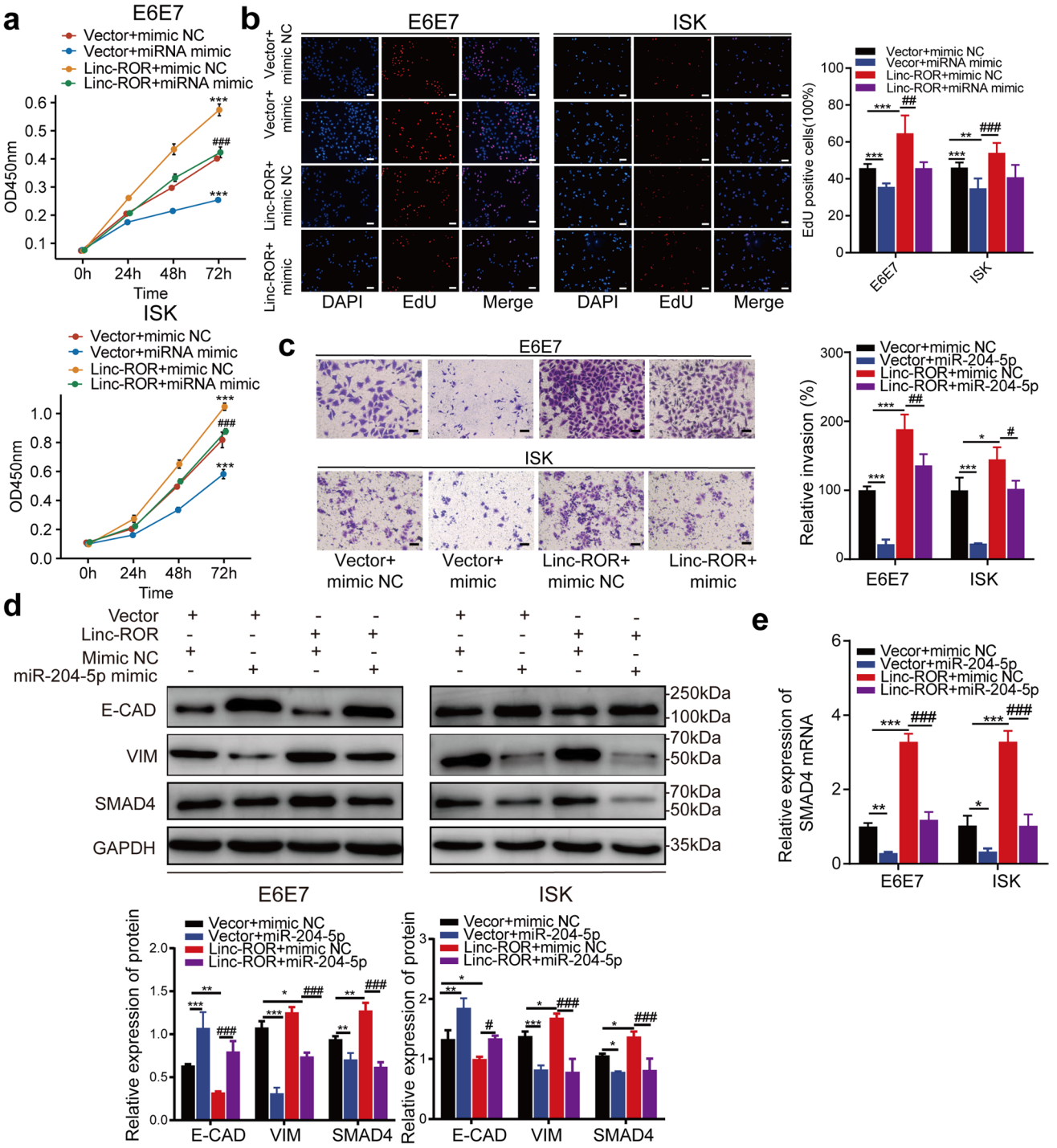
## Linc-ROR Effects Cellular Changes by Targeting the miR-204-5p/SMAD4 Axis

To further explore the role of Linc-ROR in the regulation of miR-204-5p/SMAD4 and induce changes in the EMT and cell function, we conducted a rescue experiment.

E6E7 and ISK cells were transfected with different gene products, included control products, miR-204-5p mimic, Linc-ROR plasmid, and co-transfected Linc-ROR plasmid and miR-204-5p mimic. Firstly, we conducted the cellular function assays, notably, as shown in Fig. 4a–c, the enhanced proliferation and invasion, promoted by Linc-ROR, was significantly reduced after the cells were co-transfected with the miR-204-5p mimic. Secondly, the markers of EMT were detected via immunoblot analysis, concurrently, E6E7 and ISK cells co-transfected with Linc-ROR and the miR-204-5p mimic showed higher level of E-cadherin (E6E7, $P < 0.001$; ISK, $P = 0.021$) and lower level of vimentin ($P < 0.001$) compared to cells transfected with plasmid Linc-ROR and the mimic NC (Fig. 4d). Furthermore, the expression level of SMAD4 protein and mRNA was measured; the expression of SMAD4 was significantly down-regulated, compared to cells in Linc-ROR group, after the cells were co-transfected with plasmid Linc-ROR and the miR-204-5p mimic (Fig. 4d, e, $P < 0.001$). These results further confirmed the essential role of Linc-ROR as a ceRNA in the cell proliferation, invasion, and EMT process via targeting the miR-204-5p/SMAD4 axis in EMs.

## Linc-ROR Effects the miR-204-5p/SMAD4 Axis in EMs Nude Mice

An EMs-nude-mice model was established to confirm the function of Linc-ROR in vivo. Histological examination was conducted to confirm the establishment of endometriosis in nude mice. As shown in Fig. 5a, the presence of endometrial tissue in the EMs-nude-mice model was identified, as the glandular epithelium and stroma components were identified and presented. As shown in Fig. 5b, compared to the mice in control group, qRT-PCR confirmed the significant down-regulated Linc-ROR ($P = 0.010$) in the EMs-nude mice that were injected with si-Linc-ROR; meanwhile, Fig. 5c showed elevated miR-204-5p ($P < 0.001$). As shown in Fig. 5d, e, decreased SMAD4 mRNA levels ($P = 0.010$) and protein expression level were detected in si-Linc-ROR group by qRT-PCR and IHC




assay. As shown in Fig. 5f, the IHC assay further confirmed that si-Linc-ROR repressed the EMT process, with lower expression of Ki-67 and vimentin and up-regulated expression of E-cadherin in EMs-nude mice, compared with that in control group. However, the lesion volume and weight in si-Linc-ROR group only presented a decreased trend, but the difference was not statistically significant compared to the control group (data not shown).

## Discussion

To date, EMs reaches an inadequate understanding and no known cure; thus, there is an urgent need to elucidate the underlying mechanisms and developing novel treatments [9]. EMT was identified necessary for normal female reproductive organ function, and its dysregulation promotes endometriosis, adenomyosis, and carcinogenesis. Consistently,

◂**Fig. 4** Linc-ROR promotes EMT and enhanced cell proliferation and invasion by targeting miR-204-5p/SMAD4. **a** CCK-8 assay showed cell proliferative capacity of End1/E6E7 and Ishikawa cells transfected with control, miR-204-5p mimic, Linc-ROR, or co-transfected with miR-204-5p mimic and Linc-ROR, the measurement time point was set at 0, 24, 48 and 72 h, the wavelength was set at 450 nm. Two-way repeated measures ANOVA was conducted for statistical analysis. **b** EdU assay showed cell proliferative capacity of End1/E6E7 and Ishikawa cells transfected with control, miR-204-5p mimic, Linc-ROR, or co-transfected with miR-204-5p mimic and Linc-ROR. One-way ANOVA test was conducted for statistical analysis. **c** Transwell invasion assay evaluated cell invasion capacity of End1/E6E7 and Ishikawa cells transfected with control, miR-204-5p mimic, Linc-ROR, or co-transfected with miR-204-5p mimic and Linc-ROR. One-way ANOVA test was conducted for statistical analysis. **d** immunoblot analysis showed protein expression level of E-cadherin, Vimentin and SMAD4 in End1/E6E7 and Ishikawa cells transfected with control products, miR-204-5p mimic, Linc-ROR, or co-transfected with miR-204-5p mimic and Linc-ROR. One-way ANOVA test was conducted for statistical analysis. **e** *SMAD4* mRNA expression was detected using qRT-PCR in End1/E6E7 and Ishikawa cells transfected with control, miR-204-5p mimic, Linc-ROR, or co-transfected with miR-204-5p mimic and Linc-ROR. One-way ANOVA test was conducted for statistical analysis. E6E7, End1/E6E7; ISK, Ishikawa; mimic, miR-204-5p mimic; E-CAD, E-cadherin; VIM, vimentin; scale bars in white, 100 μm; scale bars in black, 50 μm

distant metastasis and implantation were particular and significant features of EMs [9], in which EMT plays a central role. Studies have also revealed the abnormal expression of EMT-marked transcription factors (EMT-TFs) in EMs, including Snail, TWIST, and zinc-finger E-box-binding (ZEB). However, it is unclear the exactly modulator of EMT in EMs. In our study, we identified the high expression of Linc-ROR in the ectopic endometrium tissue and Linc-ROR could facilitated the cellular invasion and proliferation, as well as EMT progression in EMs, which enriched our understanding of the pathogenesis of endometriosis.

With multiple studies done, cross-talk between noncoding RNAs become one of the driving factors of EMT in diseases; therefore, we aimed to corroborate the potential binding of Linc-ROR's microRNAs and their contribution to EMT in EMs. We reviewed the scientific literature for reinforced evidence on possible binding microRNAs in their regulation on EMT and forecasted their binding with Linc-ROR using databases. Inhibition of miR-124-3p on EMT was proven as in the case of clear cell renal cell carcinoma via the modulation of ZEB2 [39], GC via regulation of Snail2, bladder cancer [40], and glioma cells [41]. Similarly, inhibition of miR-138-5p on EMT was proven in HCC [42], lung carcinoma [43–45], and breast cancer [46, 47]. The miR-195-5p displayed control of EMT in multiple carcinomas, including in prostate cancer [48], colorectal cancer [49], endometrial carcinoma [50], and colon cancer cells [51]. The miR-204-5p was associated with EMT inhibition in head and neck squamous cell carcinoma [52], laryngeal squamous cell carcinoma [53], human tenon capsule fibroblasts [54], and glioma cells [55]. Overexpression of miR-205-5p inhibited the EMT in renal cell carcinoma [56], glioma cells, anaplastic thyroid carcinoma cells, HCC, glioblastoma cells, and colorectal cancer cells [57–61].

In our study, we finally confirmed the combination of Linc-ROR and miR-204-5p; similarly, a previous study also proved the combination of Linc-ROR and miR-204-5p in esophageal squamous cell carcinoma [31]. It is worthy to note that the conventional post-transcriptional silencing mechanisms of miRNA-mRNA interaction leads the forecast of regulation towards target proteins once ceRNAs mechanism worked [62]. As research has progressed, activation of protein translation has been proposed after miRNA regulated[63, 64]. Although it remains to be determined whether this represents a general phenomenon or is simply an exception to the miRNA regulatory mechanism, studies on the novel mechanisms of miRNA-target gene action are beneficial to further understand the differential regulation of target genes following miRNA adsorption by LncRNA. Additionally, the ceRNA mechanism has been shown to contribute to the progress of EMT in EMs as well [7]. NcRNAs can also regulate EMT by directly affecting EMT-TFs or related cofactors. For example, down-regulated miR-141 [38] and miR-200b [32, 39] in ectopic EMs lesions were also verified to inhibit EMT by inhibiting TGF-β signaling pathway and inhibiting ZEB1 and ZEB2, respectively.

Of notes, we first validated the combination of miR-204-5p and SMAD4, and their regulation on EMT in EMs. Consistently, Peng et al. demonstrated that the miRNA-204-5p/SMAD4 axis regulated the EMT process in lens epithelial cells [66]; studies have proven that Linc-ROR, as well as SMAD4, promotes the EMT process in several diseases[32, 65]. Similarly, transfection of SMAD4-siRNA in endometrial interstitial cells inhibited the cytosolic aggregation of p-SMAD3 caused by activin A and suppressed the activity of the P450arom promoter, suggesting that the ALK4-SMAD pathway may promote the survival and development of ectopic lesions [67, 68]. The TGF-β/SMAD signaling pathway is one of the core signaling pathways governing EMs, promoting adhesion, angiogenesis, vascular remodeling, and cell proliferation [34]. Importantly, this results in our study also reinforce the importance of TGF-β/SMADs pathway in EMs; in another words, the up-regulated SMAD4 by Linc-ROR might create an active environment for TGF-β related responses in EMs, thus participated in the pathogenesis of EMs.

Recently, the targeting of ncRNA has shown to be a positive, therapeutic modality for disease treatment. Thus, in our study, we used in vivo si products to briefly explore treatment efficiency of Linc-ROR in EMs. The results (Fig. 5) further validated the regulation of Linc-ROR in EMs. Unfortunately, EMs-nude mice in si-Linc-ROR group showed no significant decrease in lesion weight,

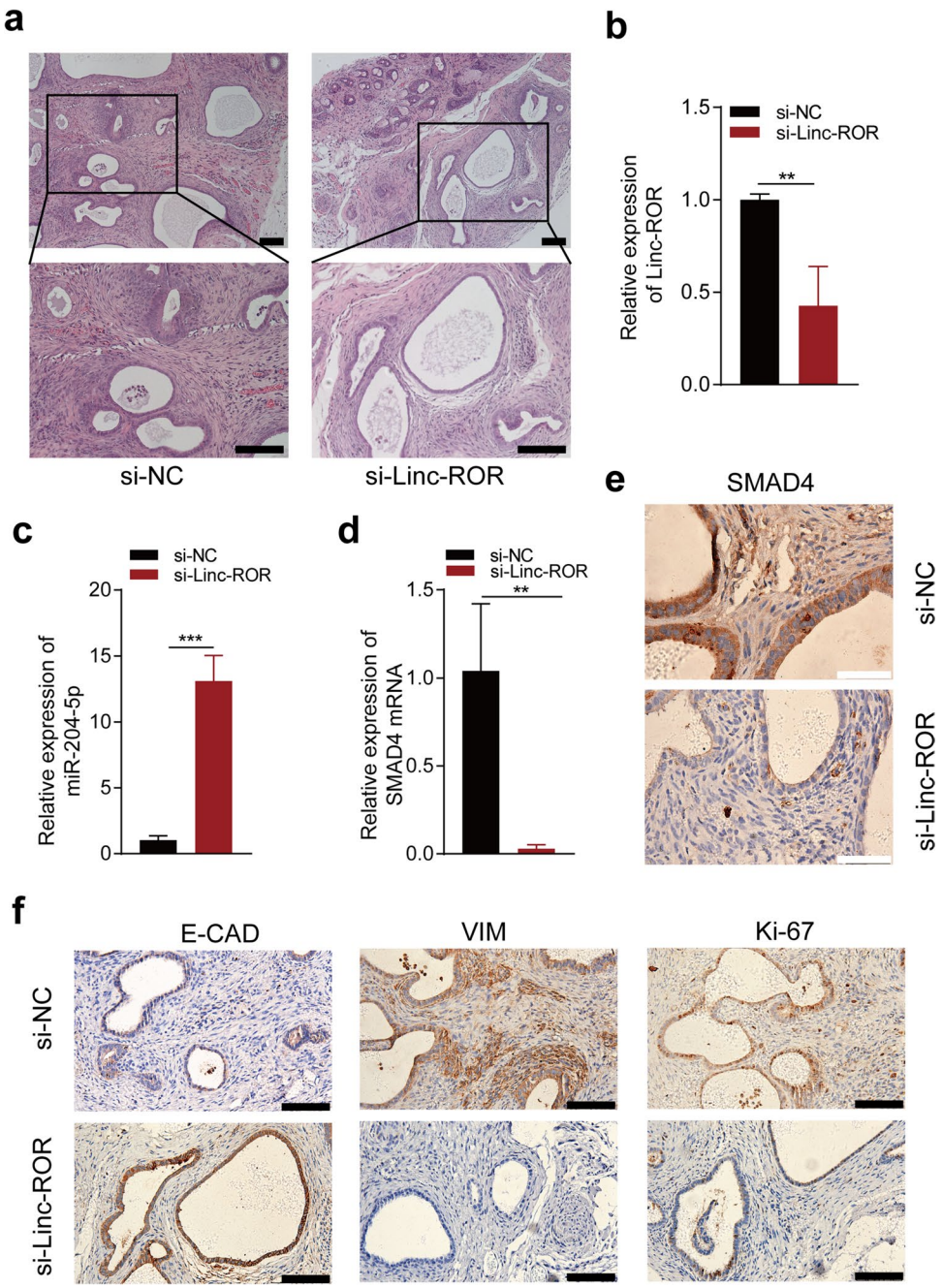



**Fig. 5** Linc-ROR effects the miR-204-5p/SMAD4 axis in endometriosis nude mice. **a** hematoxylin–eosin staining of endometriosis in nude mice. **b** qRT-PCR detected relative expression of Linc-ROR. **c** qRT-PCR detected relative expression of miR-204-5p. Two-tailed Student's *t*-test was conducted for statistical analysis. **d** Bar graph showing *SMAD4* mRNA in endometriosis nude mice intervened with si-NC or si-Linc-ROR detected by qRT-PCR. Two-tailed Student's *t*-test was conducted for statistical analysis. **e** images showing immunohistochemistry image of SMAD4 in endometriosis nude mice intervened with si-NC or si-Linc-ROR. **f** Images showing immunohistochemistry image of Ki-67, E-cad and vimentin in endometriosis nude mice intervened with si-NC or si-Linc-ROR. Scale bars in black, 100 μm; scale bars in white, 50 μm

compared with the control group. The results might partly be due to the small lesion size, the lack of a visual tracking method, and timing of si-Linc-ROR treatment in EMs.

There are several limitations in our study. In our study, it would be more comprehensive to evaluate the function of Linc-ROR/miR-204-5p/SMAD4 in primary ectopic and eutopic endometrial cells, to corroborate whether aberrant Linc-ROR triggered before metastatic implantation, or whether there was an exert influence on the maintenance of ectopic lesions. Secondly, while collecting ectopic endometrium, chocolate cyst wall was carefully scraped and cut into small pieces for different experiments; however, it would be ideal to conducted histological verification to confirm that the samples did not contain normal ovarian tissue, and then used for further RNA extraction and protein extraction. Thirdly, while analyzing the expression level, we did not analyze the expression according to the menstrual cycle since the proportion of proliferative cycle/secretory cycle was similar in patience with and without endometriosis; moreover, in study conducted by X. Y. Xu et al. confirmed that no relationship was observed between Linc-ROR expression and menstrual cycles [29]. However, considering that the EMT process participated in the normal female reproductive organ

function, it would be ideal detect the variance between different menstrual cycles. Thus, more lesions in proliferation or secretory cycle, including the lesions from phase I and II, as well as serum samples, should be further collected to analyze the expression trend of Linc-ROR, miR-204-5p and other meaningful ncRNAs to evaluate their diagnostic efficiency; it might be feasible to establish a model of clinical EMs diagnosis based on several vital ncRNAs.

## Conclusion

In summary, we identified that Linc-ROR promoted cell proliferation, invasion, and EMT process in EMs. MiR-204-5p was down-regulated by Linc-ROR, and SMAD4 was a target of miR-204-5p. The interaction of Linc-ROR, miR-204-5p and SMAD4 was verified in EMs for the first time. In addition, Linc-ROR might represent a promising biomarker for patients with EMs.

**Abbreviations** EMs: Endometriosis; EMT: Epithelial–mesenchymal transition; ncRNA: Non-coding RNA; ISK: Ishikawa; E6E7: End1/E6E7; E-cad: E-cadherin; VIM: Vimentin; ceRNA: Competing endogenous RNA; LncRNA: Long non-coding RNA; PBS: Phosphate-buffered saline; CCK-8: Cell counting kit-8; qRT-PCR: Quantitative reverse-transcription polymerase chain reaction; IHC: Immunohistochemistry; EC: Ectopic endometrium; EU: Eutopic endometrium; NC: Endometrium from the control group; R-SMAD: Receptor-regulated SMAD

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**Author Contribution** Mingyu Yi: Data curation, methodology, software, writing—original draft preparation. Sixue Wang: Data curation, Writing—Original draft preparation. Xinyue Zhang: Data curation, writing—original draft preparation. Li Jiang: Resources, Investigation, validation. Xiaomeng Xia: Resources, investigation, validation. Tingting Zhang: Resources, investigation, validation. Xiaoling Fang: Supervision, writing—reviewing and editing, funding acquisition.

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**Data Availability** Not applicable.

**Code Availability** Not applicable.

## Declarations

**Ethics Approval** This study was approved by the Institutional Ethics Review Committee of Second Xiangya Hospital of Central South University (#2016243).

**Consent to Participate** Not applicable.

**Consent for Publication** Not applicable.

**Competing Interests** The authors declare no competing interests.

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